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Tyraminergic signaling in phytophagous insects: from physiology to pest control

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Abstract

The biogenic amines tyramine (TA) and octopamine (OA) are neurochemicals essential in invertebrates that act as adrenaline and noradrenaline substitutes. They exert their effects by binding specific receptor proteins that belong to the superfamily of G-protein coupled receptors. Tyramine (TAR) and octopamine (OAR) receptors play important roles in modulating the biology, physiology and behavior of insect. This PhD thesis describes the characterization of the type 1 tyramine receptor (TAR1) in two insect pests, *Drosophila suzukii* and *Halyomorpha halys*, to dissect the receptor role in controlling physiological and behavioral traits as well as to examine TAR1 as possible target for biopesticides. *D. suzukii* TAR1 proved to be an interesting target for biopesticides, such as monoterpenes. These compounds were in fact able to modulate directly TAR1-controlled physiology and behavior. In *H. halys*, RNAi-mediated *TAR1* downregulation suggested that the receptor involvement in pheromone perception. Together, the data described emphasize TAR1 as crucial in controlling and defining physiological and behavioral aspects in insects. Furthermore, this receptor appears an interesting target for innovative and environmental friendly in pest control.

Abstract

Le ammine biogene tiramina (TA) ed octopamina (OA) sono sostanze neurochimiche, essenziali negli invertebrati, che agiscono come sostituti dell'adrenalina e della noradrenalina. TA ed OA esercitano i loro effetti legando specifici recettori che appartengono alla superfamiglia dei recettori accoppiati a proteine G. I recettori tiramici (TAR) ed octopaminici (OAR) svolgono un ruolo importante nel modulare la biologia, la fisiologia e il comportamento degli insetti. In questa tesi di dottorato viene descritta la caratterizzazione del recettore della tiramina di tipo 1 (TAR1) in due insetti nocivi per le piante, *Drosophila suzukii* e *Halyomorpha halys*, con lo scopo di investigare il ruolo che il recettore esercita nel controllo dei tratti fisiologici e comportamentali oltre che testare TAR1 come possibile bersaglio per biopesticidi. Il TAR1 di *D. suzukii* si è rivelato un interessante bersaglio per biopesticidi, nello specifico i monoterpeni. Questi composti sono stati in grado di modulare quegli aspetti fisiologici e comportamentali di *D. suzukii* direttamente controllati da TAR1. In *H. halys*, la downregolazione di TAR1 attraverso RNAi ha evidenziato un possibile coinvolgimento del recettore nella percezione dei feromoni. Insieme, i dati presentati mostrano come TAR1 sia cruciale nel controllo di numerosi aspetti fisiologici e comportamentali degli insetti. Inoltre, questo recettore sembra essere un bersaglio per approcci innovativi e rispettosi dell'ambiente nel controllo dei parassiti.

List of publications

This thesis is mainly based on the results described in the following articles:

1. **Finetti L.**, Ferrari F., Calò G., Cassanelli S., De Bastiani M., Civolani S. & Bernacchia G. (2020). Modulation of *Drosophila suzukii* type 1 tyramine receptor (DsTAR1) by monoterpenes: a potential new target for next generation biopesticides. *Pesticide Biochemistry and Physiology* 165: 104549. doi.org/10.1016/j.pestbp.2020.02.015.
2. **Finetti L.**, Tiedemann L., Zhang X., Civolani S., Roeder T. & Bernacchia G. (2021). Monoterpenes alter TAR1-driven physiology in *Drosophila* species. *Journal of Experimental Biology*, 224 (1): jeb232116. doi:10.1242/jeb.232116.
3. **Finetti L.**, Pezzi M., Civolani S., Calò G., Scapoli C. & Bernacchia G. (2021). Characterization of *Halyomorpha halys* TAR1 reveals its involvement in (*E*)-2-decenal pheromone perception. *Journal of Experimental Biology*, 224 (7): jeb.238816. doi:10.1242/jeb.238816.

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Chapter I: General overview

Insect Pest Management

Insects (phylum: Arthropoda; class: Insecta) represent the largest group of animals on Earth. The exact number of insect species is only predicted; the annual Catalogue of Life (Catalogue of Life: checklist 2019) reports 927.346 insect species identified but the estimate total number could be around 5.5 million (Stork, 2018). Among these, only a small percentage (around 1%) is considered plant pest (Kumar & Omkar, 2018). However, it is able to destroy about 10 % of the agricultural yield, every year. Despite the use of chemical pesticides to control pests has increased by 20% in the last 40 years, the crop damage caused by insects has nevertheless increased (Oerke, 2006). This spreading can be attributed to several reasons:

- Climate change. Drastic and fast environmental changes, such as increased temperature and reduced water availability, have enhanced plant susceptibility to insect pest attack (Bjerke et al., 2014).
- Decrease in biodiversity. Loss of non-target organisms, due to uncontrolled use of pesticide and extensive monoculture crop favoured pest growth (Sanchez-Bayo & Wyckhuys, 2019).
- Introduction of new pests (accidental or not). The absence of natural enemies often leads to an uncontrolled and extremely fast multiplication of an alien insect pest, with the growth of a new stable population difficult to control (Gippet et al., 2019)

Overall, pest management requires different components, such as the agronomic practices, mechanical and physical control, behavioral control, biological control, biotechnological control and chemical control (Stenberg, 2017). Agronomic practices reduce pest populations, making the field less favourable, by planting pest-resistance cultivars (Pretty, 2008). Mechanical and physical control aims to limit the access of pests to plants (e.g. nets or barriers between plant and insect) as well as directly modify the environment to slow down their growth (Vincent et al., 2003). The behavioral control tries to reduce the pest populations by changing their life cycle through chemical stimuli (pheromones, used in mating disruption methods), visual stimuli (wavelengths that attract insects), sound stimuli (sounds that mimic those emitted during mating, causing disorientation) as well as molecules with repellent properties (Polajnar et al., 2015; Yew & Chung, 2015; Kim et al., 2019). Biocontrol is based on the artificial introduction of the natural enemies of insect pests (Jones et al., 2005).

Biotechnological control

In pest management the use of biotechnological tools can be defined as the controlled manipulation of biological systems to achieve efficient insect pest control. Based on the persistence in the environment or the biological modification introduced, the biotechnological approaches can be divided into two major categories: those that act by suppressing local populations and are themselves self-limiting (self-limiting methods) and those in which the population of parasites is replaced by a more benign form (self-sustainable methods) (Alphey, 2014).

- Self-limiting methods

These methods are based on the introduction into the environment of insects with characteristics unsuitable for the growth of the population. This is the case of the Sterile Insect Technique (SIT) by which a large number of males are reared in laboratory, irradiated with UV rays to make them sterile and then introduced into the environment. The coupling of sterile males with wild type females leads to a reduction in the population size (Hendrichs et al., 1995). In the Release of Insect Carrying a Dominant Lethal (RIDL), females are created in the laboratory not able to express a tetracycline-repressible transactivator fusion protein (tTA), causing a lethal phenotype. The addition of tetracycline to the diet suppresses lethality but, in the field, where tetracycline is absent, the lethality kills the progeny and the pre-adult stages (Fu et al., 2007). In the Incompatible Sterile Technique (IST), males are infected with *Wolbachia* and their mate with uninfected females results in female-sterile progeny (Brelsfoard & Dobson, 2009).

- Self-sustainable methods

In this case, it is necessary to develop a drive gene system to spread a specific character in the pest populations. The first mechanism developed is the gene drive system based on *Wolbachia*. *Wolbachia* is a maternally inherited intracellular parasite, which gives females a better fitness than wild type ones, resulting in a rapid spread of *Wolbachia* in the population. In addition, it has been observed that the presence of this parasite in females make them less prone to carry dangerous viruses such as Dengue. The *Wolbachia* infection results therefore in a self-sustainable method to control the spreading of insect-mediated disease (Hoffman et al., 2011). Another method is the drive system gene based on homing endonuclease genes (HEGs). These genes are naturally present in the original genome and can be inserted within a target gene required for reproduction or involved in the pest development. Homology-directed repair (HdR) of the double-strand break generated by the endonuclease causes therefore the interruption of the target gene (Burt,

2003). The techniques described so far were successfully employed especially in the control of mosquitoes (Leftwich et al., 2015). On the other hand, there are new biotechnology-based techniques being developed for the control of many pests, such as the RNA interference (RNAi) and the CRISPR-Cas9 gene-editing tool (**Figure 1**).

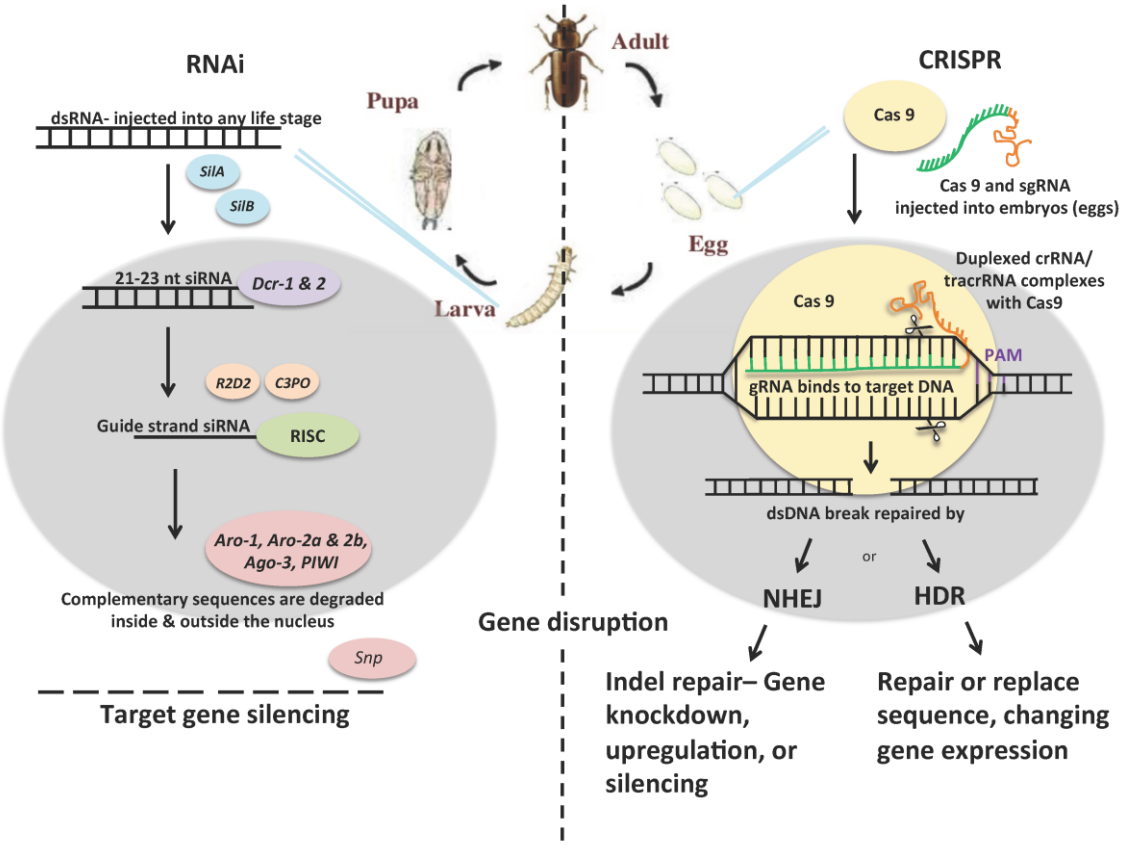

Figure 1. Schematic representation of RNA interference (RNAi) and CRISPR-Cas9 technologies in insects (Perkin et al., 2016).

The Clustered Regularly Interspaced Palindromic Repeats (CRISPR) and the endonuclease CRISPR-associated protein 9 (Cas9) are used by bacteria as a defence mechanism to ward off infections from viruses (Mojica et al., 2005). The CRISPR-Cas9 machinery uses an RNA guide (gRNA) to lead the Cas9 endonuclease to the DNA target sequence. Here, the endonuclease cuts the double stranded DNA, causing a DNA double strand break that can be repaired by non-homologous end joining (NHEJ) or by homology-directed repair (HDR). In the first case, HNEJ inserts or deletes single or multiple nucleotides causing a shift in the reading frame of the gene, turning it off. In the second case, thanks to the addition of a template containing the altered sequence desired, HDR replaces the wild type sequence, changing the gene expression profile or its sequence (**Figure 1**) (Garneau et al., 2010; Jinek et al., 2012). The CRISPR-Cas9 has

been recently used to control several insect pests such as *Tribolium castaneum* and *Drosophila suzukii* (Gilles et al., 2015; Li & Scott, 2016).

The RNA interference (RNAi) technology uses an endogenous, post-transcriptional and highly conserved immune mechanism based on a double-stranded RNA molecule (dsRNA) and the highly specific degradation response against the complementary cytoplasmic mRNA. Once the dsRNA enters the cell, it is cut by the endonuclease Dicer into 21 bp-long fragments called siRNA (short interfering RNA). These molecules are then loaded as single helices onto the RNA-induced silencing complex (RISC) and then guided and paired to the complementary endogenous mRNA sequences. Finally, this new double stranded RNA complex is degraded with the contribute of the RISC catalytic component such as Argonaute (**Figure 1**) (Perkin et al., 2016). The entire RNAi process was firstly discovered in *Caenorhabditis elegans* (Fire et al., 1998) and then described in many other species, but it is not yet fully characterized in insects. Dicer, RISC and Argonaute orthologs have been identified but the other components participating in this phenomenon remain less understood. Furthermore, the RNAi technique does not always trigger the expected silencing of the target gene. In fact, some insect orders, such as lepidoptera, appear recalcitrant to RNAi-induced silencing (Terenius et al., 2011). Several studies have reported that insensitivity to RNAi was sometimes caused by a poor expression of the core machinery genes (Swevers et al., 2011; Davis-Vogel et al., 2018). Another factor that interferes with RNAi efficiency is the delivery mechanism necessary to introduce dsRNA inside the insect cells. Over the years, two main delivery techniques have been developed, applicable only in the laboratory or directly in the field: microinjection and ingestion (Yu et al., 2012). Microinjection is the most efficient delivery approach because the dsRNA molecule is directly injected into the insect and the exact amount of dsRNA introduced is known. On the other hand, the microinjection is limited to the laboratory and the procedure could triggers inevitable immune reactions that interfere with the RNAi-mediated gene silencing (Joga et al., 2016). The dsRNA delivery by food ingestion can be achieved by two main strategies: dsRNA can be expressed in bacteria and then introduced with the diet, or it can be synthesized in vitro and mixed with food (Liu et al., 2020). Furthermore, recent studies demonstrated that transgenic plants can be engineered to express dsRNA targeting genes from insects to increase their resistance to herbivorous insects (Baum et al., 2007; Mao et al., 2011). RNAi silencing through transgenic plants has been studied in several insect orders, such as Lepidoptera, Coleoptera and Hemiptera, with positive effects (Baum et al., 2007; Pitino

et al., 2011; Zha et al., 2011). On the other hands, the different gut environment within the insect species could be interfering with the RNAi efficiency delivered by ingestion. In fact, the dsRNA could be degraded by nucleases in some tissues or extracellular matrices such as saliva and haemolymph (Christiaens et al., 2020). Another dsRNA oral delivery limiting issue is the difficulty to quantify the precise amount of dsRNA ingested by the insect. In fact, the RNAi efficiency is clearly dependent on the dsRNA dose (Liu et al., 2020). Other dsRNA delivery methods have recently been explored including electroporation, soaking application, dsRNA inclusion in nanoparticles and topical application (Yu et al., 2013; Joga et al., 2016). The topical delivery consists in a drop containing the dsRNA directly dispensed on the body of the insect. In this case, the dsRNA containing solution is absorbed and the dsRNA molecules incorporated in the insect cells. For this reason, the drop is usually dispensed on those body areas devoid of chitinous barriers such as the intersegmental membranes (Romeis & Widmer, 2020). The dsRNA topical delivery has been recently tested in two Hemiptera species, *Diaphorina citri* and *Acyrthosiphon pisum*. In *D. citri*, 20 ng of dsRNA solution topically delivered on the abdomen were able to silence several *Cyp* genes by about 70-90% (Killiny et al., 2014). In *A. pisum*, 120 ng of dsRNA induced a downregulation of a target gene by 90% after 24-36 hours (Niu et al., 2019). Similarly, a 100 ng/µl dsRNA topically applied on sunn pest, *Eurygaster integricep*, penetrated the cuticle and affected nymphal stage development (Amiri et al., 2015). These studies indicate that the exogenous application of dsRNA might be used to silence target genes of insects in the field, as it has advantages such as the non-alteration of plant genomes and the short half-life of the molecules in the environment (Jain et al., 2020). For instance, 5 µg $leaf^{-1}$ of dsRNA applied to leaves were able to protect potato plants for at least 28 days under greenhouse conditions against the Colorado potato beetle (San Miguel & Scott, 2016). Nevertheless, topical delivery presents risks related to the non-specific target of action of RNAi. In a famous study it was observed that the dsRNA sequence for the *Leptinotarsa decemlineata* vATPaseA was able to reduce the fitness of *Diabrotica virgifera virgifera* as two genes shared more than 80% of identity (Baum et al., 2007). However, recent software has been developed to evaluate the possible non-target effects of a dsRNA sequence on species different from the one to control (Zhu et al., 2020; Romeis & Widmer, 2020). Furthermore, UV rays, light and microorganisms present on the insect skin can lead to the degradation of dsRNA in the environment before its incorporation (Christiaens et al., 2020).

Chemical control

For the National Institute of Environmental Health Science, pesticides are compounds that directly destroy the pests and are generally divided based on their target. The primary classes include pesticides targeting insects (insecticides), weeds (herbicides), fungi and moulds (fungicides) and rodents (rodenticides) (https://www.niehs.nih.gov). The pesticides era started with the identification of the insecticidal properties of the dichlorodiphenyltrichloroethane (DDT) in the 1930s by the scientist Paul Hermann Muller. The subsequent twenty years have seen an uncontrolled use of synthetic chemical compounds but also a growing awareness of the damage caused by these molecules to the environment and human health (Alavanja et al., 2004; Grandjean & Landrigan, 2006). Although DDT has helped eradicate malaria in Europe and North America, it has been banned in the USA since 1972 and in Europe since 1978 (Guimarães et al., 2007). On the other hand, the synthesis of new pesticides has continued globally and they continue to be used in large quantities today; worldwide pesticide usage approaches almost 8 billion pounds (3.6 billion kg) of active ingredient per year (Carvalho, 2017).

Insecticides are classified as pyrethroids, carbamates, organophosphates, organochlorines and neonicotinoids based on their chemical composition (Kumar & Omkar, 2018). Pyrethroids are the synthetic analogues of pyrethrin, a natural compound with insecticidal properties produced by *Chrysanthemum cinerariaefolium* (**Figure 2**). As insecticides, pyrethroids block the voltage-gated sodium channels in the axonal membranes, paralyzing the organism. Furthermore, these compounds show low levels of mammalian toxicity and a fast-environmental biodegradation (Matsuo, 2019; Mohammad et al., 2019).

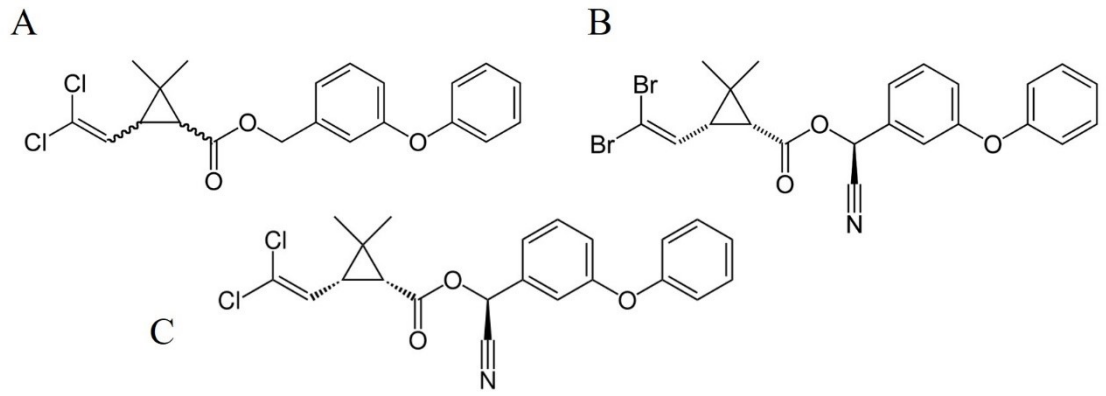

Figure 2. Example of pyrethroids commonly used. **A**: permethrin; **B**: deltamethrin; **C**: cypermethrin.

Carbamates, as shown in **Figure 3**, derive from the carbamic acid (Casida & Durkin; 2013) kill insects by the reversible inactivation of the acetylcholinesterase enzyme.

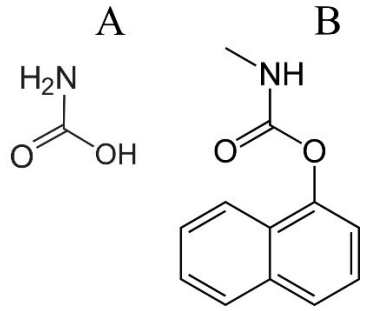

Figure 3. Panel **A** shows the carbamic acid, the structure from which the carbamates derive. Panel **B** shows the structure of carbaryl, one of the most widely used carbamate.

The organophosphates, esters of phosphoric acid, are the most used insecticide class (**Figure 4**). Like carbamates, organophosphates exert their toxic actions by binding and inactivating the acetylcholinesterase. This bond is, however, irreversible causing high toxicity and it is often toxic for humans as well (Suratman et al., 2015).

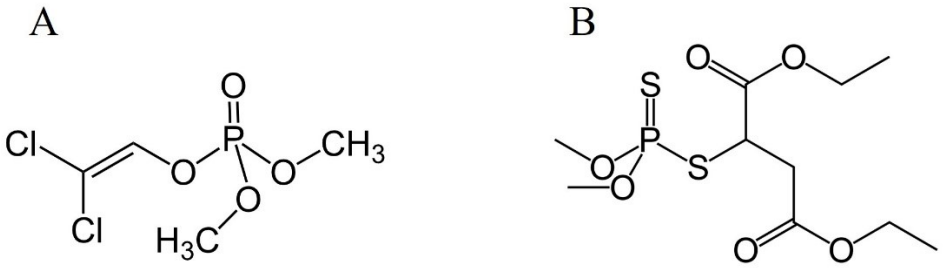

Figure 4. Two commonly used organophosphates. **A**: Dichlorvos **B**: Malathion.

Organochlorines were the first insecticides used in agriculture pest control. They work by disrupting the nervous system of the insect through axon hyperpolarization or by binding $GABA_A$ receptors. Unfortunately, these compounds showed a slow biodegradation in the field and higher stability in the environment. The main organochlorine used as insecticide is the DDT (**Figure 5)** (Coats, 1990).

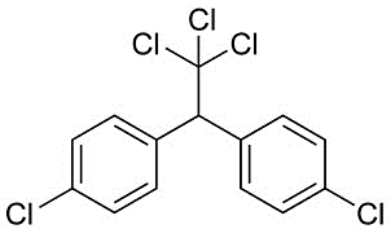

Figure 5. The DDT structure.

Neonicotinoids are insecticides that interact with insect nicotinic acetylcholine receptors (nAChRs), exhibiting high selective toxicity to insects over vertebrates. Neonicotinoids currently make up 30 % of insecticide worldwide sales and represent the new insecticide generation. However, due to their high toxicity on pollinators such as honeybees, neonicotinoids are being banned in the EU and in other countries. (Millar & Denholm, 2007; Decourtye & Devillers, 2010). Since its advent, the use of synthetic products has been the main strategy for pest control. On the other hand, the persistent environmental and human toxicity of the active ingredients, the uncontrolled use and the appearance of insect-resistances caused the chemical control to become ineffective and prohibitive (Norris et al., 2018). Thus, the development of new pest management strategies becomes more and more important in order to control diffusion and damage of insect pests. In this scenario, chemical control is moving towards the identification of natural elements with insecticidal activity as well as sustainable environmental and human impact.

Essential oils

As defined in the European Pharmacopoeia, essential oils (EOs) are the products obtained from hydro-distillation, steam distillation, dry distillation or mechanical cold pressing of plants. EOs are produced in few family plants, including Myrtaceae, Lauraceae, Lamiaceae and Asterceae (Regnault-Roger et al., 2012). They are characterized by two predominant components with different biosynthetic origins: phenylpropanoids and terpenes (Pichersky & Gang, 2000). In particular, the terpenes represent the most diversified chemical class among the compounds product by plants (Ayvaz et al., 2010). They are made from the combination of several 5-carbon-base (C5) units called isoprene and they are classified according to the number of isoprene units (**Table 1**).

Classification	Isoprene units	Carbon atoms
Emiterpenes	1	5
Monoterpenes	2	10
Sesquiterpenes	3	15
Diterpenes	4	20
Triterpenes	6	30
Politerpenes	>6	>30

Table 1. Classification of terpenes on the basis of the number of isoprene units and carbon atoms.

The terpenes present in EOs is variable among different plant species but monoterpenes are usually more abundant, accounting up to the 90 % of EOs (Croteau, 2000). Terpenes, and essential oils in general, are widely used in cosmetic, sanitary, pharmaceutical, agricultural and food industries for their bactericidal, viricidal and insecticidal features (Singh et al., 1980; Sangwan et al., 1990). In particular, the use of plant essential oils in pest control is an important tool already known and applied in agriculture. EOs have traditionally been used to protect stored agriculture products, such as grain and legumes, repelling flying insects (Sarac et al., 1995; Shaaya & Kostjukovsky, 1998). On the other hand, recent investigations confirm that some EOs also hold fumigant and contact insecticidal actions against plant pathogens (Isman, 2000). For example, in a detailed study, Kim et al. (2016) have tested 22 essential oils as fumigants and direct contact insecticides against *Drosophila suzukii*.

The use and marketing of EOs as biopesticides would have important advantages:

- With the progress of chemical, biological and genomic technologies, today it is possible to produce different EOs or specific terpenes on a large scale and in large

quantities. In this case, genetic engineering can help produce high value terpenoids in recombinant plants (Tholl, 2015).

- In general, EOs and their components show low toxicity to mammals with LD_{50} in the order of 1 g / kg in humans (O'Neil et al., 2006). Furthermore, they are biodegradable mixtures with a short half-life (up to a maximum of a few days) which tend not to bioaccumulate in the soil (Misra & Pavlostathis, 1997).

Although the EOs toxic effects against pest insects are known, the precise molecular and physiological mechanism of their action is still unclear (Blenau et al., 2011). Typically, they exert their toxic effects by reducing or disrupting insect growth at several life stages (Konstantopoulou et al., 1992). For instance thymol, a constituent of the essential oil from the plant *Thymus vulgaris L.*, is a monoterpene known for its pesticide action against numerous arthropods and is widely used to control *Varroa destructor* (Imdorf et al., 1999; Floris et al., 2004). It is thought that several terpenes can interact with P450 cytochromes, an enzymatic class involved in the insecticide detoxification processes (Jensen et al., 2006; Liao et al., 2016). Some monoterpenes, for example thymol, can cause neuronal degeneration through a direct binding with GABA receptors (Priestley et al., 2003). Linalool, thymol, carvacrol and 1,8-cineole inhibit acetylcholinesterase (Houghton et al., 2006; Park et al., 2016) while eugenol and geraniol inhibit neuronal activity (Price & Berry, 2006). Monoterpenes are also volatile molecules and it has been described that odorant binding proteins (OBP) present in the insect's sensilla are able to respond and interact with them. For example, females of *Bombyx mori* have sensilla that respond to linalool (Anderson et al., 2009). In addition, monoterpenes are known to be effective repellents against some insects and the interaction with their olfactory systems is not surprising (Isman, 2006; Nerio et al., 2010). Recently it was also described that some monoterpenes can interact with the octopamine / tyramine system, analogous to the adrenergic system present in the vertebrates (octopamine: Enan, 2001; Kostyukovsky et al., 2002; Enan, 2005a; Price & Berry, 2006; tyramine: Enan, 2005b; Gross et al., 2017; Ma et al., 2019; Ocampo et al., 2020).

Tyramine and octopamine

In insects the main biogenic amines are dopamine (DA), serotonin (5-HT), tyramine (TA) and octopamine (OA). Together, they control and modulate a broad range of biological functions essential for the life of the insects. While DA and 5-HT functions and pathways are highly conserved in both vertebrates and invertebrates, TA and OA (**Figure 6**) on the other hand can be considered the invertebrate counterparts of the catecholamines epinephrine and norepinephrine of vertebrates (Neckameyer & Leal, 2017).

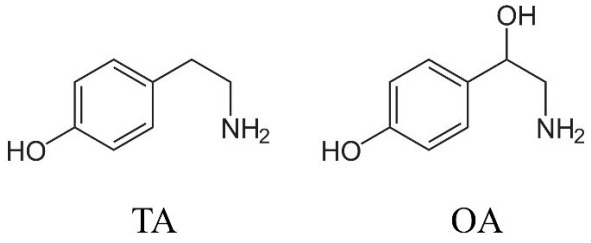

Figure 6. Chemical structure of the biogenic amines TA and OA.

TA, OA and the catecholamines epinephrine and norepinephrine have many features in common: they are synthetized from the same precursor amino acid (tyrosine), share both structural and functional characteristics such as interaction with G protein-coupled receptors (GPCRs) and regulate similar behavioral and physiological traits (Bauknecht & Jékely, 2017). To generate OA and TA, tyrosine is decarboxylated by the tyrosine decarboxylase (*Tdc*) which give rise to TA. This is then hydroxylated to OA by the tyramine β-hydroxylase (*Tβh*) as shown in **Figure 7** (Roeder et al., 2003).

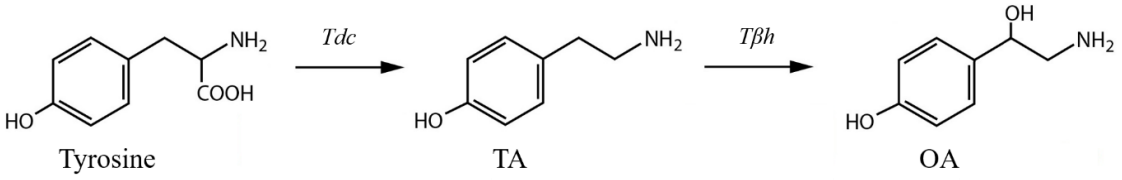

Figure 7. Biosynthetic pathway of TA and OA from the amino acid tyrosine.

The nervous tissue of insects contain high levels of both OA and TA, supporting the view that they act as neurotransmitters (Zhang & Blumenthal, 2017), but also as neuromodulators and neurohormones in a wide variety of physiological processes, acting in a paracrine, autocrine, and endocrine fashion also in peripheral organs (Roeder, 2005; Pauls et al., 2018). Originally TA was considered only as the intermediate product necessary for the production of OA (Downer et al., 1993). Nowadays, through studies on a variety of insects, it is known that TA and OA perform important functions

independently of each other (Lange, 2009). In many cases, TA and OA operate as antagonist modulators in a coordinated way (Roeder et al, 2003). For example, in *C. elegans* it has been observed that TA signalling reduces locomotion to allow feeding whereas OA signalling increases locomotion to allow searching of new food sources (Churgin et al., 2017). Given that TA and OA are produced via an enzymatic cascade and therefore TA is present in cells containing OA, the metabolic regulation by TA and OA is complex and still uncharacterized. Most studies examining this regulation were performed on *Drosophila melanogaster* strains genetically modified, i.e. Tβh mutant flies (lacking tyramine β-hydroxylase and therefore OA) and Tdc mutant flies (lacking Tyrosine decarboxylase therefore both TA and OA) (Monastirioli et al., 1996; Cole et al., 2005). The Tβh *D. melanogaster* mutant larvae showed reduced locomotor activity, rescued by OA administration through larvae feeding. Interestingly, this motor impairment was observed only in Tβh mutant flies whereas Tdc showed a normal activity (Hardie et al., 2007). The same OA-mediated role on locomotor activity was observed in *Carausius morosus* (Westmark et al., 2009) and *Apis mellifera* (Fussnecker et al., 2006; Zhao et al., 2014). Although the Tdc *D. melanogaster* mutants do not have major motor dysfunctions, they exhibit a drastic decrease in flight initiation and maintainence (Brembs et al., 2007). Furthermore, Tβh mutants have shorter life span in comparison to wild type flies but higher starvation resistance and higher fat accumulation (Hardie et al., 2007; Li et al., 2016; Roeder, 2020). Tdc mutant flies showed no difference in phenotype compared with wild type animals, thus suggesting a central role for OA in controlling metabolic traits. In terms of olfactory perception, TA modulates responses to repellents whereas OA modulates attractants (Zhukovskaya & Polyanovsky, 2017). In fact, OA enhances behavioral responses to attractants, making neutral odours attractive and repellent odours neutral (Zhukovskaya, 2012). For example, OA modulates the sensitivity of the pheromone-receptor neurons in the moth *Antheraea polyphemus* (Pophof, 2000) and the decision to visit flowers in *Mamestra sexta* (Riffell et al., 2013). In addition to olfactory perception, OA is also essential for olfactory memory, as observed in *D. melanogaster* (Schwaerzel al., 2003). In this study, Tβh mutant flies showed impaired sugar memory in comparison to wild type insects. The role of OA in olfactory memory has been also observed in honeybees (Hammer & Menzel, 1998; Farooqui et al., 2003). In contrast, TA does not appear to play a significant role in learning acquisition.

OA and TA exert most of their physiological actions by interacting with and activating specific receptors, which are G protein-coupled receptors (Ohta & Ozoe, 2014).

G protein-coupled receptors

G protein-coupled receptors (GPCRs) are the largest and most diverse group of membrane receptors in eukaryotes. GPCRs recognize a wide number of different extracellular stimuli, including photons, ions, small molecules, peptides and proteins and transmit the resulting extracellular signals to elicit intracellular responses.

Structurally, GPCRs are transmembrane receptors, consisting of a single polypeptide chain that forms seven different transmembrane α-helices, with N-terminal extracellular and C-terminal intracellular domains (**Figure 8**) (Katritch et al., 2013).

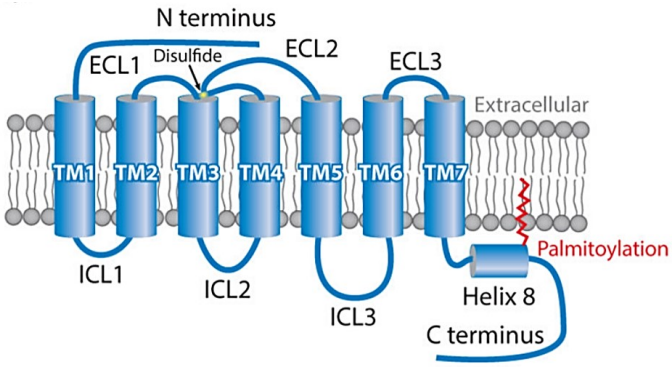

Figure 8. Overview of a typical GPCR structure organization. The extracellular loop, intracellular loop and the transmembrane domains are indicated with ECL, ICL and TM, respectively.

Activating ligands stabilize a GPCR conformation that results in the activation of associated heterotrimeric G protein. This activation promote the exchange of GTP for GDP by the G_α subunit of the G protein, leading to dissociation of the heterotrimeric protein complex into Gα and Gβγ subunits. This dissociation then promotes the production of and consequent signalling by second messenger systems, such as those involving cyclic AMP, diacylglycerol and calcium (**Figure 9**) (Weis & Kobilka, 2018).

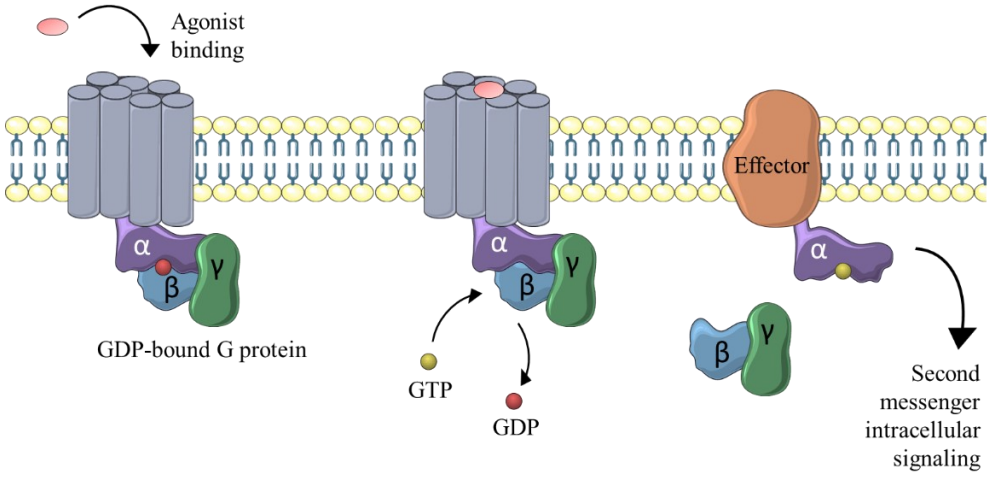

Figure 9. The classical G protein pathway. Exchange of GDP for GTP in the G protein α subunit leads to dissociation and interaction with downstream effectors.

G proteins are classified into four families according to their α subunit: Gαi, Gαs, Gαo, and Gαq. The Gαs and Gαi families regulate adenylyl cyclase activity, while Gαq activates phospholipase C and Gαo can activate small GTPase families (Neves et al., 2002).

Tyramine and octopamine receptors

The study of TA and/or OA deficient animals has revealed that the corresponding receptors play important roles in modulating the biology, physiology and behavior of invertebrates (Roeder et al., 2003). Altering the normal function of these receptor classes, blocking or overstimulating them, can lead to the death of an insect, or interfere with the physical fitness and reproductive capacity (Audsley & Down, 2015). These receptors are classified into five main groups based on their structure and pharmacological properties: α-adrenergic-like receptors (OctαR also known as OAMB or OA1), β-adrenergic-like receptors (OctβR, also known as OA2), type 1 tyramine receptors (TA/OA, Tyr1-R or TAR1) and tyramine type 2 and 3 receptors (Tyr2-R or TAR2 and Tyr3-R or TAR3) (**Figure 10**) (Wu et al., 2014). The gene coding for TAR3 was identified only in *Drosophila* (Bayliss et al., 2013).

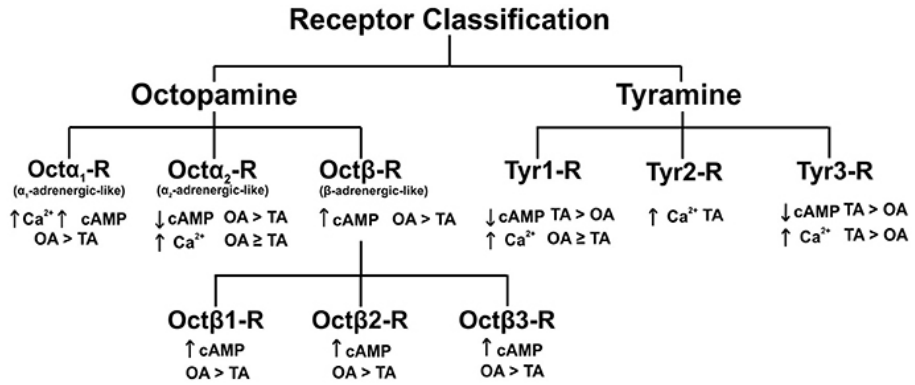

Figure 10. The most recent TA/OA receptors classification based on their sensitivity to ligands and their downstream effects (Wu e al., 2014).

Both Octα$_1$-R and Octα$_2$-R have structural similarities to mammalian α-adrenergic receptors. Octα$_1$-R, characterized for the first time in *D. melanogaster* (Han et al., 1998) is expressed mostly in the insect brains (Balfanz et al. 2005) and it has been identified in other insects, such as in *Apis mellifera* (Grohmann et al., 2003), *Periplaneta americana* (Bischof & Enan, 2004) and *Bombix mori* (Ohtani et al., 2006). Studies have demonstrated that activating this OA receptor class leads to increases in both intracellular Ca^{2+} and cAMP levels. On the other hand, the Octα$_2$-Rs have been shown to decrease forskolin-stimulated intracellular cAMP levels, as observed in *D. melanogaster* (Qi et al., 2017). All α-adrenergic-like OA receptors play key roles in appetitive olfactory learning (Kim et al., 2013) reproduction (Lee et al., 2003; Avila et al., 2012) circadian clock and sleep modulation (Crocker et al., 2010). The other OA receptors (Octβ-R) share

similarities with mammalian β-adrenergic receptors. Octβ-R do not alter intracellular Ca^{2+} levels but their mode of action is via an increase in cAMP levels (Balfanz et al., 2005; Maqueira et al., 2005). Furthermore, it seems that these receptors are able to directly control different functions including ovulation (Li et al., 2015), locomotor activity (Sujkowski et al., 2017) and feeding (Zhang et al., 2013). The TAR1 receptor group, showing a limited selectivity for TA and the ability to couple couple with G_i and G_q proteins, will be describes in the next chapter. On the other hand, TAR2 is thought to be a receptor highly specific for TA, and its activation elicits a selective stimulation of Ca^{2+} release. TAR2 seems to be involved in the regulation of renal function due to its high expression in Malpighian tubules (Cabrero et al., 2013). TAR3 is activated by TA and to a lesser extent by OA and decreases intracellular cAMP but also increases Ca^{2+} levels (Bayliss et al., 2013; Wu et al., 2014). The five OARs and three TARs have been shown to be differentially expressed in *Drosophila* (El-Kholy et al., 2015) therewith confirming their multiple, and often unique, roles in controlling physiology and behavior in insects. Since OARs and TARs play pivotal roles in insect physiology, they are also possible targets for insecticides used in pest control. For example Amitraz, an acaricide for several tick species (Davey et al., 1984), targets both OA and TA receptors (Jonsson et al., 2018).

TAR1: Molecular cloning

TAR1s, like all GPCRs, consists of a single polypeptide chain containing seven hydrophobic transmembrane domains, connected by six hydrophilic loops, along with an extracellular N-terminal and intracellular C-terminal regions (Ohta & Ozoe, 2014). To date, TAR1s have been characterized in fifteen insects (**Table 2**). The first TAR1 was described in *D. melanogaster* and called Tyr-dro (Saudou, 1990). The amino acid sequence, composed by 601 residues, is significantly longer in comparison to TAR1 from other insects (**Table 2**). In fact, in *D. melanogaster* TAR1, a putative 8th transmembrane domain was found close to the N-terminal region (Saudou et al., 1990). The same domain was reported also in *Phormia regina* TAR1 (Ishida & Ozaki, 2011) but it seems to be exclusive to the order Diptera. As suggested by Baxter & Barker (1999), this 8th domain might be a cleavable signal sequence or leader peptide, a sequence that plays a key role during the first steps of the GPCRs intracellular transport (Rutz et al., 2015). However, the exact function of this domain, present only in the TAR1 of Diptera, still remains to be clarified.

Several sequence motifs essential for correct receptor folding, ligand binding and signal transduction are well conserved within the TAR1 family. Between the 5th and 6th transmembrane domain there is a long intracellular loop 3 (IL3), composed of around 150 amino acid residues (Ohta & Ozoe, 2014). Interestingly, in Diptera the TAR1 IL3 is longer than in other insects. In particular, IL3 is 237 and 246 residues long in *D. melanogaster* and *P. regina*, respectively (Saudou et al., 1990; Ishida & Ozaki, 2011). In the β-adrenergic receptors, the IL3 is involved in intracellular signalling activation (Moukhametzianov et al., 2011). Given the evolutionary proximity between TAR1 and adrenergic receptors, it cannot be excluded that the IL3 might play the same role. Braza and colleagues have observed that, in the *Sitophilus oryzae* TAR1, the IL3 region is a very flexible element and is stabilized by TA binding, a key event in signalling for the G-protein (Braza et al., 2019). *A. mellifera* TAR1 has a relatively short IL3, composed of 110 amino acids. Blenau and colleagues linked this unusual aspect to *A. mellifera* TAR1 to the fact that this receptor couples with G_i but not G_q (Blenau et al., 2000). The GPCRs are subjected to a variety of post-translational modifications, among which are glycosylation, phosphorylation and palmitoylation are the prominent ones. In all the TAR1s characterized, two putative N-glycosylation sites on the N-terminal region have been identified (**Table 2**). The number of phosphorylation sites ranges from four (*H. virescens* TAR1) to nineteen (*Rhodnius prolixus* TAR1) (Von Nickisch-Rosenegk et al.,

1996; Hana & Lange, 2017). These sites are generally phosphorylated by protein kinase C (PKC) and protein kinase A (PKA), modulating the sensitivity of the receptor to coupling with G-proteins (Yang et al., 2017). Palmitoylation is the addition of a palmitic acid (at 16C saturated fatty acid) and occurs on one or more cysteines on the intracellular side of GPCRs, typically in the C-terminal region (Chini & Parenti, 2009). In TAR1s, putative palmitoylation sites have not been identified. This could be explained by the extremely short C-terminal region (15-20 aa) which do not contain cysteine residues. This aspect is in common with α2-adrenergic receptors (Alexander et al., 2014). In fact, palmitoylation is an event that generally influences the choice of coupling by G-protein as well as receptor phosphorylation and internalization (Chini & Parenti, 2009). A conserved domain, coding for the amino acids DRY, immediately downstream of 3rd transmembrane domain, was identified in all TAR1s examined. This motif appears important for the stabilization of GPCRs between inactive and activate conformation and its typical of catecholaminergic receptors. The DRY domain and a glutamate residue of the 6th transmembrane domain creating an ionic lock that stabilizes the inactive conformation of the receptor (Vogel et al., 2008). Through site-directed mutagenesis, Ohta and colleagues were the first to identify the amino acid residues involved in TA-binding of *B. mori* TAR1 (Ohta et al., 2004). In particular, in the mutant D134A the TA-mediated cAMP reduction observed in wild type *B. mori* TAR1 was completely abolished. Furthermore, the double mutant S218A and S222A, was also not able to attenuate cAMP levels after stimulation with TA. The authors suggested a binding scheme by which the carboxylic group of D^{134} residue forms an ion-pair between the protonated amine of TA and the S^{218} and S^{222} are involved in H-bond between the hydroxyl groups (**Figure 11**).

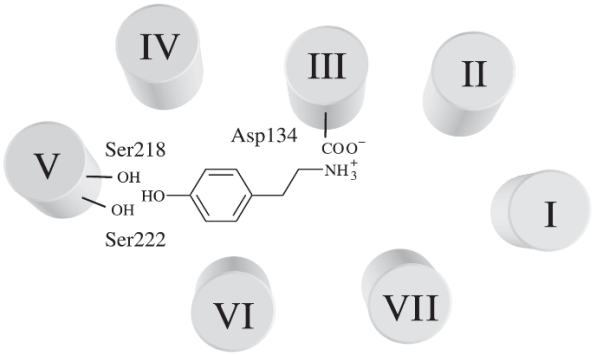

Figure 11. Extracellular view of the putative TA-binding site in *B. mori* TAR1. The even transmembrane domains are numbered as I - VII (Ohta et al., 2004).

Through molecular docking approaches, Braza and colleagues have confirmed that *S. oryzae* TAR1 binds TA forming H-bonds with D^{114} (in the 3rd transmembrane domain) and with N^{427} (in the 7th transmembrane domain) (Braza et al., 2019). Furthermore, other amino acid residues, such as V^{83}, C^{118}, W^{394} and S^{428}, are involved in TA binding. Together, this evidence suggests a more complex binding pocket for TA.

Species	Accession number	Aminoacid sequence lenght	Residues interacting with TA	Potential N-linked glycosilation	P-glycosilation sites	Intracellular loop 3 (IL3) lenght	Reference
D. melanogaster	AAA28731	601 aa	/	N^{11}; N^{57}	T^{136}; T^{296}; S^{375}; S^{397}; S^{406}; S^{482}; S^{507}	237 aa	Saudou et al., 1990
L. migratoria	X69520	484 aa	D^{130}	N^{13}; N^{198}	T^{78}; T^{164}; T^{238}; T^{300}; S^{304}; S^{365}; S^{372}	174 aa	Vanden Broeck et al., 1995
A. mellifera	AJ245824	399 aa	D^{116}; S^{200}; S^{201}; S^{204}	N^{2}	T^{63}; T^{149}; T^{223}; S^{241}; T^{265}; S^{291}; S^{292}; T^{296}	110 aa	Blenau et al., 2000
B. mori	X95607	479 aa	D^{134}; S^{218}; S^{222}	N^{11}; N^{16}	T^{81}; T^{241}; T^{258}; T^{302}; T^{306}	162 aa	Von Nickisch-Rosenegk et al., 1995 Ohta et al., 2004
P. americana	AM990461	441 aa	D^{115}; S^{199}; W^{381}; N^{418}	N^{12},N^{17}	T^{61}; T^{222}; S^{275}; S^{285}; S^{326}; T^{334}; S^{341}	144 aa	Rotte et al., 2009
C. suppressalis	AFG26689.1	478 aa	D^{135}; S^{219}; S^{223}	N^{11}; N^{16}; N^{347}	T^{205}; T^{267}; S^{274}; T^{304}; S^{315}; T^{371}; S^{396};	170 aa	Wu et al., 2013
R. prolixus	MF377527	447 aa	/	N^{14}; N^{17}	T^{75}; T^{235}; S^{246}; S^{259}; S^{265}; S^{271}; S^{274}; S^{295}; S^{298}; S^{311}; T^{313}; S^{319}; S^{320}; S^{322}; S^{338}; S^{339}; T^{354}; S^{371}; S^{373}	161 aa	Hana & Lange, 2017
P. xylostella	MK166023	467 aa	D^{127}; S^{211}; S^{215}	N^{5}; N^{10}	S^{252}; S^{268}; S^{271}; T^{296}; S^{307}; S^{322}; S^{349}; S^{352}; S^{385}	168 aa	Ma et al., 2019
M. brassicae	AF343878	477 aa	D^{136}	/	/	174 aa	Brigaud et al., 2009
P. regina	AB621975	607 aa	/	/	/	246 aa	Ishida & Ozaki, 2011
A. ipsilon	FJ640850	477 aa	D^{149}; S^{216}; S^{217}; S^{220}	N^{11}; N^{16}; N^{345}	T^{79}; T^{165}; T^{239}; T^{265}; S^{314}; S^{333}; S^{383}	177 aa	Duportets et al., 2010
S. oryzae	A0A0S1VX60	455	V^{83}; D^{114}; C^{118}; W^{394}; N^{427}; S^{428}	/	/	158 aa	Braza et al., 2019
H. virescens	CAA64864	477	D^{132}	N^{11}; N^{16}	T^{78}; T^{238}; T^{298}; T^{302}	165 aa	Von Nickisch-Rosenegk et al., 1995

Table 2. Molecular features of OA and TA receptors cloned from insects.

TAR1: pharmacology

The characterization of a receptor downstream signalling and cascade requires a precise study of its pharmacological profile. The TAR1 pharmacology is quite intriguing since it was initially characterized as an OA receptor capable of interacting with TA too. Arakawa was the first to pharmacologically characterize a TAR1, by cloning and expressing the *D. melanogaster* TAR1 in Chinese Hamster Ovary (CHO)-K1 cells (Arakawa et al., 1990). In this study, several biogenic amines were tested as putative agonists including 5-HT, adrenaline, and OA, but not TA, concluding that the receptor was an OA receptor given its high affinity to OA. However, in two separate studies, the same receptor was further investigated (Saudou et al., 1990; Robb et al., 1994) and when, expressed in mammalian cells (Cos-7), it showed a TA-mediated inhibition of adenylate cyclase activity, proving its G_i coupling activity. In particular, TA was able to reduce forskolin-stimulated cAMP levels in a dose-dependent manner with a pEC_{50} of 5.62 (**Table 3**). Conversely, OA was less potent with a pEC_{50} of 4.52 (Saudou et al., 1990). Furthermore, Robb and colleagues investigated the TA and OA responses *D. melanogaster* TAR1 upon cloning into CHO cells. In particular, this work clearly demonstrated that *D. melanogaster* TAR1 is more sensitive to TA than OA and that it activates its signalling cascade not only through G_i-coupling but also via G_q proteins (Robb et al., 1994). TAR1 signalling is, therefore, far more complex than initially thought. Over the years, several TAR1s have been cloned and pharmacologically characterized from other insects, providing a well-grounded description of the receptor pharmacology. TA appears to be significantly more potent than OA in activating the receptor, in terms of both G_i and G_q-mediated intracellular cascades. In particular, in *A. mellifera* and *D. melanogaster* TA appeared to be one order of magnitude more potent than OA while in *R. prolixus*, *L. migratoria* and *Plutella xylostella* TA was twice as effective as OA (**Table 3**). These variations in potency might be truly species specific or they might be traced back to the different cell lines used: most of the studies stably expressed TAR1 in the Human Epithelial Kidney (HEK) 293 cell line, while others used insect cell lines (S2 and Sf9), CHO or Murine Erythroleukaemia cells. Furthermore, another reason for variation in the pharmacological profile might be linked to the different experimental approaches used. In fact, for each TAR1 studied, either the G_i or G_q-mediated intracellular pathway was investigated, with a preference towards G_i. The reason for investigating the G_i-mediated transduction pathway is perhaps due to the fact that the α2-adrenergic receptors are coupled exclusively to G_i (Alexander et al., 2014). Studies investigating the G_q-mediated intracellular pathway were performed on

TAR1 from *L. migratoria* and *R. prolixus* (Poels et al., 2001; Hana & Lange, 2017). In the study of Blenau and colleagues, *A. mellifera* TAR1, expressed in HEK 293 cells, was tested for its ability to activate intracellular signalling via both G_i and G_q proteins and it was found able promote its downstream cascade exclusively via G_i activation. As discussed above, this receptor peculiarity may be due to the shorter IL3, but further investigation might be necessary (Blenau et al., 2000).

In terms of TAR1 antagonist pharmacological profile, yohimbine showed the highest affinity for this receptor class (**Table 3**). Yohimbine is an α2-adrenergic receptor antagonist and concentrations up to 1 μM were able to antagonise TA in TAR1s (**Table 3**). When the *R. prolixus* TAR1 was expressed in HEK 293, the rank order of antagonist potency was yohimbine > metoclopramide > phenoxybenzamine > phentolamine > cyproheptamide > gramine > mianserin > chlorpromazine. Similar results i.e. yohimbine > mianserin > phentolamine > chlorpromazine were obtained investigating the *P. xylostella* TAR1 expressed in HEK 293T cells. When biogenic amines different from TA and OA, such as dopamine, adrenaline, noradrenaline, L-DOPA and histamine, were tested on TAR1s no significant agonist effects were observed, suggesting that this receptor class is selectively responsive to TA and OA (Ohta et al., 2004; Rotte et al., 2009; Wu et al., 2014; Hana & Lange, 2017; Ma et al., 2019). The pharmacological profile of TAR1 has been characterized especially for the antagonist, whereas our knowledge of alternative agonists is almost completely lacking. It remains in fact to be elucidated which is the preferred receptor coupling between G_i and G_q proteins or to whether TA preferably activates the G_i or G_q-mediated transduction pathway and how much OA, even though less potent, contributes to this. Nevertheless, it is obvious that TAR1s are GPCRs able to couple with both G_i and G_q proteins as summarized in **Figure 12**.

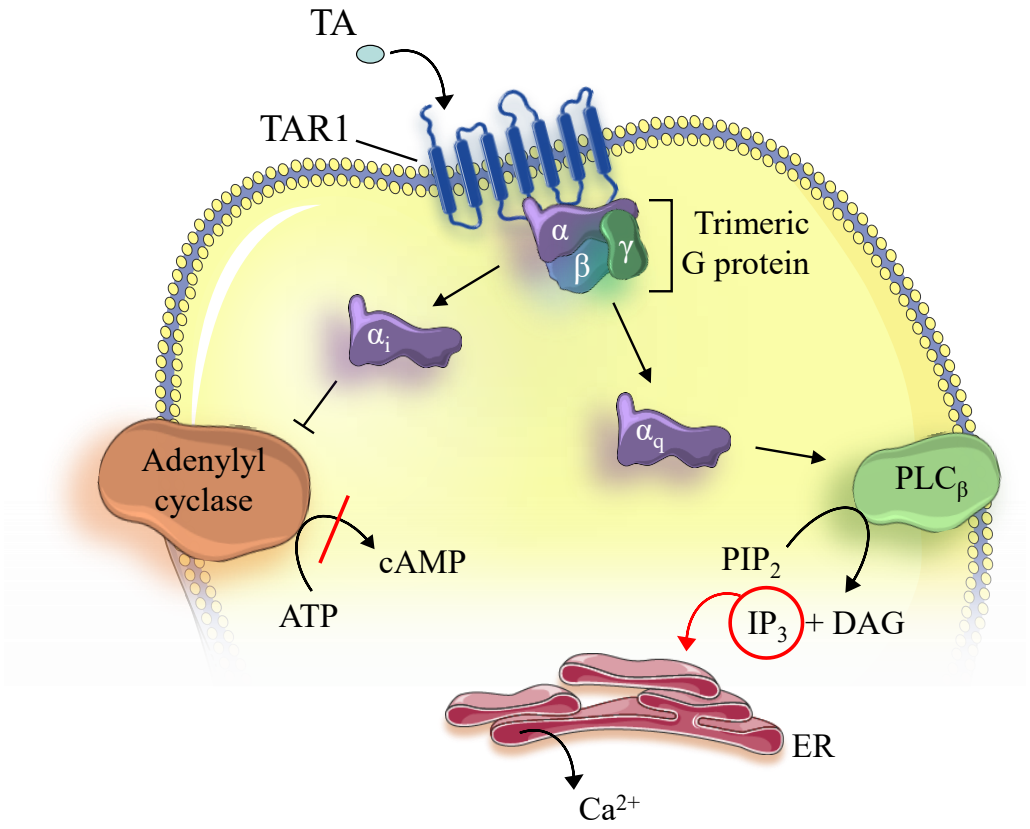

Figure 12. Intracellular signalling pathways trigged by TAR1 activation. ATP (Adenosine triphosphate), cAMP (Cyclic adenosine monophosphate), PLC_β (phospholipase Cβ), PIP_2 (Phosphatidylinositol 4,5-bisphosphate), IP3 (Inositol trisphosphate), DAG (Diacylglycerol).

Species	G-protein	pEC_{50} TA	pEC_{50} OA	Cell line used	Antagonist	Notes	Reference
D. melanogaster	G_i	5.62	4.52	Cos-7	Yohimbine (tested at 1 µM)		Saudou et al., 1990
	G_i	5.24	/	S2	/		Enan, 2005
L. migratoria	G_q	7.33	Detectable starting from 10 µM	Murine Erythroleukaemia	Yohimbine (tested at 2.5 µM)		Poels, 2001
	G_i	8.40	/		/		
A. mellifera	G_i	6.86	5.56	HEK 293	/		Blenau et al., 2000
	G_i	7.07	/	Sf9	/		Mustard et al., 2005
B. mori	G_i	8.28	5.85	HEK 293	Yohimbine > Chlorpromazine > Metoclopramide > Mianserin (tested at 10 µM in cAMP assay)	Radio-ligand binding used for agonist assay. DA, NE: tested as agonist at 100 µM	Ohta et al., 2003
P. americana	G_i	6.46	/	HEK 293	Yohimbine and chlorpromazine > mianserin (tested at 10 µM)	DA, 5-HT: tested as agonist at 10 µM	Rotte et al., 2009
C. suppressalis	G_i	6.43	6.01	HEK 293	Yohimbine > chlorpromazine > Cyproheptadine (tested at 10 µM)	DA, 5-HT: tested as agonist at 10 µM	Wu et al., 2013
R. prolixus	G_q	7.29	5.16	HEK 293- CNG	Yohimbine > Metoclopramide > Phenoxybenzamine > Phentolamine > Cyproheptamide > Gramine > Mianserin > Chlorpromazine (tested at 10 µM)	DA, 5-HT: tested as agonist at 10 µM	Hana & Lange, 2017
P. xylostella	G_i	6.35	4.86	HEK 293T	Yohimbine > Mianserin > Phentolamine > Chlorpromazine (tested at 10 µM)	DA, 5-HT: tested as agonist at 10 µM	Ma et al., 2019

Table 3. Functional and pharmacological properties of OA and TA receptors cloned from insects.

TAR1s: physiology

TAR1 transcript localization analysis provides information on the expression profile of the receptor and helps to better understand its physiological functions. In *D. melanogaster*, the receptor is mainly expressed in the central nervous system (CNS) (Saudou et al., 1990; El-Kholy et al., 2015). Trough Gal4/UAS technology, *D. melanogaster TAR1* transcripts were found abundant in the *pars intercerebralis*, in the mushroom bodies and in the antennal and olfactory lobes (El-Kholy et al., 2015). A higher expression of *TAR1* in nervous tissues, compared to periphery, was also observed in *R. prolixus*, *Chilo suppressalis*, *P. xylostella*, *Mamestra brassicae* and *Agrotis ipsilon*, suggesting a crucial role of the receptor in controlling a broad range of physiological functions (Brigaud et al., 2009; Duportets et al., 2010; Wu et al., 2014, Hana & Lange, 2017; Ma et al., 2019). Interestingly, *TAR1* was also strongly expressed in the antennae of *M. brassicae* and *A. ipsilon*, where it could regulate olfactory-mediated behaviors (Brigaud et al., 2009; Duportets et al., 2010). A possible correlation between TAR1 and olfaction was established for the first time in 2000 by Kutsukake and colleagues (Kutsukake et al., 2000). This study characterized a *D. melanogaster TAR1*-defective line, called *honoka*, whose behavioral responses to repellents were reduced in comparison to wild type flies. Furthermore, using *in situ* hybridization, Brigaud and colleagues observed that *TAR1* was expressed at the base of the olfactory sensilla trichodea, pheromone-sensitive sensilla, rather than in sensilla chaetica, which are mechano-sensitive in *A. ipsilon*(Brigaud et al., 2009). The role of TAR1 in olfactory perception was further confirmed by imaging analysis performed on *A. mellifera*. In two studies, conducted in 2017 on the honeybee brain, the authors showed that *TAR1* is mainly expressed at the presynaptic sites of olfactory receptor neurons (ORNs) innervating the antennal lobes and the mushroom bodies, which are essential structures for the olfactory system (Sinakevitch et al., 2017; Thamm et al., 2017). A similar *TAR1* mRNA localization was observed by Mustard and colleagues via *in situ* hybridization in honeybees (Mustard et al., 2005). *TAR1* showed a higher expression in the antennae of pollen foragers in comparison to nurse ones. In contrast, *OAR1* exhibited the opposite expression profile (McQuillan et al., 2012). Therefore, it can be proposed that in social insects TAR1 could represent a key element in defining the castes identity and modulating behavioral features such as olfaction. Behavioral alterations caused by TAR1 modulation have been observed in several studies performed with *L. migratoria* and *D. melanogaster.* In locusts, the ratio between *TAR1* and *OAR1* expression levels influenced olfactory preferences during the

solitary-gregarious phase transition. In fact, high levels of *TAR1* promoted solitary behavior by inducing the perception of gregarious pheromones as repellent while RNAi-mediated TAR1 downregulation in solitary locusts was able to mediate the transition to the gregarious-like behavior (Ma et al., 2015). In a subsequent study the same authors observed that TAR1 mediates the olfactory responses between the solitary-gregarious phases by modulating the *tspo* transport protein (Ma et al., 2020). It is evident that TAR1 is not only important in olfactory regulation but also in locomotor control. In *A. mellifera* movement impairment could be attributed to TAR1 (Fussnecker et al., 2006) since the topical application of yohimbine on the abdomen caused a massive movement alteration, owing to the selective antagonism of the receptor. However, yohimbine antagonises also TAR2 and further studies are necessary to evaluate which tyramine receptor is essential in this response. A TAR1-mediated role in locomotion has been hypothesized by a few studies reporting a high *TAR1* expression in leg muscles of *P. americana*, *D. melanogaster* and *A. ipsilon* (Rotte et al., 2009; Duportets et al., 2010; El-kholy et al., 2015). Furthermore, *Tβh*-deficient flies having no OA but high levels of TA showed a severe locomotion deficit, partially rescued by diet-fed TAR1 antagonist yohimbine (Saraswati et al., 2004). However, these observations do not rule out the possibility that TAR1-mediated movement alterations could be controlled by other nervous areas rich in TAR1 transcripts such as the central complex (Thamm et al., 2017). TAR1 has also been shown to influence the gustatory responses. The *D. melanogaster* $TAR1^{PL00408}$ defective line exhibited higher body fat accumulation, starvation resistance and food intake in comparison to wild type flies (Li et al., 2017) thus suggesting that nutritional constraints work through a functional TA-dependent pathway, even if the precise mechanism by which TAR1 modulates these essential metabolic traits is still unknown. Some indications came from *D. melanogaster*, where, like many other insect, lipids are mainly stored in the fat body. Their storage and release are mainly controlled by two hormones, the insulin-like peptides (mainly ILP_2) and the adipokinetic hormone (AKH, analogous to the mammalian glucagon) (roeder, 2020). Under acute stress, mobilization of lipids from the fat body is essential for survival. This mechanism appears to be also controlled by both OA and TA, presumably through modulation of ILP_2 secretion (Fields & Woodring, 1991; Orchard et al., 1993). Therefore, the increased triglycerides (TG) level observed in $TAR1^{PL00408}$, as compared to control flies, might be related to a direct tyraminergic action on the ILP_2 release. RNAi-mediated *D. melanogaster* TAR1 silencing, targeted to the fat body, indeed triggered a ILP_2 reduction in insulin-producing cells, located in the *pars*

intercerebralis, and an increased TG accumulation (Li et al., 2017) confirming a significant role for TAR1 in lipid metabolism.

It has recently been proposed that TAR1 could be involved in processes related to sugar sensibility and food intake regulation (Ishida & Ozaki, 2010). *honoka* flies, a *D. melanogaster TAR1*-defective line, exhibited a higher starvation resistance but, in contrast to $TAR1^{PL00408}$ flies, a reduced responsiveness to sugar stimuli compared with control flies (Damrau et al., 2018). It is worth noting that *TAR1* is highly expressed in neurons located in the sub-oesophageal ganglia that are presumably associated with the salivary glands and neck muscles control, thus linking TAR1 with feeding. In honeybees, the topical administration of TA induced an increased GRS (Gustatory Response Score) that was sensitive to yohimbine (Scheiner et al., 2017a). Furthermore, foraging honeybees showed a higher GRS as well as higher *TAR1* expression level in the fat body in comparison to nurses, suggesting a correlation between the receptor and sugar responsiveness (Scheiner et al., 2017b).

Last but not least, in *R. prolixus* TAR1 is expressed in the reproductive organs (Hana & Lange, 2017). In particular, *R. prolixus* ovaries display higher *TAR1* transcript levels in comparison to other reproductive tissues such as the lateral oviduct and common oviduct, suggesting its importance in modulating reproductive processes.

TAR1s: insecticide target

In addition to their role in the physiology and behavioral control of insects, TAR1s have proven to be interesting targets for insecticides. Amitraz is an acaricide and non-systemic insecticide that targets OA receptors (Jonsson & Hope, 2007). However, recent studies have demonstrated that amitraz can exert its toxic effect also through TAR1(Wu et al., 2014, Kumar, 2019). When the *C. suppressalis* TAR1 was expressed in HEK 293 cells, 10 μM of amitraz was able to inhibit forskolin-stimulated intracellular cAMP, mimicking TA effects (Wu et al., 2014). Amitraz was initially thought to work only on OA receptors, however TAR1s have been wrongly classified as OA receptors (Baxter & Barker, 1999; Chen et al., 2007). Through phylogenetic analyses, Baron and colleagues classified the receptor as Oct /Tyr, until Farooqui review where TAR1 was described as a TA receptor (Baron et al., 2015; Farooqui et al., 2012). On the other hand, ambiguities and annotation errors still persist in public databases. Further evidence supporting the hypothesis that amitraz could interact with TAR1 was provided by Gross and colleagues on *Rhipicephalus microplus* TAR1. When expressed in the CHO cell line, TAR1 was allosterically positively modulated by BTS-27271, an amitraz metabolite (Gross et al., 2015). Even if it remains to be elucidated whether the biological effects of the insecticide are really due to the activation of TAR1, it has been shown that two amino acid substitutions in the *R. microplus* TAR1 (T8P and L22S) could be responsible for a lower susceptibility, or even resistance, to the amitraz insecticide action (Chen et al., 2007), supporting the hypothesis that the amitraz-mediated toxicity was mediated by TAR1.

The tyraminergic and octopaminergic systems are interesting targets for natural insecticides, such as monoterpenes. These molecules are the main components of plant essential oils and have long been used as phagodeterrents and biopesticides in the pest control (Isman, 20069. In the last few years, several studies have showed that the monoterpenes could directly activate TAR1. Enan (Enan, 2005b) was the first to describe an agonist effect of several monoterpenes (thymol, carvacrol, α-terpineol, eugenol) on the *D. melanogaster* TAR1. However, the same monoterpenes exhibited a different pharmacological effect on *R. microplus* TAR1 receptors. In fact, they appeared able to increase the TA potency acting as positive allosteric modulators and not as agonists (Gross et al., 2017). Recent data have revealed that in silico prediction of the structural interaction between monoterpenes and *S. oryzae* TAR1 might provide new insights and possibly new molecules for TAR1-related pest control (Ocampo et al., 2020).

Drosophila suzukii

Drosophila suzukii (Matsumura) belongs to Drosophila genus, order Diptera. *D. suzukii* adults are 2-3 mm length and presenting a wingspan of 5-6.5 mm. Furthermore, they present short antennae, big and red eyes as well as a light brown chest and abdominal black stripes. Sexual dimorphism is highlighted by peculiar features:

- Males: presenting black spots on both wings (**Figure 13**), which appear 24-48 hours after the pupae-adult transition (Cini et al., 2012).
- Females: exhibiting a large and robust ovipositor with several dark and sclerified bristles in the distal area, usually absent in other Drosophila species. These structures are used to scratch the fruits epidermis and allow eggs to be deposited directly in the pulp of healthy fruits (Cini et al., 2012; Hamby et al., 2016) (**Figure 13**).

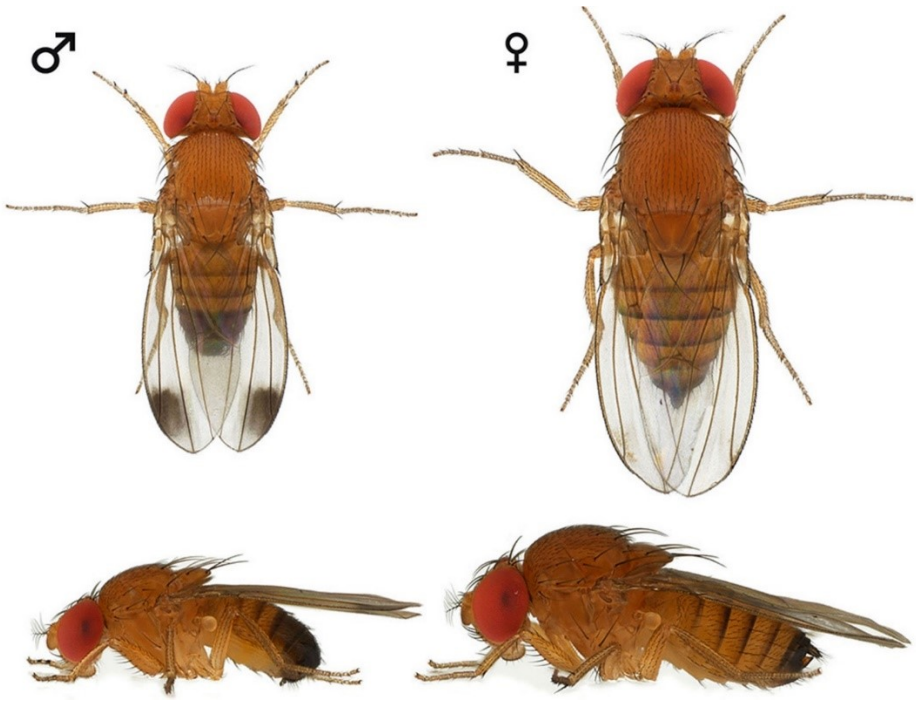

Figure 13. Adults of *D. suzukii* male and female.

D. suzukii shows a holometabolic development, characterized by complete metamorphosis: a larva emerges from the egg, a different organism from the adult that evolves through a series of larval stages. The latter are followed by a quiescent stage, called pupa that develops in adult stage by metamorphosis (**Figure 14**).

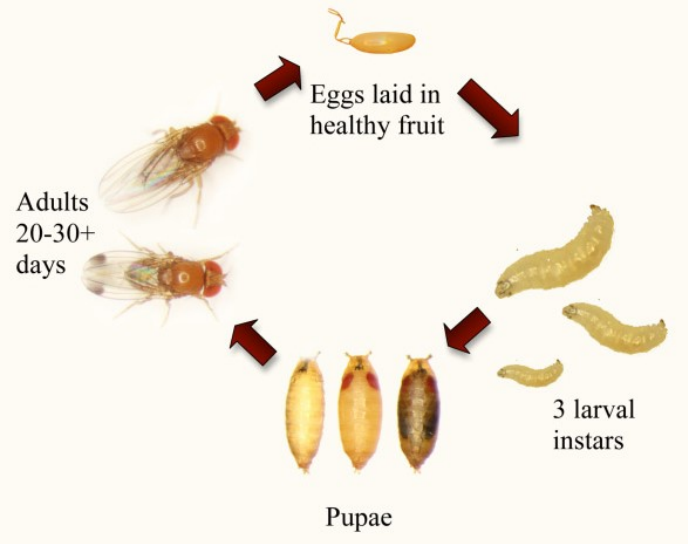

Figure 14. Representation of the *D. suzukii* developmental cycle.

D. suzukii shows a rapid life cycle that thus completing several generations per year. This ability depends on several factors, such as temperature, humidity, and the availability of essential nutrients (Wiman et al., 2016). The development time, in fact, is inversely proportional to the increase in temperature; however, too high temperature might induce stress and cause death (Hamby et al., 2016). The eggs hatch between 12 and 72 hours and the larvae, feeding the fruit pulp, emerge, reaching the third and last larval stage after 3-15 days. The larva subsequently undergoes pupation, a stage preceding the adult stage, which lasts between 4 and 15 days. Adults have a life span from 3 to 9 weeks and can give birth to numerous generations a year, up to 13 in ideal conditions (Tochen et al. 2014).

The presence of *D. suzukii* was documented for the first time in 1916 in Japan. Its spread reached the eastern lands: China, Taiwan, North, and South Korea (Cini et al., 2012). In 2008 *D. suzukii* was identified for the first time in North America, especially in California (Asplen et al., 2015), and then spread to the east and west coasts. Later, it also appeared in Canada and Mexico (Cini et al., 2012). The European presence of *D. suzukii* was attested in 2008 both in Spain (Calabria et al., 2012) and in Italy (Cini et al., 2012). The damage caused in all the affected countries mainly concerns small fruits, causing huge economic losses. In Japan, for example, the greatest losses, even up to 100%, involved cherries and blueberries (Shimizu, 2004). In Europe, however, the heaviest damage was recorded in France and Italy, with losses of 100 % of blackberries, strawberries and sweet cherries with some attacks also on apples and peaches, without significant damage (Cini et al. , 2012). The rapid and wide spread of *D. suzukii* is caused by its high voracity and

a good resistance and adaptability to environments. It is also able to carry out rather long migrations both actively (it can cover 1400 km per year) and passively, through the transport of infested commercial products (Calabria et al., 2012).

D. suzukii is particularly difficult to control due to its peculiar ability to lay eggs inside the fruit, its high reproductive potential, its ability to spread rapidly and its adaptability to different spatial-temporal conditions (Mazzetto et al., 2015). Currently, the most effective management strategy is based on chemical control. Unfortunately, the *D. suzukii* control by chemical compounds is rather complex given the need to intervene in proximity and during the harvest. Therefore, it is preferred the use of active substances with short deficiency times. The active substances authorized in *D. suzukii* control are: Acetamiprid on cherry and Spinosad on grapevine, while in provisional authorization Spinetoram (peach and raspberry in greenhouses) and Cyantaniliprole (cherry) (Cini et al., 2012). However, the use of chemical pesticides in the *D. suzukii* control is disadvantageous because it is associated with the development of resistance in target parasites, caused by frequent use of the product. Another aspect, certainly disadvantageous, is the non-specificity of the products used. Many of the insecticides adopted worldwide against *D. suzukii* have, in fact, a broad spectrum of action, so they are not in line with the "IPM" program (Haye et al., 2016). Furthermore, the difficulty to find insecticides against the larvae in fruit has shifted scientific attention to the search for active molecules targeting adults (Cini et al., 2012).

Halyomorpha halys

Halyomorpha halys (Pentatomidae; Order Rhynchota) is native of Asia (China, Japan and Korea) (Haye et al., 2015). In 1998 it made its first appearance on the American coasts, spreading among the various states, until it was considered a stable pest in orchards in 2010 (Rice et al., 2014). *H. halys* was detected for the first time in 2004 in Switzerland, and then spread to neighbouring states, arriving in Italy in 2012 (Cesari et al., 2018). *H. halys* is responsible for significant damage to many economically relevant crops such as apples, pears, peaches, and soybeans (Leskey & Nielsen, 2018). The damage is caused by the perforation of the external integuments of fruits or plants by the rostrum, a specialized sucking system typical of this order of insects. As a consequence, necrotic areas or spots on fruits and leaves develop, along with the transmission of other phytopathogens, leading to a conspicuous depreciation of the product (Rice et al., 2014). In Asia, *H. halys* presents a life cycle of only one new generation per year (Rice et al., 2014). On the other hand, the diffusion in regions with more subtropical climates has led to four annual generations, significantly increasing the number of individuals present on the territory (each female is able to deposit between 100 and 500 eggs) (Rice et al., 2014). The eggs laid by the females are mainly positioned on the underside of the leaves, are green coloured and 1 mm in size (Nielsen et al., 2016). *H. halys* undergoes to an incomplete metamorphosis (hemimetabolic) in which, starting from the egg, the insect goes through five distinct stages as nymphs before becoming an adult. After birth, the nymphs tend to stay close to the brood and then move away following the first moult. The *H. halys* juvenile stages have small spines at the level of the thorax (**Figure 15**) which are not found in other Pentatomids. In youth stages, they also have a higher level of mobility compared to the adults (Rice et al., 2014).

Figure 15. Young stage (2nd instar nymph) and adult of *H. halys*.

H. halys presents high resistance towards the most common chemical insecticides, thus making its control and elimination difficult (Bergmann and Raupp, 2014). Since the beginning of its diffusion in Europe, pheromone traps or light baits were used to monitor its spread (Morrison III et al., 2015). Therefore, biological control appeared as a possible approach given the severity of the pest. This is based on *H. halys* natural competitors, such as the samurai wasp (*Trissolcus japonicus*), an ooparasitoid (Rice et al., 2014). On the other hand, identifying innovative chemical control approaches is also important in order to contain the *H. halys* populations. Innovative molecules with an insecticidal action toward *H. halys*, such as essential oils, represents a significant added value. The natural origin, in fact, reduces the risk of possible toxicity for both the environment and men, reducing the possible limitations to its use.

Aim of study

This thesis describes a comprehensive molecular and physiological characterization of type 1 tyramine receptor (TAR1) in phytophagous insects to dissect the receptor role in controlling physiological and behavioral traits. Furthermore, the work examines TAR1 as possible target for biopesticides.

The thesis is organized in the format "three papers": the first two articles describe *D. suzukii* TAR1 (DsTAR1) molecular and pharmacological characterization. Furthermore, the cellular and molecular events underlying the interaction between monoterpenes and DsTAR1 will be studied. In particular, monoterpenes appeared able to modulate directly DsTAR1-controlled physiology and behavior. The first article was accepted for publication in *Pesticide Biochemistry and Physiology* while the second was published in *Journal of Experimental Biology*.

The third article, also published on the *Journal of Experimental Biology*, investigates the role of *H. halys* TAR1 (HhTAR1) in the alarm pheromone olfactory perception. In particular, a direct RNAi-mediated TAR1 downregulation and specific behavioral assay development will be presented.

Chapter II: Results

Modulation of *Drosophila suzukii* type 1 tyramine receptor (DsTAR1) by monoterpenes: a potential new target for next generation biopesticides.

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Abstract

This study proposes a biochemical and molecular model for the interaction between the *Drosophila suzukii* type 1 tyramine receptor (DsTAR1) and monoterpenes. A preliminary molecular and functional characterization of DsTAR1 cDNA revealed that a 1.8kb long ORF codes for a 600 amino acid polypeptide featuring seven transmembrane domains, as expected for a GPCR. A stable HEK 293 cell line expressing DsTAR1 was tested for responsiveness to tyramine (TA) and octopamine (OA). In intracellular calcium mobilization studies, TA led to a concentration-dependent increase in $[Ca^{2+}]_i$ (pEC_{50} ~ 6.40), completely abolished by pre-incubation with the antagonist yohimbine 1 μM. Besides, in dynamic mass redistribution (DMR) studies, TA evoked a positive DMR signal in a concentration-dependent manner (pEC_{50} ~ 6.80). The recombinant cell line was then used to test three monoterpenes (thymol, carvacrol and α-terpineol) as putative ligands for DsTAR1. The terpenoids showed no agonist effects in both DMR and calcium mobilization assays, but they increased the potency of the endogenous ligand, TA, acting as positive allosteric modulators. Moreover, expression analysis on adults *D. suzukii*, exposed for 24, 72 or 120 h to a sublethal concentration of the three monoterpenes, showed a downregulation of *DsTAR1*. This evidence has led to hypothesize that the downregulation of *DsTAR1* might be a compensatory mechanism in response to the positive allosteric modulation of the receptor induced by monoterpenes. Therefore, these findings might be useful for the development of a new generation of biopesticides against *Drosophila suzukii*, targeting TAR1.

Keywords: *Drosophila suzukii*; Tyramine receptor; Essential oils; Monoterpenes; Biopesticides; Dynamic mass redistribution

Introduction

The Spotted Wing Drosophila (*Drosophila suzukii*, Matsumara, 1931) is a phytophagous pest native of Asia, that prefers ripe fruits where the females lay the eggs through a robust ovipositor (Mitsui et al., 2006; Rota-Stabelli et al., 2013). The fruits damaged by larvae developing inside lose their commercial value (Lee et al., 2011). Furthermore, the oviposition wounds allow for secondary infections by other insects and pathogens, including fungi, yeast and bacteria (De Camargo and Phaff, 1957; Louise et al., 1996).

Since *D. suzukii* arrival in Europe and North America in 2008 (Walsh et al., 2011; Cini et al., 2012; Asplen et al., 2015), numerous chemical compounds have been tried as insecticides, including organophosphates (malathion, diazinon, dimethoate), pyrethroids (permethrin, pyrethrin, zeta-cypermethrin), neonicotinoids (acetamiprid, imidacloprid, thiamethoxan), diamides (cyantraniliprole, chlorantraniliprole) and spinosyns (spinosad, spinetoram) (Bruck et al., 2011; Cuthbertson et al., 2014; Profaizer et al., 2015; Shawer et al., 2018). Spinosad, a biopesticide produced by the metabolism of *Saccharopolyspora spinosa*, is the most effective chemical tool against *D. suzukii* (Van Timmeren and Isaacs, 2013). Unfortunately, cases of low susceptibility and/or resistance in some populations in North America have recently been described (Grees and Zalom, 2018).

In the last few years, essential oils (EOs) have received a growing interest: they are natural, volatile and complex compounds accumulated by aromatic plants as secondary metabolites. Indeed, since the 80's it is known that they can be used in insect pest control (Regnault-Roger, 1997). EOs are characterized by two predominant components with different biosynthetic origins, phenylpropanoids and terpenes (monoterpenes and sesquiterpenes) (Pichersky and Gang, 2000). Terpenes are molecules made from the combination of several 5-carbon-base (C_5) units called isoprene. Phenylpropanoids in plants are synthesized from the amino acids tyrosine and phenylalanine. Their structure is characterized by a phenylic unit (C_6) bounds with a propanoic unit (C_3). Phenylpropanoids occur less frequently than terpenes and they serve as essential components of a number of structural polymers (Regnault-Roger et al., 2012).

The terpenes composition of EOs is variable between different plant species but monoterpenes are usually more abundant, accounting up to the 90 % of them (Bakkali et al., 2008). Typically, they exert their toxic effect by reducing or disrupting invertebrate growth at several life stages (Konstantopoulou et al., 1992). For instance thymol, a constituent of the essential oil from the plant *Thymus vulgaris L.*, is a monoterpene known

for its pesticide action against numerous arthropods and is widely used to control *Varroa destructor* (Calderone et al., 1997).

As far as *Drosophila suzukii* is concerned, several EOs, and their major terpenic components, showed toxic activity toward the pest, including the EO from *Mentha piperita* (menthol), *Perilla frutescens* (perilla aldehyde) and *Thymus zygis* (thymol and carvacrol) (Park et al., 2016).

The precise mechanism of action of EOs is still unclear. It is thought that several terpenes can interact with P450 cytochromes, an enzymatic class involved in the insecticide detoxification processes (Jensen et al., 2006). Some monoterpenes, for example thymol, cause neuronal degeneration through direct binding to GABA receptors (Priestley et al., 2003). Other terpenes, such as linalool and 1,8-cineole, inhibit acetylcholinesterase (Mills et al., 2004) while eugenol and geraniol inhibit neuronal activity (Price and Berry, 2006).

Moreover, several monoterpenes have been shown to interact with the octopaminergic/tyraminergic system, analogous to the adrenergic system present in the vertebrates. The biogenic amines tyramine (TA) and octopamine (OA), present in traces in the vertebrate nervous system, are important neurochemical modulators in invertebrates (David and Coulon, 1985). Their biosynthetic pathway begins with the decarboxylation of tyrosine to TA by tyrosine decarboxylase. Thereafter, TA is hydroxylated into OA by tyramine β-hydroxylase (Roeder, 2005). Several studies, initially focused on OA and then extended to TA, have shown that both these amines are neurotransmitter controlling numerous insect physiological processes such as reproduction (Clark and Lange, 2003; Donini and Lange, 2004; Da Silva and Lange, 2008), locomotion (Saraswati et al., 2004; Fox et al., 2006), immune functions (Baines and Downer, 1994; Adamo, 2009) and smell and related learning (Kutsukake et al., 2000; Pophof, 2002; Farooqui et al., 2003; Schwaerzel et al., 2003). In most cases, OA and TA exert their action by interacting and activating the corresponding receptors, which are G-Protein Coupled Receptors (GPCRs). These receptors are classified into five main groups based on their structure: α-adrenergic-like receptors (OctαR also known as OAMB or OA1), β-adrenergic-like receptors (OctβR, also known as OA2), tyramine receptors type 1 (TA/OA or TAR1) and tyramine receptors type 2 and 3 (TAR2 and TAR3), although TAR3 was identified only in *D. melanogaster* (Bayliss et al., 2013; Wu et al., 2014).

Several studies have revealed that natural molecules with insecticidal activity, such essential oils, can interact with octopaminergic (Enan, 2001; Enan 2005a) and with tyraminergic receptors in *D. melanogaster* (Enan, 2005b). In particular, TAR1 can be

stimulated by several monoterpenes in *D. melanogaster* as well as in *Rhipicephalus microplus* (Enan, 2005b, Gross et al., 2017), suggesting that these natural molecules might exert their insecticidal activity through the interaction with TAR receptors.

TAR1 has been characterized in several insects (Saudou et al., 1990; Blenau et al., 2000; Ohta et al., 2003; Rotte et al., 2009; Wu et al., 2013; Gross et al., 2015; Hana and Lange, 2017; Ma et al., 2019) and demonstrated to be involved in important physiological processes such as olfactory response, control of metabolism (obesity) and locomotor activity (Kutsukake et al., 2000; Roeder, 2005; Li et al., 2017).

This paper describes the structural and functional features of TAR1 (DsTAR1) from *Drosophila suzukii* and its sensitivity to three monoterpenes, thymol, carvacrol and α-terpineol. These information might therefore help to shed some light on the possible use of monoterpenes as biopesticides against this pest.

Materials and Methods

Insects and Reagents

Drosophila suzukii flies were reared on an artificial diet with a photoperiod of 16 h light: 8 h dark, at a temperature of 23 ± 1 °C.

Endothelin-1 (ET-1), tyramine hydrochloride, octopamine hydrochloride, yohimbine hydrochloride, brilliant black, Bovine Serum Albumin (BSA), probenecid, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), *p*-menth-1-en-8-ol (α-terpineol), *p*-cymene (carvacrol) and 3-hydroxy *p*-cymene (thymol) were all obtained from Sigma-Aldrich (St Louis, USA). Pluronic acid and fluorescent dye Fluo-4 AM were purchased from Thermo Fisher Scientific. All compounds were dissolved in dimethyl sulfoxide (10 mM) and stock solutions were kept at -20 °C until use. Serial solution were made in the assay buffer (Hanks' Balanced Salt solution (HBSS)/HEPES 20 mM buffer, containing 0.01 % BSA and 0.1 % DMSO).

Isolation and cloning of the full-length *Drosophila suzukii* tyramine receptor (DsTAR1)

Sequence alignment by BLASTN performed with the orthologous gene *DmTAR1* (Accession: X54794) from *D. melanogaster*, suggested that the putative transcript XM_017071090 predicted in the *D. suzukii* genome project (Accession: PRJNA325161) might code for the putative DsTAR1 (Accession: XP_016926579).

Total RNA was extracted from six adult flies using High Pure RNA Tissue Kit (Roche, Switzerland), quantified in a micro-volume spectrophotometer Biospec-Nano (Shimadzu, Japan) and analysed by 0.8 % w/v agarose gel electrophoresis. One µg of RNA was treated with DNase I (New England Biolabs, USA) and used for the synthesis of cDNA, carried out with the RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific). For amplification of the full *DsTAR1* open reading frame (ORF), specific primers were designed based on the annotated *DsTAR1* sequence (Table 1). High fidelity amplification was achieved using Herculase II Fusion DNA Polymerase (Agilent, USA) and a touchdown thermal profile: predenaturation at 95 °C for 3 mins, followed by 5 cycles at 95 °C for 20 s, 70-60 °C for 30 s (minus 2 °C/cycle), 68 °C for 2 mins, 30 cycles at 95 °C for 20 s, 60 °C for 30 s, 68 °C for 2 mins and a final extension at 68 °C for 5 mins. PCR product was gel purified used Wizard SV Gel and PCR Clean-Up System (Promega, USA), cloned into pJET 1.2/blunt vector (Thermo Fisher Scientific) and transformed into *E.coli* SIG10 5-α Chemically Competent Cells (Sigma-Aldrich).

Positive clones were selected using LB broth agar plates with 100 µg/ml ampicillin. Plasmid was then extracted and verified by DNA sequencing (BMR Genomics, Italy). The sequence, named *DsTAR1*, was deposited in GenBank with the accession number MK405664.

For expression in Human Embryonic Kidney (HEK 293) cells, the open reading frame of *DsTAR1* was excised from pJET 1.2 vector and inserted into the pcDNA 3.1 (+) Hygro vector using *NotI* and *XbaI* restriction sites.

Multiple sequence alignment and general bioinformatics analysis

Multiple protein sequence alignments between the deduced amino acid sequence of DsTAR1 and other type 1 tyramine receptors sequences were performed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and BioEdit Sequence Alignment Editor 7.2.6.1. Phylogenetic neighbour-joining analysis was performed by MEGA software (version 7) with 1000-fold bootstrap resampling. The *Drosophila melanogaster* GABA B receptor (GABABR) was used as an outgroup to root the tree.

Expression in HEK 293 and stable line creation

HEK 293 cells were grown at 37 °C and 5 % CO_2 in Dulbecco's modified Eagles medium high glucose (D-MEM) supplemented with 10 % fetal bovine serum (Microtech, Italy). To prevent bacterial contamination, penicillin (100 U/ml) and streptomycin (0.1 mg/ml) were added to the medium. The cells were transfected with pcDNA 3.1 (+)/DsTAR1 using lipofectamin 2000 (Invitrogen, USA). Stably transfected cells were selected with Hygromycin B 100 µg/ml supplemented medium. After two weeks, the resistant colonies were treated with trypsin and separately propagated in 24-well plates. These individual cell lines were analysed for the stable integration of the recombinant DNA by RT-PCR. The clonal cell line most efficiently expressing DsTAR1 was chosen for these studies.

Calcium Mobilization Assay

When confluence was reached, cells were seeded at a density of 50,000 cells per well, total volume of 100 µl, into poly-D- lysine coated 96-well black, clear-bottom plates. After 24 h incubation at normal cell culture condition, the cells were incubated with HBSS 1X supplemented with 2.5 mM probenecid, 3 µM of the calcium sensitive fluorescent dye Fluo-4 AM and 0.01 % pluronic acid, for 30 mins at 37 °C. After that, the loading solution was removed and HBSS 1X supplemented with 20 mM HEPES, 2.5 mM probenecid and

500 µM brilliant black was added. Cell culture and drug plates were placed into the fluorometric imaging plate reader FlexStation II (Molecular Devices, Sunnyvale, CA) and fluorescence changes were measured after 10 mins of stabilization at 37 °C. On-line additions were carried out in a volume of 50 µl/well after 20 s of basal fluorescence monitoring. To facilitate drug diffusion into the wells the present studies were performed at 37 °C with three cycles of mixing (25 µl from each well moved up and down 3 times). The fluorescence readings were measured every 2 s for 120 s.

Dynamic Mass Redistribution assay

For DMR measurements the label-free EnSight Multimode Plate Reader (Perkin Elmer, MA, US) was used. When confluence was reached, cells were sub-cultured as required using trypsin/EDTA and used for experiments. Cells were seeded into Enspire TM -LC 384-wells fibronectin-coated plates and cultured 20 hours to form a confluent monolayer in the cell culture medium. Cells were seeded at a density of 20,000 cells/well/30 µl. The day of the experiment cells were manually washed twice and maintained with the assay buffer (Hank's Balanced Salt Solution (HBSS) 1X with 20 mM HEPES, 0.01 % Bovine Serum Albumin) for 90 mins before DMR experiment.

Agonism protocol: a 5 mins baseline was first established, followed by adding compounds manually in a volume of 10 µl and recording compounds triggered DMR signal for 60 mins.

Antagonism / modulation protocol: antagonists / modulators were added manually 30 mins before reading the 5 mins baseline. After baseline establishment, TA or ET-1 were injected and DMR signal was recorded for 60 mins. The antagonist / modulator properties of ligands were measured by assessing the concentration-response curve to TA and ET-1 in the absence and in presence of a fixed concentration of antagonist / modulator. All the experiments were carried out at 37 °C.

Data analysis and terminology

All data were elaborated using Graph Pad Prism 6.0 (La Jolla, USA). Concentration-response curves were fitted using the four parameters log logistic equation:

$$\text{Effect} = \text{Baseline} + \frac{(E_{max} - \text{Baseline})}{(1+10^{(LogEC_{50} - Log[compound])*Hillslope})}$$

Data are expressed as mean ± SEM of n experiments performed in duplicate and were analysed using one- or two-way analysis of variance (ANOVA) followed by Dunnett's

or Turkey's test for multiple comparison. Agonist potency was expressed as pEC_{50}, which is the negative logarithm to base 10 of the agonist molar concentration that produces 50 % of the maximal possible effect of that agonist. Antagonists / modulators potencies were assayed at single concentrations against the concentration-response curve to TA.

Monoterpenes exposure bioassay

A 15 cm x 2 cm petri dish was used to expose adults *D. suzukii* to monoterpenes. 8 ml of a 1 % agar and 5 % sucrose solution were placed on the bottom of the petri. Monoterpenes (lipophilic in nature) stock solutions were prepared at a standard concentration of 10 mg/ml in acetone to ensure complete solubilization and stored at -20 °C. Dilutions to 1 mg/l final concentration were then made in water for each monoterpene, this concentration being close to the LD_{50} of all three terpenes tested (Kim et al., 2016; Park et al., 2016). A similar solution of water and acetone was used as negative control. 300 µl of the diluted compounds were then used to soak a 12 cm diameter paper disc positioned in the petri dish on top of the agar/sucrose gel.

Thirty adult flies (fifteen males and fifteen females 3-5 days-old) were placed inside the petri dish. The insects were incubated for 24, 72 or 120 h at a photoperiod of 16 h light: 8 h dark, at 23 ± 1 ° C. The effect of monoterpenes exposure on *DsTAR1* mRNA levels was evaluated, by RT-qPCR, after each time point.

Quantitative real-time PCR analysis

Total RNA was extracted from *Drosophila suzukii* samples at various developmental stages (1^{st} to 3^{rd} instar larvae, pupae, adult males and females), different tagmas (head, thorax and abdomen, dissected from adults) or adult flies subjected to the monoterpene exposures using High Pure RNA Tissue Kit (Roche, Switzerland). The three tagmas of *D. suzukii* (head, thorax and abdomen) were dissected out in a RNA preservation medium (20 mM EDTA disodium (pH 8.0), 25 mM sodium citrate trisodium salt, 700 g/l ammonium sulphate, final pH 5.2). The thorax presented all associated appendix (wings and legs). One µg of RNA was treated with DNase I (New England Biolabs) and used for cDNA synthesis, carried out with iScript Reverse Trascription Supermix (Bio-Rad, USA), according to the manufacturer's instructions. Real time PCR was performed using a CFX Connect Real-Time PCR Detection System (Bio-Rad) in 12 µl reaction mixture containing 0.8 µl of total cDNA obtained from one µg of RNA, 6 µl Sybr Green (SIGMA), 0.4 µl forward primer (10 µM), 0.4 µl reverse primer (10 µM) and 4.4 µl of

nuclease free water. Thermal cycling conditions were: 95 °C for 2 mins, 40 cycles at 95 °C for 15 s and 60 °C for 20 s. After the cycling protocol, a melting-curve analysis from 55 °C to 95 °C was applied. In expression analysis on tissues and development stages of *D. suzukii*, *DsTAR1* was quantified utilizing the relative quantification method (Larionov et al., 2005). After monoterpenes treatment the *DsTAR1* and *PKA* (accession number: NW_016019885.1) expression were quantified using qBase+ algorithm (Hellemans et al., 2007). AK and TBP were used as reference genes in both of the expression studies for the normalization (Zhai et al., 2014). Gene-specific primers (**Table 1**) were used and for each sample three independent biological replicates, made in triplicate, were performed.

Results

cDNA cloning and molecular characterization of *DsTAR1*

The ORF of *DsTAR1* is 1803 bp long and codes for a 600 aa polypeptide with a predicted molecular mass of 64.31 kDa (**Figure 1**).

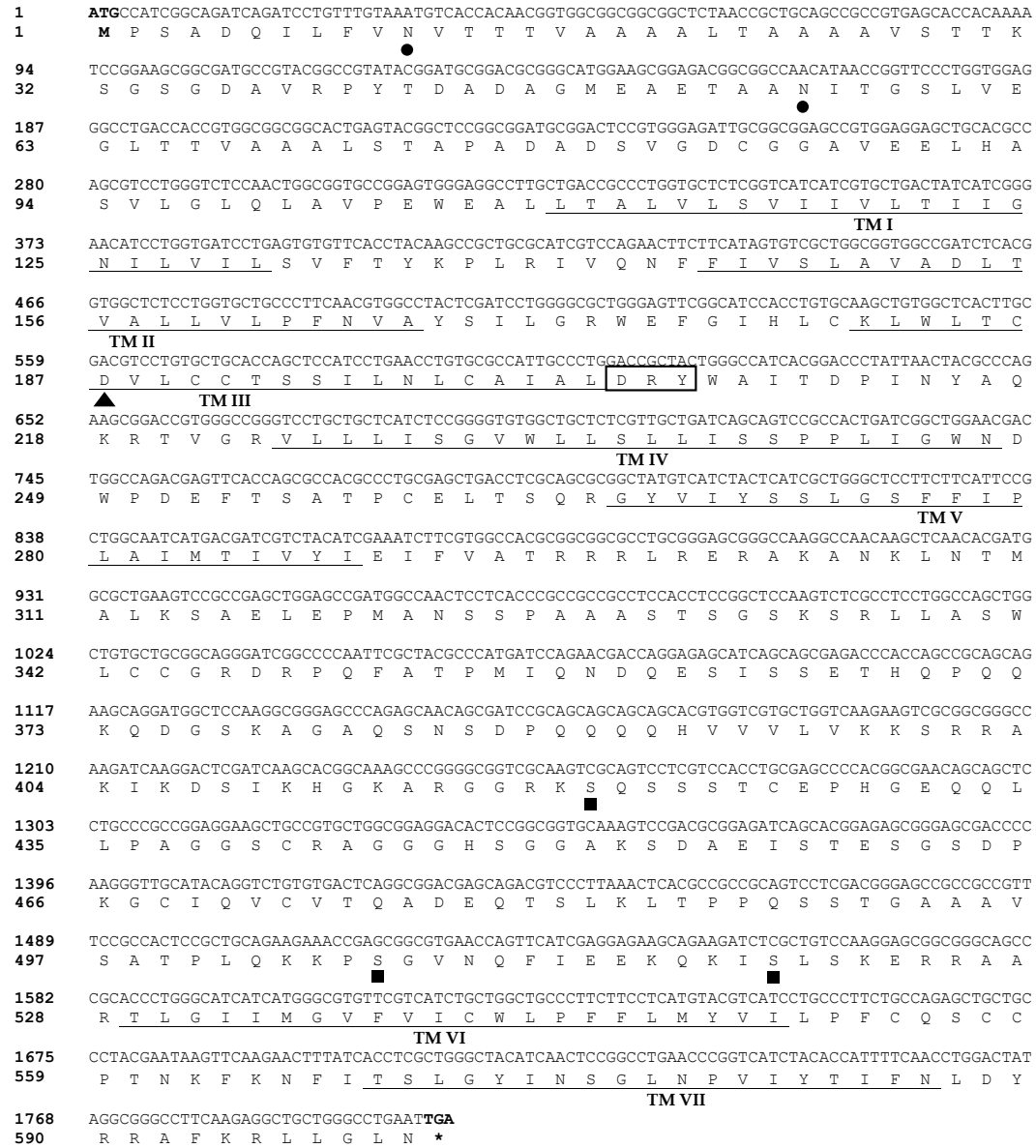

Figure 1. Nucleotide sequence of the tyramine receptor open reading frame cloned from *Drosophila suzukii* and deduced amino acid sequence. Prediction of the DsTAR1 transmembrane segments (underlined and numbered from I to VII) was obtained with TMHMM v. 2.0 software. After the third transmembrane domain there is the DRY motif (highlighted with a black box) important for the stabilization of GPCRs between inactive and activate conformation. Potential sites for N-linked glycosylation (predicted with NetNGlyc 1.0 server) are shown with a dot (●) and potential sites for PKA or PKC phosphorylation (predicted with NetPhos 3.1 server) are shown with a square (■). The black triangle (▲) represents an aspartic acid in TM3 (D_{187}) highly conserved in TAR1 family.

In terms of genomic structure *DsTAR1* appears to be encoded by four different exons (**Supplementary figure S1, panel A**), separated by three long introns. The same genomic organization is observed in the *D. melanogaster* counterpart coding for type 1 tyramine receptor (Chromosome 3L – NT_037436.4).

The analysis of the expected TAR1 polypeptide by TMHMM v. 2.0 software predicted the existence of seven transmembrane domains, a typical feature of GPCRs (**Figure 1**). Hydropathy profile analysis, according to the Kyte and Doolittle method (Kyte and Doolittle, 1982), further confirmed the presence of seven transmembrane helices, along with an eighth domain located close to the amino-terminal end of the polypeptide (**Supplementary figure S1, panel B**). This extra domain has been found in other biogenic amine receptors, in particular in the TAR1s from *D. melanogaster* (Saudou et al., 1990) and *B. microplus* (Baxter and Barker, 1999). This extra domain might be a cleavable signal sequence or leader peptide, a sequence that plays a key role during the first steps of the intracellular transport of G Protein-Coupled Receptors (Rutz et al., 2015). In *DsTAR1* sequence there are two asparagine residues, located in the N-terminal domain before the first transmembrane domain, that form the classic motif N-X-S/T for N-glycosylation sites (Nørskov-Lauritsen and Bräuner-Osborne, 2015). Furthermore, several serines were identified as putative phosphorylation sites, targeted by Protein kinase C or Protein kinase A, in the intracellular loops, especially in the loop between TMV and TMVI. The aspartic acid in TM3 (D_{187}), indicated by a black triangle in **Figure 1**, is conserved in all the members of the TAR1 family, because it interacts with the amino group of TA, the principal agonist of these receptors (Ohta and Ozoe, 2014).

The amino acid sequences of several insect biogenic amine receptors were used for multiple sequence alignment and to construct a neighbour-joining phylogenetic tree with MEGA 7 server. The results indicate that DsTAR1 clusters in the family of TAR1s, phylogenetically close to its orthologue from *D. melanogaster* (**Figure 2**).

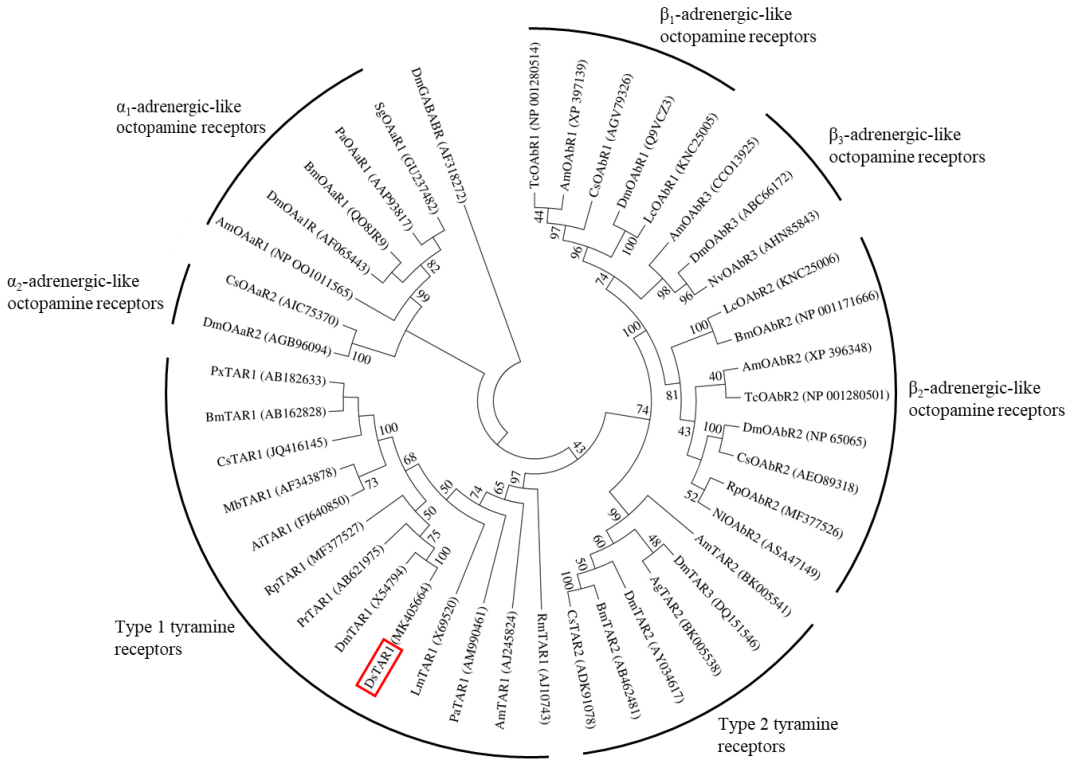

Figure 2. Phylogenetic relationships resulting from the neighbour joining analysis of DsTAR1 (highlighted with a box) and other insect amine receptors. The values shown at the nodes of the branches are the percentage bootstrap support (1000 replications) for each branch. *Drosophila melanogaster* GABA-B receptor (DmGABABR) was chosen as outgroup. Dm, *Drosophila melanogaster*; Ds, *Drosophila suzukii*; Pr, *Phormia regina*; Rp, *Rhodnius prolixus*; Px, *Papilio xuthus*; Cs, *Chilo suppressalis*; Bm, *Bombyx mori*; Ai, *Agnotis ipsilon*; Mb, *Mamestra brassicae*; Pa, *Periplaneta americana*; Lm, *Locusta migratoria*; Am, *Apis mellifera*; Rm, *Rhipicephalus microplus*; Sg, *Schistocerca gregaria*; Ag, *Anopheles gambiae*; Tc, *Tribolium castaneum*; Nv, *Nilaparvata lugens*; Lc, *Lucilia cuprina*.

Multiple sequence alignment between DsTAR1 amino acid sequence and TAR1s from *D. melanogaster*, *P. regina* and *C. suppressalis* revealed a similarity in the transmembrane domains, while less conserved regions were found in the N-terminal region of the proteins and in the intracellular loop between the 5th and 6th transmembrane segments (**Figure 3**). The highest protein identity was found, as expected, with *Dm*TAR1 (*Drosophila melanogaster* TAR1) with more than 93 % sequence identity (**Figure 3**).

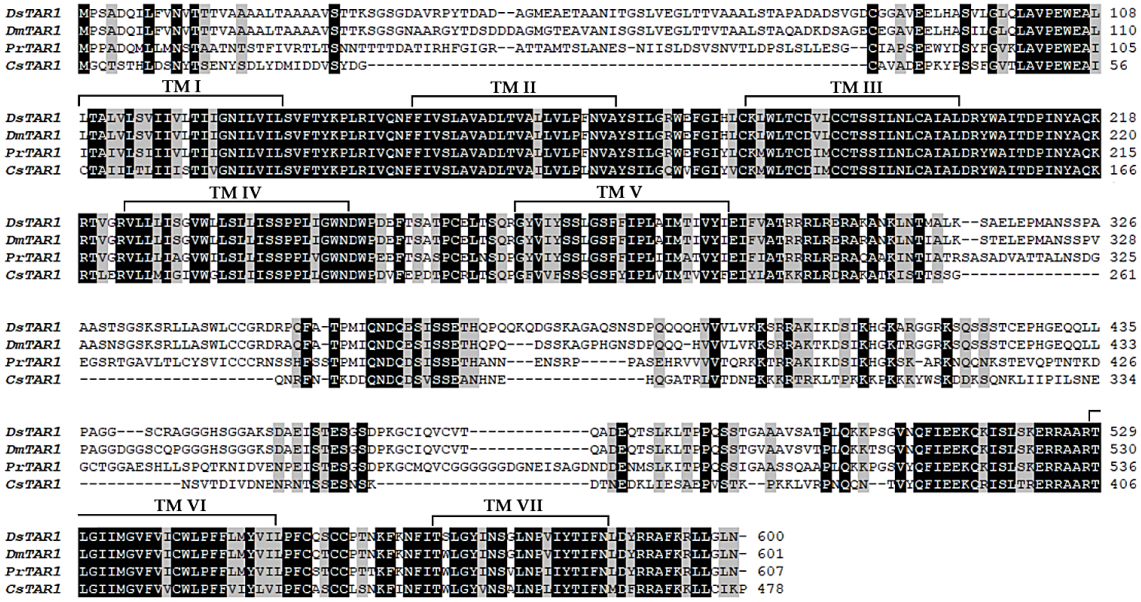

Figure 3. Amino acid sequence alignment of DsTAR1 with orthologous receptors from *D. melanogaster* (DmTAR1), *P. regina* (PrTAR1) and *C. suppressalis* (CsTAR1). The putative seven transmembrane domains (TM I-VII) are indicated with a black line. Identical residues are highlighted black while conservative substitutions are shaded.

Expression pattern of *DsTAR1*

Tissue localization analyses of the receptor were performed by RT-qPCR to understand the function of *DsTAR1* in *D. suzukii*. Total RNA was extracted from different development stages (first to third larvae, pupae and adult) and from three different tagmas (head, thorax and abdomen). The analysis revealed that *DsTAR1* was expressed in all developmental stages of *D. suzukii*, with high levels found in the first instar larvae and in the adult males (**Figure 4, panel A**). Furthermore, in adults, the expression levels in whole males were about twice than in females. The head of males and females accumulated higher levels of *DsTAR1* mRNA than the two other tagmas. In particular, the heads of males accumulated much higher mRNA levels of *DsTAR1* in comparison to the female counterparts. Interesting, the male abdomens shown a significant difference in the *DsTAR1* expression levels as compared to females, suggesting a possible role of TA in male reproductive system (**Figure 4, panel B**).

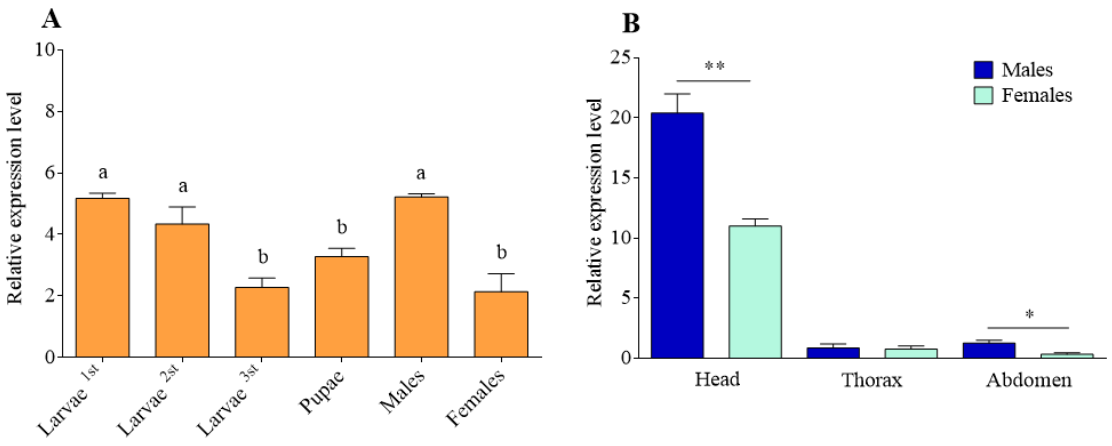

Figure 4. mRNA expression levels of *DsTAR1* gene in *D. suzukii*. (**A**) Expression of *DsTAR1* gene in different development stages (first to third larvae, pupae and adult). *DsTAR1* in first larvae was used as a comparator for the LSD (Least Significant Difference) statistical. (**B**) Expression of *DsTAR1* in tagmas (head, thorax and abdomen) on female and male adult flies. Data represent means ± SEM of three independent experiments performed in triplicate. * $p < 0.05$ ** $p < 0.01$ according to Student's t-test. Arginine kinase (*AK*) and TATA Box Protein (*TBP*) were used as housekeeping genes.

Pharmacological data

To confirm the function of DsTAR1 as TA receptor and its possible interaction with monoterpenes, the cloned cDNA was expressed in HEK 293 cells and tested for the ability to respond to TA and OA, like other TAR1s studied so far in insects, as well as to several monoterpenes. The responses were evaluated with two different assays, the calcium mobilization assay and the dynamic mass redistribution (DMR) assay, the latter being a label free technique that has been previously demonstrated to be useful for investigating the functional profile of G Protein-Coupled Receptors (Grundmann and Kostenis, 2015). In the calcium mobilization assay performed on HEK 293_{DsTAR1} cells, TA evoked the release of intracellular calcium in a concentration-dependent manner with pEC_{50} and E_{max} values of 6.35 (6.07 - 6.62) and 105 ± 15 % over the basal values, respectively (**Figure 5, panel A**). On the contrary, OA did not stimulate any intracellular calcium release when tested in the concentration range 10 µM - 100 pM (data not shown). ET-1, chosen as external control (Atwood et al., 2011), was able to increase in HEK 293_{DsTAR1} cells the intracellular calcium mobilization with a potency value of 7.02 (6.88 - 7.15) and maximal effects of 152 ± 12 % (**Figure 5, panel B**). Yohimbine, tested as agonist up to 10 µM on the DsTAR1, did not elicit the intracellular calcium mobilization (data not shown). Yohimbine 1 µM was also tested as antagonist against TA and ET-1. The molecule was able to rightward shift the concentration response curve to TA (**Figure 5, panel A**), while the same concentration did not significantly affect the concentration response curve to

ET-1 (**Figure 5, panel B**). A pA_2 of 7.87 was calculated for yohimbine against TA assuming a competitive type of antagonism. In wild type HEK 293 cells, TA and OA were completely inactive (data not shown), while ET-1 stimulated calcium mobilization in a similar manner observed in HEK 293_{DsTAR1} cells (data not shown).

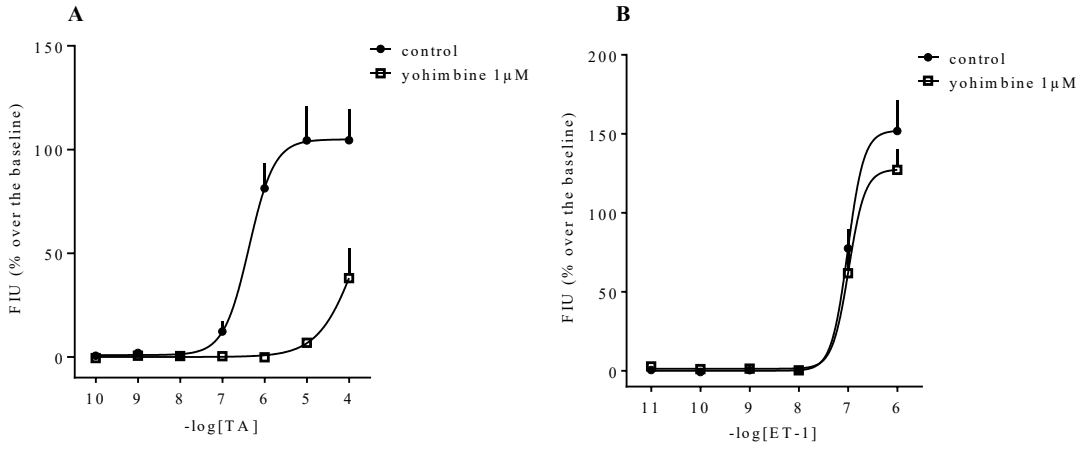

Figure 5. Concentration-response curves, by calcium mobilization assay, to TA **(A)** or ET-1 **(B)** in the absence (control) and in presence of 1 µM yohimbine in DsTAR1 transfected HEK293 cells. Data represent means ± SEM of four separate experiments performed in duplicate.

The same cell lines were then treated with three monoterpenes (α-terpineol, carvacrol and thymol) to evaluate their possible agonist effect. α-terpineol did not elicit $[Ca^{2+}]_i$ mobilization in any cell line **(Supplementary figure S2, panel C and F)**, while carvacrol and thymol stimulated $[Ca^{2+}]_i$ mobilization only at 100 µM in both HEK 293 wild type and DsTAR1 transfected cells (**Supplementary figure S2, panels A, B, D and E**).
DMR assays revealed that TA is able to evoke a positive concentration dependent signal (**Figure 6, panel A)** in HEK 293_{DsTAR1} while OA can elicit an intracellular Ca^{2+} release only at 10 µM (data not shown). In these experiments, TA showed a potency value of 6.87 (6.46 - 7.28) and maximal effect of 164 ± 25 pm (**Figure 6, panel C**). The DsTAR1 antagonist yohimbine, tested as agonist up to 1 µM did not modify per se the DMR signal (data not shown). However, yohimbine 1 µM was able to rightward shift the concentration response curve to TA without changing the agonist maximal effect with a pA_2 value of 7.24 (6.56 - 7.92) (**Figure 6, panel B and C**).

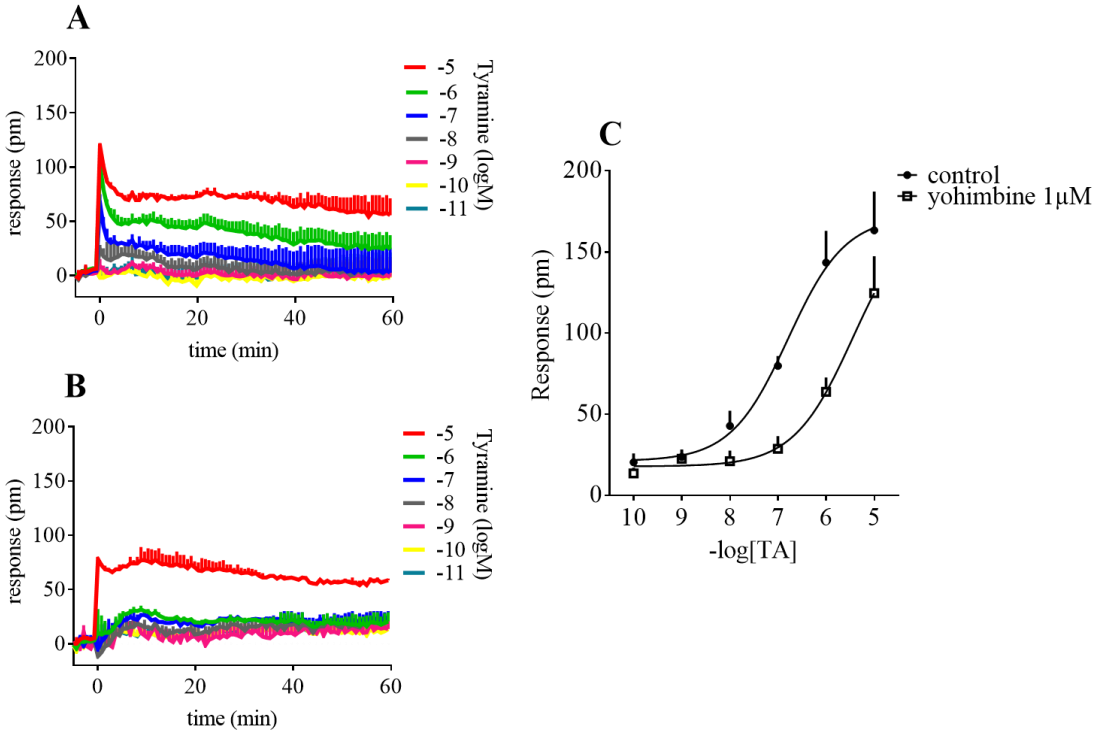

Figure 6. Baseline corrected DMR traces of TA in the absence (**panel A**) and in presence (**panel B**) of 1 µM yohimbine and concentration-response curve to TA (**panel C**) in the absence (control) and in presence of 1 µM yohimbine, in HEK293$_{DsTAR1}$ cells. Data are the means ± SEM of four experiments performed in duplicate.

ET-1 elicited concentration-response curves with or without yohimbine 1 µM (**Supplementary figure S3**). In wild type HEK 293 cells, TA and OA were completely inactive while ET-1 evoked a concentration-dependent DMR response (pEC$_{50}$ 7.86 (7.46 - 8.27) and maximal effects of 440 ± 27 pm). The effects of the highest concentrations of the two agonists tested in the two HEK 293 cell lines are summarized in **Table 2**. The DMR analyses also confirmed that the three monoterpenes tested do not act as agonist of DsTAR1. Two (100 and 10 µM) or four (up to 0.1 µM) different monoterpenes concentrations were tested on HEK 293 wild type or DsTAR1 stably transfected cells, respectively. All three terpenes at the highest concentration appeared to be able to stimulate a DMR signal in HEK 293$_{DsTAR1}$ comparable to the one observed in HEK 293 wild type cells. Lower concentrations were, on the other hand, completely inactive in both cell types **(Supplementary figure S4)**.

Recently, several monoterpenes have been also shown to act as modulators of TAR1 from *Rhipicephalus (Boophilus) microplus*, increasing the in vitro TA response.

To investigate the possible antagonist/modulatory action of the three monoterpenes towards DsTAR1 in DMR experiments, HEK 293_{DsTAR1} cells were pre-incubated for 30 mins with 10 µM, 1 µM or 0.1 µM of each terpene.

Upon the addition of TA (10 µM - 10 pM) all three monoterpenes, at 10 µM and 1µM, were able to increase TA potency (**Figure 7**; **Table 3**).

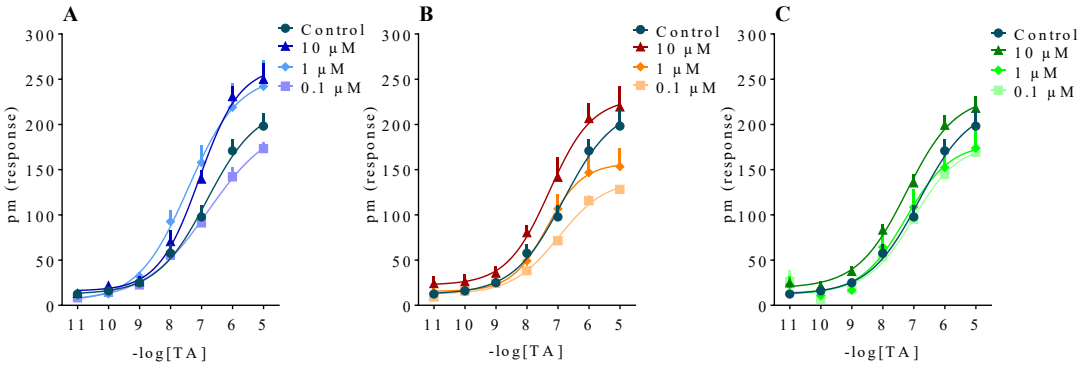

Figure 7. Concentration-response curves, by DMR assays, to TA after recording in HEK 293_{DsTAR1} cells pre-incubated with 10 µM, 1 µM or 0.1 µM of carvacrol (**panel A**), α-terpineol (**panel B**) or thymol (**panel C**). Data are means ± SEM of at least three separate experiments made in duplicate.

In particular, α-terpineol 1 µM was able to shift the concentration response curve to TA by 5 folds. The higher potency was observed for the endogenous agonist with a pEC_{50} of 7.44 (7.28 - 7.59) in HEK 293_{DsTAR1} pre-incubated with the monoterpene as compared to 6.81 (6.66 - 6.89) in the control. (**Table 3**). All three monoterpenes, in the same concentration ranges, did not modify the concentration response curve to ET-1 (Data not shown).

Taken together these experiments confirm that, as predicted by structural analysis, DsTAR1 is a functional type 1 tyramine receptor sensitive to TA. In both calcium mobilization and DMR assays, TA stimulated DsTAR1 causing significant changes in $[Ca^{2+}]_i$ mobilization and dynamic mass redistribution. Furthermore, DMR experiments revealed that three monoterpenes do no act as DsTAR1 agonists but rather as positive allosteric modulators of the receptor.

Expression of *DsTAR1* and *PKA* genes after monoterpenes exposure

To evaluate the effect of the exposure to monoterpenes on the expression levels of *DsTAR1* and *PKA* genes, adult flies of *D. suzukii* were exposed to 1 mg/l of carvacrol, α-terpineol and thymol and the mRNA levels analyzed by qPCR. The concentration tested was close to the LD_{50} of each monoterpene (Kim et al., 2016; Park et al., 2016).

The exposure induced an interesting downregulation of *DsTAR1* gene expression. Significant differences were observed for α-terpineol at 24 h (5.2-fold lower than control), for thymol at 24 h (3.5 fold lower than control) and 72h (12.9 fold lower than control) and for carvacrol at 120 h (5.2 fold higher than control) (**Figure 8, panel A**). On the other hand, the mRNA levels of *PKA* (chosen as internal unrelated control) were not significantly altered by any treatment (**Figure 8, panel B**). This gene was chosen as an internal control, given propensity of TAR1 to couple with G_i proteins. These G proteins, in fact, have PKA as the last transduction effector.

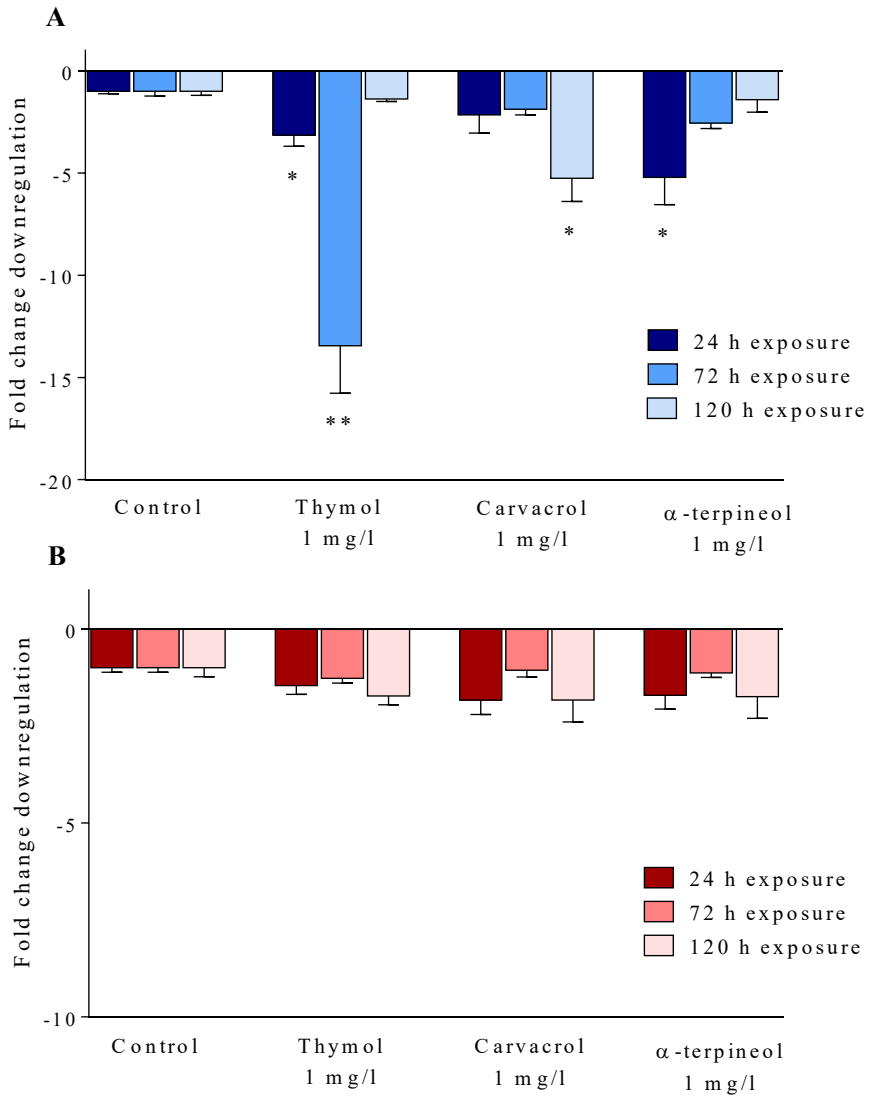

Figure 8. *DsTAR1* (**A**) and *PKA* (**B**) expression levels in *D. suzukii* adult flies after 24, 72 or 120h of continuous exposure to monoterpenes. Data represent means ± SEM of three independent experiments performed in triplicate. * $p < 0.05$ ** $p < 0.01$ vs control according to one-way ANOVA followed by Dunnett's test for multiple comparisons. Arginine kinase (*AK*) and TATA Box Protein (*TBP*) were used as housekeeping genes.

Discussion and Conclusions

This study describes the first molecular and functional characterization of DsTAR1, a type 1 tyramine receptor from the phytophagous *D. suzukii*.

The structural analysis of DsTAR1 predicted amino acid sequence revealed many features shared with other insect type 1 tyramine receptors. The hydropathy profile revealed the typical organization of a GPCR with seven transmembrane segments along with an additional region also found in *D. melanogaster* TAR1 (Saudou et al., 1990). This putative TM VIII, located at the beginning of the amino-terminal end, is not unusual in these receptors. In several studies, it was postulated that this short amino acid sequence might be a signal peptide, necessary for the correct vesicular transport of the protein (Saudou et al., 1990; Baxter and Barker, 1999). At position 187 in DsTAR1 predicted amino acid sequence there is an aspartic acid residue highly conserved among TAR1s, believed to interact with the amine group of TA. This binding is strengthened through weak interactions with three serine residues presents in TM V: S_{271}, S_{272} and S_{275} in DsTAR1 (Ohta et al., 2004). Amino acid sequence alignment revealed similarity between DsTAR1 and other insect type 1 tyramine receptors, especially with *D. melanogaster* TAR1. The two proteins differ only for 44 different residues, localized in the N-terminus and in the intracellular loop between TM V and TM VI. Interestingly, these two regions are the least conserved among all TAR1 sequences analyzed, therefore suggesting a role in defining the different molecular and functional characteristics of the receptors.

To shed some light on the role that DsTAR1 plays in *D. suzukii*, an expression profile analysis of the receptor was performed. RT-qPCR revealed that *DsTAR1* is significantly more expressed in male adult flies than in females. *DsTAR1* mRNA accumulates especially in the head of males as compared to female adult flies and to the other two tagmas (thorax and abdomen). The significant difference between the male and female abdomens is an interesting observation that suggests a possible role of TA in male reproductive system. A recent study in *D. melanogaster* has indeed shown that this receptor is mainly expressed in the brain and in the male reproductive organs (El-Kholy et al., 2015). This expression profile is in accordance with other studies on type 1 tyramine receptors that have described a higher expression in brain and nerve cords, while the mRNA is almost absent in other districts or organs (Rotte et al., 2009; Wu et al., 2013; Ono and Yoshikawa, 2004). Furthermore, TAR1 mRNA has been described in other districts located in the head, such as the antennas and the maxillary palps, suggesting a role for type 1 tyramine receptors in neurons responsible for olfactory and taste responses

(Kutsukake et al., 2000). The marked difference in expression between adult males and females suggests that *D. suzukii* type 1 tyramine receptor is probably involved in the control of male specific functions such as the development and function of male reproductive organs and the search for female partners through specific behavioral or physiological processes (El-Kholi et al., 2015; Hana and Lange, 2017).

Structural data suggest that DsTAR1 codes for a TA receptor. To confirm its function the cDNA was cloned, stably expressed in HEK 293 cells and tested in functional studies for its sensitivity to TA and OA. Moreover, the pharmacological effects of yohimbine were also investigated since this compound has been reported to act as a TAR1 antagonist in previous studies (Gross et al., 2015; Hana and Lange, 2017).

The *D. suzukii* cloned receptor was functionally studied using two different pharmacological assays. In the calcium mobilization assay, TA was able to increase the intracellular calcium mobilization with a pEC_{50} of 6.35 while OA was inactive. The effect of TA was sensitive to the antagonist yohimbine similarly to the orthologous type 1 tyramine receptor of *Drosophila melanogaster* (Saudou et al., 1990; Enan, 2005a).

The *D. suzukii* receptor was further studied by DMR assay, which is based on an optical biosensor technology. This recently developed analysis does not employ labelled molecules and therefore it monitors integrated receptor signaling responses including those mediated by GPCRs (Ferrie et al., 2011; Tran et al., 2012; Carter et al., 2014; Grundmann and Kostenis, 2015). DMR has never been applied to type 1 tyramine receptors before.

In the DMR assay, TA activated DsTAR1 with a pEC_{50} of 6.87 while OA showed a signal only at 10 μM concentrations. Yohimbine was able to rightward shift the dose-response curve to TA showing a competitive type of interaction and a pA_2 value of 7.24.

Collectively, the receptor cloned from *D. suzukii* displays the pharmacological profile expected for a member of the TAR1 family in terms of rank order of potency of agonists, i.e. TA > OA and sensitivity to the selective and competitive antagonist yohimbine, that displayed nanomolar potency. These features were detected not only at the level of the calcium pathway but also, for the first time, in terms of dynamic mass redistribution. Therefore the functional data presented here not only confirm the structural analysis on the predicted protein but expand the biochemical knowledge on TAR1 receptors as well.

Studies carried out on TAR1 have shown that monoterpenes interact with the receptor, either by acting as agonist (*Drosophila melanogaster* Enan, 2005b) or as modulator *Rhipicephalus* (*Boohilus*) *microplus* (Gross et al., 2017). Therefore, HEK 293 cells stably

expressing DsTAR1 were used to verify whether these biochemical interactions could be also observed in *Drosophila suzukii*.

When tested as agonists in DMR experiments, thymol, carvacrol and α-terpineol were not able to generate pharmacological responses attributable to the interaction with DsTAR1. Similar results were obtained in the calcium mobilization assay, where monoterpenes were tested on both HEK wild type and HEK 293_{DsTAR1} cells at 1, 25 and 100 μM, that is the same concentration range tested on TAR1 from *D. melanogaster* in a similar calcium mobilization assay (Enan, 2005b). Thymol and carvacrol stimulated a release of $[Ca^{2+}]_i$ but only at 100 μM, and this signal was detected in both HEK 293 wild type and stably transfected DsTAR1 cells. However, α-terpineol was inactive at all concentrations tested (100 μM, 25 μM and 1 μM) and on both cell lines.

These monoterpenes are known to elicit calcium release in many different tissues and cell types and are agonists for many different ion channels and receptors (Magyar et al., 2002; Krizaj et al., 2003; Szentesi et al., 2004; Vogt-Eisele et al, 2007; Sarkozi et al., 2007). Therefore, it is not surprising that they could induce a remarkable elevation in $[Ca^{2+}]$ levels and an intense DMR signal in both stably transfected and wild type HEK 293 cells at high concentration. On the other hand, at lower concentrations (25 μM) all monoterpenes were unable to stimulate the release of $[Ca^{2+}]$ in HEK 293 stably expressing DsTAR1, contrary to what observed by Enan (Enan, 2005b) for the type 1 tyramine receptor from *D. melanogaster*. These functional differences observed between the two phylogenetically close receptors might be structurally connected to 44 amino acids that differentiate the amino acid sequences of the two receptors (as judged by alignment between DmTAR1 and DsTAR1). Unfortunately, there are no structural studies that might shed some light on how amino acid changes in these regions could be directly related to a different receptor sensitivity. Therefore, the structural reasons of the different sensitivity of DmTAR1 and DsTAR1 to monoterpenes remains to be assessed.

A possible modulatory action of the three monoterpenes on DsTAR1 was investigated by DMR assay. Pre-treatment of the transfected DsTAR1 cells with 10 μM or 1 μM of the three monoterpenes promoted an increase in agonist potency; the larger effect was obtained with 1 μM α-terpineol that produced a 5-fold increase in TA potency.

It has been reported that carvacrol can induce a positive allosteric modulation on type 1 tyramine receptor from *Rhipicephalus* (*Boophilus*) *microplus*, allowing the terpene to stabilize and enhance the pharmacological activity of TA, through a conformational

change in the receptor (Gross et al., 2017). Therefore, the monoterpenes might interact with DsTAR1 with a similar biochemical mechanism.

Furthermore, a similar modulatory effect was observed by Gross in 2015 on the RmTAR1 (Gross et al., 2015) after treatment with a metabolite of the amitraz insecticide (BTS-27271) that causing an increased the TA response when tested in vitro at 10 µM. This similarity in mechanism might suggest that monoterpenes modulate DsTAR1 comparable to that of an insecticide molecule.

The three monoterpenes were also tested on whole insects at sublethal concentrations to reveal possible effects on *DsTAR1* transcription. A transcriptional downregulation of the receptor was noticeable for all treatments, which was not observed for the *PKA* control gene, thus ruling out a generalized downregulation. This monoterpene-induced transcriptional effect might be either exerted directly on *DsTAR1* or by means of an adjustment carried out on the regulatory pathway of the receptor.

Taken together, one might hypothesize that the downregulation of *DsTAR1* represents a compensatory mechanism in response to the enhanced DsTAR1 signalling due to positive allosteric modulatory effect of monoterpenes. Furthermore, it will be fundamental to understand how the downregulation of DsTAR1 could interfere with the normal *Drosophila suzukii* behavior and physiology.

In conclusion, the present data contribute to widen the existing knowledge about the role of TA and particularly DsTAR1 in insect physiology. Moreover, the identification of a role for monoterpenes in DsTAR1 action, through a set up and validated in vitro system, will allow the pharmacological quest for biomolecules that may possibly acts as innovative biopesticides.

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Table 1. Primer use in this study.

Primers	Primer sequence (5'-3')
cDNA cloning	
DsTAR1-For	TTCCGTCCGCCATTCAACC
DsTAR1-Rev	TCAATTCAGGCCCAGCAGC
Quantitative RT-PCR	
Fw-*DsTAR1*-RT	GCAGTCCTCGTCCACCTG
Rev-*DsTAR1*-RT	TTAAGGGACGTCTGCTCGTC
AK-Fw	CTACCACAACGATGCCAAGA
AK-Rev	AAGGTCAGGAAGCCGAGA
TBP-Fw	CCACGGTGAATCTGTGCT
TBP-Rev	GGAGTCGTCCTCGCTCTT
PKA-Fw	CGGAGAACCTGCTAATCGAC
PKA-Rev	CCATTTCGTAGACGAGCACA

Table 2. Effects of the highest concentrations tested for the ligands in HEK 293 and HEK 293_{DsTAR1} cells.

	HEK 293 wt E_{max} (pm ± SEM)	HEK 293_{DsTAR1} E_{max} (pm ± SEM)
Buffer	6 ± 14	-8 ± 15
TA 10 µM	-2 ± 33	164 ± 25*
ET-1 1 µM	440 ± 27*	263 ± 37*

*$p < 0.05$ vs buffer according to one-way ANOVA followed by the Dunnett's test for multiple comparisons.

Table 3. pEC_{50} and E_{max} values of TA, in HEK 293_{DsTAR1} cells, after a pre-incubation with buffer (control) or 10 μM, 1 μM or 0.1 μM of monoterpenes.

Chemical	Concentration	TA	
		pEC_{50} ± SEM	E_{max} (pm ± SEM)
	Control	6.81 ± 0.07	223 ± 25
Carvacrol	10 μM	7.15 ± 0.06 *	230 ± 14
	1 μM	7.28 ± 0.09 *	179 ± 26
	0.1 μM	6.95 ± 0.13	184 ± 17
α-terpineol	10 μM	7.13 ± 0.09 *	261 ± 22
	1 μM	7.44 ± 0.06 *	253 ± 33
	0.1 μM	6.86 ± 0.16	198 ± 5
Thymol	10 μM	7.23 ± 0.11 *	232 ± 21
	1 μM	7.37 ± 0.08 *	156 ± 22
	0.1 μM	6.79 ± 0.16	152 ± 13

* $p < 0.05$ vs control according to one-way ANOVA followed by the Dunnett's test

Supplementary figures

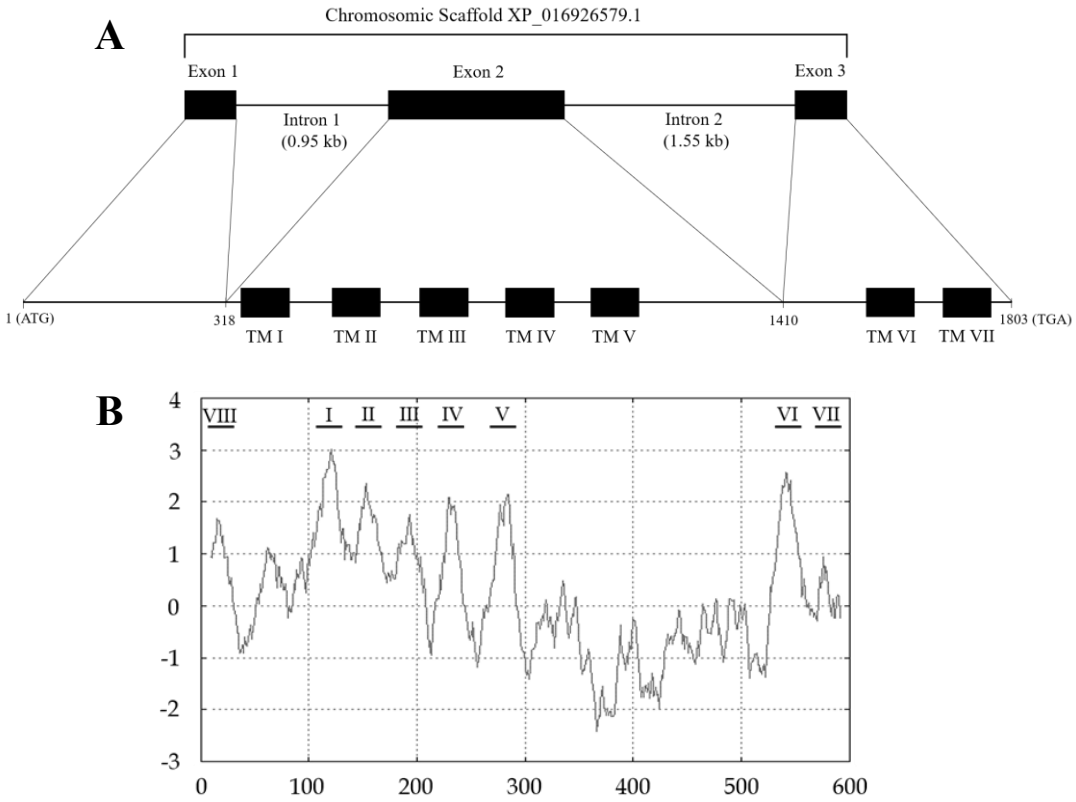

Figure S1. Schematic representation of the *DsTAR1* genomic exon-intron organization (**A**). The genomic sequence is in the top line and the open reading frame sequence in the bottom line. The genomic exon-intron organization was determined by BLAST comparison between *D. suzukii* genome and the cloned *DsTAR1* sequence. The predicted transmembrane domains are indicated as TM I to VII.
Hydropathy profile of the predicted amino acid sequence (**B**). The hydropathy profile of the predicted amino acid sequence of DsTAR1 was calculated with the method of Kyte and Doolittle using the software ExPAsy-ProtScale with a window size of 17 amino acids. Amino acids numbers are given on the axis. The transmembrane segments are indicated with a black bar and numbered I-VIII.

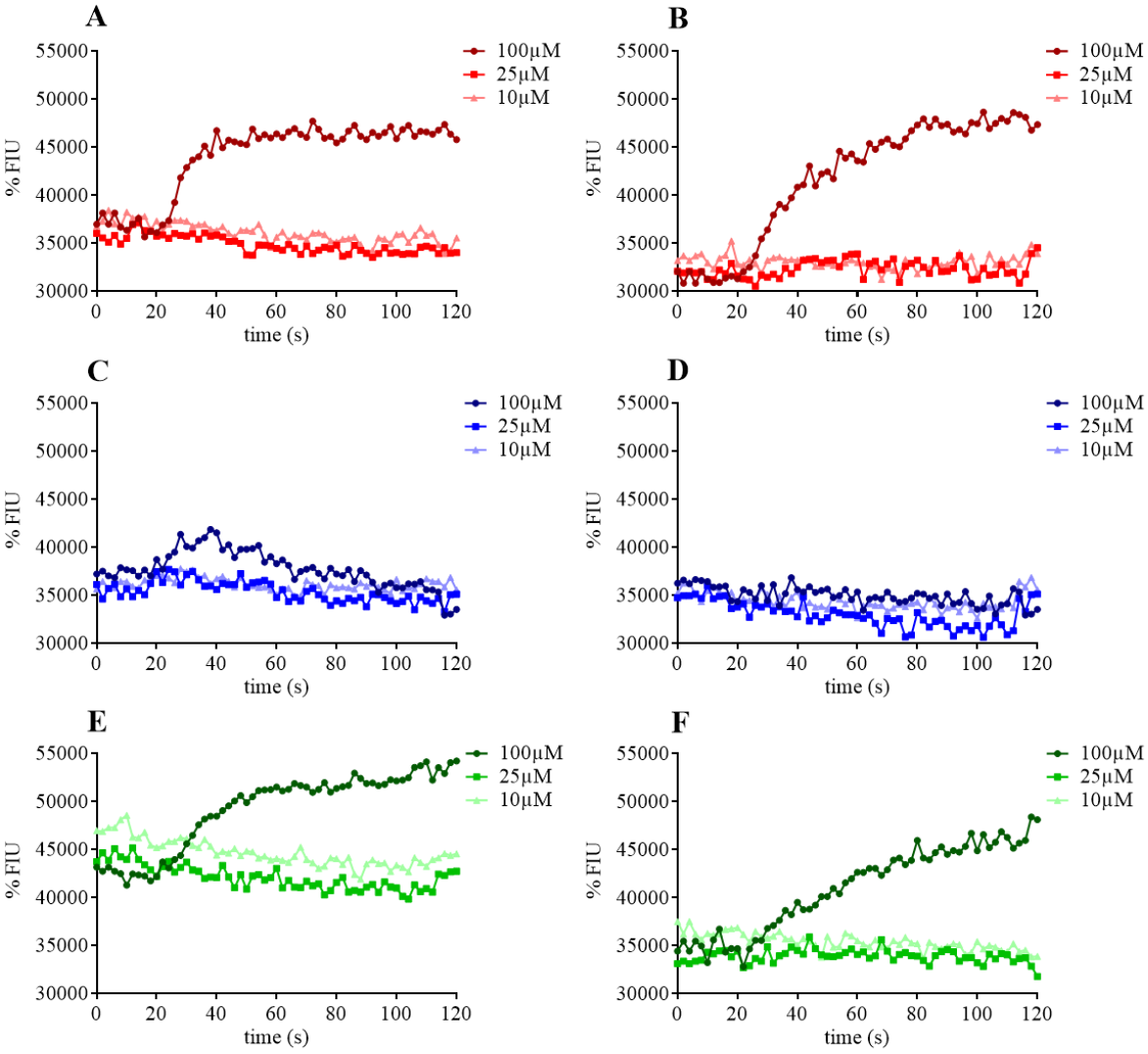

Figure S2. The effects of monoterpenes on [Ca2+]$_i$ mobilization in HEK 293 wild type (**panel A, C and E**) and HEK 293$_{DsTAR1}$ cells (**panel B, D and F**). The cells were incubated 20 s before the addition of the tested carvacrol (**panel A and B**) α-terpineol (**panel C and D**) and thymol (**panel E and F**). These data represent one of three independent experiments performed in duplicate and typical result are reported.

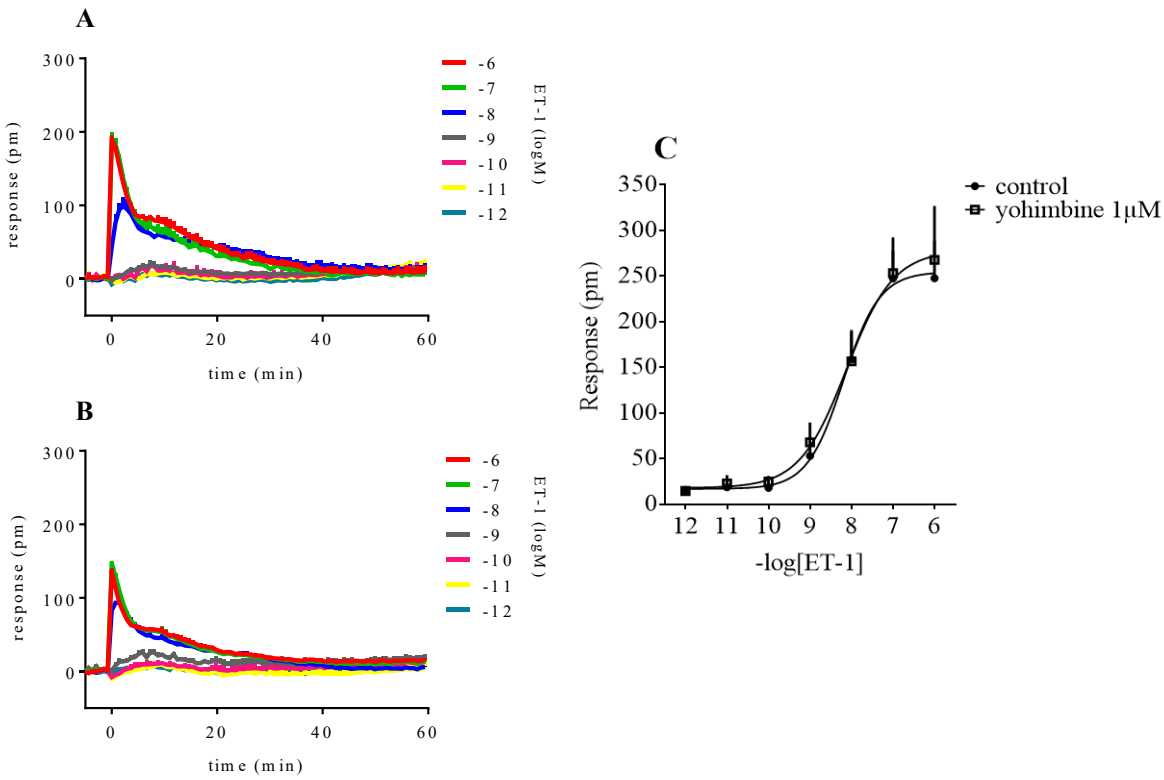

Figure S3. Baseline corrected DMR traces of ET-1 in the absence (**panel A**) and in presence (**panel B**) of 1 µM yohimbine and concentration-response curve to ET-1 (**panel C**) in the absence (control) and in presence of 1 µM yohimbine, in HEK293$_{DsTAR1}$ cells. Data are the means ± SEM of 4 experiments performed in duplicate.

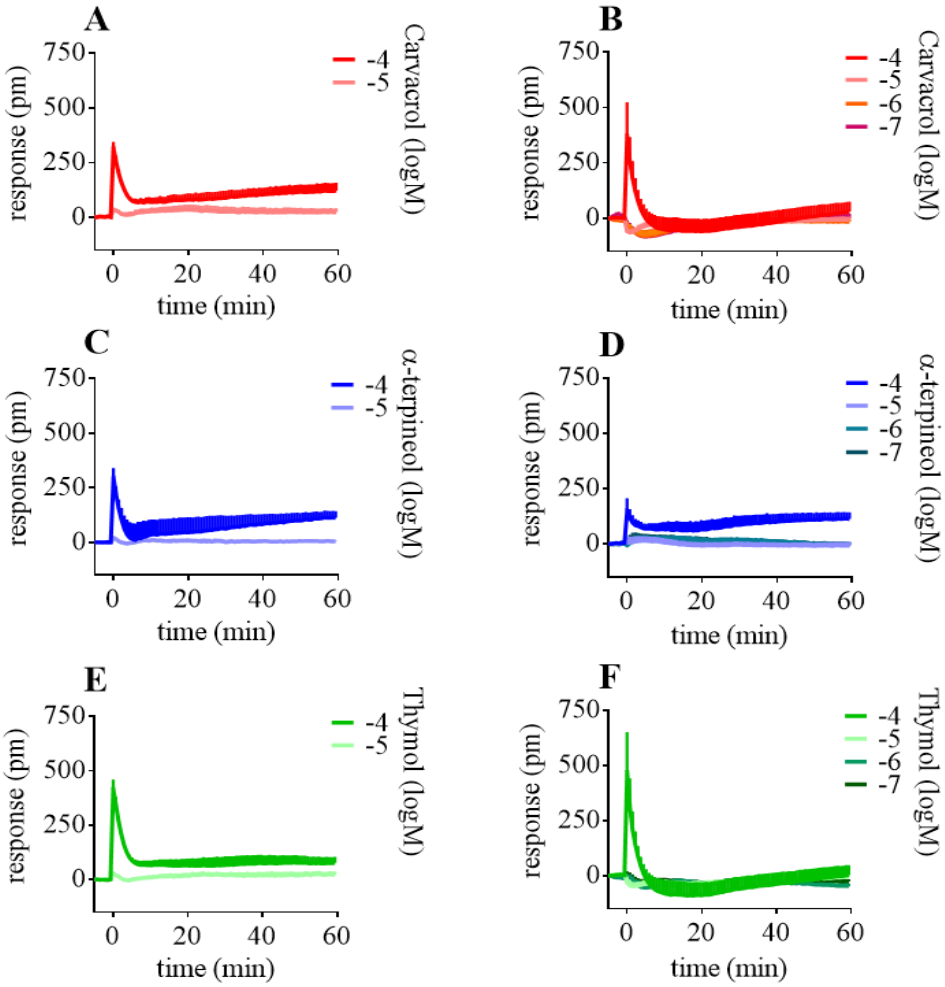

Figure S4. The effects of monoterpenes, by DMR assays, on HEK 293 wild type (**panel A, C and E**) and HEK 293_{DsTAR1} cells (**panel B, D and F**). The cells were incubated 20 s before the addition of the tested carvacrol (**panel A and B**), α-terpineol (**panel C and D**) and thymol (**panel E and F**). These data represent one of three independent experiments performed in duplicate and typical result are reported.

Monoterpenes alter TAR1-driven physiology in *Drosophila* species

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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 can cause complex behavioural alterations that require a 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 mutant *D. melanogaster* strain (*TAR1*^{PL00408}) was used to better understand the relationships between the receptor and monoterpenes-related behavioural changes. Immunohistochemistry analysis revealed that, in the *D. melanogaster* brain, TAR1 appeared to be expressed in the *Pars Intercerebralis*, mushroom bodies and olfactory lobes. In comparison to the *D. melanogaster* wild type, 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 *D. suzukii* and *D. melanogaster* wild types 24 h after a continuous monoterpene exposure. Interestingly, they were ineffective in modifying the physiological performances of TAR1 mutant flies. In conclusion, it appears that monoterpenes not only act as biopesticides for *Drosophila* but they can also interfere with its behaviour and metabolism in a TAR1-dependent fashion.

Keywords: *Drosophila*, Monoterpenes, Tyramine receptor, Metabolism, Behaviour

Introduction

Drosophila suzukii Matsumura (Diptera: Drosophilidae), commonly known as "Spotted Wing Drosophila", is one of the few Drosophilidae that can lay its eggs on healthy fruits before they becomes fully ripe (Walsh et al., 2011; Lee et al., 2011). *D. suzukii* is able to infest most of the fruit and vine species worldwide with a particular preference for small fruits (Rota-Stabelli et al., 2013). This species causes serious damages to the horticultural economy especially in South-East Asia and its presence has been recently reported also in North America and Europe (Asplen et al., 2015). Moreover, *D. suzukii* can spread rapidly (seven to fifteen generations - 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 enforcements due to 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 & Beers, 2012). Alternative and more sustainable control strategies are constantly under investigation (Schetelig et al., 2017). Currently, research on the biology, genetics, as well as physiology of *D. suzukii* has gained interest in order to develop new tools for a 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 essential oils 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 of 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; Enan, 2005b; Price & 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's nervous system contains high levels of OA and TA, suggesting a role as neurotransmitters (Ohta & Ozoe, 2014), but also as neuromodulators and neurohormones in a wide variety of physiological processes (Pauls et al., 2018).

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 Receptors (GPCR) family (Evans & Maqueira, 2005). Tyramine receptors (TARs) play important roles in modulating the biology, physiology and behaviour of invertebrates (Ohta & 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 & Down, 2015). These receptors are classified into two main groups based on their structure and activity: tyramine receptors type 1 (TA/OA or TAR1) on one hand and tyramine receptors type 2 and 3 on the other (TAR2 and TAR3) (Wu et al., 2014). TAR1 transcripts localization analysis provides clues to understand 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 been observed also 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 & 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 & 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), in egg-laying behaviour (Donini & Lange, 2004; Fuchs et al., 2014), in sex pheromone production (Hirashima et al., 2007), in 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 tyramine receptors. In 2000 Kutsukake and co-workers characterized *D. melanogaster hono*, a mutant line with an impaired TAR1, exhibiting a different behaviour towards repellent odours. Furthermore, Li et al. (2017) have showed that TAR1 deficient flies exhibit significant changes in the metabolic control such as higher body fat, lower starvation resistance and movement activity. Similar TAR1-mediated metabolic alterations were observed by Ishida & Ozaki (2011) in starved flies. Nevertheless, the existence of a crosstalk between the tyraminergic system and other systems, such as the octopaminergic and dopaminergic, 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 on *D. suzukii* and *Rhipicephalus microplus* TAR1 receptors. 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 the 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 monoterpenes treatment, in order to understand whether the *DsTAR1* downregulation could affect fitness and physiology. Furthermore, a TAR1 *D. melanogaster* mutant line was used as a control to compare the effects of chronic TAR1 impairment on the physiology in *D. melanogaster* with monoterpenes-treated *D. suzukii* flies.

Material and methods

Fly stocks

Drosophila suzukii was 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 16:8 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, Indiana, USA; 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} mutant line was backcrossed several time with y^1w^{1118}, the control line for all corresponding experiments, as described previously (Li et al., 2017). All *D. melanogaster* flies were raised on standard food at 25 ± 1 °C (12:12 light-dark photoperiod).

Fumigant toxicity assay

A glass cylinder (10 cm in height, 4.5 cm inner diameter; 150 ml) was employed to calculate the monoterpenes LC_{50} values on *D. suzukii* and *D. melanogaster* y^1w^{1118} and to perform the monoterpenes exposure. Monoterpenes including thymol, carvacrol, and α-terpineol were dissolved in acetone and applied to a filter paper (2 cm x 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 - 67 µl/L and acetone alone was used as negative control. After CO_2 anesthetization, thirty flies (fifteen males and fifteen 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 the LC_{50} values calculation, at least one hundred flies were tested, in four replicates.

Quantitative real-time PCR analysis

Total RNA was extracted from *D. suzukii* or *D. melanogaster* y^1w^{1118} adult flies subjected to the monoterpene exposures using Aurum Total RNA Mini Kit (Bio-Rad, USA). One µg of RNA was treated with DNase I (Thermo Fisher, USA) and used for cDNA synthesis, carried out with the OneScript ® cDNA Synthesis Kit (Abm, Canada), according to the manufacturer's instructions. Real time PCR was performed using a CFX Connect Real-Time PCR Detection System (Bio-Rad, USA) in a 12 µl reaction mixture

containing 1.6 µl cDNA (diluted 1:2), 6 µl Sybr PCR Master Mix (Vazyme, China), 0.4 µl forward primer (10 µM), 0.4 µl reverse primer (10 µM) and 3.6 µl nuclease free water. Thermal cycling conditions were: 95 °C for 2 mins, 40 cycles at 95 °C for 15 s and 60 °C for 20 s. After the cycling protocol, a melting-curve analysis from 55 °C to 95 °C was applied. In *D. suzukii* expression of *TAR1* was normalized using *AK* and *TBP* genes that 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

The TAR1-Gal4 *Drosophila* line was crossed with an 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 mins at room temperature. The samples were then washed three times in PBST and blocked for 30 min in blocking buffer (1X PBS + 2 % NP-40 + 10 % goat serum) at room temperature. The samples were incubated with the primary antibodies in blocking buffer (anti-GFP rabbit 1:300 (Sigma-Aldrich, code: AB3080) and anti-Nc82 mouse 1:20 (Developmental Studies Hybridoma Bank, University of Iowa)) overnight at 4 °C and washed three times for 5 min in PBST. Subsequently, the samples were incubated with the secondary antibodies in blocking buffer (donkey anti-rabbit IgG Alexa Fluor-488 1:300 (Jackson ImmunoResearch, code: 711-545-152) and goat anti-mouse IgG Alexa Fluor 555 1:300 (Jackson ImmunoResearch, code: 115-165-003) 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, Germany).

Body fat quantification

Total body triglyceride (TG) content was estimated using the Triglyceride (TG) colorimetric assay kit GPO-PAP method (Elabscience, China). Three flies were accurately weighted and homogenation medium (9 times the volume, phosphate buffer 0.1 mol/L, pH 7.4) was added. The sample was mechanically homogenized on ice with a motorized pestle and centrifugated (at 2500 rpm for 10 min). 7 µl of the supernatant were added to 700 µl of working solution kit, 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 blank. Triglyceride content was estimated using a glycerol solution (2.26 mmol/L) as standard. Five independent biological replicates was performed for each sex and genotype.

Dye-labelling food intake quantification

The dye-labelling food intake quantification was performed as described by Deshpande and co-workers (Deshpande et al., 2014), with minor modifications. In brief, five flies of each sex and genotype were placed into a vial with 2 ml of 1 X dyed medium (2.5 % yeast, 2.5 % sucrose, 1 % agar and 1 % Brilliant Blue FCF – Sigma Aldrich, USA). 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 ul of 1 % PBST and centrifugated for 1 min at 12000 g to clear the debris. The supernatant absorbance was measured at 630 nm on a label-free EnSight Multimode Plate Reader (Perkin Elmer, USA). The values obtained from flies fed with non-labelled food were used as 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

The measurement of the metabolic rate was assessed as described (Yatsenko et al., 2014). In brief, three adult flies were placed in each vial and the metabolic rate was measured for 2 h using the respirometry. The CO_2 yield during the test was calculated based on the µl produced per h 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 two times to move flies to the bottom and the climbing height of flies was photographed after 2 s. The average distance climbed in cm for each fly was measured using Image J software. Five independent biological replicates per sex and genotype were performed.

Starvation resistance assay

The starvation resistance assay was performed placing twenty-five flies of each sex and genotype in 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 one hundred flies).

Statistical analyses

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

Results

Monoterpenes LC_{50} calculation

The results of the LC_{50} estimation as obtained by POLO-plus analyses for each monoterpene, performed on both *D. suzukii* and *D. melanogaster* y^1w^{1118} flies, are summarized in **Table 2**. The table reports the $LC_{50\text{-}90}$ values, the 95% confidence limits (Robertson et al., 2017), the slopes (angular coefficients) of lines and the values of χ^2 for each monoterpene.

TAR1 expression analysis after monoterpenes exposure

To evaluate the effect of the 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 LC_{50} concentrations of thymol, carvacrol and α-terpineol, respectively, and the mRNA levels analyzed 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 (**Figure 1, panel A**) but not for α-terpineol. On the other hand, in *D. melanogaster* y^1w^{1118} all three monoterpenes induced a significant downregulation of *TAR1* although less marked as compared to *D. suzukii* (**Figure 1, panel B**).

Figure 1.

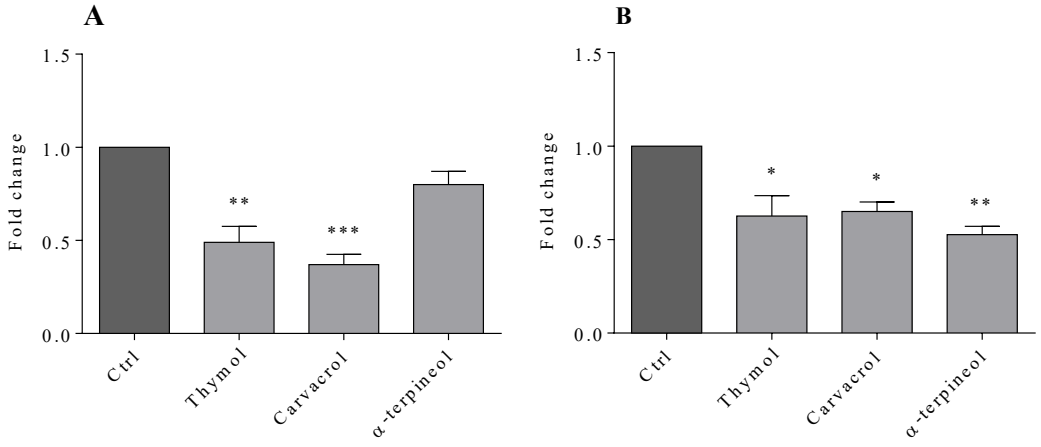

Figure 1. *D. suzukii* (panel A) and *D. melanogaster* y^1w^{1118} (panel B) *TAR1* expression levels after 24 h of continuous exposure to the LC_{50} of thymol, carvacrol and α-terpineol. Data represent means ± SEM of four independent experiments performed in triplicate. *p < .05 **p < .01 ***p< .005 vs 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 gene in *D. melanogaster* y^1w^{1118} analysis (Ponton et al., 2011).

TAR1 expression in *D. melanogaster* brain

In order to determine the physiological functions controlled by TAR1, the receptor accumulation in *D. melanogaster* brains was investigated by immunohistochemistry. The Gal4-UAS system was used to selectively mark TAR1 with the GFP reporter protein, then recognized by the anti-GFP antibody. The receptor showed specific expression in the *pars intercerebralis* as well as lateral horn, sub-esophageal ganglia, mushroom bodies, and antennae mechanosensory - motor center (**Figure 2**), suggesting that TAR1 might be implicated in important physiological traits in *Drosophila*.

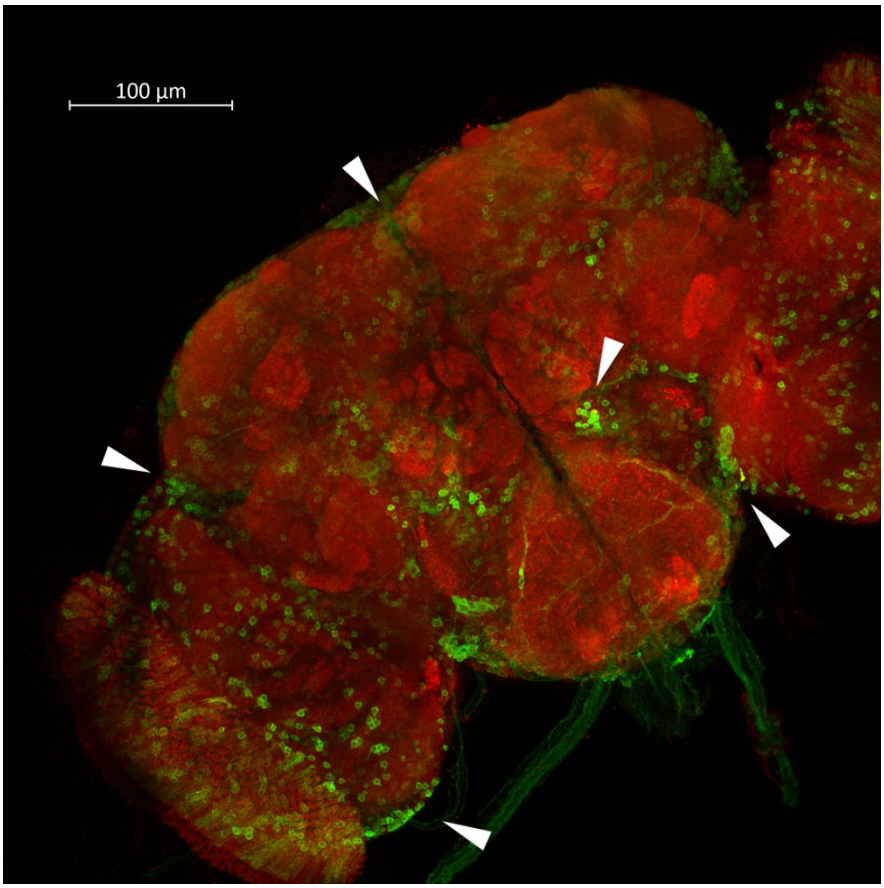

Figure 2. Activity of the TAR1 promoter in the *D. melanogaster* brain. Representative confocal image of GFP driven by TAR1-Gal4: synaptic regions are labelled with the presynaptic marker Nc82 (anti-Bruchpilot), TAR1 is marked by anti-GFP antibody. TAR1 is mainly localized in the *pars intercerebralis*,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

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. Flies with the same genetic background (y^1w^{1118}) were used as controls. In general, the reduced expression of *TAR1* translates into a higher propensity to triglycerides accumulation in male flies (**Figure 3, panel A**) and a greater food intake in both sexes (**Figure 3, panel B**). Therefore, $TAR1^{PL00408}$ flies show higher resistance to

starvation than control (**Figure 3, panel E, F**). These changes are furthermore associated with a slower metabolism in TAR1 impaired insects (**Figure 3, panel C**). The increased triglycerides accumulation and the slower metabolism could also be related to the lower propensity to movement of the *TAR1*^{PL00408} flies (**Figure 3, panel D**).

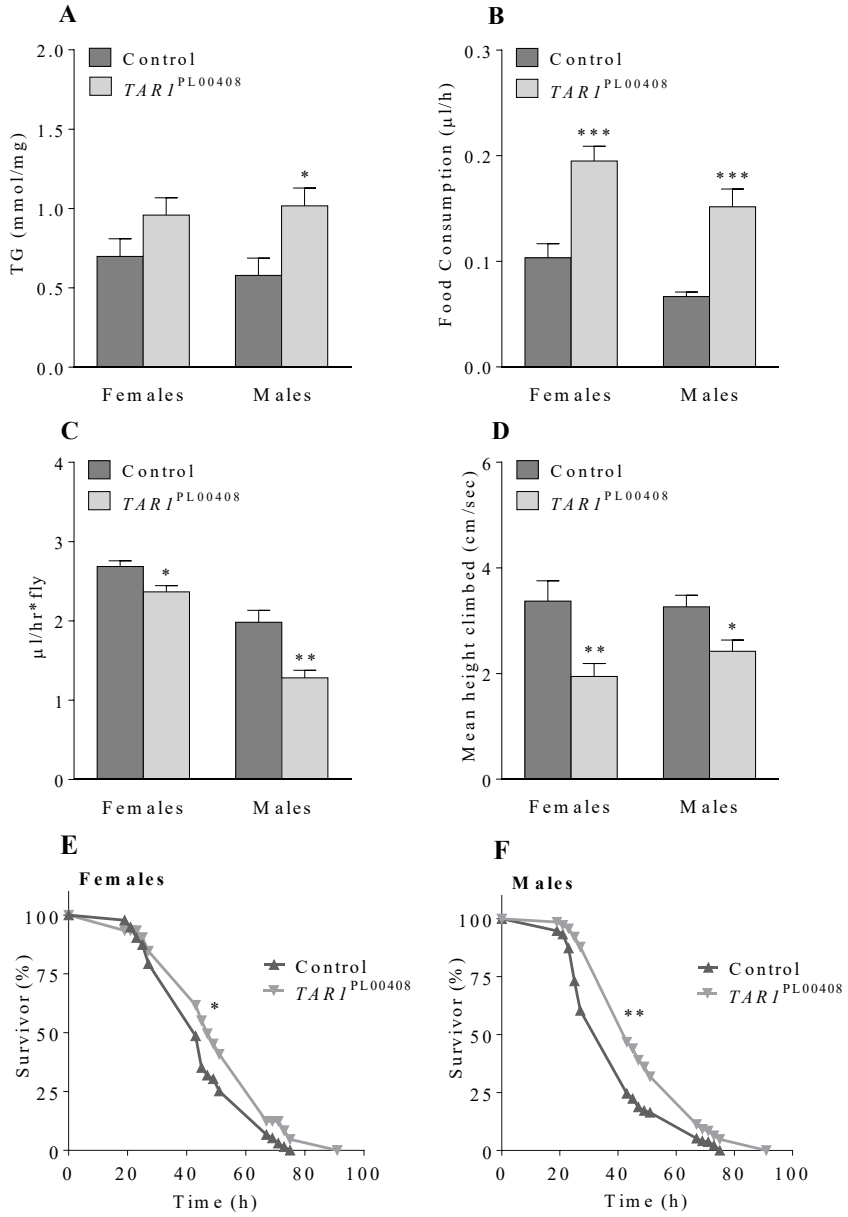

Figure 3. Physiological, metabolic and behavioural alterations in flies with an impaired TAR1. Total body triglyceride (TG) content (panel A), food intake quantification (panel B), metabolic rate (panel C), climbing activity measured by RING assay (panel D) and starvation resistance (panel E,F) were tested in control and TAR1$^{-/-}$ animals of both sexes. For all experiments, means of at least four independent biological replicates ± SEM are shown. *p < .05 **p < .01 ***p< .005 vs control according to student's *t*-test. In starvation resistance, statistical analyses were performed using the log-rank test.

To test whether monoterpenes, besides downregulationg *TAR1*, might also alter the physiology of *D. suzukii* and *D. melanogaster* (wild type or *TAR1*^{PL00408}), flies 24 h after

the continued monoterpenes LC_{50} exposure were challenged with several behavioural tests.

Monoterpenes treatment - effects on total body triglyceride (TG) content

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

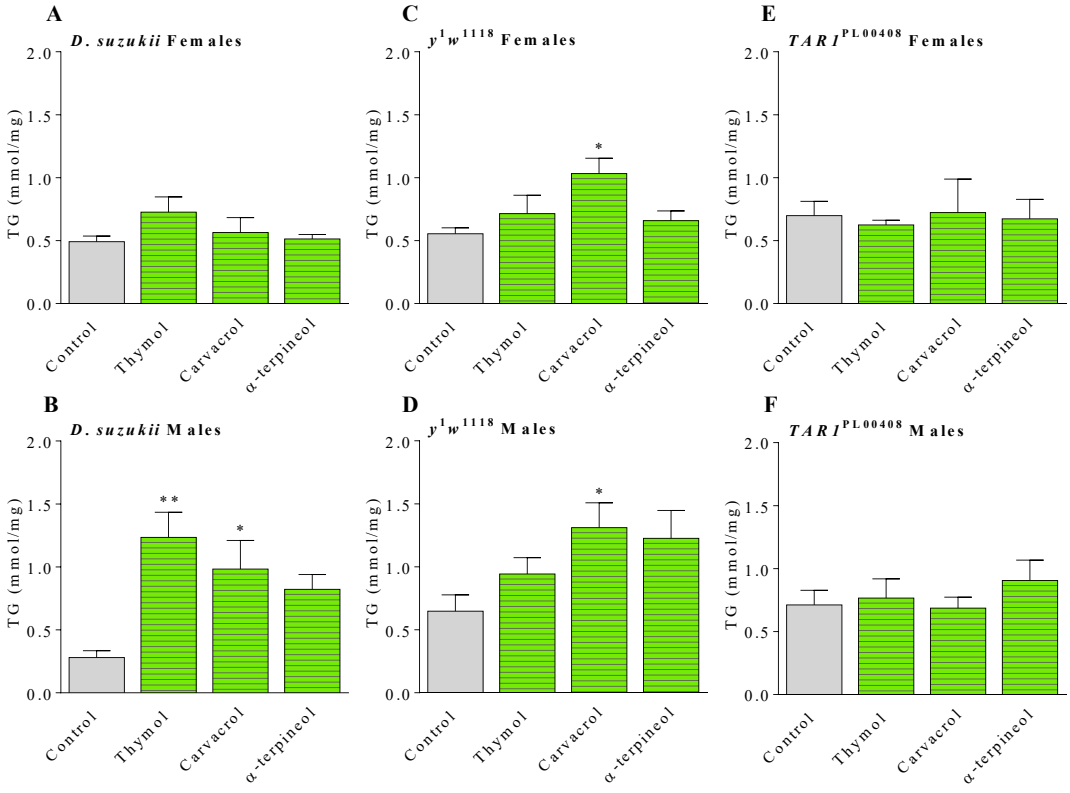

Figure 4. Total body triglyceride (TG) content, after 24 h of exposure to monoterpenes, in *D. suzukii* (panels A and B), *D. melanogaster* y^1w^{1118} (panels C and D) and *D. melanogaster TAR1*^{PL00408} (panels E and F). Data shown are the means ± SEM of four independent biological replicates. *p < .05 **p < .01 vs control according to one-way ANOVA followed by Dunnett's test for multiple comparisons.

Monoterpenes treatment - effects on food intake

The food consumption was quantified after two hours of feeding on a dye-labelled diet. A significantly high food intake was observed only after α-terpineol exposure in both *D. suzukii* and *D. melanogaster* y^1w^{1118} of both sexes (**Figure 5, panels A, B, C and D**). The increased food intake might explain the high triglyceride levels observed in both *D. suzukii* and *D. melanogaster* y^1w^{1118} sexes after monoterpenes exposure. On the other hand, the monoterpene treatments did not cause any change in food consumption in *D. melanogaster TAR1*^{PL00408} mutant flies (**Figure 5, panels E and F**) further suggesting the requirement for an active TAR1.

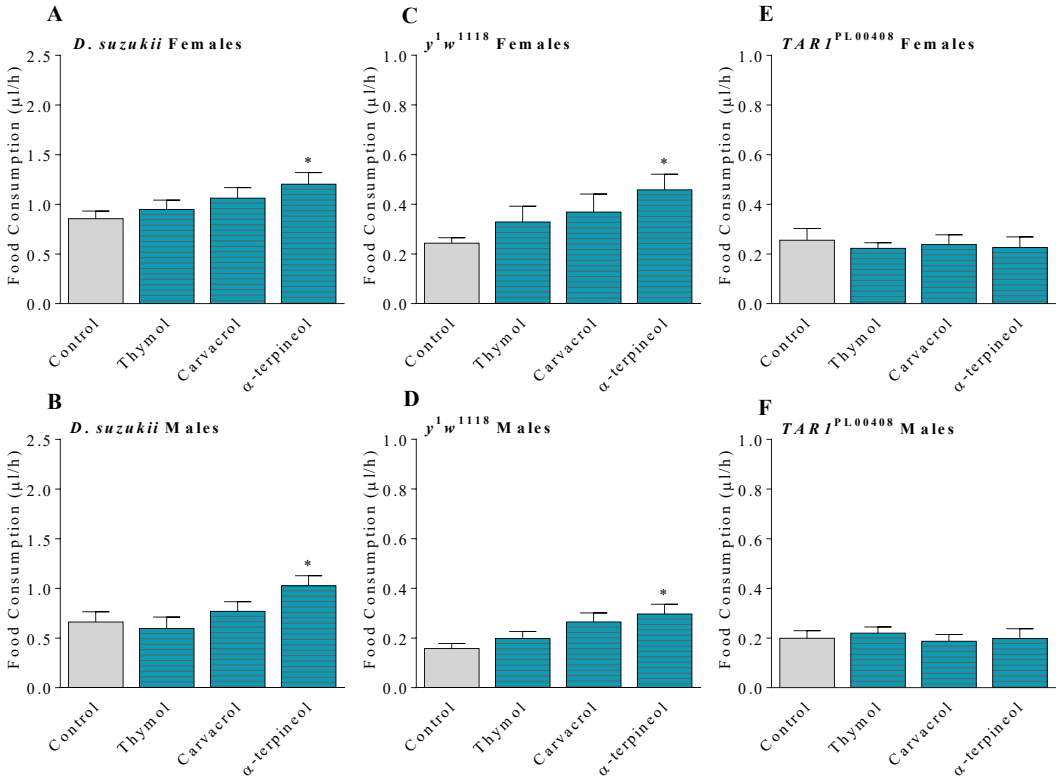

Figure 5. Food intake, after 24 h of exposure to monoterpenes, in *D. suzukii* (panels A and B), *D. melanogaster* y^1w^{1118} (panels C and D) and *D. melanogaster TAR1*^{PL00408} (panels E and F) measured as µl of diet per hour. Data shown are the means ± SEM of five independent biological replicates. *p < .05 vs control according to one-way ANOVA followed by Dunnett's test for multiple comparisons.

Monoterpenes treatment - effects on metabolic rate

In order to determine if the monoterpenes and the *TAR1* downregulation might affect the metabolism, the metabolic rate was analysed in all *D. suzukii* and *D. melanogaster* genotypes after treatment with the different monoterpenes. In *D. suzukii*, only males treated with the three monoterpenes showed a significantly lower metabolic rate than control flies (**Figure 6, panels A and B**). Carvacrol and α-terpineol were able to reduce the metabolic rate in *D. melanogaster* y^1w^{1118} males and females as well (**Figure 6, panels**

C and D). Conversely, *D. melanogaster TAR1*^{PL00408} metabolic rate appeared unaffected by the treatments therefore undistinguishable from that of the untreated controls (**Figure 6, panels E and F**).

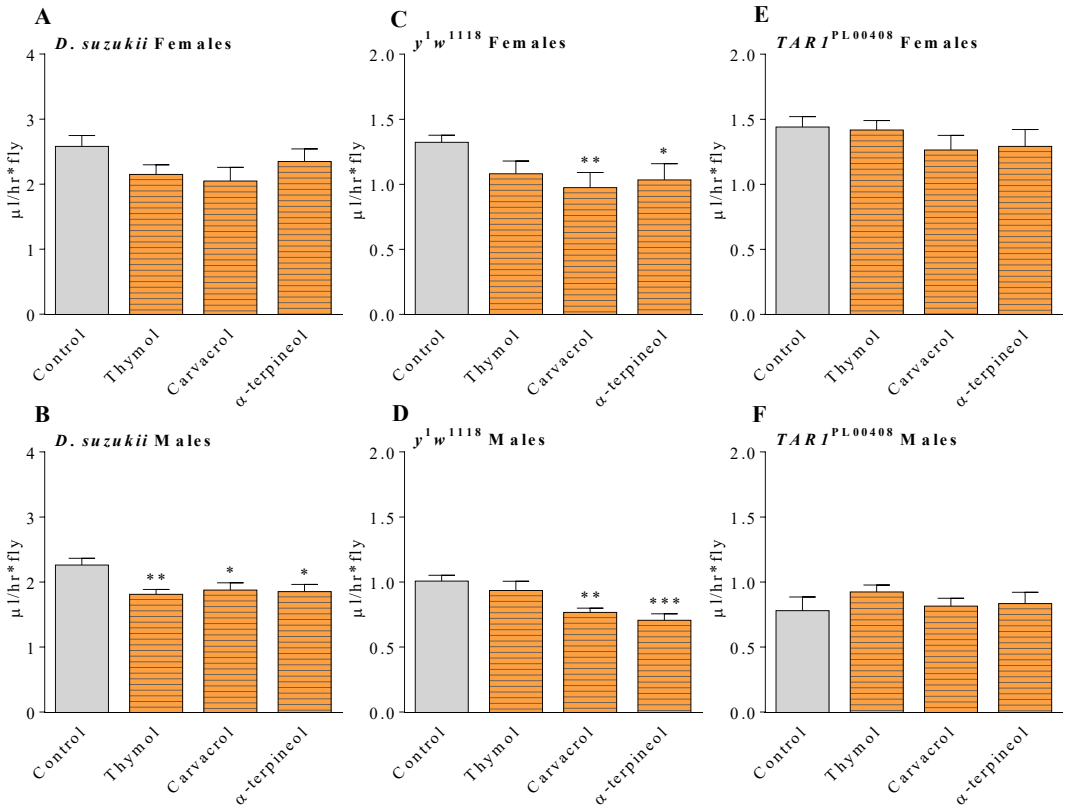

Figure 6. Metabolic rate, after 24 h of exposure to monoterpenes, in *D. suzukii* (panels A and B), *D. melanogaster* y^1w^{1118} (panels C and D) and *D. melanogaster TAR1*^{PL00408} (panels E and F). Data shown are the means ± SEM of five independent biological replicates. *p < .05 **p < .01 ***p< .005 vs control according to one-way ANOVA followed by Dunnett's test for multiple comparisons.

Monoterpene treatment - effects on locomotory activity

The observed metabolic changes in terms of energy expenditure and TG content might also affect flies physical activities. 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 controls, *D. suzukii* and *D. melanogaster* y^1w^{1118} males showed a statistically significant reduction in climbing ability only after α-terpineol treatment (**Figure 7, panels B and D**). *D. melanogaster* y^1w^{1118} females motility was negatively affected only by thymol (**Figure 7, panel C**), while *D. suzukii* females did not respond to the RING assay at all, in both control and treated samples (**Figure 7, panel A**). The climbing ability in both *D. melanogaster TAR1*^{PL00408} sexes was unaffected by the exposure to monoterpenes, confirming the hypothesis of TAR1 involvement in this behavioural trait.

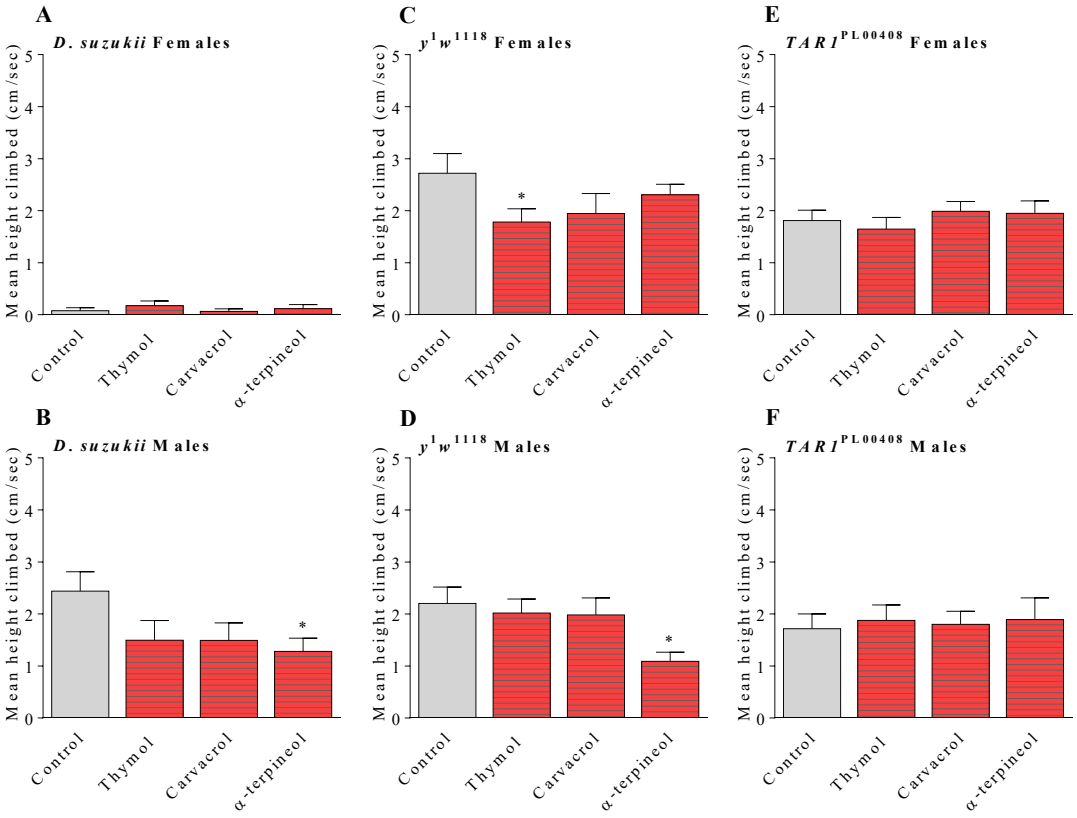

Figure 7. RING assay, after 24 h of exposure to monoterpenes, on *D. suzukii* (panels A and B), *D. melanogaster* y^1w^{1118} (panels C and D) and *D. melanogaster* *TARI*^{PL00408} (panels E and F). The vertical movement capacity for each insect is expressed in cm per second. Data shown are the means ± SEM of five independent biological replicates. *p < .05 vs control according to one-way ANOVA followed by Dunnett's test for multiple comparisons.

Monoterpene treatment - effects on starvation resistance

Finally, a starvation resistance assay was performed to investigate whether the monoterpene-mediated metabolic modifications could affect the general fitness. Given the higher food intake and TG content caused by the treatment, an enhanced starvation resistance was expected. *D. suzukii* and *D. melanogaster* y^1w^{1118} showed different results depending on the monoterpene used as compared to control (**Figure 8, panels A, B, C and D**). According to log-rank statistical analysis, a significant reduction in starvation resistance was detected in *D. suzukii*, both males and females, after carvacrol treatment (**Figure 8, panels A and 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 flies (**Figure 8, panels C and D**). Conversely, the carvacrol exposure significantly increased the starvation resistance in *D. melanogaster* y^1w^{1118} males (**Figure 8, panel C**). *D. melanogaster* *TARI*^{PL00408} mutant

were again unaffected by the treatment, thus showing starvation resistance comparable to controls (**Figure 8, panels E and F**).

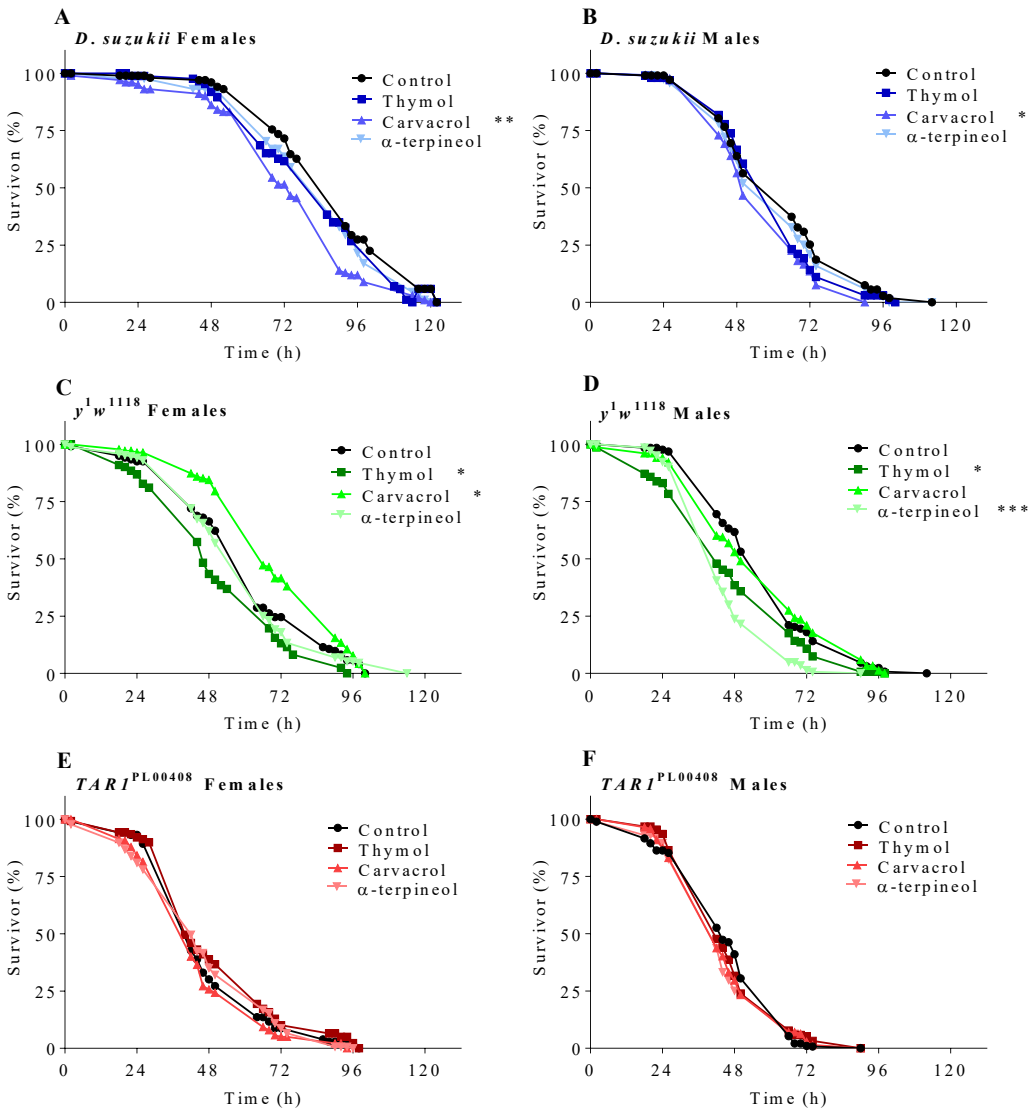

Figure 8. Starvation resistance, after 24 h of exposure to monoterpenes, on *D. suzukii* (panels A and B), *D. melanogaster* y^1w^{1118} (panels C and D) and *D. melanogaster* $TAR1^{PL00408}$ (panels E and F). Five independent biological replicates were performed with the log-rank test statistical analysis. *p < .05, **p<.01, ***p<.005 vs control.

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, the tyramine receptors TAR (Saudou et al., 1990; Roeder et al., 2003). TAR1 is highly expressed in the central nervous system (CNS) of numerous insects, thus suggesting its involvement in essential behavioural processes (El-Kholy et al., 2015; Hana & Lange, 2017; Finetti et al., 2020). Furthermore, several studies showed that TAR1 could be a direct target for biomolecules with insecticidal action, such as monoterpenes. In fact, it has been reported that the *D. melanogaster* and *R. microplus* TAR1s, 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 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 mutant line (*TAR1*^{PL00408}) was evaluated together with matching controls and *D. suzukii*. Comparative studies using these two *Drosophila* species are possible since 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 a LC_{50} of 0.844 µl/L for *D. suzukii* and 0.592 µl/L 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 % reduced mortality was observed as compared to 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, after 24 h of fumigant treatment, were able to induce a TAR1 downregulation not only in *D. suzukii* (as already established, Finetti et al., 2020) but also in *D. melanogaster*. Since 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 confirmed also 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 lateral horn, sub-esophageal 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 center should be composed by 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 (Velasco et al., 2006). $TAR1^{PL00408}$ 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; Li et al., 2017). These metabolic alterations were not sex dependent, although the effects in $TAR1^{PL00408}$ males appeared to be more pronounced as compared to those seen in females. This could be related to sex-dependent differences in TAR1 expression, whose mRNAs accumulated at higher levels in males than in females (Finetti et al., 2020). Despite all this, little is still known on 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, at the same time, one of the most important metabolic centers (Arrese & Soulages, 2010). Lipid storage and release are mainly controlled by two hormones, the *Drosophila* insulin-like peptides (mainly dILP2) and the AKH (Adipokinetic hormone, analogous to the 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 & Woodring, 1991; Orchard et al., 1993). In fact, it has recently been observed that in *C. elegans*, during acute stress, TA accumulates, which in turn modulates insulin signal (De Rosa et al., 2019). Therefore, increased TG level observed in $TAR1^{PL00408}$, as compared to 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 reduction of dILP2 in insulin-producing cells in the *D. melanogaster pars intercerebralis* and an increased 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 & Ozaki, 2010). For example, *honoka* flies showed a reduced sugar response (Damrau et al., 2019) linked to differences in food intake. It is worth noting that TAR1 is highly expressed in neurons located in the sub-esophageal ganglia that are presumably associated with the salivary glands and neck muscles control, thus linked with feeding.

After monoterpene treatments, 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 monoterpenes-induced behavioural changes and TAR1. TAR1-mediated physiological alterations due to monoterpenes were also observed in *P. regina*. In fact, D-limonene treatment decreased TA levels in *P. regina* brain, causing a direct modification of the food intake (Nishimura et al., 2005). This different response to food stimuli was subsequently attributed to a probable alteration of the TAR1 expression at the level of the sub-exophageal ganglion (Yshida & 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 demonstrated that monoterpenes, in addition to their insecticidal properties, can modify the metabolism and fitness of surviving *D. suzukii* opening to innovative applications of these molecules in the pest control.

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Table 1. Primers used in this study.

Primers	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

Table 2.

D. suzukii				
Compound	Slope (± SE)	LC_{50} (95% CI) µl/L	LC_{90} (95% CI) µl/L	χ^2
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}				
Compound	Slope (± SE)	LC_{50} (95% CI) µl/L	LC_{90} (95% CI) µl/L	χ^2
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

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

Supplementary figure

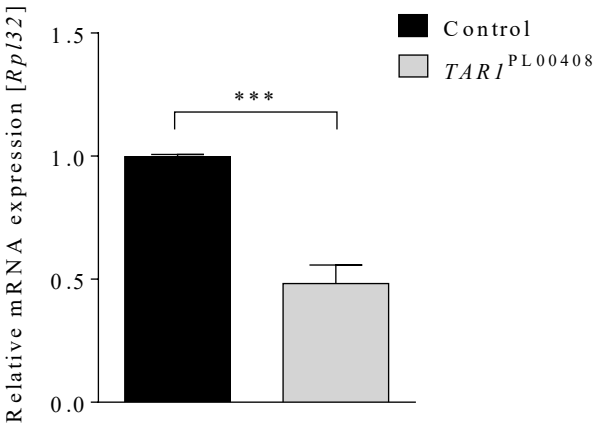

Figure S1. *TAR1* expression levels in D. melanogaster y^1w^{1118} and $TAR1^{PL00408}$. Data represent means ± SEM of five independent experiments performed in triplicate. ***p< .005 vs control according to student's t-test. *Rpl32* was used as reference genes (Li et al., 2017).

Characterization of *Halyomorpha halys* TAR1 reveals its involvement in (*E*)-2-decenal pheromone perception.

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Abstract

In insects, the tyramine receptor 1 (TAR1) has been shown to control several physiological functions, including olfaction. We investigated the molecular and functional profile of the *Halyomorpha halys* type 1 tyramine receptor gene (*HhTAR1*) and its role in olfactory functions of this pest. Molecular and pharmacological analyses confirmed that the *HhTAR1* gene codes for a true TAR1. The RT-qPCR analysis revealed that *HhTAR1* is expressed mostly in adult brain and antennae as well as in early development stages (eggs, 1st and 2nd instar nymphs). In particular, among the antennomeres that compose a typical *H. halys* antenna, *HhTAR1* was more expressed in flagellomeres. Scanning electron microscopy (SEM) investigation revealed the type and distribution of sensilla on adult *H. halys* antennae: both flagellomeres appear rich in trichoid and grooved sensilla, known to be associated with olfactory functions. Through a RNAi approach, topically delivered *HhTAR1* dsRNA induced a 50 % gene downregulation after 24 h in *H. halys* 2nd instar nymphs. An innovative behavioral assay revealed that *HhTAR1* RNAi-silenced 2nd instar nymphs were less susceptible to the alarm pheromone component (*E*)-2 decenal as compared to control. These results provide critical information concerning the TAR1 role in olfaction regulation, especially alarm pheromone reception, in *H. halys*. Furthermore, considering the emerging role of TAR1 as target of biopesticides, this work opens the way for further investigation on innovative methods for controlling *H. halys*.

Keywords: Brown Marmorated Stink Bug, TAR1 receptor, Antennae, Olfaction, Behavior, RNAi.

Introduction

Identifying volatile compounds through the olfactory system allows insects to find food sources, avoid predators as well as localize putative partners and oviposition habitats (Gadenne et al., 2016). Furthermore, the olfactory modulation by volatile molecules with repellent activity could be a promising strategy for pest control (Carey & Carlson, 2011). The basic organization of the olfactory system begins with the antennae, organs possessing cuticular structures, the sensilla, innervated by olfactory sensory neurons (OSNs) (Amin & Lin, 2019). The OSNs recognize different molecules through special olfactory receptors. Each OSN expresses only one type of olfactory receptor, ensuring the specificity of signal for a single odour (Zhao & McBride, 2020). When an OSN is activated, it sends the output signal through the axon to the antennal lobe. Here, excitatory projection neurons (PNs) transport the olfactory information to brain centres such as the mushroom body and the lateral horn (Tanaka et al., 2012). The mushroom body plays an important role in the olfactory learning and memory (Caron et al., 2013) while the lateral horn controls innate olfactory response functions (Jefferis et al., 2007). In insects, the olfactory system can be modulated by exogenous (photoperiod, temperature) and endogenous (hormones) factors.

The biogenic amines tyramine (TA) and octopamine (OA) are present in high levels in the nervous tissue of insects, suggesting their roles as neurotransmitters (Roeder, 2005). Furthermore, TA and OA act also as neurohormones and neuromodulators in a wide variety of physiological processes, acting in a paracrine, endocrine and autocrine way on the cells of the organism (Pauls et al., 2018).

Initially, TA was considered only as a biosynthetic intermediate of OA (Lange, 2009), but later numerous studies showed that TA is indeed an important neurotransmitter (Blenau & Baumann, 2003; Roeder, 2005; Lange, 2009; Roeder, 2020). Among invertebrates, TA is the endogenous agonist of the tyramine receptors (TARs). Structurally, TARs receptors are part of the superfamily of G protein-coupled receptors (GPCR) sharing the typical structure with seven transmembrane domains (Ohta & Ozoe, 2014). Several studies have highlighted that TARs can by coupled with both G_q (increasing intracellular calcium levels) and G_i proteins (decreasing cAMP levels) (Saudou et al., 1990; Blenau et al., 2000; Enan, 2005; Rotte et al., 2009). Based on the rank order of potency of agonists, the TAR receptors have been classified into three different types (Wu et al., 2014): TAR1, coupled with G_q and G_i proteins and TAR2, coupled only with G_i protein, while TAR3 has been so far described only in *Drosophila*

melanogaster (Bayliss et al., 2013; Wu et al., 2014). The first TAR1 was characterized in 1990 in *D. melanogaster* (Saudou et al., 1990). The receptor, called Tyr-dro, showed higher affinity (12-fold) for TA than for OA and was mainly expressed in heads. Since then the same receptor has been characterized in several orders of insects: Hymenoptera (Blenau et al., 2000), Orthoptera (Poels et al., 2001), Lepidoptera (Ohta et al., 2003), Hemiptera (Hana & Lange, 2017a) and Diptera (Finetti et al., 2020).

Several physiological and behavioral functions are controlled by TAR1, including olfaction. In 2000 Kutsukake et al. (2000) characterized *honoka*, a *D. melanogaster* strain that presented a TAR1 mutation and a compromised olfactory profile. These insects were not able to localize repellent stimuli suggesting that TAR1 could be involved in this physiological response. Furthermore, RNAi-mediated modulation of TAR1 expression was shown to affect the gregarious and solitary phase change through a different olfactory sensibility to attractive and repulsive volatiles (Ma et al., 2015). In honeybee antennae, an upregulation of TAR1 was observed during the transition from nurses to pollen foragers, suggesting a TAR1-regulation in their behavioral plasticity (McQuillan et al., 2012). High TAR1 levels were also found in the antennae of *Mamestra brassicae* and *Agrotis ipsilon*, further suggesting a pivotal role of this receptor in olfactory modulation (Brigaud et al., 2009; Duportets et al., 2010).

The TAR1s are considered interesting target for insecticides, especially bioinsecticides. Amitraz is an acaricide and non-systemic insecticide that targets the OA receptors. However, recent studies have shown that Amitraz can exert its toxic effect also through TAR1 activation (Wu et al., 2014; Kumar, 2019). Furthermore, a secondary metabolite of Amitraz, BTS-27271, increases the TA response on the *Rhipicephalus microplus* TAR1 (Gross et al., 2015). Concerning biopesticides, in the last years several studies have showed that monoterpenes could interact and activate, directly or indirectly, TAR1s. In detail, Enan (2005) was the first to describe an agonist effect of several monoterpenes (thymol, carvacrol, α-terpineol, eugenol) on the *D. melanogaster* TAR1. However, the same monoterpenes did not show the same pharmacological profile on *D. suzukii* and *R. microplus* TAR1 receptors where they act as positive allosteric modulators (Gross et al., 2017; Finetti et al., 2020).

Halyomorpha halys (Rhyncota; Pentatomidae), an insect typical of the Eastern Asia (China, Japan, Taiwan, and Korea) (Haye et al., 2015), was detected for the first time in USA in 1998 (Hoebeke & Carter, 2003) and became a stable presence in orchards since 2010 (Rice et al., 2014). Its first European appearance was reported in 2004 in

Switzerland then leading to its spread across the continent (Cesari et al., 2018). *H. halys* is responsible for major damages to many economically relevant crops (Leskey & Nielsen, 2018). The damages are caused by the perforation of the external integuments of fruits by the rostrum, the specialized sucking apparatus typical of Rhynchota. This causes necrotic areas on fruits, as well as the transmission of other phytopathogens, leading to a relevant decrease in the product value (Peiffer & Felton, 2014). In the Asiatic regions, the life cycle of *H. halys* consists of only one generation per year (Lee et al., 2013). However, in warmer regions, the insect is able to complete up to four annual generations, significantly increasing its number in the area (each female is able to lay between 100 and 500 eggs for cycle) (Nielsen et al., 2016). This relevant pest shows high resistance to common pesticides, making difficult its control and elimination (Bergmann & Raupp, 2014).

The present work aims to characterize the *H. halys* TAR1. Based on studies performed in *D. melanogaster* (Kutsukake et al., 2000), *M. brassicae* (Brigaud et al., 2009) and *A. mellifera* (Mustard et al., 2005; Thamm et al., 2017; Sinakevitch et al., 2017) we advanced the hypothesis that the *H. halys* TAR1 could be involved in the olfactory perception. First, we identified and characterized *H. halys* TAR1 (*HhTAR1*) gene. Afterwards, we tested the ability of RNAi *HhTAR1*-mediated downregulated insects to respond to the alarm pheromone (*E*)-2-decenal compared to control insects. These findings may shed light on the TAR1 importance in *H. halys* pheromone perception and contribute to develop new TAR1-targeting control tools.

Materials and Methods

Insects and reagents

Individuals of *H. halys* were reared on green beans and kiwi with a photoperiod of 16 h light: 8 h dark, at a temperature of 24 ± 1 °C. Tyramine hydrochloride, octopamine hydrochloride, yohimbine hydrochloride, γ-aminobutyric acid, serotonin hydrochloride, epinephrine, norepinephrine, brilliant black, Bovine Serum Albumin (BSA), probenecid, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), (*E*)-2-decenal were all obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Dopamine was obtained from Tocris Bioscience (Bristol, United Kingdom). Pluronic acid and fluorescent dye Fluo-4 AM were purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). All compounds were dissolved in dimethyl sulfoxide (10 mM) and stock solutions were kept at -20 °C until use. Serial solutions were made in the assay buffer (Hanks' Balanced Salt solution (HBSS)/HEPES 20 mM buffer, containing 0.01 % BSA and 0.1 % DMSO.

Isolation and cloning of full-length HhTAR1

Sequence alignment by BLASTN performed with the orthologous gene RpTAR1 (GenBank Accession: MF377527.1; Hana & Lange, 2017) from *Rhodnius prolixus*, suggested that the putative transcript (Accession: XM_014422850.2) predicted in the *H. halys* genome project (Accession: PRJNA298780) may code for a putative HhTAR1 (Accession: XP_014278336.1).

Total RNA was extracted from four adults of *H. halys* using RNAgent® Denaturing Solution (Promega, Madison, Wisconsin, USA), quantified in a micro-volume spectrophotometer Biospec-Nano (Shimadzu, Kyoto Japan) and analysed by 0.8 % w/v agarose gel electrophoresis. One μg of RNA was treated with DNase I (Thermo Fisher Scientific) and used for the synthesis of cDNA, carried out with the OneScript® Plus cDNA Synthesis Kit (ABM, Richmond, Canada). For amplification of the full *HhTAR1* open reading frame (ORF), specific primers were designed based on the annotated transcript (XM_014422850.2). The Kozak translation initiation sequence (GCCACC) was inserted at 5' end of the receptor (**Table 1**). High fidelity amplification was achieved using Herculase II Fusion DNA Polymerase (Agilent, Santa Clara, California, USA) and a touchdown thermal profile: predenaturation at 95 °C for 3 mins, followed by 10 cycles at 95 °C for 20 s, 65-55 °C for 20 s (minus 1 °C/cycle), 68 °C for 2 mins, 30 cycles at 95 °C for 20 s, 55 °C for 20 s, 68 °C for 2 mins and a final extension at 68 °C for 4 mins. The PCR product was gel purified by Illustra GFX PCR DNA and Gel Band Purification

Kits (GE Healthcare, Chicago, Illinois, USA), cloned into pJET 1.2/blunt vector (Thermo Fisher Scientific) and transformed into *Escherichia coli* SIG10 5-α Chemically Competent Cells (Sigma-Aldrich). Positive clones were selected using LB broth agar plates with 100 µg/ml ampicillin. Plasmid was then extracted by GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich) and verified by DNA sequencing (BMR Genomics, Padua, Italy). The sequence, named *HhTAR1*, was deposited in GenBank with the accession number MT513133. For expression in Human Embryonic Kidney (HEK 293) cells, the open reading frame of *HhTAR1* was excised from pJET 1.2 vector and inserted into the pcDNA 3.1 (+) Hygro expression vector using *Xho I* and *Xba I* restriction sites.

Multiple sequence alignment and general bioinformatics analysis

Multiple protein sequence alignments between the deduced amino acid sequence of HhTAR1 and other type 1 tyramine receptor sequences were performed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and BioEdit Sequence Alignment Editor 7.2.6.1. Phylogenetic neighbour-joining analysis was performed by MEGA software (version 7) with 1000-fold bootstrap resampling. The *D. melanogaster* GABA B receptor (GABABR) was used as an outgroup to root the tree.

HhTAR1 transient expression in HEK 293

HEK 293 cells were grown at 37 °C and 5 % CO_2 in Dulbecco's modified Eagles medium high glucose (D-MEM) supplemented with 10 % fetal bovine serum (Euroclone, Milan, Italy). To prevent bacterial contamination, penicillin (100 U/ml) and streptomycin (0.1 mg/ml) were added to the medium. The cells were transiently transfected with pcDNA 3.1 (+) / HhTAR1 in T75 cell culture flasks (Euroclone) using JetOPTIMUS (Polyplus-Transfection, New York, New York, USA), following the manufacturer's protocol. Cells were incubated in the transfection medium for 24 h at normal cell growth conditions before their use for the calcium mobilization assay.

Calcium mobilization assay

Cells were seeded at a density of 50,000 cells per well, total volume of 100 µl, into poly-D- lysine coated 96-well black, clear-bottom plates. After 24 h incubation at normal cell culture condition, the cells were incubated with HBSS 1X supplemented with 2.5 mM probenecid, 3 µM of the calcium sensitive fluorescent dye Fluo-4 AM and 0.01 % pluronic acid, for 30 mins at 37 °C. After that, the loading solution was removed and

HBSS 1X supplemented with 20 mM HEPES, 2.5 mM probenecid and 500 µM brilliant black were added. Cell culture and drug plates were placed into the fluorometric imaging plate reader FlexStation II (Molecular Devices, Sunnyvale, California, USA) and fluorescence changes were measured after 10 mins of stabilization at 37 °C. On-line additions were carried out in a volume of 50 µl/well after 20 s of basal fluorescence monitoring. In antagonism protocols, to facilitate drug diffusion into the wells the assays were performed at 37 °C with three cycles of mixing (25 µl from each well moved up and down three times). The fluorescence readings, expressed in FIU (fluorescent intensity unit), were measured every two s for 120 s.

Quantitative real-time PCR analysis

Total RNA was extracted from whole bodies of *H. halys* samples at various developmental stages (eggs, 1st to 5th instar nymphs, adult males and females) and different organs (antennae, brain, midgut, reproductive organs) using RNAgent® Denaturing Solution (Promega). Total RNA for each biological replicate was extracted from 20 eggs, ten 1st instar nymphs, six 2nd or 3rd instar nymphs, three 4th or 5th instar nymphs or adults, 30 antennae, 6 brains, 6 midguts, 12 testicles or ovary, 40 antennae regions, respectively. The organs of *H. halys* were dissected in a RNA preservation medium (20 mM EDTA disodium (pH 8.0), 25 mM sodium citrate, 700 g/l ammonium sulphate, final pH 5.2). One µg of purified RNA was then treated with DNase I (Thermo Fisher Scientific) and used for cDNA synthesis, carried out with OneScript® Plus cDNA Synthesis Kit (ABM), according to the manufacturer's instructions. Real time PCR was performed using a CFX Connect Real-Time PCR Detection System (Bio-Rad) in a 12 µl reaction mixture containing 0.8 µl of the cDNA obtained from 1 µg of total RNA, 6 µl ChamQ SYBR qPCR Master Mix (Vazyme, Nanchino, China), 0.4 µl forward primer (10 µM), 0.4 µl reverse primer (10 µM) and 3.6 µl nuclease free water. Thermal cycling conditions were: 95 °C for 2 mins, 40 cycles at 95 °C for 15 s and 60 °C for 20 s. After the cycling protocol, a melting-curve analysis from 60 °C to 95 °C was applied. Expression of *HhTAR1* was normalized in accordance with the relative quantitation method (Larionov et al., 2005) using *ARP8* and *UBE4A* as reference genes (Bansal et al., 2016). Gene-specific primers (**Table 1**) were used and at least three independent biological replicates, each made in triplicate, were performed for each sample.

Antennae preparation and SEM analysis

Preliminary morphological investigations were performed on ten adults of *H. halys* (five males and five females) using a Nikon SMZ 800 stereomicroscope (Nikon Instruments Europe, Amsterdam, The Netherlands), provided with a Nikon Digital Sight Ds-Fil camera (Nikon Instruments Europe, Amsterdam, The Netherlands) and connected to a personal computer with the imaging software NIS Elements Documentation (Nikon Instruments Europe, Amsterdam, The Netherlands). Based on stereomicroscope observations, the head was dissected from body and prepared for scanning electron microscopy (SEM), according to previously published procedures (Pezzi et al. 2015, 2016). Afterwards, samples were critical point dried in a Balzers CPD 030 dryer (Leica Microsystems, Wetzlar, Germany), glued on stubs, and coated with gold-palladium in an S150 Edwards sputter coater (HHV Ltd, Crawley, United Kingdom). The SEM observations were conducted at the Electronic Microscopy Centre of the University of Ferrara, using a Zeiss EVO 40 SEM (Zeiss, Milan, Italy).

Synthesis of dsRNA and *H. halys* treatment

For RNAi silencing, *HhTAR1* and *LacZ* (control) amplicons, 400-500 bp long, were generated by PCR using primers with 5' extensions containing T7 promoters (**Table 1**). These products were cloned into pJET 1.2 vector (Thermo Fisher Scientific) and then used as templates for *in vitro* dsRNA synthesis performed by T7 RNA Polymerase (Jena Bioscience, Jena, Germany), according to the manufacturer's protocol. After one hour of synthesis at 37 °C, a DNase I (Thermo Fisher Scientific) treatment was performed and the dsRNA was cleaned up by ammonium acetate precipitation (Rouhana et al., 2013). Finally, the dsRNA was resuspended in ultrapure water and quantified by Biospec-Nano spectrophotometer. To induce RNAi silencing 2nd stage nymphs of *H. halys* 3 days post-ecdysis were treated with 100 ng of dsTAR1 or dsLacZ in 1 µl of solution using a 0.1-2 µl micropipette. The dsRNA molecules were topically delivered through a drop placed on the abdomen of nymphs (**Supplementary figure 4**). Insects were tested by behavioral assay after 24 h while the *HhTAR1* transcript level was measured by RT-qPCR, as described above.

Repellence assay

An open petri dish (90 mm x 15 mm), containing 24 h starved *H. halys* 2nd instar nymphs and a green bean, was placed inside a plexiglass box (50 cm each side) with two lateral openings covered by nets to allow air circulation. The negative control acetone or the

positive repellent control (*E*)-2-decenal were applied to a filter paper (1 cm x 1 cm) that was placed under the green bean. The positive control (*E*)-2-decenal, dissolved in acetone, was tested at a fixed quantity of 10 µg, a value ensuring the maximum repellence activity against the *H. halys* nymphs (Zhong et al., 2018). The number of *H. halys* nymphs standing and feeding on the green bean was monitored every ten minutes for one hour. Four biological replicates were made, each including at least ten insects, for both untreated and dsRNA treated *H. halys* nymphs. All experiments were performed in the morning in a behavioral room with a controlled temperature of 24 ± 1 °C.

Data analysis and terminology

All data were elaborated using Graph Pad Prism 6.0 (La Jolla, California, USA). Data are expressed as mean ± SEM of n experiments and were analysed using one- or two-way analysis of variance (ANOVA) followed by Dunnett's or Turkey's test for multiple comparison. In the pharmacological assays, the concentration-response curves were fitted using the four parameters log logistic equation:

$$\text{Effect} = \text{Baseline} + \frac{(E_{max} - \text{Baseline})}{(1+10^{(LogEC_{50} - Log[compound])*Hillslope})}$$

Agonist potency was expressed as pEC_{50}, defined as the negative logarithm to base 10 of the agonist molar concentration that produces 50% of the maximum possible effect of that agonist. Antagonist potency was derived from Gaddum-Schild equation:

$$pA_2 = -\log\left[\frac{CR - 1}{\text{antagonist}}\right]$$

assuming a slope value equal to unity, where CR indicates the ratio between agonist potency in the presence and absence of antagonist (Kenakin, 2014).

Results

Molecular characterization of HhTAR1

The amplified *HhTAR1* sequence was 1347 bp long and coded for a 449 aa polypeptide with a predicted MW of 50.97 KDa and pI of 9.41. About structural domains, both TMHMM v 2.0 software and the Kyte and Doolittle method (Kyte & Doolittle, 1982) suggest seven putative transmembrane domains, as expected for a GPCR. The helixes are flanked by an extracellular N-terminus of 51 residues and an intracellular C-terminus of 18 residues. Furthermore, the HhTAR1 sequence contains a DRY conserved sequence in the TM3, several N-glycosylation sites in the extracellular N-terminus and P-glycosylation sites, 2 specific for PKA and 10 specific for PKC (**Supplementary Figure 1**). These features are important for the correct folding and function of GPCRs (Nørskov-Lauritsen & Bräuner-Osborne, 2015). Moreover, at position 128 in TM3 there is a conserved aspartic acid (D^{128}, shown in **Figure 1, panels A and B**) responsible for the interaction with TA, the endogenous agonist of the TAR1s (Ohta & Ozoe, 2014).

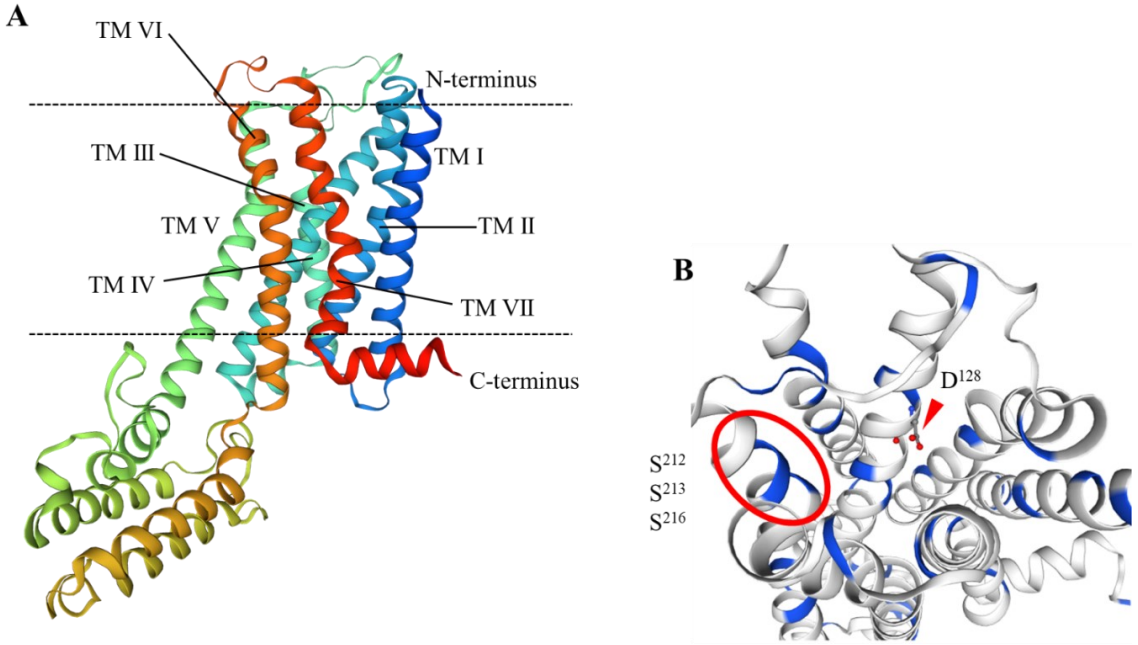


Figure 1. Structural overview of HhTAR1 predicted by SWISS-MODEL. (**A**) Model of the whole receptor showing the transmembrane domains. (**B**) Detail of the putative ligand binding pocket, seen from the extracellular side, of HhTAR1. All serine residues are highlighted in blue. The aspartic acid in TM3 (D^{128}) is shown by a triangle and the three serine residues interacting with TA are highlighted by a circle.

To study the binding site structure, the HhTAR1 aminoacidic sequence was analysed by SWISS-MODEL (Waterhouse et al., 2018). The model was created based on the crystal structure of the human α2A adrenergic receptor (Template code: 6kux.1.A) that shares

33.51 % of sequence identity with HhTAR1. The three-dimensional model of the whole receptor and the putative ligand binding pocket are shown in **Figure 1**. In 2004, several serine residues in TM V were found to play a key role in stabilizing the interaction with TA in *Bombyx mori* and *Sitophilus oryzae* (Ohta et al., 2004; Braza et al., 2019) TAR1. These serine residues localized in the TM V are also conserved in HhTAR1 at positions S^{212}, S^{213} and S^{216} (**Figure 1, panel B**). MolProbity model quality investigation (**Table 2**) confirmed the validity of the SWISS-MODEL 3D model of HhTAR1 (Chenn et al., 2010). The HhTAR1 deduced amino acid sequence was then compared to several OA and TA receptors allowing the construction of a neighbour-joining phylogenetic tree by MEGA 7 server (**Supplementary Figure 2**). As expected, HhTAR1 grouped in the TAR1 family, the main monophyletic group, and shared the highest percentage of identity with the *Rhodnius prolixus* TAR1 (Accession number: MF377527.1), another Pentatomidae. Based on the phylogenetic results, a multiple sequence alignment was performed between the HhTAR1 deduced amino acid sequence and TAR1 from other insects (**Figure 2**). The analysis further strengthen the similarity of HhTAR1 with known TAR1 receptors showing the typical GPCR structure with highly conserved domains corresponding to the transmembrane regions as well as the TA binding site.

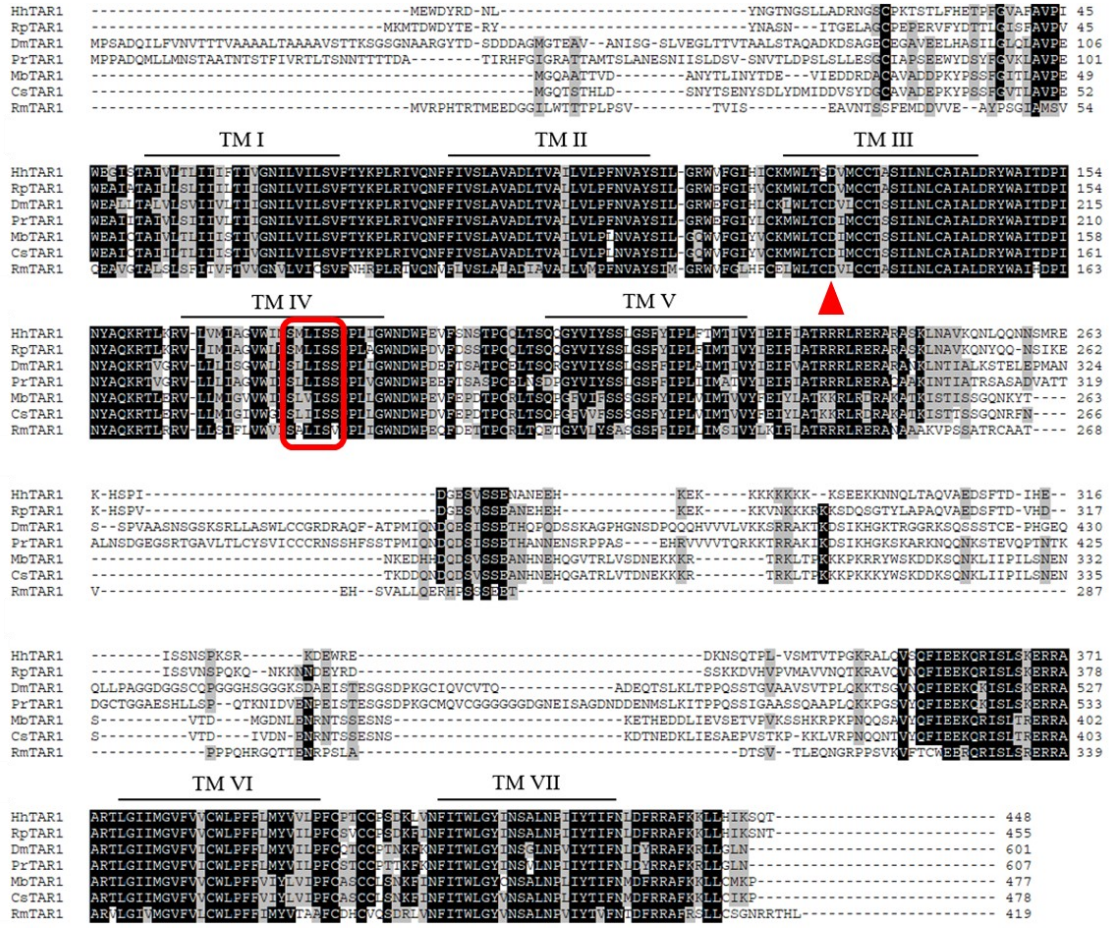


Figure 2. Amino acid sequence alignment of HhTAR1 with orthologous receptors from *R. prolixus* (RpTAR1), *D. melanogaster* (DmTAR1) *Phormia regina* (PrTAR1), *Mamestra brassicae* (MbTAR1), *Chilo suppressalis* (CsTAR1) and *Rhipicephalus microplus* (RmTAR1). The putative seven transmembrane domains (TM I-VII) are indicated with a black line. Identical residues are highlighted in black while conservative substitutions are shaded in grey. A red triangle indicates the conserved aspartic acid D^{128} and the serine residues that could interact with TA are shown by a red box.

HhTAR1: pharmacological validation

In the calcium mobilization assay HhTAR1 was activated by both TA and OA in a concentration-dependent manner (**Figure 3, panel A**). TA evoked the release of intracellular calcium with pEC_{50} values of 5.99 ($CL_{95\%}$ 5.32-6.66) and E_{max} of 109.33 ± 14.86 FIU (fluorescence intensity unit), while OA resulted less potent with a pEC_{50} of 4.41 (4.17-4.64) calculated assuming the TA maximum effect (**Figure 3, panel A**). In wild type HEK 293 cells, TA and OA were completely inactive when tested in the same concentration range (from 10^{-10} M to 10^{-4} M) (data not shown). Afterwards, antagonist studies were performed using yohimbine, the standard α2-adrenergic receptor antagonist, that was demonstrated to antagonize TAR1, in previously studies (Le Corre et al., 2004; Hana & Lange, 2017; Finetti et al., 2020). When tested as HhTAR1 agonist, yohimbine

was completely inactive as agonist, while, at 1 μM, elicited a rightward shift of the concentration response curve to TA (**Figure 3, panel B**); a pA_2 of 8.26 was calculated from these experiments.

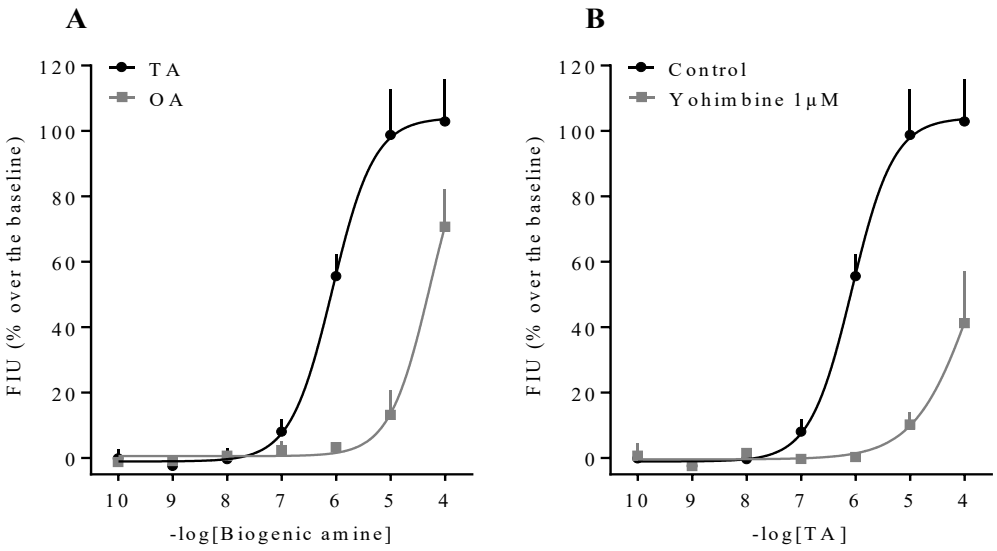


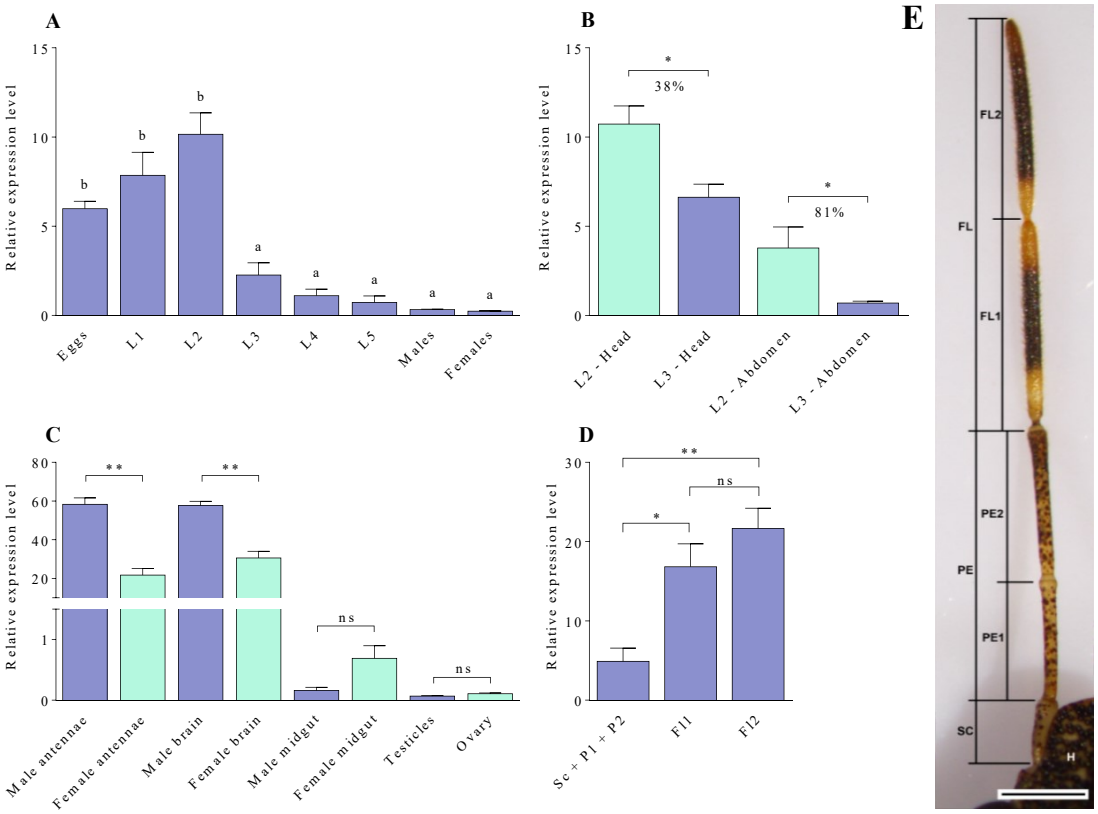
Figure 3. Calcium mobilization assay in HhTAR1-transfected HEK293 cells. Concentration-response curves to TA and OA (**A**). Concentration-response curves to TA in the absence (control) and in presence of 1 μM yohimbine (**B**). Data are means ± S.E.M of three separate experiments performed in duplicate.

In order to confirm the HhTAR1 sensitivity to TA and OA, other biogenic amines such as dopamine, L-DOPA, epinephrine, norepinephrine and serotonin or important neurotransmitter like γ-aminobutyric acid were tested at 10^{-4} M as putative ligands. The amines TA and OA were able to generate a large and robust effect as HhTAR1 agonist while the other molecules, including DA, elicited a negligible calcium release (**Supplementary Figure 3**). DA was able to induce a response when tested at 10^{-4} M on the *Periplaneta americana*, *Chilo suppressalis* and *R. prolixus* TAR1s (Rotte et al., 2009; Wu et al., 2014; Hana & Lange, 2017). However, even in these studies the signal was modest, and it was hypothesized that the responses were probably due to the activation of endogenous dopaminergic receptors present in the cell lines used to express TAR1s.

HhTAR1 expression pattern

Given the importance of TAR1s in insect physiology and behavior, *HhTAR1* expression profile was studied in all *H. halys* development stages (egg, 1st to 5th instar nymphs, L1 to L5, and adult) as well as in the major organs of the adult. The analysis revealed that *HhTAR1* was mostly expressed in eggs and in 1st and 2nd instar nymphs, with a dramatic

decrease in receptor mRNA levels in the later stages from the 3rd instar nymph to adult (**Figure 4, panel A**). This mRNA reduction in 2nd and 3rd instar nymphs was further investigated. The nymphs were divided in two parts: head + antennae and thorax + abdomen and the *HhTAR1* expression levels analysed. The *HhTAR1* mRNA level decrease affected both sections of 2nd and 3rd instar nymphs with different intensity (**Figure 4, panel B**): the level in head/antennae decreased only by 38% between L2/L3, while it dropped by 82 % in thorax/abdomen. This reveals that *HhTAR1* levels remain high in the nervous tissues while they decrease significantly in the rest of the nymph body.



Among the different organs analysed (antennae, brains, midguts and gonads), the highest levels of *HhTAR1* transcript were detected in the brains and the antennae of both sexes, even if they were statistically more abundant in male tissues (**Figure 4, panel C**). Furthermore, *HhTAR1* expression was investigated in all antennomeres of *H. halys*. The antenna is in fact composed by a scape (SC), two pedicels (PE1 and PE2) and two flagellomeres (FL1 and FL2) (**Figure 4, panel E**). The *HhTAR1* mRNA was detected in all antennomeres but it was 2-3 times more abundant in FL1 and FL2 in comparison to SC and both elements of pedicel (**Figure 4, panel D**).

Figure 4. mRNA expression levels of *HhTAR1* gene. (**A**) Expression of *HhTAR1* gene in all development stages: eggs, 1st to 5th instar nymphs (L1 to L5), adult male and female. (**B**) Expression of *HhTAR1* in

different parts of 2nd (L2) and 3rd (L3) *H. halys* instar nymphs. (**C**) Expression of *HhTAR1* gene in organs of both sexes. (**D**) Expression of *HhTAR1* in different parts of adult *H. halys* antennae. Data represent means ± S.E.M of at least three independent experiments performed in triplicate. * $p < 0.05$ ** $p < 0.01$ according to one-way ANOVA followed by multiple comparisons Bonferroni post-hoc. (**E**) Antenna structure of the adult *H. halys* observed on a stereomicroscope. FL, flagellum; FL1, first segment of flagellum; FL2, second segment of flagellum; H, head; PE, pedicel; PE1, first segment of pedicel; PE2, second segment of pedicel; SC, scape.

Sensilla investigation by S.E.M

The different expression of *HhTAR1* in antennomeres required a further characterization of the antenna. The antennae, the main organs of the olfactory system in insects, are rich in sensilla whose morphology correlates with their physiological role. We investigated by scanning electron microscopy (S.E.M) the morphology and distribution of sensilla in the different parts of adult *H. halys* antennae: scape (SC), two pedicels (PE1 and PE2) and two flagellomeres (FL1 and FL2) (**Figure 4, panel E**). In the SC and both PEs, sporadic basiconic sensilla (BS) (**Figure 5, panels A, B and E**) were visible along with particular perforations classified as pit sensilla (PT), or coeloconic sensilla, found in both PEs (**Figure 5, panels F and G**). Several chaetic sensilla (CH) were observed in the PE2-FL1 junction area (**Figure 5, panel C**). A high number of sensilla was found in both FLs, classified as trichoid (TR) (**Figure 5, panels D and I**), basiconic (BS), or grooved sensilla (**Figure 5, panel H**).

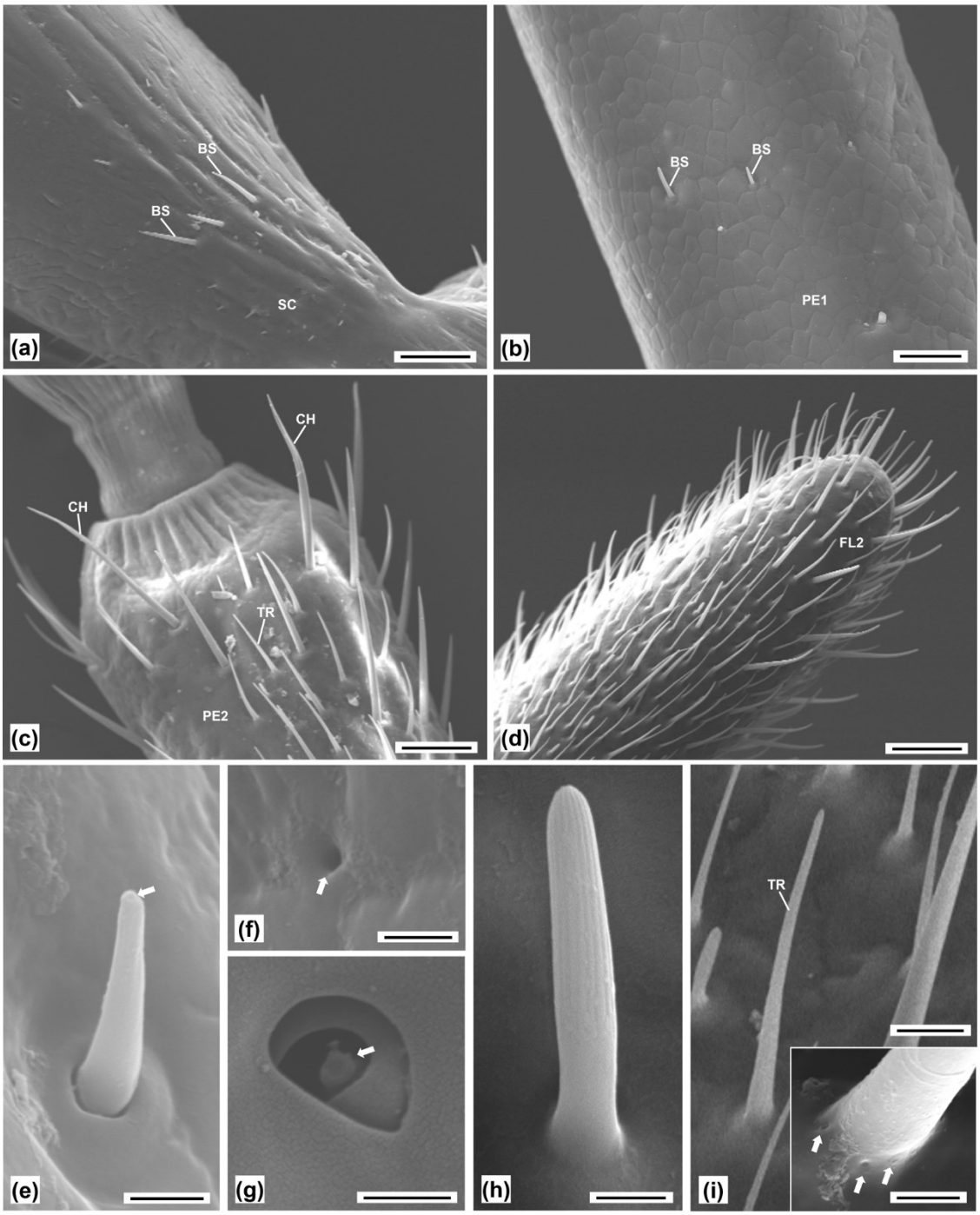


Figure 5. Antennae of adult *H. halys* observed at the scanning electron microscope (S.E.M) (**A-I**). (**A**) Female antenna, detail of the base of the scape. Scale bar 50µm. (**B**) Male antenna, detail of the first segment of the pedicel base of the scape. Scale bar 25µm. (**C**) Male antenna, distal part of the second segment of the pedicel. Scale bar 50µm. (**D**) Male antenna, tip of the second segment of the flagellum. Scale bar 50µm. (**E**) Female antenna, basiconic sensillum of the scape with a tip perforation (arrow). Scale bar 2.5µm. (**F**) Male antenna, perforation of the pedicel (arrow). Scale bar 1.5µm. (**G**) Female antenna, pit sensillum of the pedicel, containing a peg (arrow). Scale bar 1.5µm. (**H**) Female antenna, grooved sensillum of the flagellum. Scale bar 2.5µm. (**I**) Female antenna, trichoid sensillum of the flagellum. Scale bar 10µm. Inlay: detail of the base of the trichoid sensillum, showing microperforations (arrows). Scale bar 2.5µm. Abbreviations: BS, basiconic sensillum; CH, chaetic sensillum; FL2, second segment of flagellum; GR, grooved sensillum; PE1, first segment of pedicel; PE2, second segment of pedicel; SC, scape; TR, trichoid sensillum.

H. halys dsRNA treatment and repellency assay

To investigate the functional role of HhTAR1 in *H. halys* behavior and chemosensory recognition, a behavioral repellence assay was first set up. To *H. halys* 2nd instar nymphs, the developmental stage that showed the highest *HhTAR1* expression levels, a green bean was offered in the presence or absence of the alarm pheromone component (*E*)-2-decenal and the number of individuals feeding or standing on the bean was measured during a period of an hour. The (*E*)-2-decenal, as expected, was able to repel approximately 50 % of the nymphs compared to the acetone-treated group, used as control (**Figure 6, panel A).** Subsequently, to assess the physiological relevance of HhTAR1 in repellency, a RNAi -silencing approach was applied to 2nd instar (L2) nymphs. HhTAR1 dsRNA was administered by topical delivery (**Supplementary Figure 4**) to *H. halys* 2nd instar nymphs and the silencing effect on *HhTAR1* transcript levels was evaluated by RT-qPCR 24 h after the treatment. The dsTAR1 treatment did induce a gene silencing effect, with a 50 % decrease in transcript abundance while the dsLacZ negative control RNA did not cause any variation (**Figure 6, panel B**). Interestingly, the insects treated the *HhTAR1*-dsRNA exhibited a reduced sensitivity to (*E*)-2-decenal, i.e. they moved towards and fed on green bean in the presence of (*E*)-2-decenal in a similar manner to the acetone-only control (**Figure 6, panel C**). On the other hand, the behavior of nymphs treated with *LacZ*-dsRNA was unmodified, therefore the alarm pheromone correctly repelled the insects (**Figure 6, panel D**).

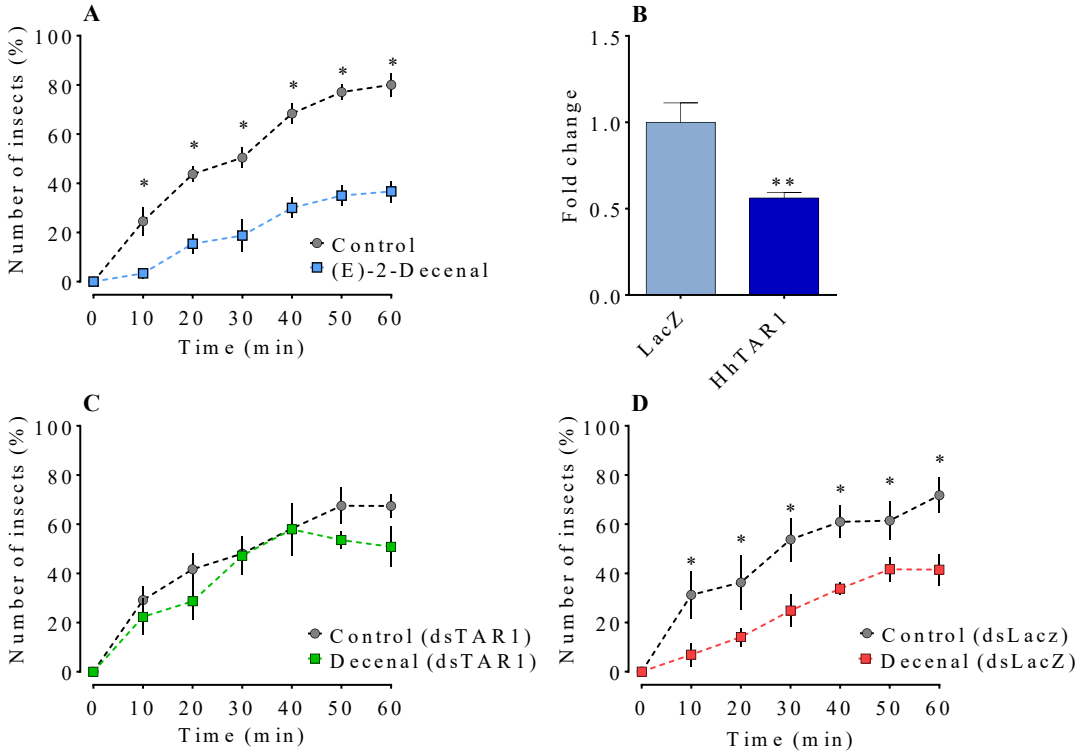


Figure 6. Olfactory modulation of *H. halys* 2nd instar nymphs. (**A**) Behavioral repellence assay on *H. halys* 2nd instar in the presence or absence of the alarm pheromone (*E*)-2-decenal. (**B**) Reduction in *HhTAR1* transcript levels by RNAi. Each bar shows the mean fold change ± S.E.M (standard error) of four independent replicates of *H. halys* 2nd instar nymphs 24 h after gene-specific dsRNA treatment, topically delivered. LacZ specific dsRNA treatment was used as a negative control. ** $p < 0.01$ vs control according to student's t test. (**C**) Behavior assay after dsHhTAR1 administration or (**D**) dsLacZ. Data are means ± S.E.M of four independent replicates for a total of at least 50 insect tested. * $p < 0.05$ vs control according to two-way ANOVA (time x treatment) followed by Dunnett post-hoc.

Discussion

Since its appearance in Europe and in America, *Halyomorpha halys* has caused serious damage to agriculture (Rice et al., 2014; Valentin et al., 2017). Due to its reduced susceptibility to traditional control strategies, new methods for *H. halys* containment need to be developed, identifying innovative chemical compounds as well as new targets based on biochemistry, physiology and behavior of this insect.

This study deals with the molecular and pharmacological characterization of the *H. halys* type 1 tyramine receptor (HhTAR1). Through a RNAi silencing of *HhTAR1* it was possible to reveal the important role of HhTAR1 in physiological aspects of *H. halys*, such as the olfactory response to the alarm pheromone (*E*)-2-decenal.

The HhTAR1 polypeptide shares many structural features with TAR1s from other insects (Ohta & Ozoe, 2014). HhTAR1 contains seven highly conserved transmembrane segments, as expected for a GPCR, as well as phosphorylation and glycosylation sites, typical for this receptor class and essential for the correct protein folding and receptor signaling (Nørskov-Lauritsen & Bräuner-Osborne, 2015; Alfonzo-Mèndez et al., 2017). Most of these sites (seven phosphorylation sites - T^{235} and $S^{246, 260, 294, 319, 321, 364}$) are localized in the long intracellular loop between TM V and VI and are probably involved in receptor signaling and regulatory processes such as desensitization and internalization. Concerning the TA binding site, the main amino acid residue interacting with the endogenous agonist is an aspartic acid located in TM III and well conserved in all insect TAR1s, based on alignment studies (Braza et al., 2019). In HhTAR1 this Asp residue is found at position 128 (D^{128}). The D^{128} involvement in ligand binding has been confirmed in a mutation study performed on *B. mori* TAR1 that showed that the orthologous Asp residue binds the TA-amine group with an ionic bond reinforced by H-bond (Ohta et al., 2004). The same study also showed that several serine residues in TM V stabilise the interaction between TAR1 and TA. The HhTAR1 molecular model furthermore suggests that three serine residues (found at positions 212, 213 and 216 and well conserved within TAR1 insects family might be involved in generating the receptor binding pocket (Ohta et al., 2004; Braza et al., 2019).

The structural description encouraged to proceed towards a functional characterization. The HhTAR1 coding region was cloned and expressed into HEK 293 cells and the recombinant receptor tested for its ability to respond to known TAR1 ligands. In the calcium mobilization assay, TA was significantly more potent than OA, as observed for other TAR1s (Gross et al., 2015; Hana & Lange, 2017a; Finetti et al., 2020). Furthermore,

the effect of TA was sensitive to the antagonist yohimbine, as observed in other orthologous TAR1s (Saudou et al., 1990; Gross et al., 2015; Hana & Lange, 2017a; Finetti et al., 2020). Other biogenic amines, such as dopamine and adrenaline, were not able to activate HhTAR1 in a massive manner, as also shown in *R. microplus* TAR1 (Gross et al., 2015) (**Supplementary figure 3**).

Many studies support the physiological role of TAR1 in processes such as locomotion (Saraswati et al., 2004; Schützler et al., 2019), metabolic control (Nishimura et al., 2005; Li et al., 2017; Roeder, 2020), reproduction (Hana & Lange, 2017a; Hana & Lange, 2017b) and olfaction (Kutsukake et al., 2000; Brigaud et al., 2009; Duportets et al., 2010; McQuillan et al., 2012; Ma et al., 2015; Zhukovskaya & Polyanovsky, 2017; Ma et al., 2019b). The *TAR1* expression patterns mirror its functional roles because the *TAR1* gene is highly expressed in the CNS, salivary glands and antennae in different insect species (Duportets et al., 2010; McQuillan et al., 2012; Wu et al., 2014; El-Kholy et al., 2015; Hana & Lange, 2017a; Ma et al., 2019a; Finetti et al., 2020). Two studies conducted in 2017 on the honeybee brain showed that *TAR1* was mainly expressed at the presynaptic sites in antennal lobe OSNs and in the mushroom bodies PNs, which are essential structures for the olfactory system in insects (Sinakevitch et al., 2017; Thamm et al., 2017). Similarly, in *H. halys HhTAR1* appeared strongly expressed in brain and antennae but was less expressed in the midgut and reproductive systems of adults. Furthermore, *HhTAR1* mRNA was more abundant in the male brain than in the female one. This sex-dependent *TAR1* expression was also detected in *D. suzukii* (Finetti et al., 2020) and *P. xylostella* (Ma et al., 2019a) suggesting that TAR1 could be involved in male specific functions such as development as well as reproduction. The high brain expression of *HhTAR1* correlates well with the abundance of TAR1 in CNS of numerous insect species (El-Kholy et al., 2015; Hana & Lange, 2017a; Finetti et al., 2020) where it regulates several sensory processes (Roeder et al., 2003; Lange, 2009; Ohta & Ozoe, 2014; Neckameyer & Leal, 2017). Interestingly, *HhTAR1* was also highly abundant in the antennae. Actually, several studies have shown that TAR1 is expressed in antennae although its role in these structures is still unclear. A possible correlation between TAR1 and olfaction was established for the first time in 2000 (Kutsukake et al., 2000). This study characterized a *D. melanogaster* TAR1-mutant line, called *honoka*, whose behavioral responses to repellents were reduced in comparison to wild type flies. Our data also revealed that *HhTAR1* is more expressed in the male antennae of *H. halys* than in female ones. These results suggest that TAR1, besides being associated with olfactory

repellence processes, could also play a role in responses to olfactory-reproductive stimuli, such as pheromones, or in mating behaviors (Mazzoni et al., 2017). The *HhTAR1* mRNA resulted more abundant in the two flagellomeres FL1 and FL2 with a 6-fold difference in comparison to the other antennal structures. A typical insect antenna contains numerous sensilla, essential structures for smell, taste, mechanoreception and thermo-hygro perception (Zacharuk, 1985). The great number of sensilla in the apical parts of the *H. halys* antennae correlates with the high *HhTAR1* expression level in the same areas, further strengthening a role for TAR1 in olfaction. Since the physiological role of each sensilla may be predicted based on their morphology, size and distribution (Keil, 1999), the *H. halys* sensilla were investigated by SEM. Different types of sensilla have been classified in the Pentatomidae, including basiconic, trichoid, coeloconic and chaetic sensilla (Brèzot et al., 1997). The most abundant structures in FL1 and FL2 segments of the adult *H. halys* were trichoid sensilla (TR) followed by basiconic sensilla (BS) and grooved sensilla as observed also by Ibrahim et al. (2019) in the same insect. Both TR and BS-C share olfactory functions (Toyama et al., 2006) as suggested by the presence, on the surface, of distinctive microperforations necessary to connect the odorous molecules with the olfactory receptors in the OSNs (Zacharuk, 1985). It is difficult to associate each type of sensillum to a specific olfactory-mediated behavior, but the removal of both FLs completely inhibited the adult *H. halys* aggregation, indicating that these structures, and probably also TR and BS are necessary to perceive the aggregation pheromone (Toyama et al., 2006). On the other hand, sporadic BS have been observed in SC and both segments of the pedicel, PE1 and PE2 along with structures identified as pit sensilla or coeloconic sensilla that could be involved in the thermos-hygro perception (Altner & Prillinger, 1980). It is interesting to note that *HhTAR1* is more expressed in the flagellum as compared to the scape and pedicel, suggesting a correlation between TAR1 and olfactory sensilla. These data would therefore suggest an important role for HhTAR1 in olfactory processes. Interestingly, *HhTAR1* showed high expression levels also in eggs and in 1st and 2nd instar nymphs, followed by a dramatic decrease from the 3rd instar nymphs onwards. The high *TAR1* expression level in eggs has been observed also in *P. xylostella* (Ma et al., 2019). However, information about TA and TAR1 role in embryogenesis are also limited on *D. melanogaster*, in which TAR1 gene exhibits a dynamic expression pattern during embryo maturation inside the eggs with a peak in correspondence to the nervous tissue formation (Hannon & Hall, 1996). In this study, the authors hypothesized that the decline in TAR1 expression in larval stages may be

explained by the relative decrease in the ratio of neuronal versus non-neuronal tissue. The results also revealed that between 2nd and 3rd instar nymphs the *HhTAR1* expression decreased more (about 80 %) in the abdomen and thorax tissues in comparison to the head. A similar result was observed also in adults, where *HhTAR1* levels remained high in brain and antennae in comparison to other tissues. Previous studies observed that *H. halys* nymphs exhibited a 4-times higher mortality than adults after treatment with essential oils for 1 or 48 h (Bergmann & Raupp, 2014) The high *HhTAR1* expression in CNS and antennae of nymphs could explain the greater sensitivity to volatile compounds with insecticide properties, such as essential oils. It is known that TAR1 is a putative target for biopesticides, such as monoterpenes (Gross et al., 2017, Finetti et al., 2020). Although their toxicity in vertebrates has not been ascertained, monoterpenes are currently used as repellents against insect pests (Reis et al., 2016).

The analysis on *HhTAR1* expression patterns together with the SEM observations on *H. halys* antennae strongly suggests a connection between HhTAR1 and *H. halys* olfactory regulation. To better investigate this aspect, *HhTAR1* was silenced by RNAi in young nymphs. In recent years, several Hemiptera genes have been successfully silenced through this method (Christiaens & Smagghe, 2014; Bansal et al., 2016; Ghosh et al., 2017; Lu et al., 2017; Mogilicherla et al., 2018, Riga et al., 2019). In these studies, RNAi silencing has been successfully performed on *H. halys* using microinjection and feeding as delivery methods. One µg of dsRNA injected in *H. halys* adults was able to silence several target genes by 60 - 80 % after 72 h (Mogilicherla et al., 2018). On the contrary, when the dsRNA solution was delivered by feeding to *H. halys* 2nd and 4th instar nymphs, some target genes were silenced only by 40 - 80 % (Kumar et al., 2017). Here, the dsRNA was delivered exclusively by microinjection or by feeding but both these delivery methods are problematic. Microinjection requires experience and specific instruments to control the injected volume, as well as minimizing the wound that often causes a drastic increasing in mortality (Christiaens et al., 2020). Actually, through the microinjection we were able to obtain a *HhTAR1* RNAi downregulation in *H. halys* 2nd instar nymphs (data not shown) but with an extremely high mortality. On the other hand, the dsRNA delivery by feeding requires a large amount of dsRNA and it does not allow to control the amount of dsRNA ingested by each insect (Joga et al., 2016). The dsRNA topical delivery has been recently tested in two Hemiptera species, *Diaphorina citri* and *Acyrthosiphon pisum*. In *D. citri*, 20 ng of dsRNA solution topically delivered on the abdomen were able to silence several *Cyp* genes by about 70 - 90 % (Killiny et al., 2014). In *A. pisum*, 120 ng

of dsRNA solution induced a downregulation of a target gene by 90 % after 24-36 hours (Niu et al., 2019). Accordingly, in *H. halys* 2nd instar nymphs (rich in *HhTAR1* mRNA), a 100 ng dose of *HhTAR1* dsRNA topically delivered appeared sufficient to silence *HhTAR1* by about 50 % after 24 hours, as verified by RT-qPCR. The different RNAi efficiency observed between *D. citri*, *A. pisum* and *H. halys* could be based on the different body structure: the abdominal cuticle of *H. halys* nymphs is thicker than that of *D. citri* and *A. pisum*, an aspect that could limit absorption of dsRNA solution. At any rate, this is the first time that RNAi mediated gene silencing is induced by topical delivery in *H. halys*. Although the dsRNA topically delivered is less efficient as gene silencer in *H. halys*, the administered amount of dsRNA is lower compared to the microinjection and the feeding applications. Reducing the dsRNA amount could be an effective strategy to prevent off target effects (Romeis & Widmer, 2020).

Upon *HhTAR1* silencing, *H. halys* 2nd instar nymphs were tested in their olfactive performances by an innovative behavioral assay. This assay measured the repellent effect of (*E*)-2-decenal, one of the main alarm compounds released by *H. halys* under threats, on 2nd instar nymphs (Zhong et al., 2017; Zhong et al. , 2018; Nixon et al., 2018). The *HhTAR1*-dsRNA treatment caused a reduced sensitivity to (*E*)-2-decenal in comparison to the *LacZ*-dsRNA control nymphs, suggesting that the (*E*)-2-decenal-mediated alarm requires a functional TAR1. Based on the *HhTAR1* expression pattern, it is possible that RNAi-mediated downregulation of the receptor might affect both *H. halys* brain and the antennae, the regions showing the highest receptor expression. Sinakevitch et al. (2017) observed that the *A. mellifera* TAR1 was expressed in the presynaptic regions of the ORN (olfactory receptor neuron) axons that innervate the antennal lobe glomeruli and that could control the transduction signal through TA. Based on this study it might be that, after HhTAR1-RNAi treatment, also the presynaptic regions of the ORN axons could have a lower *TAR1* abundancy in *H. halys* and, accordingly, show an impairment in the transmission of the pheromone-stimulated signal.

However, in a study performed on *M. sexta*, the injection of TA directly into the sensillum modulated the response to sexual pheromones. The authors advanced the hypothesis that TA, through TARs-binding, regulated the levels of both Ca^{2+} and cAMP, which in turn regulated the olfactory receptors (ORs) sensibility to pheromones (Flecke & Stengl, 2009). Therefore, the downregulation of *HhTAR1* could affect the intracellular cascade involving Ca^{2+} and cAMP content triggered by the pheromone-mediated ORs activation.

Since *H. halys* is a relatively new research target and many tools available for model species cannot be applied to this species, we cannot reasonably attribute the reduced sensibility to the alarm pheromone (*E*)-2-decenal to the neural or the antennal regions. Further investigation will be needed to understand whether the RNAi-mediated TAR1 downregulation affects the complex pheromone olfactory perception in a peripheral or central way.

In conclusion, HhTAR1 could play a relevant role in the *H. halys* olfactory network, contributing to modulate olfaction-mediated behaviors, such as reception of alarm pheromone compounds. A more detailed characterization of the interconnections between TAR1 and the olfactory system will open the way for developing TAR1-targeting volatile compounds, such as essential oils, with both repellent and insecticidal properties against *H. halys*.

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Table 1. Primers used in this study.

Primer	Primer sequence (5'-3')
Cloning	
HhTAR1-Fw	TTAGTGCGGTGAGGAAGGTT
HhTAR1-Fw-Kozak	GCCACCATGGAGTGGGACTATAGAG
HhTAR1-Rev	CGATTTTCATGGAGAAGTGGA
RT-qPCR analysis	
HhTAR1-Fw	CTCATTGGCTGGAACGACTG
HhTAR1-Rev	CCCGTTCACGTAACCTCCTC
ARP8-Fw	TTGATGCTGACTGGCCCTAA
ARP8-Rev	GGCCTCCTTCGTTGGTACAG
UBE4A-Fw	CGCCAGCTGACTTTTCCTCT
UBE4A-Rev	GACAGCAGTGGCTCCATCAG
dsRNA synthesis	
HhTAR1-Fw	GAATTAATACGACTCACTATAGGGAGACCGGAAGTCTTCAGCAACT
HhTAR1-Rev	GAATTAATACGACTCACTATAGGGAGACGTGACTTAGGGGAATTGG
LacZ-Fw	GAATTAATACGACTCACTATAGGGAGATGAAAGCTGGCTACAGGA
LacZ-Rev	GAATTAATACGACTCACTATAGGGAGAGCAGGCTTCTGCTTCAAT

Table 2. MolProbity results based on the HhTAR1 3D model obtained by SWISS-MODEL software.

MolProbity Parameter	**Result**
MolProbity Score	1.95
ClashScore	3.07 (M^{260}, K^{263})
Ramachandran Favoured	97.94 % (goal: > 98 %)
Ramachandran Outliers	0.51 % (D^{331}, P^{77}) (goal: < 0.2 %)

Supplementary figures

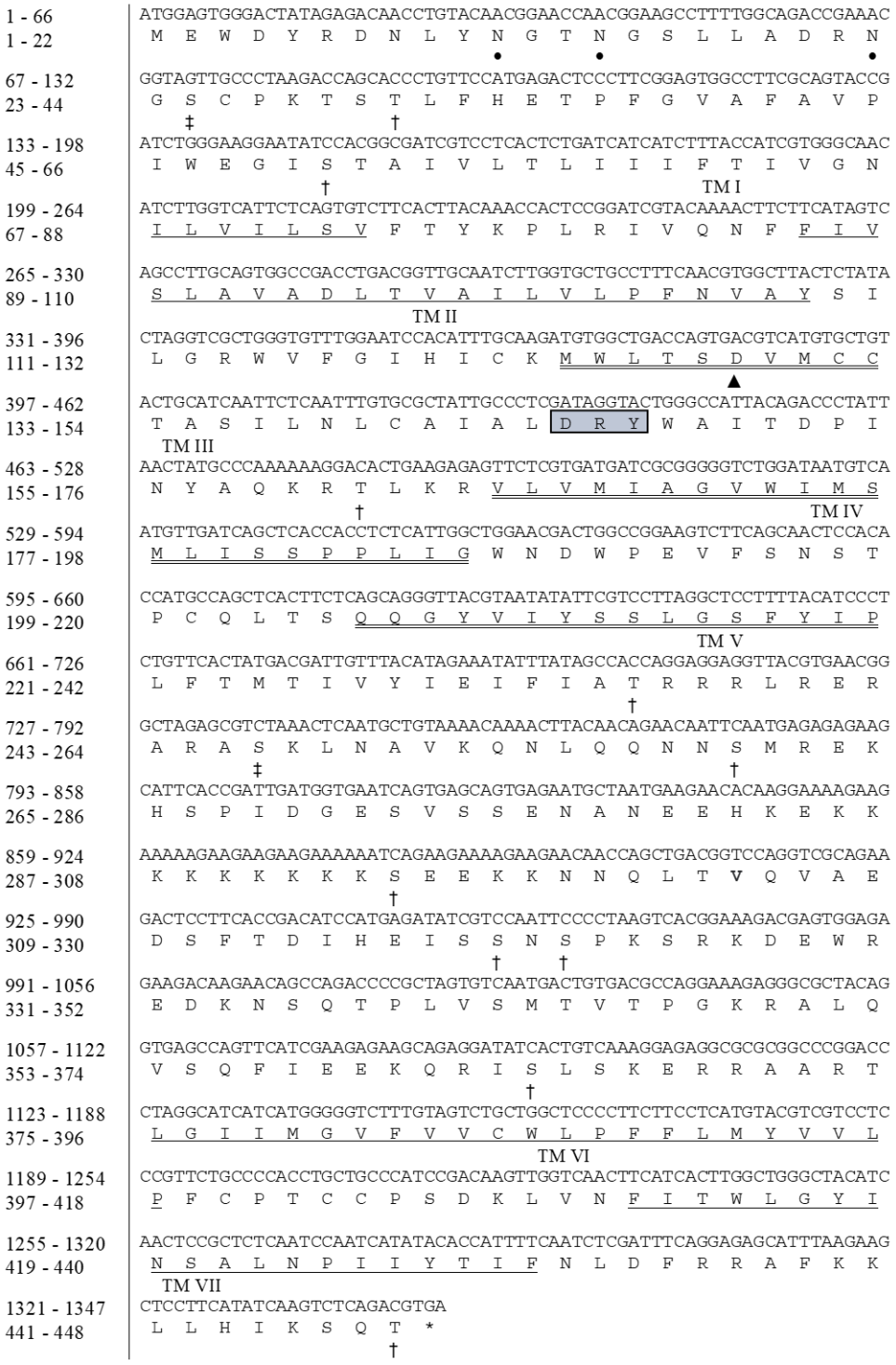

Figure S1. Nucleotide sequence of the TAR1 open reading frame cloned from *Halyomorpha halys* and deduced amino acid sequence. Prediction of the transmembrane segments (underlined and numbered from I to VII) was obtained with TMHMM v. 2.0 software. After the third transmembrane domain there is the DRY motif (highlighted with a box). Potential sites for N-linked glycosylation (predicted with NetNGlyc 1.0 server) are shown with a ● and potential sites for PKA or PKC phosphorylation (predicted with NetPhos 3.1 server) are shown with a † and a ‡ respectively.

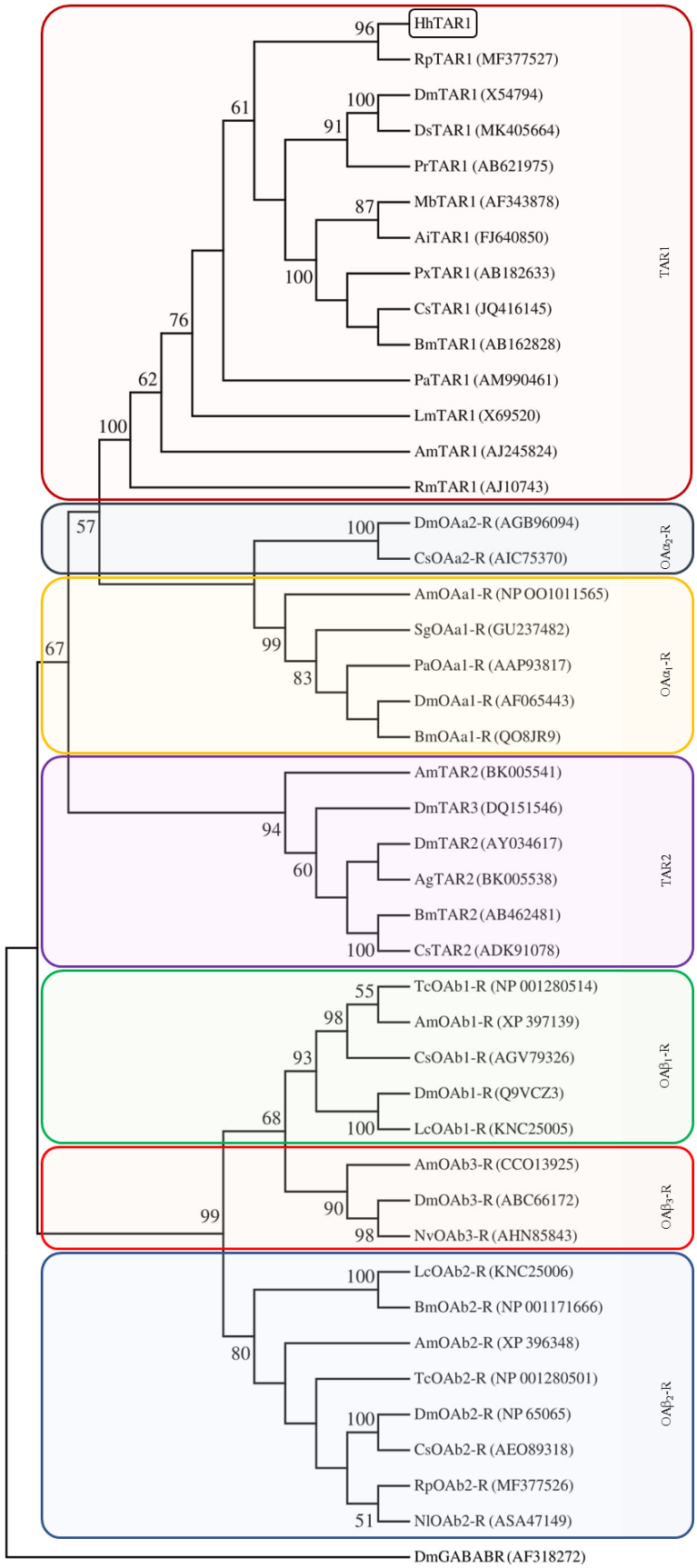
HhTAR1
RpTAR1 (MF377527)
DmTAR1 (X54794)
DsTAR1 (MK405664)
PrTAR1 (AB621975)
MbTAR1 (AF343878)
AiTAR1 (FJ640850)
PxTAR1 (AB182633)
CsTAR1 (JQ416145)
BmTAR1 (AB162828)
PaTAR1 (AM990461)
LmTAR1 (X69520)
AmTAR1 (AJ245824)
RmTAR1 (AJ10743)
TAR1
DmOAa2-R (AGB96094)
CsOAa2-R (AIC75370)
OAα2-R
AmOAa1-R (NP OO1011565)
SgOAa1-R (GU237482)
PaOAa1-R (AAP93817)
DmOAa1-R (AF065443)
BmOAa1-R (QO8JR9)
OAα1-R
AmTAR2 (BK005541)
DmTAR3 (DQ151546)
DmTAR2 (AY034617)
AgTAR2 (BK005538)
BmTAR2 (AB462481)
CsTAR2 (ADK91078)
TAR2
TcOAb1-R (NP 001280514)
AmOAb1-R (XP 397139)
CsOAb1-R (AGV79326)
DmOAb1-R (Q9VCZ3)
LcOAb1-R (KNC25005)
OAβ1-R
AmOAb3-R (CCO13925)
DmOAb3-R (ABC66172)
NvOAb3-R (AHN85843)
OAβ3-R
LcOAb2-R (KNC25006)
BmOAb2-R (NP 001171666)
AmOAb2-R (XP 396348)
TcOAb2-R (NP 001280501)
DmOAb2-R (NP 65065)
CsOAb2-R (AEO89318)
RpOAb2-R (MF377526)
NlOAb2-R (ASA47149)
OAβ2-R
DmGABABR (AF318272)

Figure S2. Phylogenetic relationships of HhTAR1 and other insect amine receptors resulting from neighbour joining analysis, using MEGA7. The values shown at the nodes of the branches are the percentage bootstrap support (1000 replications) for each branch. Alignment was performed using the amino acid sequences found in GenBank (accession number are indicated). *Drosophila melanogaster* GABA-B receptor (DmGABABR) was chosen as outgroup. Dm, *Drosophila melanogaster*; Ds, *Drosophila suzukii*; Pr, *Phormia regina*; Hh, *Halyomorpha halys*; Rp, *Rhodnius prolixus*; Px, *Papilio xuthus*; Cs, *Chilo suppressalis*; Bm, *Bombyx mori*; Ai, *Agnotis ipsilon*; Mb, *Mamestra brassicae*; Pa, *Periplaneta americana*; Lm, *Locusta migratoria*; Am, *Apis mellifera*; Rm, *Rhipicephalus microplus*; Sg, *Schistocerca gregaria*; Ag, *Anopheles gambiae*; Tc, *Tribolium castaneum*; Nv, *Nilaparvata lugens*; Lc, *Lucilia cuprina*; Nl, *Nilaparvata lugens*.

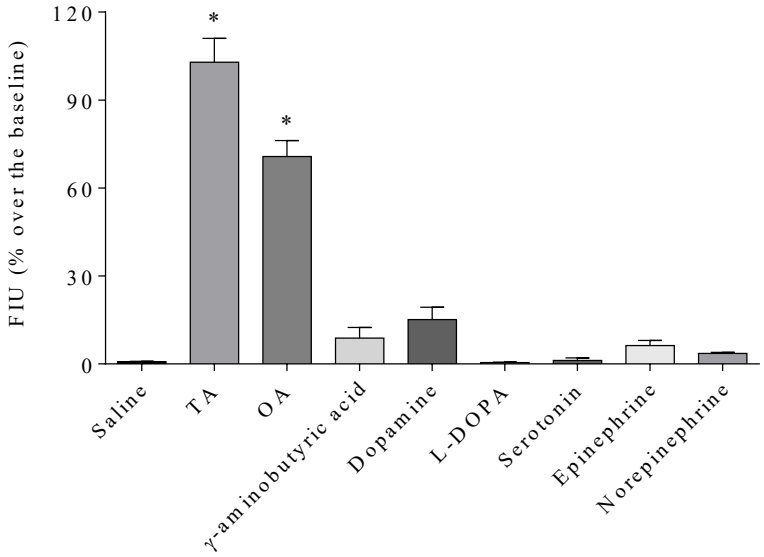

Figure S3. Effect of biogenic amines and γ-aminobutyric acid on the intracellular calcium release in HEK 293 stably expressing HhTAR1. All compounds were tested at 10^{-4} M. Data represent means ± S.E.M of three separate experiments performed in duplicate. * $p < 0.001$ vs saline according to one-way ANOVA followed by Dunnett's multiple comparison test.

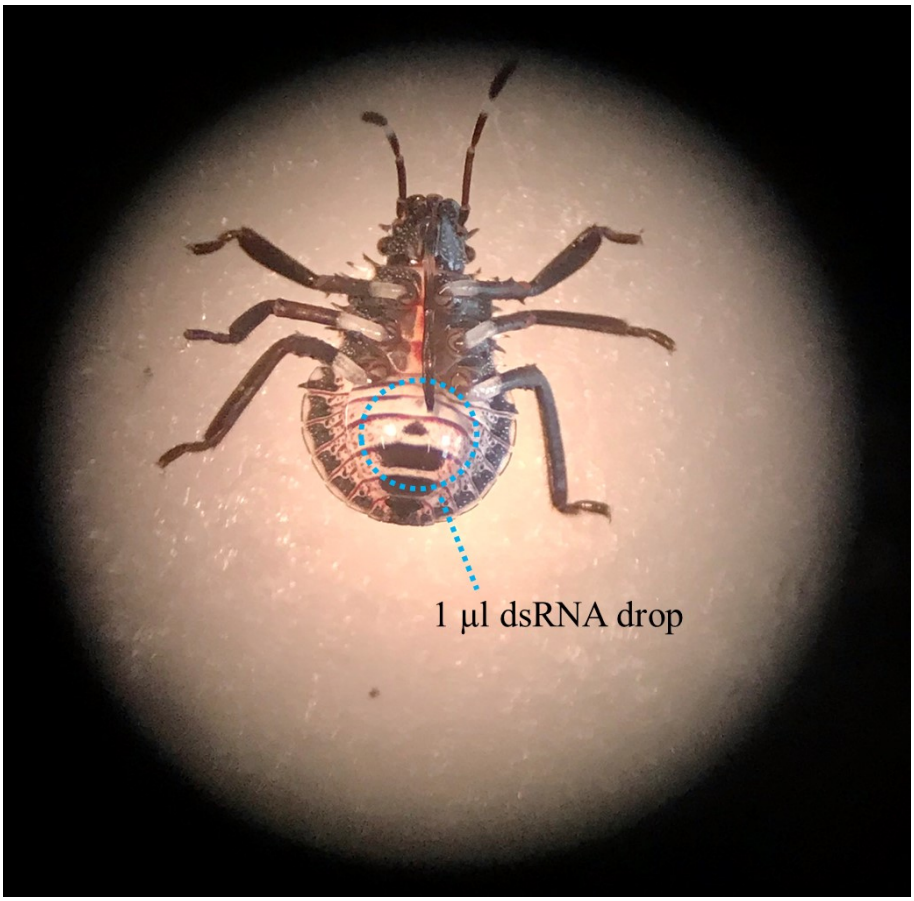

Figure S4. Image of dsRNA topically delivered on a *H. halys* 2nd instar nymph. The 2nd instar nymphs were collected 3 days post-ecdysis and placed on double-sided adhesive tape to avoid movements. One µl of the dsRNA solution was placed on the dorsal side of the abdomen. When the dsRNA solution was completely absorbed, the nymphs were put back in the nursery cage.

Chapter III: General discussion

TAR1 appears central in controlling physiological processes and defining specific behavioral traits in insects. In recent years, the number of studies showing that TAR1 plays a role in physiology has increased, helping to shed some light on the role of this receptor. Furthermore, there is several evidence that TAR1 may be the main target of action for bioinsecticides, such as the monoterpenes and essential oils. There still remains much to learn about the role of TAR1 in insects and its value as potential target for biopesticides.

Based on these information, my research aims to investigate the TAR1 role in controlling physiological and behavioral traits in two phytophagous insects, *Drosophila suzukii* and *Halyomorpha halys*. In fact, the characterization of this receptor and its potential roles may be beneficial to understand the multiple mechanisms by which TA orchestrates physiology and behaviours, as well as develops innovative pest management TAR1-targeting approaches.

Studies performed on *D. melanogaster* and *B. microplus* TAR1 (Enan, 2005; Gross et al., 2017) observed that monoterpenes might interact with TAR1. However, despite a range of studies, the location of the monoterpenes binding site on the insect TAR1s remains elusive. Therefore, the research I presented here aims to investigate and develop the first cellular mechanism between monoterpenes and TAR1 interaction. In particular, the data showed that the monoterpenes appeared able to increase TA potency acting as positive allosteric modulator. As a consequence, the increased TA potency could induce a receptor downregulation which was correlated with an alteration of physiology and fitness in both *D. suzukii* and *D. melanogaster* species. Although this cellular pattern about monoterpenes and TAR1 interaction deserves other validation in different insects, it paves the way to develop innovative management approaches based on natural compounds. Furthermore, the research performed on *D. suzukii* allowed to confirm that TAR1 is essential in the TAergic control of crucial physiological and behavioral aspects such as movement and metabolic TG control in *Drosophila*, as previously investigated by Li and colleagues (Li et al., 2017).

In *H. halys*, through RNAi-mediated TAR1 gene silencing, it was possible to observe that the receptor plays a crucial role in controlling the pheromone perception of (*E*) -2-decenal. Furthermore, TAR1 appeared to be more expressed in those regions of the antennae (flagellomeres) rich in sensilla trichodea, important structures involved in olfaction perception. These results might have interesting implications in the *H. halys*

control. Indeed, by combining the information collected on *D. suzukii* TAR1, it is possible to hypothesize that volatile molecules, such as monoterpenes, could be able to act as *H. halys* repellents through interaction by TAR1.

Based on the TAR1 pharmacology information present in the literature and the data shown in this doctoral thesis about the *D. suzukii* and *H. halys* TAR1 intracellular signaling, the TA/OA receptors scheme proposed by Wu and colleagues should be revised, defining TAR1s more sensitive to TA in both Ca^{2+} and cAMP intracellular variations (**Figure 16**).

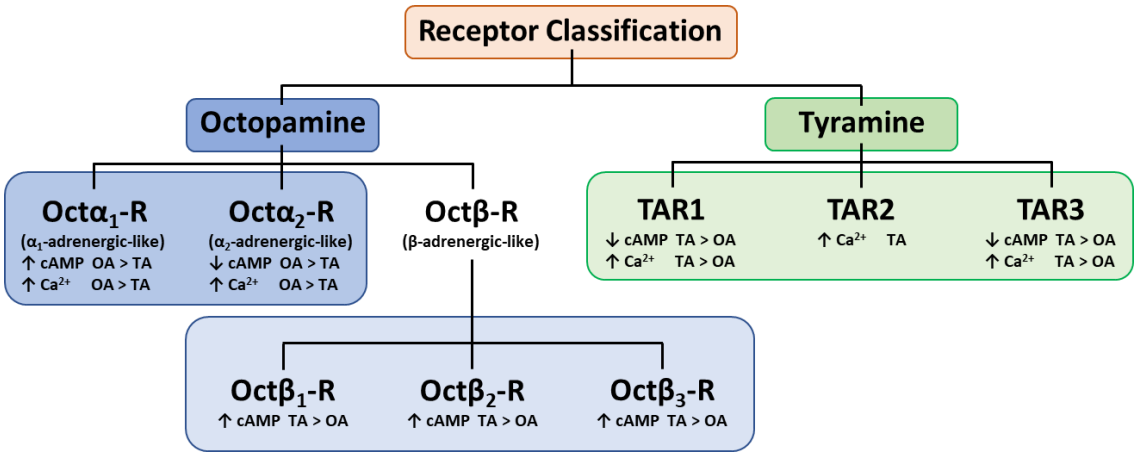

Figure 16. Revised scheme, based on Hana and Lange, describing the TA/OA receptors classification based on their sensitivity to ligands and their downstream effects (Hana & Lange, 2017).

The *D. suzukii* and *H. halys* TAR1 characterization will be the starting point for future studies.

The development of cell lines stably expressing both *D. suzukii* and *H. halys* TAR1s will allow to perform *in vitro* screening of molecules that act either as TAR1 agonists or modulators, in order to identifying interesting TAR1-targeting insecticidal compounds.

Site-specific mutagenesis studies, based on CRISPR-Cas9 approach, will be performed in order to identifying the amino acid residues involved in TAR1-monoterpenes interaction. This research might be helpful for prevent resistance toward monoterpenes, based on aminoacid changes in TAR1 polypeptide sequence.

The RNAi-mediated TAR1 gene silencing protocol, assessed in *H. halys*, will be used as a tool for investigating the TAR1 role in controlling specific physiological and behavioral aspects such as reproduction, movement and aggressivity. Furthermore, the topical application of the dsRNA targeting TAR1 will be test in field in order to investigate new biotechnological approaches for *H. halys* control.

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Appendix I: General methods

Isolation and cloning of the TAR1 full-length

RNA extraction

RNAgents® Denaturing Solution (Promega, USA) was employed for RNA extraction from large pools of insect material, following the manufacturer's recommended protocol. 50-100 mg of frozen samples were homogenised in 100 µl of RNAgents® Denaturing Solution and 400 µl of the same solution was added. After 15 minutes at RT the homogenized mixture was centrifuged at 12,000 g for 10 minutes at 4 °C to remove parts of the insect and other insoluble materials. The supernatant was transferred to a fresh centrifuge tube, chloroform added in a ratio of 200 µl / ml of RNAgents® Denaturing Solution. Then, the tube has been shaken and incubated at room temperature for 2 minutes before the centrifugation at 12,000 g for 15 minutes at 4 °C. The upper phase was collected into a new tube and 500 µl of cold isopropanol added per 1 ml of RNAgents® Denaturing Solution used. The obtained solution was gently mixed and incubated in ice for 10 minutes followed by centrifugation at 12,000 g for 10 minutes at 4 °C. The RNA pellet obtained was washed with 1ml of ice-cold 75% ethanol per 1ml of initial reagent volume and centrifuged at 12,000 g for 5 minutes at 4 °C. The supernatant was then removed, the RNA pellet briefly air-dried and re-suspended in 30 – 50 µl of RNase-free water. All RNA samples were stored at -80 °C. The quality of RNA was checked using a NanoDrop1000 spectrophotometer (Thermo Scientific, USA). Samples were considered pure if the A260/A280 and A230/A260 ratios were above 1.8. Potential DNA contamination was removed from the RNA eluate using the DNase I (Thermo Scientific, USA), following the manufacturer's recommended protocol. A 1 µg of total RNA was treated with 1 µl of DNase I enzyme (1 U/µl). The solution was mixed gently and incubated at 37 °C for 30 minutes. The reaction was stopped by incubation at 70 °C for 10 minutes, in presence of 1 µl of 50 mM EDTA.

Synthesis of cDNA

OneScript® Plus cDNA Synthesis Kit (ABM, USA) was used to synthesize cDNA. The cDNA synthesis was carried out in 20 µl reactions as follows: 1 µg of total RNA after DNAse I treatment, 1 µl dNTPs mix (10 mM each), 1µl OligodT (10 µM) and up to 14.5 µl of RNase free water. Reaction mixtures were preincubated at 65 °C for 5 minutes to resolve any RNA secondary structure and then the following reagents were added: 1 µl

of RNase OFF Ribonuclease Inhibitor (200 U/µl), 4 µl of 5X buffer and 1 µl reverse transcriptase enzyme (200 U/µl). The tube contents were mixed and incubated at 50 °C for 15 minutes. The reactions were terminated by incubating the tubes at 85 °C for 5 minutes. The synthesised cDNA was subsequently used as a template in PCR reactions.

Polymerase Chain Reaction (PCR)

Primer design

All gene specific primers were designed according to the sequence to be amplified (**Table 1**). The properties of the primers, including the sequence, the melting temperature (Tm) and possible secondary structures, were checked using the online software Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Primers were synthesised by Thermo scientific and shipped desalted and dry. 100 µM primer stocks were stored at -20 °C and before use primer stocks were diluted in nuclease-free water to give 10 µM working concentrations.

Amplification of TAR1 by High-Fidelity PCR enzyme

All PCR reactions using cDNA as a template aimed to amplify TAR1 sequences were set up using the pfu enzyme Herculase II Fusion DNA polymerase (Agilent, USA). A typical reaction mix included: 10 µl 5X buffer, 2 µl dNTPs mix (10mM each), 1.25 µl forward primer (10 pmol), 1.25 µl reverse primer (10 pmol), 1 µl Herculase II Fusion DNA polymerase (3 U/µl), 0.5 µl DMSO, 2 µl of template cDNA and nuclease-free water to 50 µl. Typical PCR cycling conditions were: predenaturation at 95 °C for 3 minutes, followed by 10 cycles at 95 °C for 20 s, 70-60 °C for 20 s (minus 1 °C/cycle), 68 °C for 2 minutes, 30 cycles at 95 °C for 20 s, 60 °C for 20 s, 68 °C for 2 minutes and a final extension at 68 °C for 4 minutes. Specific primer were designed for amplification of both *D. suzukii* and *H. halys* TAR1.

Colony PCR

Prior to propagation of *E. coli* transformants in liquid cultures, PCR was used to test for the presence of inserts in the bacterial plasmids using small samples taken from colonies of the plasmid containing bacteria (colony PCR). The PCR reactions were performed with REDTaq® ReadyMix™ PCR Reaction Mix (Sigma Aldrich, USA). The templates were cells re-suspended in 25 µl of LB medium. The PCR reactions were set up in a total volume of 20 µl as follows: 10 µl of 2 X REDTaq, 1 µl of forward primer (10 pmol), 1 µl

of reverse primer (10 pmol), 7 µl of sterile water and 1 µl of template. Typical PCR cycling conditions were: predenaturation at 94 °C for 3 minutes, followed by 10 cycles at 94 °C for 20 s, 70-60 °C for 20 s (minus 1 °C/cycle), 72 °C for 1 minutes, 30 cycles at 94 °C for 20 s, 60 °C for 20 s, 72 °C for 2 minutes and a final extension at 72 °C for 7 minutes. 12 µl of product from each PCR reaction was run on a 1% (w/v) TAE agarose gel.

Purification of PCR products

PCR products were run on 1% w/v 30ml TAE-agarose gel in TAE buffer at 50V/200mA for at least 1hour. DNA fragments were subsequently purified from excised gel slices using Illustra GFX PCR DNA and gel band (GE Life Sciences, USA). 10 µl of capture buffer type 3 was added for each 10 mg of gel slice. If the gel slice weight was less than 300 mg, 300 µl of Capture buffer type 3 was added. Samples were mixed, incubated at 60 °C until the agarose was completely dissolved. The whole mixture was transferred to a GFX Microspin column (pre-ass.e.mbled in a 2 ml collection tube), incubated at room temperature for 1 minute and centrifuged at 16,000 g for 30 seconds. 500 µl of wash buffer type 1 was added to the column and the wash was repeated with the same wash buffer type 1 volume and the same centrifugation. The collection tube was discarded and the column was placed in a new 1.5 ml collection tube. DNA was eluted from the column after 1 minute of pre-incubation of the column with 30 µl of elution buffer type 4 followed by 1 min centrifugation at 16,000 g. The quality of PCR products were checked using a NanoDrop1000 spectrophotometer (Thermo scientific, USA). Samples were considered pure if the A260/A280 and A230/A260 ratios were above 1.8.

Clone JET PCR Cloning Kit

Type 1 tyramine receptors PCR products were ligated into the pJET1.2 blunt vector, which is a part of the cloneJET™ PCR cloning kit (Thermo-Fermentas, USA), in 20 µl reactions as follows: 10 µl of 2 X reaction buffer, 1 µl of pJET vector (50 ng/µl), 1 µl of T4 DNA ligase (5 U/µl), 100 ng of insert DNA and nuclease-free water up to 20 µl. Ligations were incubated at room temperature for 30 minutes and then subjected to bacterial transformation.

Bacterial transformation

50 µl of SIG10 Chemically Competent cells (Sigma Aldrich, USA) were used per reaction. Cells were thawed on ice for at least 10 minutes. Typically 5µl of ligation reaction was added to the cells and the mixture incubated on ice for 30 minutes. Cells were then heat-shocked at 42 °C for 30 seconds and immediately placed on ice for a 3 minutes. 950µl of 37 °C pre-warmed S.O.C. medium was then added to each tube and the tubes placed in a shaking incubator for 1 hours at 30 °C and 350 rpm. Up to 200 µl of cells were then plated onto LB agar plates containing 100 µg/ml of ampicillin for colony selection and incubated at 37 °C for approximately 16 hours.

Miniprep

A colony of *E. coli* with desired plasmid was inoculated into a 6 ml of LB broth containing 100 µg/ml of ampicillin and propagated in a shaking incubator at 37 °C, 350 rpm. After approximately 16 hours, 4 ml of the culture was used to purify the plasmid. GenElute™ Plasmid Miniprep Kit (Sigma Aldrich, USA) was used for the purification of plasmid DNA. Cells were harvested by centrifugation at 16,000 g for 1 minute, the supernatants removed and the pellets re-suspended (by pipetting and vortexing) in 250 µl of 4 °C resuspension buffer. The cell lysis was performed by adding 250 µl of lysis buffer to each tube followed by gentle mixing until a homogenous solution was obtained. After 5 minutes, the lysis reaction was terminated by adding 350 µl of Neutralisation solution. The mixtures were then centrifuged for 10 minutes at 16,000g at room temperature to pellet the cell debris and denatured proteins. Supernatants were then transferred to DNA binding columns. A brief 1 minute centrifugation at 16,000 g allowed DNA to bind to the column resin which was then washed with both 700 µl of wash buffer 1 and 500 µl of wash buffer 2. Columns were then spun for a further 1 minute at max speed to remove any residual wash buffer. DNA was eluted from the columns with 30 µl of nuclease-free water. The quantity and the quality of the DNA was checked by A260/A280 measurements using a NanoDrop1000 (Thermo Scientific, USA) spectrophotometer. The TAR1 sequences have been verified by DNA sequencing (BMR Genomics, Italy).

Restriction digests

Expected restriction fragment sizes of each TAR1 cloned were predicted in silico using Webcutter 2.0 software. Typical diagnostic digest was set up in a 15 µl reaction as follows: 1.5 µl of 10 X buffer, 1 µl of enzyme (10 U/µl), 1 µg of plasmid DNA, nuclease

free water up to 15 µl. Reactions were briefly vortexed and then incubated at 37 °C for one hour. Products were run on 1 % w/v agarose gels for visualization and analysis.

TAR1s general bioinformatic analysis

TAR1 nucleotide sequences and the related protein sequences, obtained from sequencing experiments, has been subjected to bioinformatics studies to characterize structural and functional aspects. The software used in bioinformatics analyses were:

• TMHMM Version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) used to predict the transmembrane segments in the TAR1 polypeptides.

• NetNglyc Version 1 (http://www.cbs.dtu.dk/services/NetNGlyc/) and NetPhos Version 3.1 (http://www.cbs.dtu.dk/services/NetPhos/) used to identify respectively the possible glycosylation and phosphorylation sites present in the amino acid chains.

• Phyre 2 (http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index), free online software used for the creation of the presumably tertiary structure of TAR1s as well as the study of the TA binding site inside the receptor pore.

• Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) has been used in the Multiple protein sequence alignments between *D. suzukii* and *H. halys* TAR1 and other tyramine receptors, with the aim of identifying possible conserved amino acid regions.

• MEGA 7 and BioEdit Version 7.2.6.1 are free software used for the phylogenetic study of TAR1s. In particular, phylogenetic trees were created with the aim to investigate the possibility of the receptors to localize within the family of type 1 tyramine receptors, as well as to have information about its evolutionary history towards other species of insects. The phylogenetic neighbour-joining analysis were performed with 1000-fold bootstrap resampling.

Functional expression of TAR1s

Propagation of HEK 293 cell cultures

The HEK 293 cultures were maintained in complete high glucose Dulbecco's Modified Eagle Medium (DMEM) (EuroClone, Italy) containing 10% FBS and penicillin-streptomycin (50 U/50 µg/ml), in a sterile dedicated 37 °C, 5 % CO_2 incubator. For cell propagation, the media surrounding cells grown to near confluence in T-75 flasks was removed from the flask and the cells washed once with 5 ml of phosphate-buffered saline pH 7.4 (PBS). To detach the cells from the flask, 3 ml of trypsin-EDTA (Life Technologies, USA), pre-warmed to 37 °C was added, spread out to coat all the cells and

left for 5 minutes. 7 ml of DMEM at 37 °C was then added to stop the reaction; cells were pipetted up and down to break up any clumps and the cells transferred to a 15 ml sterile centrifuge tube. Cells were centrifuged at 500 g for 5 minutes at room temperature, the supernatant removed and the cells re-suspended in fresh complete DMEM at a density of $1x10^6$ cells/ml. One ml of cells was then seeded into a new T-75 flask in a total volume of 13 ml complete DMEM.

Transfection protocols

For the TAR1s transfection the JetOPTIMUS® kit (Polyplus, USA) was used. JetOPTIMUS® is a lipid-based reagent that ensure high levels of exogenous protein expression and a low cytotoxicity. Experiments with JetOPTIMUS® were done in 60-mm dish plates and in T-75 flasks. The protocol for the 60-mm dish plates was as follows: $6x10^5$ cells in 8 ml of high glucose DMEM medium containing 10 % FBS were seeded into the well to reach 60 to 80 % confluency at the time of transfection. On the day of transfection the medium was replaced with 5 ml of serum-free high glucose DMEM and 4 µg of DNA was diluted in 500 µl of JetOPTIMUS® buffer in a 1.5 ml tube. Then, 5 µl of the JetOPTIMUS® reagent was diluted in the same tube, vortexed and incubated at room temperature for 10 minutes. After incubation, the transfection mixture was added dropwise to the cells, the plate gently rocked to evenly distribute the solution, and left to transfect in a 37 °C incubator for at least 24 h before any experiments. For the large-scale transfection, a T-75 flask was used. One day prior to transfection $3x10^6$ cells were seeded into a T-75 flask with 13 ml of complete high glucose DMEM. On the day of transfection the medium was replaced with 10 ml of serum-free high glucose DMEM and 10 µg of DNA was diluted in 1000 µl of JetOPTIMUS® buffer in a 2 ml tube. Then, 11 µl of the JetOPTIMUS® reagent was diluted in the same tube, vortexed and incubated at room temperature for 10 minutes. After incubation, the transfection mixture was added dropwise to the medium, the T-75 gently rocked to evenly distribute the solution and left to transfect in a 37 °C incubator for at least 24 h before any experiments.

Pharmacological assays

Calcium mobilization assay

The FlexStation II (Molecular Devices) fluorometer was used for the calcium mobilization assay. With the FlexStation is possible to measure fluorescence variations related to level changes of cytosolic calcium. Fluorescence levels are expressed as

fluorescence intensity units (FIU). FlexStation II is equipped with an optical system consisting of a double monochromator and a xenon lamp with excitation frequency from 250 to 850 nm and emission frequency from 360 to 850 nm. The xenon lamp emits light which is filtered by the monochromator at the wavelength desired and then reflected on the wells of the plate. The cells inside the plate, previously incubated with calcium-dependent fluorophore Fluo-4 AM (Invitrogen, USA), are affected by the light (λ 488 nm) and emit radiation at a λ of 525 nm. The lipophilic nature of the Fluo-4 AM fluorophore allows it to penetrate inside the cells by passive diffusion. Here, the AM portion of the fluorophore was degraded by endogenous esterases which hydrolyse the ester bonds. In this condition, the fluorophore is able to chelate calcium ions but no longer able to passively exit the cell.

For the intracellular calcium mobilization experiments, wild type or TAR1-transfected HEK 293 were seeded in black 96-well plates (Corning Costar, USA) to minimize non-specific fluorescence with a clear bottom to allows the reading of the fluorescence. The day before the experiment, the media was removed from the flask and the cells washed once with 5 ml of PBS. To detach the cells from the flask, 3 ml of trypsin-EDTA (Life Technologies, USA), pre-warmed to 37 °C was added, spread out to coat all the cells and left for 5 minutes. 7 ml of DMEM at 37 °C was then added to stop the reaction; cells were pipetted up and down to break up any clumps and the cells transferred to a 15 ml sterile centrifuge tube. Cells were centrifuged at 500 g for 5 minutes at room temperature, the supernatant removed and the cells re-suspended in fresh complete DMEM in order to seed 50,000 cells per 100 µl of medium in the black 96-well plate. After 24 h of incubation at 37 °C and 5 % of CO_2 the culture medium was replaced with 100 µl of loading solution containing the Fluo-4 AM 3µM fluorophore and then the plate was incubated at 37 ° C, 5 % of CO_2. After 30 minutes of incubation with the fluorophore, the loading solution was aspirated from the wells and replaced with 100 µl of Brilliant-Black solution for 10 minutes before starting the reading. The black colour of this solution is essential to shield the background fluorescence due to the fluorophore which has failed to penetrate inside the cells. After the incubation with Brilliant-Black, the basal fluorescence of each well of the plate was read. These values is useful for evaluating the degree of homogeneity and the quality of the cells. Then, the compounds to be tested, provide in a second 96-wells plate and placed in the instrument, were injected onto the cells automatically, for successive columns, according to the protocol established by the operator. Each well was

read for 120 seconds. The fluorescence values obtained was expressed as the peak of fluorescence calculated as a percentage of the basal fluorescence.

Dynamic Mass Redistribution (DMR) assay

The DMR assay was used to study the activation of TAR1s by estimating the dynamic redistribution of the masses over time, i.e. the displacement of the different biomolecules within the cell that occurs following the agonist-receptor bond. These displacements cause the variation of the cellular optical density, a parameter that can be measured using an RWG (Resonant Waveguide Grating) optical biosensor type. In the DMR technology, the RWG biosensor was incorporated into the bottom of a 384-well cell microplate. When cells are seeded in these particular plates, the wavelength of the light outgoing from the bottom of the biosensor depends on the density of the cell mass located up to 150 nm above the sensor. When a receptor is activated by the agonist, the consequent rearrangement of intracellular structures induce a change in the optical density of the mass above the biosensor and consequently the wavelength of the light outgoing from the RWG biosensor. This change in wavelength is called "DMR response" (**Figure 17**). The DMR response is due to different cellular events, not all known, such as protein trafficking, morphological changes, cytoskeleton rearrangements, receptor internalization and cell adhesion. The DMR response can be both positive and negative, depending on whether these events are associated with a decrease or an increase in the optical density of the cellular portion above the sensor. For these reasons, the DMR response is a complex response that collects all the cellular processes that occur following the activation of the receptor studied.

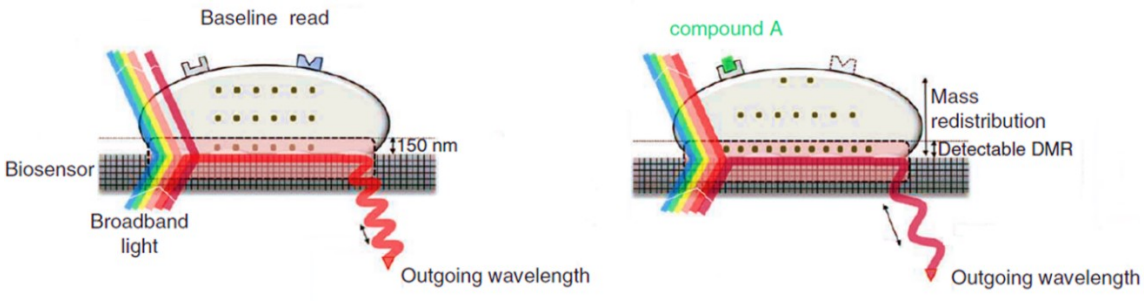

Figure 17. Principle of DMR detection. In the baseline read polarized light illuminates the bottom of the microplate in which a RWG biosensor is present. The biosensor interacts with the cell layer on its surface, thereby forming an optical complex able to propagates and reflects the specific wavelength that is in resonance to it. The propagated light generates a wave that penetrates the bottom portion of the cell layer with a penetration depth of 150 nm into the cell and the outgoing wavelength is recorded and normalized to zero. On addition of a test compound (for example the compound A), the OD of the cell layer changes

near the biosensor because of a redistribution of intracellular mass. This results in a shift of outgoing wavelength relative to the baseline value and is recorded in picometers (Schröder et al., 2011).

24 hours before the experiment, the cells was seeded in an Epic 384- well cell assay microplate fibronectin coated (Corning Costar, USA). For each well, 20,000 cells was seeded in 30 ul of complete medium. Then, the plate was centrifuged for 30 seconds at 800 g to allow the cells to settle on the bottom and it was left in an incubator at 37 °C in the presence of 5 % CO_2. The DMR experiment was performed in the EnSight multimodal plate reader (PerkinElmer, USA) using the Label Free module. In the first step of the experiment each well was washed twice with DMR buffer. The medium and after the buffer were aspirated from each well through a comb that does not touch the bottom of the well to prevent both cells and biosensor from being damaged. Then, two washes were carried out, each with 30 µl of buffer. A third wash with 20 µl of buffer was performed to bring the final volume to 30 µl and the cells were placed 90 minutes inside the instrument previously set at 37 °C. The experiment was taken in two phases. In the first step, the basal signal was measured for 5 minutes. The second phase begun to the addition of the agonist compounds, examinated for 60 minutes. A single reading was made every 22 seconds both during the baseline reading and during the experiment. In the antagonism / modulation experiments, fixed concentration antagonists were added 30 minutes before the agonists. The raw data of each experiment was collected by the Kaleido software but required complex processing to obtain the concentration-response curves. First of all, the last reading of the basal signal was subtracted from each raw signal to obtain the DMR response, i.e. the shift of the wavelength emitted with respect to the basal value. Then, the response produced by the addition of the buffer (negative control) was subtracted from each DMR response. Finally, to obtain the concentration-response curve, the peak of response produced was calculated for each concentration of each tested molecule. This value was reported in a graph where the logarithm of the concentration was indicated on the abscissa and the response in pm on the ordinate. Concentration-response curves were fitted using the four parameters log logistic equation:

$$\text{Effect} = \text{Baseline} + \frac{(E_{max} - \text{Baseline})}{(1+10^{(LogEC_{50} - Log[compound])*Hillslope})}$$

Data are expressed as mean ± S.E.M of n experiments performed in duplicate and were analysed using one- or two-way analysis of variance (ANOVA) followed by Dunnett's or Turkey's test for multiple comparison. Agonist potency was expressed as pEC_{50}, which is

the negative logarithm to base 10 of the agonist molar concentration that produces 50 % of the maximal possible effect of that agonist. Antagonists / modulators potencies were assayed at single concentrations against the concentration-response curve to TA.

Quantitative real-time PCR

RT-qPCR was used to examine TAR1s expression levels in different life stages of *D. suzukii* and *H. halys* as well as in different organs. Furthermore, this technique was used to determine the effects in terms of mRNA levels of TAR1 after *D. suzukii* exposure to monoterpenes as well as the efficiency in term of gene silencing in the RNAi experiments on *H. halys* L2 nymphs. Total RNA extraction and cDNA synthesis were performed as described below. Real time PCR was performed using a CFX Connect Real-Time PCR Detection System (Bio-Rad, USA) in a 12 µl reaction mixture containing 0.8 µl of the cDNA obtained from 1 µg of total RNA, 6 µl ChamQ SYBR qPCR Master Mix (Vazyme, China) or 6 ul SsoAdvanced Universal SYBR Green Supermix (BioRad, USA), 0.4 µl forward primer (10 µM), 0.4 µl reverse primer (10 µM) and 4.4 µl nuclease free water. Thermal cycling conditions were: 95 °C for 2 mins, 40 cycles at 95 °C for 10 s and 60 °C for 30 s. After the cycling protocol, a melting-curve analysis from 60 °C to 95 °C was applied. Expression of TAR1s were normalized in accordance with the relative quantitation method (Larionov et al., 2005) or using the qBase+ algorithm (Hellemans et al., 2007) using specific housekeeping genes. Gene-specific primers were used (**Table 1**) and for each sample at least three independent biological replicates, made at least in triplicate, were performed.

Drosophila brain dissection and immunohistochemistry

The Drosophila brain were dissected and post-fixed by adding a 2 % of PFA solution in S2 medium for 55 mins. Brains were then rinsed three times in PBST (0.5 % Triton-X in PBS) and blocked in 5% normal goat serum (NGS) in PBST for 90 mins. Then, the samples were incubated with the primary antibody in bocking buffer overnight at 4 °C. After overnight incubation, brains were rinsed three times in PBS and then, incubated with the secondary antibody overnight at 4 °C in the dark. Finally, brains were washed in PBT for 30 minutes three times, mounted on slides with Rapiclear 1.47 and analysed by a Zeiss Axio Imager Z1 microscope equipped with an apotome (Zeiss, Germany). The antibodies used in this study were summarized in **Table 2**.

Monoterpenes LC_{50} determination

A glass cylinder (10 cm in height, 4.5 cm inner diameter; 150 ml volume) was employed to calculate the monoterpene median lethal concentration (LC_{50}) values for *D. suzukii* and *D. melanogaster*. 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} calculation, at least 100 flies were tested, in four replicates.

Behavioural assays:

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 composed by agar 1 %, Yeast 5 %, sucrose 5 % and blue dye 1 %. After 2 h of feeding, the flies were collected and frozen at −80 °C (**Figure 18).** 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, 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.

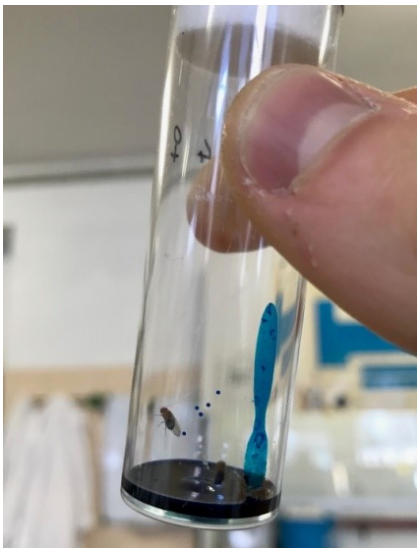

Figure 18. *D. suzukii* females during the food intake assay.

Metabolic rate determination assay

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 (**Figure 19**). The CO2 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.

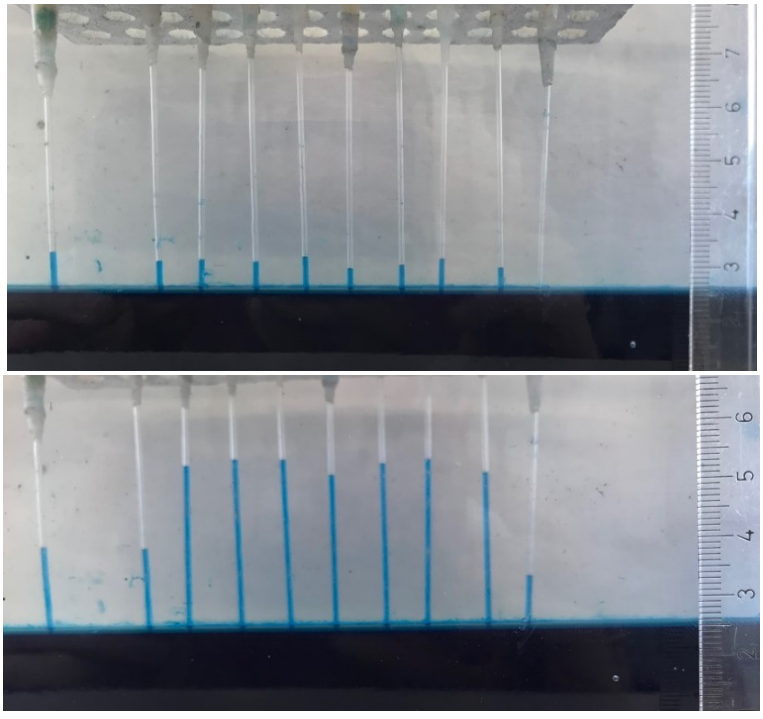

Figure 19. Example of Metabolic rate determination assay at tea. The first capillary from the left is the control, the insect-free respirometer.

To calculate the carbon dioxide produced by insects over time, this formula was used:

$$V = [(\pi R \wedge 2)(\Delta d) - (\Delta c)]\ 1000 / nh$$

Where:

V = volume of CO_2 produced by the insects per time interval

R = radius of the micropipette in cm

Δd = distance travelled by the liquid in cm

Δc = distance travelled by the liquid in cm from the negative control

n = number of insects

h = hours

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 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 (**Figure 20**). Five independent biological replicates per sex and genotype were performed.

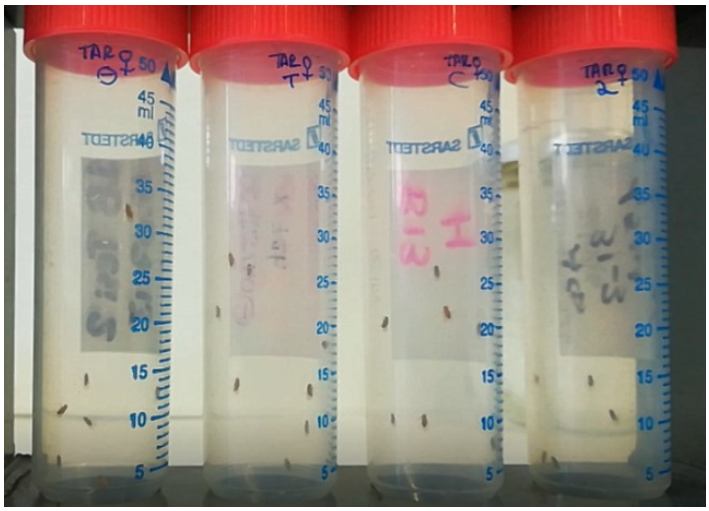

Figure 20. Example of a frame after 1 second of testing.

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).

Fab body quantification

Total body triglyceride (TG) content was estimated using the TG colorimetric assay kit GPO-PAP method (Elabscience, China). Three flies were accurately weighed and homogenization medium (9 times the volume, 0.1 mol l^{-1} phosphate buffer, pH 7.4) was added. The sample was mechanically homogenized on ice with a motorized pestle and centrifuged (at 2500 rpm 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.

In vitro synthesis of dsRNA

For RNAi silencing, TAR1 and LacZ (control) amplicons, 400-500 bp long, were generated by PCR using primers with 5' extensions containing T7 promoters (Table 1). These products were cloned into pJET 1.2 vector (Thermo Fisher Scientific), as described above, and then used as templates for in vitro dsRNA synthesis performed by T7 RNA Polymerase (Jena Bioscience, Germany), according to the manufacturer's protocol. After one hour of synthesis at 37 °C, a DNase I (Thermo Fisher Scientific) treatment was performed and the dsRNA was clean up by ammonium acetate precipitation (Rouhana et al., 2013). In brief, the dsRNA solution was brought to 100 ul with RNase-free water. An equal volume of 5 M ammonium acetate (2.5 M final concentration) was added. Then, two volumes of 100 % ethanol was added and the solution was incubated over-night at -20 °C. The solution was centrifugated at 15000 g for 15 mins at 4 °C and the pellet washed with cold 70 % ethanol. Finally, the dsRNA was resuspended in ultrapure water and quantified by Biospec-Nano spectrophotometer.

***H. halys* repellent assay**

An open petri dish (90 mm x 15 mm), containing 24 h starved H. halys 2nd instar nymphs and a green bean, was placed inside a plexiglas box (50 cm each side) with two lateral openings covered by nets to allow air circulation. The negative control acetone or the positive repellent control (*E*)-2-decenal were applied to a filter paper (1 cm x 1 cm) that was placed under the green bean. The positive control (*E*)-2-decenal, dissolved in acetone, was tested at a fixed quantity of 10 µg, a value ensuring the maximum repellence activity against the *H. halys* nymphs (Zhong et al., 2018). The number of *H. halys* nymphs standing and feeding on the green bean was monitored every ten minutes for one hour. Four biological replicates were made, each comprising at least ten insects, for both untreated and dsRNA treated *H. halys* nymphs. All experiments were performed in the morning in a behavioral room with a controlled temperature of 24 ± 1 °C.

Tables

Table 1. Primers used in these studies.

Primer name	Primer sequence (5'-3')	Species
Cloning		
DsTAR1-Fw	TTCCGTCCGCCATTCAACC	*D. suzukii*
DsTAR1-Rev	TCAATTCAGGCCCAGCAGC	*D. suzukii*
HhTAR1-Fw	TTAGTGCGGTGAGGAAGGTT	*H. halys*
HhTAR1-Fw-Kozak	GCCACCATGGAGTGGGACTATAGAG	*H. halys*
HhTAR1-Rev	CGATTTTCATGGAGAAGTGGA	*H. halys*
RT-qPCR analysis		
DsTAR1-Fw-RT	GCAGTCCTCGTCCACCTG	*D. suzukii*
DsTAR1-Rev-RT	TTAAGGGACGTCTGCTCGTC	*D. suzukii*
AK-Fw	CTACCACAACGATGCCAAGA	*D. suzukii*
AK-Rev	AAGGTCAGGAAGCCGAGA	*D. suzukii*
TBP-Fw	CCACGGTGAATCTGTGCT	*D. suzukii*
TBP-Rev	GGAGTCGTCCTCGCTCTT	*D. suzukii*
PKA-Fw	CGGAGAACCTGCTAATCGAC	*D. suzukii*
PKA-Rev	CCATTTCGTAGACGAGCACA	*D. suzukii*
DmTAR1-Fw-RT	CACTCTGGAGGCGGAAAGT	*D. melanogaster*
DmTAR1-Rev-RT	GCAACGGAGTGACAGAAACG	*D. melanogaster*
Actin-Fw	GCGTCGGTCAATTCAATCTT	*D. melanogaster*
Actin-Rev	AAGCTGCAACCTCTTCGTCA	*D. melanogaster*
Tubulin-Fw	TGTCGCGTGTGAAACACTTC	*D. melanogaster*
Tubulin-Rev	AGCAGGCGTTTCCAATCTG	*D. melanogaster*
DmTAR1-Exon1-Fw	CAACTCAAAGCGACAGACCA	*D. melanogaster*
DmTAR1-Exon2-Rev	TACATGCGTCTTGGTGGAAA	*D. melanogaster*
Rpl32-Fw	CCGCTTCAAGGGACAGTATC	*D. melanogaster*
Rpl32-Rev	GACAATCTCCTTGCGCTTCT	*D. melanogaster*
HhTAR1-Fw-RT	CTCATTGGCTGGAACGACTG	*H. halys*
HhTAR1-Rev-RT	CCCGTTCACGTAACCTCCTC	*H. halys*
ARP8-Fw	TTGATGCTGACTGGCCCTAA	*H. halys*
ARP8-Rev	GGCCTCCTTCGTTGGTACAG	*H. halys*
UBE4A-Fw	CGCCAGCTGACTTTTCCTCT	*H. halys*
UBE4A-Rev	GACAGCAGTGGCTCCATCAG	*H. halys*
dsRNA synthesis		
HhTAR1-Fw-RNAi	GAATTAATACGACTCACTATAGGGA GACCGGAAGTCTTCAGCAACT	*H. halys*
HhTAR1-Rev-RNAi	GAATTAATACGACTCACTATAGGGA GACGTGACTTAGGGGAATTGG	*H. halys*
LacZ-Fw-RNAi	GAATTAATACGACTCACTATAGGGA GATGAAAGCTGGCTACAGGA	*E. coli*
LacZ-Rev-RNAi	GAATTAATACGACTCACTATAGGGA GAGCAGGCTTCTGCTTCAAT	*E. coli*

Table 2. Antibodies used in these studies.

Antibody target	Animal	Dilution	Company
GFP	Rabbit	1:300	Sigma-Aldrich (AB3080)
Nc82	Mouse	1:20	University of Iowa-Developmental Studies Hybridoma Bank
Anti-rabbit IgG Alexa Fluor-488	Donkey	1:300	Jackson ImmunoResearch (711-545-152)
Anti-mouse IgG Alexa Fluor-555	Goat	1:300	Jackson ImmunoResearch (115-165-003)

Appendix II: Contribution in other works

- **Finetti, L.**, Roeder, T., Calò, G. & Bernacchia, G. (2021) The insect type 1 tyramine receptor: from structure to behavior. *Insects*, 12: 315. doi.org/10.3390/insects12040315.
- **Finetti, L.**, Civolani, S., Bernacchia, G. (2021). Monoterpenes-induced toxicity in nymphal stages of *Halyomorpha halys*. *Journal of Plant Diseases and Protection*. In press.
- Civolani, S., Vaccari, G., Caruso, S., **Finetti, L.**, Bernacchia, G., Chicca, M. & Cassanelli, S. (2021). Insecticide efficacy and insecticide adaptive response in Italian population of *Drosophila suzukii. Bulletin of Insectology*, 74(1): 103-114.
- Holanda, V.A.D., Pacifico, S., Azevedo Neto, J., **Finetti, L**., Lobão-Soares, B., Calo, G., Gavioli, E.C. & Ruzza, C. (2019). Modulation of the NOP receptor signaling affects resilience to acute stress. Journal of Psychopharmacoly. 33(12): 1540-1549.
- Costa, G., **Finetti, L**., Civolani, S. & Bernacchia, G. (2018) Evaluation of Brevis® as a thinning agent for 'Fuji'. EUFRIN Thinning Working Group Symposia 1221, 37-38.

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Oggetto:

Dichiarazione di conformità della tesi di Dottorato

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Biologia Evoluzionistica ed Ecologia

Ciclo di Dottorato

33

Titolo della tesi:

Tyraminergic signaling in phytophagous insects: from physiology to pest control

Titolo della tesi (traduzione):

Tyraminergic signaling in phytophagous insects: from physiology to pest control: Il segnale tiraminergico negli insetti fitofagi: dalla fisiologia alla disinfestazione

Tutore: Prof. (Cognome e Nome)

Bernacchia Giovanni

Settore Scientifico Disciplinare (S.S.D.)

BIO/04

Parole chiave della tesi (max 10):

Tiramina: Tyramine, Octopamina: Octopamine, Recettori della tiramina: Tyramine receptors, G protein-coupled receptors: Recettori accoppiati a proteine G, Drosophila suzukii, Halyomorpha halys, Monoterpeni: Monoterpenes, Essential oils: Oli essenziali, Insecticides: Insetticidi.

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