

A critical role for Dop1-mediated dopaminergic signaling in the plasticity of behavioral and neuronal responses to sex pheromone in a moth

Edmundo Gassias^a, Nicolas Durand^d, Elodie Demondion^c, Thomas Bourgeois^c, Paleo Aguilar^a, Françoise Bozzolan^b and Stéphane Debernard^b

^aInstitute of Biology, Complutense University of Madrid, Pozuelo de Alarcon, 28223 Madrid, Spain

^bSorbonne Université, INRA, CNRS, UPEC, IRD, Univ. P7, Institute of Ecology and Environmental Sciences of Paris, 75005 Paris, France

^cSorbonne Université, INRA, CNRS, UPEC, IRD, Univ. P7, Institute of Ecology and Environmental Sciences of Paris, 78026 Versailles, France

^dFRE CNRS 3498, Ecologie et Dynamique des Systèmes Anthropisés, Université de Picardie, Jules Verne, 80039 Amiens, France

Corresponding author: Stéphane Debernard, Sorbonne Université, INRA, CNRS, UPEC, IRD, Univ. P7, Institute of Ecology and Environmental Sciences of Paris, 4 place Jussieu 75005 Paris, France

Tel: +33-1-4427-3839; e-mail: stephane.debernard@upmc.fr

Keywords: sexual behavior, dopamine, Dop1 receptor, olfactory plasticity, neuromodulation, insects

Summary statement: Dopamine exerts its modulatory actions on the olfactory system through the Dop1 receptor and thus acts as a mediator of behavioral plasticity in insects.

Abstract

Most animal species, including insects, are able to modulate their responses to sexual chemosignals and this flexibility originates from the remodeling of olfactory areas under the influence of dopaminergic system. In the moth *Agrotis ipsilon*, the behavioral response of males to the female-emitted sex pheromone increases throughout adult life and after a prior exposure to pheromone signal and this change is accompanied by an increase in neuronal sensitivity within the primary olfactory centers, the antennal lobes (ALs). To identify the underlying neuromodulatory mechanisms, we examined whether this age- and experience-dependent olfactory plasticity is mediated by dopamine (DA) through the Dop1 receptor, an ortholog of the vertebrate D1-type dopamine receptors, which is positively coupled to adenylyl cyclase. We cloned *A. ipsilon* Dop1 (AiDop1) which is expressed predominantly in brain and especially in ALs and its knockdown induced decreased AL cAMP amounts and altered sex pheromone-orientated flight. The levels of DA, *AiDop1* expression and cAMP in ALs increased from the third day of adult life and at 24h and 48h following pre-exposure to sex pheromone and the dynamic of these changes correlated with the increased responsiveness to sex pheromone. These results demonstrate that Dop1 is required for the display of male sexual behavior and that age- and experience-related neuronal and behavioral changes are sustained by DA-Dop1 signaling that operates within ALs probably through cAMP-dependent mechanisms in *A. ipsilon*. Thus, this study expands our understanding of the neuromodulatory mechanisms underlying olfactory plasticity, mechanisms that appear to be highly conserved between insects and mammals.

Abbreviations: AiDop1, *Agrotis ipsilon* Dop1; AiRpL8, *Agrotis ipsilon* Ribosomal protein L8; AL, antennal lobe; BCA, Bicinchoninic Acid; cAMP, cyclic adenosine monophosphate; Ct, cycle threshold; DA, dopamine; DEPC, diethylpyrocarbonate; dNTP, deoxynucleotide triphosphate; DRD1, dopamine D1 receptor; ds-RNA, double-stranded RNA; EXL, extracellular loop; GPCR, G protein-coupled receptor; HPLC, high-performance liquid chromatography; IKCa, Ca²⁺-dependent K⁺ current; INL, intracellular loop; JH, juvenile hormone; LacZ, Beta-galactosidase; MCG, macroglomerular complex; OFR, open reading frame; ORN, olfactory receptor neuron; PCA, perchloric acid; PKC, protein kinase C; QMB, queen mandibular pheromone; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction; RNAi, RNA interference; SK, Ca²⁺-dependent K⁺ channel; TM, transmembrane domain; UPM, Universal Primer Mix; UTR, untranslated region.

Introduction

Many animal species, including insects, have developed a sophisticated olfactory system allowing them to display a wide range of vital behaviors such as locating food sources and mating partners (Brennan, 2010; Galizia and Sachse, 2010; Gadenne et al., 2016). Given that the performance of these odor-guided behaviors requires a considerable amount of time and energy, the responsiveness to biologically active chemical cues, at the behavioral and neuronal level, must occur only under certain situations in which it is beneficial. As a direct consequence, the coding and processing of odor-related information is highly malleable and appears to be dependent on external factors, previous experience, and in coordination with physiological state (Palmer and Kristan, 2011; Gadenne et al., 2016; Ross and Fletcher, 2019). For instance, responses to sexual chemosignals are restricted to the periods of fertility in order to optimize the production of offspring. This olfactory plasticity is known to be achieved through anatomical and functional modifications of the neuronal network under the influence of hormonal factors and biogenic amines (Dacks et al., 2012; Duportets et al., 2013; Schellino et al., 2016; Harvey and Heinbockel, 2018; Verlinden, 2018), and the interactions between these neuromodulators as well as their action mechanisms are still not fully explored.

In general, the organization of the monoaminergic system of insects has a high similarity with that of vertebrates (Sotnikova and Gainetdinov, 2009). The most notable difference is that in insects, dopamine (DA) is the only physiologically relevant catecholamine present at relatively large amounts throughout the peripheral tissues, sense organs and central nervous system (Blenau and Baumann, 2001; Donly and Caveney, 2005; Mustard et al., 2005). Thus, DA can act as neurohormone, neurotransmitter and neuromodulator to regulate multiple physiological functions as well as innate and cognitive behaviors. DA was found to have potential effects on the maturation of reproductive organs in eusocial hymenopterans (Sasaki et al., 2009; Sasaki and Harano, 2010) and to promote the male courtship behavior in the fruit fly *Drosophila melanogaster* (Wicker-Thomas and Hamann, 2008) and behavioral phase changes from solitariness to gregariousness in the migratory locust, *Locusta migratoria* (Ma et al., 2011). Moreover, dopaminergic signaling has been involved in regulating olfactory learning and memory within a wide range of insect species (Unoki et al., 2005; Mizunami et al., 2009; Agarwal et al., 2011; Dacks et al., 2012; Keleman et al., 2012; Klappenbach et al., 2013; Awata et al., 2016; Lenschow et al., 2018; Lim et al., 2018; Vinuager et al., 2018).

The neuromodulatory actions of DA are attributed to the multiplicity of DA receptors linked to distinct intracellular signaling pathways, a complex innervation of all major areas of brain by clusters of DA neurons and dynamic changes in the brain levels of DA (Blenau and Baumann, 2001; Yamamoto and Vernier, 2011; Verlinden, 2018). Dopaminergic signaling is mediated through the activation of G protein-coupled receptors (GPCRs) belonging to the rhodopsin-like subfamily. In vertebrates, five DA receptor subtypes have been characterized and further classified into two major groups based on their structural and pharmacological properties: D1-like (D1 and D5) and D2-like (D2, D3 and D4) receptors (Callier et al., 2003; Beaulieu and Gainetdinov, 2011). In invertebrates, specifically in insects, three distinct groups of DA receptors were identified and referred to as Dop1, Dop2 or invertebrate-type DA receptor and Dop3 according to the vertebrate DA receptor classification system (Mustard et al., 2005). Members of Dop1 and Dop2 groups are most closely related to the vertebrate D1-like DA receptors and stimulate the activity of adenylyl cyclase, resulting in increased intracellular cyclic adenosine monophosphate (cAMP) levels (Hill et al., 2016; Himmelreich et al., 2017; Xu et al., 2017), whereas Dop3 receptors are functionally D2-like and repress adenylyl cyclase (Beggs et al., 2011; Verlinden et al., 2015; Xu et al., 2017). In addition, a fourth group of DA receptors, DopEcR, was initially discovered in *D. melanogaster* and this receptor have a binding affinity for both DA and 20-hydroxyecdysone (20E) (Srivastava et al., 2005; Evans et al., 2014).

These DA receptor subtypes exhibit significant divergence in their sequence, structure and function (Xu et al., 2017; Verlinden et al., 2018). Recent studies on the functional characterization of the Dop1 receptor have been performed in a few insect species. In *D. melanogaster*, Dop1R1 has been shown to be involved in the dopaminergic pathways controlling various aspects of sexual behavior such as persistence copulation, male-male courtship, and courtship learning and memory (Keleman et al., 2012; Lim et al., 2018). In the mosquito, *Aedes aegypti*, it has been demonstrated that the female has the ability to learn odors associatively with aversive stimuli, thus contributing to shifts in host preferences and that this plasticity is modulated by DA and requires Dop1 (Vinauger et al., 2018). More recently, *L. migratoria* Dop1 was shown to facilitate the olfactory preference for gregarious volatiles and that this effect was mediated through the inhibition of microRNA-9a maturation leading to the upregulation of adenylyl cyclase expression (Guo et al., 2018).

Over the last decade, evidence has accumulated that the noctuid moths are excellent model organisms for studying the plasticity of olfactory system (Anton et al., 2007; Gadenne et al., 2016). In these species, the sexual communication relies on the attraction of males by

sex pheromones produced and emitted by conspecific females. The pheromone signal is recognized by olfactory receptor neurons (ORNs) located in the antennae and is integrated first in the primary olfactory centers of brain, the antennal lobes (ALs), which are compartmentalized into spherical neuropil structures, the olfactory glomeruli. The ALs consist of two complexes of glomeruli, the macroglomerular complex (MGC) that is devoted to processing of sex pheromone input from ORNs and the ordinary glomeruli that is involved in processing of all the other odors that the male encounters (Haupt et al., 2010). The processed information is then conveyed by different pathways to higher-order brain centers, the mushroom bodies and the lateral horn, ultimately leading to a characteristic orientation behavior of male towards the pheromone source that is supported by optomotor anemotaxis (Haupt et al., 2010). Despite its stereotypy, this behavior and the underlying processing steps exhibit high degrees of flexibility.

In addition to modulation by circadian rhythm (Groot, 2014) and mating status (Gadenne et al., 2001; Kromann et al., 2015), age- and experience-related plasticity in the male response to sex pheromone has been unveiled in noctuid moths, *Spodoptera littoralis* and *Agrotis ipsilon*. Indeed, a brief pre-exposure of *S. littoralis* males to sex pheromone induced a lowering of the threshold for the behavioral response 24h later. This long-term effect was correlated with a higher sensitivity of ORNs and AL neurons to pheromone signal accompanied by a volumetric increase of MGC glomeruli and of the calyces of the mushroom bodies (Anderson et al., 2007; Anton et al., 2016). It is also known that *A. ipsilon* males are apt to elicit the sex pheromone-mediated orientation flight only when they reach their full sexual maturity within three to four days after emergence (Gadenne et al., 1993; Duportets et al., 1998). This increase in behavioral responsiveness is paralleled by a raise in the sensitivity of sex pheromone-responding AL neurons (Gadenne and Anton, 2000). This modulation of sex pheromone perception was found to be under the control of juvenile hormone (JH) (Anton and Gadenne, 1999; Gadenne et al., 2000) and 20E (Duportets et al., 2013; Bozzolan et al., 2015) and which act in concert with octopamine (Jarriault et al., 2009) and DA respectively (Abrieux et al., 2014). Moreover, *A. ipsilon* DopEcR was recently identified as a mediator of the combined actions of both 20E and DA in the central nervous system (Abrieux et al., 2013).

To further unveil the molecular mechanisms underlying the olfactory modulation by the dopaminergic system in *A. ipsilon* males, we examined whether the age- and experience-related plasticity of behavioral and neuronal responses to sex pheromone is mediated by DA through the Dop1 receptor which is positively coupled to adenylyl cyclase. We first cloned A.

ipsilon Dop1 (AiDop1) and determined its tissue expression profile. We then quantified the levels of DA, *AiDop1* expression and intracellular cAMP in the ALs as a function of age (immature versus mature) and of experience (naive versus pre-exposed to sex pheromone) in relation to the pheromone signal responsiveness. Finally, we examined the effects of the deficiency of Dop1 signaling caused by RNAi-knockdown on the AL cAMP levels and the behavioral response to sex pheromone. Our results highlighted a major requirement of DA-Dop1 signaling in the flexibility of male sexual behavior probably by mediating the neuronal plasticity within ALs through cAMP-dependent mechanisms in the moth *A. ipsilon*.

Material and methods

Insects and tissue collection

Adult *A. ipsilon* originated from a laboratory colony established in Bordeaux and transferred to Versailles. The colony is based on field catches in southern France and wild insects are introduced each spring. Insects were reared on an artificial diet in individual cups until pupation (Poitout and Bues, 1974). Pupae were sexed, and males and females were kept separately in an inversed light/dark cycle (16 h light: 8 h dark photoperiod, with the scotophase starting at 10:00 am) at 22 °C. Newly emerged adults were removed from the hatching containers every day and were given access to a 20% sucrose solution ad libitum. The day of emergence was considered as day-0. All tissue dissections were performed in the middle of the scotophase (between 1:00 pm and 4:00 pm) when males respond maximally to the sex pheromone (Barrozo et al., 2010). For the expression profiles of *AiDop1*, antennae, ALs, brains with excised ALs, sex accessory glands, thoracic muscles, testes, fat bodies, malpighian tubules and midguts of males were dissected under Ringer's solution, then immediately flash-frozen in Eppendorf (Montesson, France) vials kept in liquid nitrogen and stored at -80°C until treatment. For the collection of ALs, brains were first dissected and ALs were then cut from the protocerebrum with a pair of fine scissors.

cDNA synthesis and cloning of *AiDop1*

Total RNAs were extracted with TRIzol reagent (TRI Reagent®, Euromedex, Souffelweyersheim, France) according to the manufacturer's instructions, and were quantified by spectrophotometry at 260 nm. DNase treatment was performed with 2 units of TURBO™ DNase 1 (Ambion, Villebon-sur-Yvette, France) for 30 min at 37 °C followed by a 10-min inactivation at 75 °C. After DNase treatment, single-stranded cDNAs were synthesized from total RNAs (1 µg) with SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA,

USA) according to the manufacturer's instructions. The reaction contained a deoxynucleotide triphosphate (dNTP) mix, RNase OUT, Oligo(dT) primer and sterile water to a final volume of 20 μ l. The mix was heated to 65 °C for 5 min before the enzyme was added and then incubated for 1 h at 42 °C.

Degenerate DNA primers Dop1Gdir1 and Dop1Grev1 were designed against the highly conserved amino acid sequences LIFLSVA and IIYSIFN located in the TM1 and TM7 transmembrane domains of *Bombyx mori* and *Chilo suppressalis* (Table S1). PCRs were carried out with 200 ng brain cDNA with 2.5 units of High Expand Fidelity DNA polymerase (Roche, Saint-Quentin Fallavier, France). Dop1Gdir1 and Dop1Grev1 were added thereafter at 0.4 μ M and each dNTP at 0.25 mM. Following an initial 5-min denaturation at 94 °C, the thermal amplification procedure consisted of 30 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s, elongation at 72 °C for 1 min 30 s and then final elongation at 72 °C for 10 min. The 5' and 3' regions of the corresponding cDNA were obtained by 5'- and 3'-RACE (SMART RACE cDNA Amplification Kit, Clontech, Mountain View, CA, USA) following the manufacturer's instructions. For 5'-RACE, we used a specific reverse primer Dop15'-RACE and Universal Primer Mix (UPM, Clontech) as the forward anchor primer (Table S1). The 3'-RACE amplification was carried out with UPM as the reverse primer and a specific forward primer Dop13'-RACE (Table S1). Touchdown PCR was performed using hot start as follows: 1 min at 94 °C, five cycles of 30 s at 94 °C, 30 s at 67 °C and 1 min at 72 °C, then five cycles of 30 s at 94 °C, 30 s at 64 °C and 1 min at 72 °C, then 25 cycles of 30 s at 94 °C, 30 s at 62 °C and 1 min at 72 °C, then 15 min at 72 °C.

PCR products were purified by agarose gel electrophoresis (NucleoSpin® Extract II, Macherey-Nagel GmbH & Co. KG, Düren, Germany) and cloned into PCRII-Topo plasmid (Invitrogen). After colony isolation, DNA minipreps were prepared (NucleoSpin® Plasmid DNA Purification, Macherey-Nagel GmbH & Co. KG) and the DNA clone containing the proper insert was then sequenced (GATC Biotech SARL, Marseille, France). By merging the overlapping sequences obtained from the 5'- and 3'-RACE, a putative full-length cDNA of 2113 bp was generated and named *AiDop1*.

Quantification of *AiDop1* expression

Real time PCR (qPCR) was performed on cDNA preparations using the ICycler IQ™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Each 12 μ l reaction consisted of 6 μ l Absolute Blue SYBR Green fluor (Thermo Scientific, Waltham, MA, USA), 4 μ l cDNA (25 ng/ μ l) and 2 μ l of qDop1dir

and qDop1rev primers (Table S1) at 10 μ M. PCR conditions were 35 cycles of 95 °C for 30 s, 67 °C for 30 s, 72 °C for 30 s and the length of amplified product was 216 bp. Fluorescence measurements over a 55-95 °C melting curve confirmed the presence of a single specific peak and the absence of primer-dimer peaks for the used primer pairs. Each run included a negative control (water) and a fivefold dilution series of pooled cDNA (from all conditions). The fivefold dilution series were used to construct a relative standard curve to determine the PCR efficiencies and for further quantification analysis. In all experiments, the primers gave amplification efficiencies of 90-100%. Each reaction was run in three technical replicates with at least five independent biological replicates using the technical platform of The Integrative Biology Institute (Sorbonne University, France). Expression levels were analysed with the ICYCLER IQ software (Bio-Rad, Marnes-la-Coquette, France) and the GENORM Visual Basic application for Microsoft EXCEL (Issy-les-Moulineaux, France) as described by Vandesompele et al. (2002). The cycle threshold (Ct) values were determined for the candidate gene and the reference gene, the *A. ipsilon* Ribosomal protein L8 (AiRpL8) gene (accession number JX975720.1), which exhibited the most stable expression levels amongst other tested control genes, Ribosomal protein L13, Glyceraldehyde-3-phosphate dehydrogenase and β -actin. The average Ct value of each triplicate reaction was used to normalize the candidate gene expression level to the geometric mean of the RpL8 level in the Q-GENE software (Simon, 2003). The sequences for specific primers of AiRpL8 are RpL8dir and RpL8rev (Table S1).

Measurement of DA amount in antennal lobes

In the middle of scotophase, the brains of males were removed in ice-cold saline solution and then the ball-shaped ALs were cut from the protocerebrum under a dissecting microscope. The ALs were immediately transferred into Eppendorf vials kept on ice and were sonicated in 100 μ l of 0.1 M perchloric acid (PCA) with antioxidants (0.2 g/l sodium metabisulfite, 0.05 g/l disodium ethylenediaminetetraacetate). PCA extraction has an excellent recovery (over 90%) and therefore no internal standard was used in this procedure. Eppendorf vials contained 10 ALs (from 5 males) and ten vials were prepared for each experimental group. After centrifugation at 20 000 g for 30 min at 4 °C, the supernatant was collected and stored at -20 °C until analysis and the protein content of pellets was measured using Pierce™ Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Fisher Scientific, Villebon-sur-Yvette, France). Fifteen microliters of supernatant was analyzed with high-performance liquid chromatography (HPLC) using a BAS 460 MICROBORE-HPLC system with electrochemical detection (Bio-analytical Systems Inc., West Lafayette, USA) together with a

Uniget C-18 reverse phase microbore column as the stationary phase and connected to a computer equipped with chromatography data station software (model D-7000 HPLC system software manager version 2.0, Merck-Hitachi). The mobile phase was pumped with a flowrate of 0.8 ml/ min and consisted of 0.16 M monochloroacetic acid, 0.5 mM disodium ethylenediaminetetraacetic acid (adjusted to pH 3.4 with glacial acetic acid), 1.4 mM sodium octylsulfonate, 10 nM sodium acetate and 8% acetonitrile. The electrochemical detector with a glassy carbon electrode was set to an oxidation potential of +0.85 V relative to an Ag/AgCl reference electrode. Under these conditions, the detection limit for DA was determined by running the known concentrations of DA in the HPLC system and was about 20 fmol. The DA peak was identified by the retention time and quantified by the area peak as related to the external standard (20 pmol). The DA levels in the AL samples were expressed as nmol per milligram of protein.

Measurement of cAMP amount in antennal lobes

The cAMP concentration was assayed using the cAMP Direct Immunoassay Kit (ab65355; Abcam) according to the manufacturer's instructions. Once dissected, ALs were transferred into Eppendorf vials and rapidly homogenized on ice in 100 μ L of 0.1 M hydrochloric acid. The homogenate was then centrifuged at 10 000 g for 30 min at 4°C and the resulting supernatant was directly used in the assay. Using two-fold serial dilutions, a set of standard solutions of cAMP was prepared for the calibration curve. A standard reaction solution was added to 100 μ L of the diluted cAMP standard solution or 100 μ L of the supernatant and cAMP levels were then determined by ELISA with the reading of absorbance at 260 nm. The cAMP values, expressed as nanograms per milligram of protein, were obtained from the luminescence measurements by reference to the cAMP standard curve. The protein content of the centrifugation pellets was measured using Pierce™ Bicinchoninic Acid (BCA) Protein Assay Kit. The cAMP and total proteins were quantified in a sample prepared from 10 ALs for each group with 10 biological replicates.

Age-related experiments

Males of different ages (0, 1, 2, 3, 4 and 5 days) were collected in the mid-scotophase and transferred to cylindrical plastic containers in a dark box, then transported from the rearing room to the wind tunnel room for the quantification of behavioral response to sex pheromone. The males were then euthanized by using liquid nitrogen, and the ALs were dissected from frozen heads for the quantification of DA and cAMP and the analysis of *AiDop1* expression.

Pre-exposure experiments

Pre-exposure treatments were performed in the wind tunnel at mid-scotophase under the same light and temperature conditions as for behavioral assays. Newly emerged males (day-0) were transferred from the rearing room to the wind tunnel room, and placed in plastic cages (maximum of 3 males per cage) before the end of the photophase. A cage was introduced in the wind tunnel. After 30 s during which the males adjusted to the airflow, a filter paper containing the stimulus was placed 160 cm upwind from the cage. Pheromone stimulation was performed with an artificial pheromone blend containing (Z)-7-dodecen-1-yl acetate (Z7-12:OAc), (Z)-9-tetradecen-1-yl acetate (Z9-14:OAc), and (Z)-11-hexadecen-1-yl acetate (Z11-16:OAc) (Sigma Aldrich, Saint-Quentin Fallavier, France) at a ratio of 4:1:4. For pre-exposure, we used a dose of 10 ng pheromone blend which was previously found to elicit the optimum behavioral response (Barrozo et al., 2010). Each male, kept within the cage, was submitted to pheromone stimulation during 3 min, after which it was removed from the wind tunnel and transferred to the rearing room. The behavioral response to sex pheromone of pre-exposed males was analyzed at 4h, 24h and 48h after the pre-exposure treatment. The males were then euthanized by using liquid nitrogen, and the ALs were dissected from frozen heads for the quantification of DA, cAMP and *AiDop1* expression. Control groups were composed of 0-, 1- and 2-day-old sexually immature males exposed to hexane for 3 min.

RNA interference experiments

The dsRNA production and injection conditions were established by following the protocols used in *A. ipsilon* for the silencing of *AiDopEcR* (Abrieux et al., 2013). The dsRNAs targeting *AiDop1* named Dop1-1-dsRNA (375 bp) and Dop1-2-dsRNA (411 bp), and designated against bacterial *LacZ* (372 bp) were produced with a MEGAscript® T7 High Yield Transcription Kit (Ambion) according to the manufacturer's instructions. A PCR was performed on 1 µl plasmid (50 ng/µl) with specific primers for each target gene (Dop1-1 T7 dir/Dop1-1 T7 rev, Dop1-2 T7 dir/Dop1-2 T7 rev and LacZ T7 dir/LacZ T7 rev) (Table S1) under the following program: 35 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min. PCR products were purified with a Nucleospin® extract II kit (Macherey-Nagel GmbH & Co. KG) and quantified by spectrometry. The transcription reaction was then performed overnight at 37 °C on 1 µg of PCR product in a reaction mix containing 2 µl of each nucleotide, 2 µl of T7 RNA polymerase enzyme and 2 µl T7 10X Buffer in a final volume of 20 µl. After spectrometry quantification and gel dock analysis, the reaction mix was incubated for 15 min at 37° C with 2 units of Turbo DNase (Ambion). The dsRNA precipitation was performed by addition of 30 µl diethylpyrocarbonate (DEPC) water and 20 µl LiCl, storage of the mixture

at -20 °C for 2 h, and then centrifugation for 30 min at 16 000 g. The pellet was washed with 1 ml 75% ethanol, centrifuged and dried before elution in 11 µl DEPC water. Samples were denatured at 95 °C for 5 min followed by a rehybridization step of 1 h 30 min at room temperature. The dsRNA integrity was then checked by loading on an agarose gel. Before injection, dsRNA was diluted at 0.5 µg/µl in saline solution (CaCl₂ 7.1 mM, Na₂ β-glycerophosphate 22 mM, MgSO₄ 13.5 mM, MgCl₂ 26.9 mM, KCl 29.5 mM, glucose 23.9 mM, pH 6.8).

Day-2 males were anaesthetized with carbon dioxide and injected with 1 µg dsRNA at the lateral region of the intersegment membrane between the second and third abdominal segments. The injections were performed with a Hamilton syringe with a 32-gauge needle. At day-5, the behavioral response of males to sex pheromone was tested in a wind tunnel. The ALs were then dissected for the *AiDop1* expression analysis and the cAMP quantification. Control groups were composed of non-injected, dsRNA-bacterial LacZ- and saline-injected males.

Behavioral response to sex pheromone in a wind tunnel

For the wind tunnel experiments, we prepared a pheromone blend diluted in hexane at 1 ng/µl containing (Z)-7-dodecenyl acetate (Z7-12: Ac), (Z)-9-tetradecenyl acetate (Z9-14: Ac) and (Z)-11-hexadecenyl acetate (Z11-16: Ac) at a ratio of 4: 1: 4, which has been used successfully in field trapping experiments (Picimbon et al., 1997; Gemenio and Haynes, 1998). Behavioral tests were performed using a 190-cm-long flight tunnel under red light illumination as previously described. Experiments were performed during the middle of the scotophase when males respond maximally to the sex pheromone (Barrozo et al., 2010). Environmental conditions during the bioassay were held constant: 23 °C, 50 ± 10% relative humidity, wind speed of 0.3 m/s. A single experimental male was introduced in the wind tunnel and placed in a cage. After 30 s, during which the male adjusted to the airflow, the pheromone blend, positioned 150 cm upwind from the cage, was delivered for 3 min. During this space of time, the behavior of males (*AiDop1*-dsRNA-injected males and control males) was observed, and partial flight (half of the distance between the source and the cage), complete flight (within 5 cm of the source) and landing on the pheromone source were considered as an orientated response towards the sex pheromone. Orientated as well as random flights were all counted together in order to quantify the general flight activity of insects. All experiments were performed double-blind to avoid partial observations. On each day of experiments, different groups of males were tested including at least one group of males that were expected to show a high response level to avoid experimental bias.

The pheromone stimulation was delivered with a programmable olfactometer adapted from Party et al. (2009). Air from the building was charcoal-filtered and re-humidified, then divided into several equal flows (300 ± 10 ml/min) using a Y connector (model 1/8" P514, Upchurch Scientific) and a 5-port manifold (model P-115, Upchurch Scientific, Silsden, United Kingdom). Each flow was connected to a miniature electrovalve (model LHDA1233115H, the Lee Company, Westbrook, Maine, USA) driven by a Valve-Bank programmer (AutoMate Scientific, Berkeley, CA, USA). Activating the appropriate valve directed the flow to a 4-ml glass vial containing the pheromone source and closed by a septum cork. The inlet and outlet of the source pheromone were made of two hypodermic needles (18-g size) inserted through the septum and connected with polytetrafluorethylene tubing (1.32 mm Inner Diameter (ID), 20 cm Length). 10 μ l of the blend pheromone at 1 ng/ μ l was deposited on a filter paper and placed inside the vial. This dose (10 ng) was shown to give the best behavioral results with sexually mature virgin males (Barrozo et al., 2010). Contaminated air was removed from the set-up by an exhaust fan after each behavioral assay.

Bioinformatics

The molecular mass of AiDop1 protein was determined with the Compute pI/MW application (<http://www.expasy.org/>). The amino acid sequences of DA receptors were aligned with MAFFT program. The transmembrane domains were predicted by TMHMM (<http://genome.cbs.dtu.dk/services/TMMH/>; <http://smart.embl-heidelberg.de/>). The potential N-linked glycosylation sites (N-x-[S/T]) and potential phosphorylation sites by PKC ([S/T]-x-[R/K]) were determined by using NetNGlyc (<http://www.cbs.dtu.dk/services/NetNGlyc/>) and NetPhos (<http://www.cbs.dtu.dk/services/NetPhos/>) respectively.

Accession numbers (IDs) for protein sequences of dopamine receptors retrieved from GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) are as follows: the lepidoptera *B. mori* Dop1 (BmDop1) ID: NP_001108459.1 and *C. suppressalis* Dop1 (CsDop1) ID: KP784317, the dipteran *D. melanogaster* Dop1 (DmDop1) ID: CAA54451.1, the coleopteran *Tribolium castaneum* Dop1 (TcDop1) ID: NP_001280543.1, the hymenopteran *Apis mellifera* Dop1 (AmDop1) ID: NP_001011595.1 and the orthopteran *L. migratoria* Dop1 (LmDop1) AKD41292.1.

Statistical analysis

The means of *AiDop1* expression and of DA and cAMP levels in ALs were compared using one-way analysis of variance followed by a Tukey's multiple comparison post-hoc test. $P < 0.05$ was accepted as statistically significant. For behavioral experiments, statistical differences between groups of males were evaluated using a R X C test of independence by means of a G-test and also by applying the Williams's correction (Sokal et al., 1995).

Results

Cloning and sequence analysis of *AiDop1*

On the basis of the amino acid sequences of Dop1 receptors cloned in the lepidopterans *B. mori* and *C. suppressalis*, we designed a pair of degenerate oligonucleotide primers from highly conserved regions in the TM1 and TM7 transmembrane domains and succeeded in amplifying a partial fragment of 864 bp via reverse transcription-polymerase chain reaction (RT-PCR) on brain total RNA extracted from 5-day-old adult males. The missing 5' and 3' ends of this fragment were then obtained by rapid amplification of cDNA ends (RACE) using gene-specific primers. The 5'-RACE and 3'-RACE reaction products were assembled with the original fragment to generate a full-length cDNA named *AiDop1* and deposited in the GenBank database under the accession number MN164482. The cDNA extends over 2113 bp and contains a 5'-untranslated region (5'-UTR) of 169 bp, an open reading frame (ORF) of 1227 bp and a 3'-UTR of 717 bp with a polyadenylation signal upstream of the poly(A) tail (Fig. S1). The *AiDop1* ORF was translated into a predicted protein sequence of 409 amino acids with a calculated molecular mass of 46.5 kDa.

Hydrophobicity analysis of the *AiDop1* protein revealed the expected conformation of GPCRs with seven transmembrane domains (TM1-7) connected with three intracellular loops (INL1-3) and three extracellular loops (EXL1-3), an extracellular N-terminal region and a cytoplasmic C-terminal region (Fig. 1). A series of functionally critical amino acid residues and sequence motifs for the aminergic GPCRs were also identified in the *AiDop1* protein (Fig. 1). The *AiDop1* receptor possesses two aspartate residues D₈₂ in TM2 and D₁₁₆ in TM3 that are shared between all catecholamines receptors and two conserved cysteine residues C₁₀₉ and C₁₉₅ that are involved in the formation of a disulfide bond between EXL1 and EXL2. As in all dopaminergic receptors, three serine residues S₂₀₇, S₂₀₈ and S₂₁₁ in TM5 with a conserved spacing pattern in the motif S₂₀₇-S-x-x-S are present in *AiDop1*. Additionally, *AiDop1* harbors the consensus sequence D₁₃₃RY located at the N-terminus of INL2. In TM6, the motif F₂₈₅-x-x-CW-x-PFF is highly conserved and the pair of phenylamine residues (F₂₉₁,

F₂₉₂) represents a unique feature of aminergic receptors. Finally, the characteristic motif N₃₂₅P-x-x-Y located at the end of TM7 is commonly found in all members of the biogenic amine receptor family. It is known that aminergic GPCRs are subject to regulatory events involving post-translational modifications, such as phosphorylation, palmitoylation and glycosylation. AiDop1 contains two potential sites for N-linked glycosylation, N₁₆ET in the extracellular N-terminal region and N₂₄₉RT in INL3, and nine potential sites for phosphorylation by protein kinase C (PKC) such as T₆₃DR in INL1, T₁₄₉RK in INL2, T₁₈₆QK in EXL2, S₂₃₆IR and S₂₅₃VR in INL3, and T₃₄₅SR, S₃₅₆VR, T₃₉₃PR and S₄₀₀VR in the intracellular C-terminal region (Fig. 1).

Comparison of the full protein sequence of AiDop1 with insect Dop1 orthologues revealed a high percentage of amino acid identity and especially with the lepidopterans *B. mori* Dop1 (BmDop1; 96%) and *C. suppressalis* Dop1 (CsDop1; 95%) followed by the coleopteran *Tribolium castaneum* Dop1 (TcDop1; 67%), the hymenopteran *A. mellifera* Dop1 (AmDop1; 60%), the orthopteran *L. migratoria* Dop1 (LmDop1, 59%) and the dipteran *D. melanogaster* Dop1 (DmDop1; 53%) (Fig. 1).

Tissue expression pattern of *AiDop1*

In order to gain some preliminary information about the functions of *AiDop1*, an initial tissue distribution screen of *AiDop1* mRNA in 5-day-old males was performed by qPCR. *AiDop1* is expressed at higher levels in both AL and the brain deprived of ALs than in peripheral tissues including sex accessory gland, testis and midgut (Fig 2). No amplification was detected in other investigated tissues (antenna, thoracic muscle, fat body and malpighian tubule) (Fig. 2).

Effect of age on the sex pheromone response and the amounts of DA, *AiDop1* transcript and cAMP in ALs

To apprehend the possible involvement of Dop1-mediated dopaminergic signaling in the age-related olfactory plasticity, we evaluated the percentage of sex pheromone-guided orientated flight as well as the levels of DA, *AiDop1* mRNA and cAMP in the ALs for 0- to 5-day-old males. The proportion of the orientated behavioral response was low (below 20%) in 0- to 2-day-old males and significantly increased at the age of 3 days to attain about 75% in 4- to 5-day-old males (Fig. 3A). DA together with *AiDop1* mRNA and cAMP were present in ALs within the first days of adult life, and increased significantly from day-3, then remained high at day-5 (Fig. 3B, C, D). The amounts of DA, *AiDop1* mRNA and cAMP in ALs of 5-day-old sexually mature males were approximately 4.6-, 3.1- and 3-fold higher than in 1-day-old sexually immature males, respectively (Fig. 3B, C, D).

Effect of pre-exposure on the sex pheromone response and the amounts of DA, *AiDop1* transcript and cAMP in ALs

To explore the possible role of Dop1-mediated dopaminergic signaling in the experience-induced olfactory plasticity, newly emerged males (day-0) were pre-exposed to sex pheromone during 3 min and we then determined the percentage of sex pheromone-guided orientated flight as well as the levels of DA, *AiDop1* mRNA and cAMP in ALs at 4h, 24h and 48h after the pre-exposure treatment. The percentage of males performing an orientated response at 24h (PE-24h) and 48h (PE-48h) following pre-exposure was significantly higher as compared with that of control males (D1 and D2) respectively (Fig. 4A). The AL DA, *AiDop1* mRNA and cAMP in PE-24h and PE-48h males were at higher levels than in D1 and D2 males, respectively (Fig. 4B, C, D). By contrast, there was no difference in the behavioral response and the amounts of AL DA, *AiDop1* mRNA and cAMP between PE-4h and D0 males (Fig. 4A, B, C, D). The quantities of DA, *AiDop1* mRNA and cAMP in ALs of PE-48h males were approximately 3-, 1.9- and 2.4-fold higher than in D2 males, respectively (Fig. 4B, C, D).

Effect of *AiDop1* silencing on the amount of AL cAMP and the behavioral response to sex pheromone

By using RNA interference technology, we tested the impact of *AiDop1* knockdown on both the cAMP level in ALs and the behavioral response of male to sex pheromone. The injection of Dop1-1-double-stranded RNA (Dop1-1-dsRNA) into 2-day-old males resulted in a decreased amount of *AiDop1* mRNA of approximately 67% in ALs three days after the administration as compared with the three control groups (saline-, bacterial *Beta-galactosidase* (*LacZ*)-dsRNA- and non injected males) (Fig. 5A). The Dop1-1-dsRNA-injected males exhibited a sharp decline in the AL cAMP level and the orientated behavioral response to sex pheromone as compared with control males (Fig. 5B, C). The general flight activity of Dop1-1-dsRNA-injected males (around 85%) was not statistically different than that of control males (around 92%), showing that the *AiDop1* silencing did not alter the flight ability of moths (Fig. 5D). In order to consolidate our RNAi results, we also tested the effect of second *AiDop1*-double-stranded RNA (Dop1-2-dsRNA). The injection of Dop1-2-dsRNA caused a decrease in the AL *AiDop1* mRNA expression and cAMP levels (Fig. S2A, B) and the resulting behavioral phenotype was similar to that induced in the Dop1-1-dsRNA-treated males (Fig. S2C).

Discussion

AiDop1 belongs to the Dop1 group of insect DA receptors

We cloned a full length cDNA fragment encoding a predicted AiDop1 protein that exhibits the structural features of GPCRs including seven hydrophobic transmembrane domains, an extracellular N-terminus, and a cytoplasmic C-terminal region. AiDop1 also contains several highly conserved amino acid residues and sequence motifs among the catecholamine receptors. The two aspartate residues (D₈₂, D₁₁₆) are thought to act as counterions in binding the protonated amine group of DA (Fraser et al., 1988; Strader et al., 1988) and the three serine residues (S₂₀₇, S₂₀₈, S₂₁₁) within the signature motif S-x-x-SS are predicted to form hydrogen bonds with the DA ring hydroxyl groups (Strader et al., 1989, 1995). The formation of a disulfide bond between EXL1 and EXL2 by the two cysteine residues (C₁₀₉, C₁₉₅) is believed to maintain the receptor under its active conformation (Karnik and Khorana, 1990). The characteristic D₁₃₃RY and F₂₈₅-x-x-CW-x-PFF sequences, which are presumed to play a key role in both receptor activation and interaction with the aromatic ring of DA (Kobilka, 2007), are also present in AiDop1. The consensus motif N₃₂₅P-x-x-Y was additionally found in TM7 of AiDop1 and this sequence is known to be critical for ligand binding, G protein coupling and sequestration of the inactive state of the receptor (Fritze et al., 2003; Kalatskaya et al., 2004; Borroto-Escuela et al., 2011). As for the aminergic GPCRs, AiDop1 has a number of consensus motifs for *N*-glycosylation sites and PKC phosphorylation sites. Thus, potential glycosylation and phosphorylation of AiDop1 might be involved in cell surface expression of the receptor as well as in its desensitization mediated by an uncoupling of associated G protein (Defea, 2008). Finally, the overall protein of AiDop1 was found to share a high degree of similarity with Dop1 receptors previously identified in the lepidopterans *B. mori*, *C. suppressalis*, with more than 94% identity, but also in other insect orders including Coleoptera, Orthoptera, Diptera and Hymenoptera. Based on all these bioinformatic data, AiDop1 is clearly assigned to the Dop1 group of insect DA receptors, and which is closely related to vertebrate D1-like receptors.

***AiDop1* is expressed within the central nervous, digestive and reproductive systems**

A single form of *Dop1* seems to be present in *A. ipsilon* as reported in the moths *B. mori* (Mitsumasu et al., 2008), *C. suppressalis* (Xu et al., 2017) and *M. sexta* (Dacks et al., 2012), the honey bee *A. mellifera* (Blenau et al., 1998) and the cricket *G. bimaculatus* (Watanabe et al., 2013) whereas two splice variants encoding *Dop1R1* and *Dop1R2* isoforms have been characterized in the fruit fly *D. melanogaster* (Sugamori et al., 1995; Han et al., 1996). As all *Dop1*-type receptors, *AiDop1* exhibits a wide tissue distribution marked by a major expression in the brain (Watanabe et al., 2013; Xu et al., 2017). This elevated transcriptional activity of *AiDop1* was measured more specifically in the ALs, indicating that this receptor is a potential target for any modulatory actions of DA within the central olfactory system. This is consistent with previous studies performed in the moth *M. sexta* and the mosquito *A. aegypti* demonstrating that *Dop1* is highly expressed in the entire glomerular volume of ALs (Dacks et al., 2012; Vinauger et al., 2018).

It is interesting to note that *AiDop1* was also detected in the organs of male reproductive tract, and especially in the testis and the sex accessory glands which produce proteinaceous material that is associated with the production and the transport of sperm within a spermatophore transferred into the female during copulation. In *A. mellifera*, *Dop1* is expressed in different parts of male reproductive apparatus such as testis, mucus glands and seminal vesicles in which the high expression of *Dop1* is coupled to increased cAMP levels in response to DA (Matsushima et al., 2019). Thus, it was proposed that DA might promote, via *Dop1*, the activities of seminal vesicles including sperm transfer and storage. In mammals, the DA testicular originates from nerve innervation, bloodstream and local synthesis by Leydig cells (Romeo et al., 2004; Davidoff et al., 2005) and the dopamine D1 receptor (also known as DRD1) has been localized in germ cells and spermatogonia nearest the basal lamina of seminiferous tubules, thus suggesting paracrine actions of DA in the spermatogenic process through the activation of DRD1 (Gonzalez et al., 2015).

As described in *G. bimaculatus* and *C. suppressalis*, *Agrotis Dop1* is expressed in the midgut (Watanabe et al., 2013; Xu et al., 2017). In insects, and especially in moths, it is known that the midgut receives catecholamine-containing nerve endings from the ventral nerve cord along its longitudinal axis (Klemm, 1979). In *L. migratoria*, DA was found to repress the spontaneous and phasic contractions of the foregut (Lange and Chan 2008). Similarly, it has been unveiled in the mouse, *Mus musculus*, that the inhibitory action of DA on the contractility of the small intestine is achieved through a DRD1/adenylyl cyclase-

dependent signaling, resulting in altered calcium mobilization and myosin ATPase activity (Zizzo et al., 2010).

Finally, this tissue-specific expression analysis of *AiDop1* highlights that the dopaminergic system is largely distributed in *A. ipsilon* and that Dop1 is probably a molecular mediator of central and peripheral dopaminergic signaling pathways modulating olfactory functions, midgut mobility as well as several aspects of male reproductive physiology, and notably spermatogenesis and seminal fluid production.

***AiDop1* is required for the display of sex pheromone-orientated flight**

In this study, we observed that the injection of dsRNAs targeting various regions of *AiDop1* induced approximately 65-75% reduction in the expression levels of transcript in ALs, and led to similar effects on both the AL cAMP levels and the sex pheromone behavioral response in dsRNA-treated males. These results consolidated our RNAi experiments even if we could not verify the decline of the corresponding protein in the ALs of *Dop1*-deficient males because the antibody against this receptor is not available.

The *AiDop1* silencing resulted in decreased AL cAMP amounts and altered sex pheromone-orientated flight with a lower percentage of responding males. Given that *AiDop1* is not exclusively expressed in ALs but is also present in other brain regions as well as in the reproductive organs such as testes and sex accessory glands, it is likely that the *AiDop1* knockdown in these tissues also contributes to the disruption of sex pheromone behavioral response in dsRNA-treated males. Our RNAi data provide evidence that *AiDop1* potentiates the orientated response of male to sex pheromone in *A. ipsilon* and probably through the activation of cAMP signaling pathway in ALs. In concordance with this finding, *Dop1R1* has been shown to be involved in many aspects of *D. melanogaster* sexual activity including male-male courtship (Chen et al., 2012) and copulation duration (Crickmore and Vosshall, 2013). Moreover, it has been demonstrated in rats that the copulatory behavior of males and their preference for female pheromones depend on the activation of DRD1 signaling in the mesolimbic dopaminergic system (Beny-Shefer et al., 2017).

In the moths, it is well described that the dopaminergic innervation is extended across the brain with a high density in all the ordinary glomeruli and the proximal region of MCG within ALs as well as in the lateral protocerebrum, including the mushroom bodies (Dacks et al., 2012). Recent studies have revealed that DA facilitates the male sexual behavior in *A. ipsilon* (Abrieux et al., 2014) and affects both the response (i.e., amplitude and temporal dynamic) and the sensibility of AL neurons to odors, thereby inducing changes in the

proportion of neurons participating in the encoding of olfactory stimuli, in *M. sexta* (Dacks et al., 2012). Considering all the above data, it is likely that *AiDop1* mediates some modulatory effects of DA on the sex pheromone-driven responses of AL neurons, thus contributing to the elicitation of sexual attraction behavior in *A. ipsilon* males. In order to decipher these central actions of DA and *AiDop1*, we are planning to evaluate the effects of DA treatment and *AiDop1* silencing on the neuronal response to pheromone signal, and in particular in the *Agrotis* MCG, using intracellular recordings.

We cannot rule out the possibility that other DA receptors than *Dop1* might be involved in the dopaminergic modulation of the sex pheromone responsiveness of the AL. Indeed, *DopEcR* has been shown to act as an up-regulator of the sex pheromone perception by transmitting the DA signals within the *Agrotis* AL (Abrieux et al., 2013, 2014) and in addition to *Dop1*, the expression of *Dop2* and *Dop3* has been detected in the *Manduca* AL (Dacks et al., 2012). It seems reasonable to hypothesize that all these receptors may coexist in the *Agrotis* AL with a distinct and/or complementary distribution pattern. Given that their activation is either negatively or positively coupled to intracellular cAMP levels, these receptors might be potential mediators of enhancing and suppressive effects of DA on the AL neuronal responses, and thus modulating the central processing of pheromone information in moths. This speculation is strengthened by recent data demonstrating in rats that dopaminergic modulation plays a role in odor detection and discrimination through opposing effects of D1- and D2- type receptors within the olfactory bulb (Yue et al., 2004; Escanilla et al., 2010). There is also a strong possibility that *AiDop1* could act in other brain areas than the primary olfactory neuropiles. This is supported by the observation that *AiDop1* was detected in the brain whose ALs were excised. In addition, several studies have shown that *Dop1* was present in second-order olfactory centers, and more precisely in the Kenyon cells of mushroom bodies (Mustard et al., 2005; Hamada et al., 2009; Dacks et al., 2012) and that its silencing impaired the ability to discriminate, learn and memorize odors (Lim et al., 2018; Vinauger et al., 2018).

DA-*Dop1* signaling in AL is potentially related to the increase in sex pheromone responsiveness throughout adulthood

Our results indicated that DA and *AiDop1* transcript as well as intracellular cAMP in ALs were at higher levels in old sexually mature males than in young sexually immature males. This brought in light an age-dependent upregulation of DA amount and *AiDop1* expression coupled to cAMP within ALs, and which coincided with the age-dependent

increase in the neuronal and behavioral responses to the pheromone signal. Such a correlation leads us to suggest that DA-Dop1 signaling is positively related to the neuronal plasticity that takes place within ALs and contributes to the emergence of sex pheromone-orientated flight behavior in sexually mature males. Similarly, a coordinated increase in both DA levels and *Dop1* expression in the adult brain was correlated with the activating effects of DA on the sexual maturation and mating flight of the male honeybees (Sasaki et al., 2012). In addition, it has been described in the male hamsters that the DA and DRD1 levels increase gradually in various brain areas throughout the pubertal transition and this molecular plasticity of dopaminergic system is associated to neuronal remodeling that accompanies the maturation of reproductive behavior (Romeo et al., 2002).

There is a growing body of evidence that biogenic amines can be themselves modulated through functional interactions with endocrine factors such as sexual hormones, and this second order modulation, termed metamodulation, has been particularly described in the context of male reproduction (Mesce, 2002). In mammals, testosterone is known to be essential for the display of male sexual behavior by enhancing the processing relevant stimuli and this action has been shown to be driven through alterations in the synthesis, release and receptor expression for neurotransmitters such as DA and serotonin (Hull et al., 1999, 2004). In insects, juvenile hormone (JH) was found to be a critical signal in the emergence of DA-mediated mating flight of *A. mellifera* drones by increasing simultaneously DA and *Dop1* expression levels in the brain (Akasaka et al., 2010; Sasaki et al., 2012). In the *A. ipsilon* male, it was observed that the levels of JH in hemolymph of old sexually mature males are higher than those of young sexually immature males and that this difference is in part due to the elevation of JH biosynthetic activity by the corpora allata, the hormone-producing gland, with aging (Duportets et al., 1998). By manipulating the JH hemolymph titers, we have previously showed that the presence of this hormone is needed for the maturation of sex pheromone responses of male. Indeed, the injection of JH into immature males resulted in an increased sensitivity of the sex pheromone-responding AL neurons, and which was sufficient to elicit the anemotactic zigzagging flight. On the contrary, the neuronal and behavioral responsiveness to sex pheromone was suppressed in mature males if they were deprived of JH by the surgical removal of the corpora allata (Anton and Gadenne, 1999; Gadenne and Anton, 2000; Jarriault et al., 2009). It is interesting to emphasize that the behavioral changes resulting from RNAi-mediated *Dop1* silencing are similar to those caused by the JH deprivation in mature males. It is also noteworthy that the age-related dynamics of DA and *AiDop1* expression amounts in ALs match with fluctuations of the JH hemolymph titers. Taken

together, these observations point a possible functional interplay between the JH signaling and the dopaminergic system operating in ALs. Thus, DA might be a mediator of enhancing effects of JH on sex pheromone responses through the activation of AL DA-Dop1 signaling via JH-evoked concomitant increases of DA and Dop1 protein in response to the elevation of circulating hormone levels throughout the adulthood of *A. ipsilon* male. To explore the existence of such a regulation of the AL dopaminergic system by JH, experiments are in progress to examine the impact of JH administration in young males and of JH deprivation in old males on the amounts of DA and *AiDop1* expression in ALs.

DA-Dop1 signaling in AL is potentially related to the increase in sex pheromone responsiveness after odor pre-exposure

We noticed that young sexually immature males exhibited an increase in their behavioral response to sex pheromone 24h and 48h after a brief pre-exposure to this odor and this long term effect was accompanied by elevated amounts of DA, *AiDop1* transcript and intracellular cAMP in ALs as compared with naive males. In concordance with these results, it has been reported in *A. mellifera* that exposing young worker bees to queen mandibular pheromone (QMB) induced fluctuations in the levels of DA, dopaminergic receptors and cAMP in the brain, and these central effects of QMB on the DA pathways strengthened their attraction towards this pheromone (Beggs et al., 2006). Histological and neurophysiological investigations performed in the moth *S. littoralis* indicated that the behavioral increase in the response of male to sex pheromone after odor pre-exposure was paralleled with a rise in both the sensitivity of AL neurons and the volume of glomeruli belonging to the MCG (Anton et al., 2016). The enlargement of AL glomeruli observed in young *Drosophila* females through olfactory pre-exposure has been found to originate from an increase in the number of neuron branching and synaptic connections, and these structural modifications of the glomerular network appeared to be dependent on the cAMP cascade (Devaud et al., 2001, 2003). By taking into account the above-mentioned data, it seems reasonable to assume that the brief pre-exposure of *Agrotis* males to the female sex pheromone, early in their adult life, undergoes concomitant increases in DA and *AiDop1* expression amounts within ALs, and thus triggering DA-Dop1 signaling which in turn governs to the anatomical and functional reshaping of neuronal circuits within glomeruli processing pheromone information and consequently facilitates the expression of orientated flight behavior. This assumption is backed up by recent data unveiling in rats that the binding of DA to DRD1 receptor contributes to the enhancement of copulatory behavior in males pre-exposed to an estrous

female, probably through the involvement of adenylyl cyclase-cAMP-protein kinase A (PKA) signaling in the medial preoptic area (McHenry et al., 2012).

A series of reports demonstrated that in young worker bees exposed to QMP, the pheromone-evoked behavioral effects were mediated by changes in the circulating JH levels via a modulation of JH biosynthesis rate, and which were correlated with the changes induced in the brain dopaminergic system (Kaatz et al., 1992; Robinson et al., 1998; McQuillan et al., 2014). An interesting observation in *A. ipsilon* is that sex pheromone pre-exposed young males have higher levels of circulating JH than in naive males (E. Gassias, unpublished data). This raises the possibility that sex pheromone pre-exposure provokes an elevation of circulating JH, which might affect the functioning of AL dopaminergic system through DA-Dop1 signaling activation, and thus promoting to the emergence of sex pheromone-guided flight behavior in young *Agrotis* males. In order to ascertain the existence of such an interaction of JH with DA signals through early olfactory experience, we will examine whether the JH deprivation may prevent the sex pheromone-induced effects on the levels of DA and *AiDop1* expression in ALs as well as on the sex pheromone behavioral response in pre-exposed males.

Hypothetical model for Dop1-mediated DA actions in the *Agrotis* AL

It is well established that D1-like receptor has the ability to activate numerous signaling cascades, via a physical and functional coupling to heterotrimeric G proteins, including the canonical adenylyl cyclase-cAMP-PKA pathway which has been found in mammals to regulate the excitability of neurons as well as their synaptic communication by modulating the trafficking and the activity of ion channels (Neve 2004; Undieh, 2010; Yang et al., 2013). *In vitro* studies performed from honeybee AL neurons provided convincing evidence that DA modifies the temporal dynamic of their odor-driven responses by attenuating the amplitude of Ca^{2+} -dependent K^{+} currents (I_{KCa}), thus reducing the duration of the postexcitatory inhibition and consequently increasing the cell excitability (Ellen and Mercer, 2012). Given that there are numerous reports showing that the inhibition of Ca^{2+} -dependent K^{+} channels (SK) also facilitates the growth and differentiation of neurons and synaptic plasticity (Faber and Sah, 2007; Ohtsuki et al., 2012), the downregulation of SK conductance by DA would be expected to play a role in developmental events occurring within ALs. Taken together, these data lead us to propose a hypothetical model of Dop1-mediated DA modulation in *Agrotis* ALs. After binding to DA, Dop1 activates the catalytic activity of adenylyl cyclase by G subunit and subsequently increases cAMP to initiate a

putative cAMP-PKA signaling which might repress the SK channel activity, and the resulting reduction of I_{KCa} would contribute to structural and functional development of MCG glomeruli, and thus promoting the neuronal and behavioral responses to pheromone signal. In *A. ipsilon* male, the response of MCG neurons to sex pheromone has been recently characterized by extracellular recordings and it exhibited the postexcitatory inhibition phase (called I) which was presumed to be generated by an intrinsic SK conductance (Martinez et al., 2013). In order to test the hypothesis that SK channels are targets of DA-Dop1 signaling, as proposed above, it would be interesting to examine whether the magnitude of I phase in the sex pheromone response of *Agrotis* MCG neurons is affected in Dop1-deficient males. A recent study highlighted a new molecular mechanism of action of Dop1 in the migratory locust adult brain. The Dop1 activation was found to inhibit the maturation of microRNA-9a leading to an increase in the expression level of adenylyl cyclase coupled with the enhancement of gregarious behavior (Guo et al., 2018). It might be conceivable that Dop1 mediates its modulatory actions in *Agrotis* AL by targeting the adenylyl cyclase through a regulation of the expression of its mRNA via microRNA-9a inhibition and of its activity via G protein activation. This double regulation of adenylyl cyclase in DA-Dop1 signaling might strengthen the activation of the down-stream cascade and thus inducing long-lasting changes in the MCG network.

In summary, we show that in the moths, Dop1 signaling in ALs mediates the behavioral response of male to the female sex pheromone, probably by promoting the central processing of pheromonal signal. Moreover, our results reveal an age- and experience-related upregulation of DA and *AiDop1* expression levels coupled to cAMP in ALs, and which is timely correlated with the upregulation of neuronal and behavioral sensitivity to sex pheromone. This relationship provides evidence that age- and experience-dependent changes in sex pheromone responsiveness are sustained by DA-Dop1 signaling which operates in the primary olfactory centers, presumably through cAMP-dependent mechanisms. Finally, this present study expands our understanding of mechanisms underlying olfactory plasticity, mechanisms that appear to be highly conserved between insects and mammals.

Acknowledgements

We thank Corinne Chauvet for her help in the insect rearing.

Competing interests

The authors disclose no potential conflicts of interest.

References

- Abrieux, A., Debernard, S., Maria, A., Gaertner, C., Anton, S., Gadenne, C. and Duportets, L.** (2013). Involvement of the G-protein-coupled dopamine/ecdysteroid receptor DopEcR in the behavioral response to sex pheromone in an insect. *PLoS One* **8**, <https://doi:10.1371/journal.pone.0072785>.
- Abrieux, A., Duportets, L., Debernard, S., Gadenne, C. and Anton S.** (2014). The GPCR membrane receptor, DopEcR, mediates the actions of both dopamine and ecdysone to control sex pheromone perception in an insect. *Front. Behav. Neurosci.* **8**, <https://doi:10.3389/fnbeh.2014.00312>.
- Agarwal, M., Giannoni, G. M., Morales-Matos, C., Del Valle Díaz, R.A., Abramson, C.I. and Giray, T.** (2011). Dopamine and octopamine influence avoidance learning of honey bees in a place preference assay. *PLoS One* **6**, <https://doi:10.1371/journal.pone.0025371>.
- Akasaka, S., Sasaki, K., Harano, K. and Nagao, T.** (2010). Dopamine enhances locomotor activity for mating in male honeybees (*Apis mellifera* L.). *J. Insect. Physiol.* **56**, <https://doi:10.1016/j.jinsphys.2010.03.013>.
- Anderson, P., Hansson, B.S., Nilsson, U., Han, Q., Sjöholm, M., Skals, N. and Anton, S.** (2007). Increased behavioral and neuronal sensitivity to sex pheromone after brief odor experience in a moth. *Chem. Senses* **32**, 483-491.
- Anton, S., Chabaud, M.A., Schmidt-Büsser, D., Gadenne, C., Iqbal, J., Juchaux, M., List, O., Gaertner, C. and Devaud, J.M.** (2016). Brief sensory experience differentially affects the volume of olfactory brain centres in a moth. *Cell. Tissue Res.* **364**, <https://doi:10.1007/s00441-015-2299-0>.
- Anton, S., Dufour, M.C. and Gadenne, C.** (2007). Plasticity of olfactory-guided behaviour and its neurobiological basis: lessons from moths and locusts. *Entomol. Exp. Appl.* **123**, <https://doi:org/10.1111/j.1570-7458.2007.00516.x>.
- Anton, S. and Gadenne, C.** (1999). Effect of juvenile hormone on the central nervous processing of sex pheromone in an insect. *Proc. Natl. Acad. Sci. U S A* **96**, 5764-5767.

- Awata, H., Wakuda, R., Ishimaru, Y., Matsuoka, Y., Terao, K., Katata, S., Matsumoto, Y., Hamanaka, Y., Noji, S., Mito, T. and Mizunami, M.** (2016). Roles of OA1 octopamine receptor and Dop1 dopamine receptor in mediating appetitive and aversive reinforcement revealed by RNAi studies. *Sci. Rep.* **6**, [https://doi: 10.1038/srep29696](https://doi.org/10.1038/srep29696).
- Barrozo, R.B., Gadenne, C. and Anton, S.** (2010). Switching attraction to inhibition: mating-induced reversed role of sex pheromone in an insect. *J. Exp. Biol.* **213**, 2933-2939.
- Beaulieu, J.M. and Gainetdinov, R.R.** (2011). The physiology, signaling, and pharmacology of dopamine receptors. *Pharmacol. Rev.* **63**, [https://doi: 10.1124/pr.110.002642](https://doi.org/10.1124/pr.110.002642).
- Beggs, K.T., Tyndall, J.D. and Mercer, A.R.** (2011). Honey bee dopamine and octopamine receptors linked to intracellular calcium signaling have a close phylogenetic and pharmacological relationship. *PLoS One* **6**, [https://doi: 10.1371/journal.pone.0026809](https://doi.org/10.1371/journal.pone.0026809).
- Beny-Shefer, Y., Zilkha, N., Lavi-Avnon, Y., Bezalel, N., Rogachev, I., Brandis, A., Dayan, M. and Kimchi, T.** (2017). Nucleus Accumbens Dopamine Signaling Regulates Sexual Preference for Females in Male Mice. *Cell. Rep.* **21**, [https://doi: 10.1016/j.celrep.2017.11.062](https://doi.org/10.1016/j.celrep.2017.11.062).
- Blenau, W. and Baumann, A.** (2001). Molecular and pharmacological properties of insect biogenic amine receptors: lessons from *Drosophila melanogaster* and *Apis mellifera*. *Arch. Insect Biochem. Physiol.* **48**, 13-38.
- Blenau, W., Erber, J. and Baumann, A.** (1998). Characterization of a dopamine D1 receptor from *Apis mellifera*: cloning, functional expression, pharmacology, and mRNA localization in the brain. *J. Neurochem.* **70**, 15-23.
- Borroto-Escuela, D.O., Romero-Fernandez, W., García-Negredo, G., Correia, P.A., Garriga, P., Fuxe, K. and Ciruela, F.** (2011). Dissecting the conserved NPxxY motif of the M3 muscarinic acetylcholine receptor: critical role of Asp-7.49 for receptor signaling and multiprotein complex formation. *Cell. Physiol. Biochem.* **28**, [https://doi: 10.1159/000335788](https://doi.org/10.1159/000335788).
- Bozzolan, F., Duportets, L., Limousin, D., Wycke, M.A., Demondion, E., François, A., Abrieux, A. and Debernard, S.** (2015). Synaptotagmin I, a molecular target for steroid hormone signaling controlling the maturation of sexual behavior in an insect. *FEBS J.* **282**, [https://doi: 10.1111/febs.13231](https://doi.org/10.1111/febs.13231).

Brennan, P.A. (2010). Pheromones and mammalian Behavior. In: The neurobiology of olfaction, chap.6 (Menini A, ed). Bota Raton: Floride University Press.

Callier, S., Snapyan, M., Le Crom, S., Prou, D., Vincent, J.D. and Vernier, P. (2003). Evolution and cell biology of dopamine receptors in vertebrates. *Biol. Cell.* **95**, 489-502.

Chen, B., Liu, H., Ren, J. and Guo, A. (2012). Mutation of *Drosophila* dopamine receptor DopR leads to male-male courtship behavior. *Biochem. Biophys. Res. Commun.* **423**, [https://doi: 10.1016/j.bbrc.2012.06.003](https://doi.org/10.1016/j.bbrc.2012.06.003).

Crickmore, M.A. and Vosshall, L.B. (2013). Opposing dopaminergic and GABAergic neurons control the duration and persistence of copulation in *Drosophila*. *Cell* **155**, [https://doi: 10.1016/j.cell.2013.09.055](https://doi.org/10.1016/j.cell.2013.09.055).

Dacks, A.M., Riffell, J.A., Martin, J.P., Gage, S.L. and Nighorn, A.J. (2012). Olfactory modulation by dopamine in the context of aversive learning. *J. Neurophysiol.* **108**, [https://doi: 10.1152/jn.00159.2012](https://doi.org/10.1152/jn.00159.2012).

Davidoff, M.S., Ungefroren, H., Middendorff, R., Koeva, Y., Bakalska, M., Atanassova, N., Holstein, A.F., Jezek, D., Pusch, W. and Müller, D. (2005). Catecholamine-synthesizing enzymes in the adult and prenatal human testis. *Histochem. Cell. Biol.* **124**, 313-323.

Defea, K. (2008). Beta-arrestins and heterotrimeric G-proteins: collaborators and competitors in signal transduction. *Br. J. Pharmacol.* **153**, 298-309.

Devaud, J.M., Acebes, A. and Ferrús, A. (2001). Odor exposure causes central adaptation and morphological changes in selected olfactory glomeruli in *Drosophila*. *J. Neurosci.* **21**, 6274-8622.

Devaud, J.M., Acebes, A., Ramaswami, M. and Ferrús, A. (2003). Structural and functional changes in the olfactory pathway of adult *Drosophila* take place at a critical age. *J. Neurobiol.* **56**, 13-23.

Donly, B.C. and Caveney, S. (2005). A transporter for phenolamine uptake in the arthropod CNS. *Arch. Insect Biochem. Physiol.* **59**, 172-183.

Duportets, L., Dufour, M.C., Couillaud, F. and Gadenne, C. (1998). Biosynthetic activity of corpora allata, growth of sex accessory glands and mating in the male moth *Agrotis ipsilon* (Hufnagel) *J. Exp. Biol.* **201**, 2425-2432.

Duportets, L., Maria, A., Vitecek, S., Gadenne, C. and Debernard, S. (2013). Steroid hormone signaling is involved in the age-dependent behavioral response to sex pheromone in the adult male moth *Agrotis ipsilon*. *Gen. Comp. Endocrinol.* **186**, [https://doi: 10.1016/j.ygcen.2013.02.024](https://doi.org/10.1016/j.ygcen.2013.02.024).

Ellen, C.W. and Mercer, A.R. (2012). Modulatory actions of dopamine and serotonin on insect antennal lobe neurons: insights from studies *in vitro*. *J. Mol. Histol.* **43**, [https://doi: 10.1007/s10735-012-9401-7](https://doi.org/10.1007/s10735-012-9401-7).

Escanilla, O., Yuhua, C., Marzan, D. and Linster, C. (2009). Dopaminergic modulation of olfactory bulb processing affects odor discrimination learning in rats. *Behav. Neurosci.* **123**, [https://doi: 10.1037/a001585](https://doi.org/10.1037/a001585).

Evans, P.D., Bayliss, A. and Reale, V. (2014). GPCR-mediated rapid, non-genomic actions of steroids: comparisons between DmDopEcR and GPER1 (GPR30). *Gen. Comp. Endocrinol.* **195**, [https://doi: 10.1016/j.ygcen.2013.10.015](https://doi.org/10.1016/j.ygcen.2013.10.015).

Faber, E.S. and Sah, P. (2007). Functions of SK channels in central neurons. *Clin. Exp. Pharmacol. Physiol.* **34**, 1077-1083.

Fraser, C.M., Chung, F.Z., Wang, C.D. and Venter, J.C. (1988). Site-directed mutagenesis of human beta-adrenergic receptors: substitution of aspartic acid-130 by asparagine produces a receptor with high-affinity agonist binding that is uncoupled from adenylate cyclase. *Proc. Natl. Acad. Sci. U S A* **85**, 5478-5482.

Fritze, O., Filipek, S., Kuksa, V., Palczewski, K., Hofmann, K.P. and Ernst, O.P. (2003). Role of the conserved NPxxY(x)5,6F motif in the rhodopsin ground state and during activation. *Proc Natl Acad Sci U S A* **100**, 2290-2295.

Gadenne, C. and Anton, S. (2000). Central processing of sex pheromone stimuli is differentially regulated by juvenile hormone in a male moth. *J. Insect Physiol.* **46**, 1195-1206.

Gadenne, C., Barrozo, R.B. and Anton, S. (2016). Plasticity in Insect Olfaction: To Smell or Not to Smell?. *Annu. Rev. Entomol.* **61**, [https://doi: 10.1146/annurev-ento-010715-023523](https://doi.org/10.1146/annurev-ento-010715-023523).

Gadenne, C., Dufour, M.C. and Anton, S. (2001). Transient post-mating inhibition of behavioural and central nervous responses to sex pheromone in an insect. *Proc. Biol. Sci.* **268**, [https://doi: 10.1098/rspb.2001.1710](https://doi.org/10.1098/rspb.2001.1710).

Gadenne, C., Renou, M. and Streng, L. (1993). Hormonal control of pheromone responsiveness in the male black cutworm *Agrotis ipsilon*. *Experientia* **49**, 721-724.

Galizia, C.G. and Sachse, S. (2010). Odor coding in insects. In: The neurobiology of olfaction, chap.2 (Menini A, ed). Bota Raton: Floride University Press.

Gemenio, C. and Haynes, K.F. (1998). Chemical and behavioral evidence for a third pheromone component in a north american population of the black cutworm moth, *Agrotis ipsilon*. *J. Chem. Ecol.* **24**, 999-1011.

González, C.R., González, B., Matzkin, M.E., Muñoz, J.A., Cadet, J.L., Garcia-Rill, E., Urbano, F.J., Vitullo, A.D. and Bisagno, V. (2015). Psychostimulant-Induced testicular toxicity in mice: evidence of cocaine and caffeine effects on the local dopaminergic System. *PLoS One* **10**, [https://doi: 10.1371/journal.pone.0142713](https://doi.org/10.1371/journal.pone.0142713).

Groot, A.T. (2014) Circadian rhythms of sexual activities in moths: a review. *Front. Ecol. Evol.* [https://doi: org/10.3389/fevo.2014.00043](https://doi.org/10.3389/fevo.2014.00043).

Guo, X., Ma, Z., Du, B., Li, T., Li, W., Xu, L., He, J. and Kang, L. (2018). Dop1 enhances conspecific olfactory attraction by inhibiting miR-9a maturation in locusts. *Nat. Commun.* **9**, [https://doi: 10.1038/s41467-018-03437-z](https://doi.org/10.1038/s41467-018-03437-z).

Hamada, A., Miyawaki, K., Honda-sumi, E., Tomioka, K., Mito, T., Ohuchi, H. and Noji, S. (2009). Loss-of-function analyses of the fragile X-related and dopamine receptor genes by RNA interference in the cricket *Gryllus bimaculatus*. *Dev. Dyn.* **238**, [https://doi: 10.1002/dvdy.22029](https://doi.org/10.1002/dvdy.22029).

Han, K.A., Millar, N.S., Grotewiel, M.S. and Davis, R.L. (1996). DAMB, a novel dopamine receptor expressed specifically in *Drosophila* mushroom bodies. *Neuron* **16**, 1127-35.

Harvey, J.D. and Heinbockel, T. (2018). Neuromodulation of synaptic transmission in the main olfactory bulb. *Int. J. Environ. Res. Public Health* **15**, [https://doi: 10.3390/ijerph15102194](https://doi.org/10.3390/ijerph15102194).

Haupt, S.S., Sakurai, T., Namiki, S., Kazawa, T. and Kanzaki, R. (2010). Olfactory Information Processing in Moths. In: The neurobiology of olfaction, chap.3 (Menini A, ed). Bota Raton: Floride University Press.

Hill, C.A., Doyle, T., Nuss, A.B., Ejendal, K.F., Meyer, J.M. and Watts, V.J. (2016). Comparative pharmacological characterization of D1-like dopamine receptors from *Anopheles gambiae*, *Aedes aegypti* and *Culex quinquefasciatus* suggests pleiotropic signaling in mosquito vector lineages. *Parasit. Vectors* **9**, [https://doi: 10.1186/s13071-016-1477-6](https://doi.org/10.1186/s13071-016-1477-6).

Himmelreich, S., Masuho, I., Berry, J.A., MacMullen, C., Skamangas, N.K., Martemyanov, K.A. and Davis R.L. (2017). Dopamine Receptor DAMB Signals via Gq to Mediate Forgetting in *Drosophila*. *Cell. Rep.* **21**, [https://doi: 10.1016/j.celrep.2017.10.108](https://doi.org/10.1016/j.celrep.2017.10.108).

Hull, E.M., Lorrain, D.S., Du, J., Matuszewich, L., Lumley, L.A., Putnam, S.K. and Moses, J. (1999). Hormone-neurotransmitter interactions in the control of sexual behavior. *Behav. Brain. Res.* **105**, 105-116.

Hull, E.M. and Muschamp, J.W. and Sato, S. (2004). Dopamine and serotonin: influences on male sexual behavior. *Physiol. Behav.* **83**, 291-307.

Jarriault, D., Barrozo, R.B., De Carvalho Pinto, C.J., Greiner, B., Dufour, M.C., Masante-Roca, I., Gramsbergen, J.B., Anton, S. and Gadenne, C. (2009). Age-dependent plasticity of sex pheromone response in the moth, *Agrotis ipsilon*: combined effects of octopamine and juvenile hormone. *Horm. Behav.* **56**, [https://doi: 10.1016/j.yhbeh.2009.04.005](https://doi.org/10.1016/j.yhbeh.2009.04.005).

Kaatz, H.H., Hildebrandt, H. and Engels, W. (1992). Primer effect of queen pheromone on juvenile hormone biosynthesis in adult worker honey bees. *J. Comp. Physiol. B*: 588-592.

Kalatskaya, I., Schüssler, S., Blaukat, A., Müller-Esterl, W., Jochum, M., Proud, D. and Faussner, A. (2004). Mutation of tyrosine in the conserved NPXXY sequence leads to constitutive phosphorylation and internalization, but not signaling, of the human B2 bradykinin receptor. *J. Biol. Chem.* **279**, 31268-31276.

Karnik, S.S. and Khorana, H.G. (1990). Assembly of functional rhodopsin requires a disulfide bond between cysteine residues 110 and 187. *J. Biol. Chem.* **265**, 17520-17524.

Keleman, K., Vrontou, E., Krüttner, S., Yu, J.Y., Kurtovic-Kozaric, A. and Dickson, B.J. (2012). Dopamine neurons modulate pheromone responses in *Drosophila* courtship learning. *Nature* **489**, [https://doi: 10.1038/nature11345](https://doi.org/10.1038/nature11345).

Klappenbach, M., Kaczer, L. and Locatelli, F. (2013). Dopamine interferes with appetitive long-term memory formation in honeybees. *Neurobiol. Learn. Mem.* **106**, [https://doi: 10.1016/j.nlm.2013.09.011](https://doi.org/10.1016/j.nlm.2013.09.011).

Klemm, N. (1979). Biogenic amines in the stomatogastric nervous system of several insect orders. *Entomol. Gen.* **5**, 113-121.

Kobilka, B.K. (2007) G protein coupled receptor structure and activation. *Biochim. Biophys. Acta* **1768**, 794-807.

Kromann, S.H., Saveer, A.M., Binyameen, M., Bengtsson, M., Birgersson, G., Hansson, B.S., Schlyter, F., Witzgall, P., Ignell, R. and Becher, P.G. (2015). Concurrent modulation of neuronal and behavioural olfactory responses to sex and host plant cues in a male moth. *Proc. Biol. Sci.* **282**: 20141884.

Lange, A.B. and Chan, K. (2008). Dopaminergic control of foregut contractions in *Locusta migratoria*. *J. Insect Physiol.* **54**, 222-230.

Lenschow, M., Cordel, M., Pokorný, T., Mair, M.M., Hofferberth, J. and Ruther, J. (2018). The post-mating switch in the pheromone response of *Nasonia* females is mediated by Dopamine and can be reversed by appetitive learning. *Front. Behav. Neurosci.* **12**, [https://doi: 10.3389/fnbeh.2018.00014](https://doi.org/10.3389/fnbeh.2018.00014).

Lim, J., Fernandez, A.I., Hinojos, S.J., Aranda, G.P., James, J., Seong, C.S. and Han, K.A. (2018). The mushroom body D1 dopamine receptor controls innate courtship drive. *Genes Brain Behav.* **17**, [https://doi: 10.1111/gbb.12425](https://doi.org/10.1111/gbb.12425).

Ma, Z., Guo, W., Guo, X., Wang, X. and Kang, L. (2011). Modulation of behavioral phase changes of the migratory locust by the catecholamine metabolic pathway. *Proc. Natl. Acad. Sci. U S A* **108**, [https://doi: 10.1073/pnas.1015098108](https://doi.org/10.1073/pnas.1015098108).

Martinez, D., Chaffiol, A., Voges, N., Gu, Y., Anton, S., Rospars, J.P. and Lucas, P. (2013). Multiphasic on/off pheromone signalling in moths as neural correlates of a search strategy. *PLoS One* **8**, [https://doi: 10.1371/journal.pone.0061220](https://doi.org/10.1371/journal.pone.0061220).

- Matsushima, K., Watanabe, T. and Sasaki, K.** (2019). Functional gene expression of dopamine receptors in the male reproductive organ during sexual maturation in the honey bee (*Apis mellifera* L.). *J. Insect Physiol.* **112**, [https://doi: 10.1016/j.jinsphys.2018.11.005](https://doi.org/10.1016/j.jinsphys.2018.11.005).
- McHenry, J.A., Bell, G.A., Parrish, B.P. and Hull, E.M.** (2012). Dopamine D1 receptors and phosphorylation of dopamine- and cyclic AMP-regulated phosphoprotein-32 in the medial preoptic area are involved in experience-induced enhancement of male sexual behavior in rats. *Behav. Neurosci.* **126**, [https://doi: 10.1037/a0028707](https://doi.org/10.1037/a0028707).
- McQuillan, H.J., Nakagawa, S. and Mercer, A.R.** (2014). Juvenile hormone enhances aversive learning performance in 2-day old worker honey bees while reducing their attraction to queen mandibular pheromone. *PLoS One* **9**, [https://doi: 10.1371/journal.pone.0112740](https://doi.org/10.1371/journal.pone.0112740).
- Mesce, K.A.** (2002). Metamodulation of the biogenic amines: second-order modulation by steroid hormones and amine cocktails. *Brain Behav. Evol.* **60**, 339-49.
- Mitsumasu, K., Ohta, H., Tsuchihara, K., Asaoka, K., Ozoe, Y., Niimi, T., Yamashita, O. and Yaginuma, T.** (2008). Molecular cloning and characterization of cDNAs encoding dopamine receptor-1 and -2 from brain-suboesophageal ganglion of the silkworm, *Bombyx mori*. *Insect Mol. Biol.* **17**, [https://doi: 10.1111/j.1365-2583.2008.00792.x](https://doi.org/10.1111/j.1365-2583.2008.00792.x).
- Mizunami, M., Unoki, S., Mori, Y., Hirashima, D., Hatano, A. and Matsumoto, Y.** (2009). Roles of octopaminergic and dopaminergic neurons in appetitive and aversive memory recall in an insect. *BMC Biol.* **7**, [https://doi: 10.1186/1741-7007-7-46](https://doi.org/10.1186/1741-7007-7-46).
- Mustard, J.A., Beggs, K.T. and Mercer, A.R.** (2005). Molecular biology of the invertebrate dopamine receptors. *Arch. Insect Biochem. Physiol.* **59**, 103-17.
- Neve, K.A., Seamans, J.K. and Trantham-Davidson, H.** (2004). Dopamine receptor signaling. *J. Recept. Signal Transduct. Res.* **24**, 165-205.
- Ohtsuki, G., Piochon, C., Adelman, J.P. and Hansel, C.** (2012). SK2 channel modulation contributes to compartment-specific dendritic plasticity in cerebellar Purkinje cells. *Neuron* **75**, [https://doi: 10.1016/j.neuron.2012.05.025](https://doi.org/10.1016/j.neuron.2012.05.025).
- Palmer, C.R. and Kristan, W.B.** (2011). Contextual modulation of behavioral choice. *Curr. Opin. Neurobiol.* **21**, [https://doi: 10.1016/j.conb.2011.05.003](https://doi.org/10.1016/j.conb.2011.05.003).

Party, V., Hanot, C., Saïd, I., Rochat, D. and Renou, M. (2009). Plant terpenes affect intensity and temporal parameters of pheromone detection in a moth. *Chem. Senses* **34**, 763-774.

Picimbon, J.F., Gadenne, C., Bécard, J.M., Clément, J.L. and Sreng, L. (1997). Sex pheromone of the french black cutworm moth, *Agrotis ipsilon* (Lepidoptera: Noctuidae): identification and regulation of a multicomponent blend. *J. Chem. Ecol.* **23**, 211-230.

Poitout, S. and Bues, R. (1974). Elevage de plusieurs espèces de lépidoptères sur milieu artificiel simplifié. *Ann. Zool. Ecol. Anim.* **2**, 79-91.

Robinson, G.E., Winston, M.L., Huang, Z. and Pankiw, T. (1998). Queen mandibular gland pheromone influences worker honey bee (*Apis mellifera* L.) foraging ontogeny and juvenile hormone titers. *J. Insect Physiol.* **44**, 685-692.

Romeo, R., Pellitteri, R., Russo, A. and Marcello, M.F. (2004). Catecholaminergic phenotype of human Leydig cells. *Ital. J. Anat. Embryol.* **109**, 45-54.

Romeo, R.D., Richardson, H.N. and Sisk, C.L. (2002). Puberty and the maturation of the male brain and sexual behavior: recasting a behavioral potential. *Neurosci. Biobehav. Rev.* **26**, 381-391.

Ross, J.M. and Fletcher, M.L. (2019). Aversive learning-induced plasticity throughout the adult mammalian olfactory system: insights across development. *J. Bioenerg. Biomembr.* **51**, [https://doi: 10.1007/s10863-018-9770-z](https://doi.org/10.1007/s10863-018-9770-z).

Sasaki, K., Akasaka, S., Mezawa, R., Shimada, K. and Maekawa, K. (2012). Regulation of the brain dopaminergic system by juvenile hormone in honey bee males (*Apis mellifera* L.). *Insect Mol. Biol.* **21**, [https://doi: 10.1111/j.1365-2583.2012.01153.x](https://doi.org/10.1111/j.1365-2583.2012.01153.x).

Sasaki, K. and Harano, K. (2010). Multiple regulatory roles of dopamine in behavior and reproduction of social insects. *Trends in entomology* **6**, 1-13.

Sasaki, K., Yamasaki, K., Tsuchida, K. and Nagao, T. (2009). Gonadotropic effects of dopamine in isolated workers of the primitively eusocial wasp, *Polistes chinensis*. *Naturwissenschaften* **96**, [https://doi: 10.1007/s00114-009-0510-4](https://doi.org/10.1007/s00114-009-0510-4).

Schellino, R., Trova, S., Cimino, I., Farinetti, A., Jongbloets, B.C., Pasterkamp, R.J., Panzica G., Giacobini, P., De Marchis, S. and Peretto, P. (2016). Opposite-sex attraction in

male mice requires testosterone-dependent regulation of adult olfactory bulb neurogenesis. *Sci. Rep.* **6**, [https://doi: 10.1038/srep36063](https://doi.org/10.1038/srep36063).

Simon, P. (2003). Q-gene: processing quantitative real-time RT-PCR data. *Bioinformatics* **19**, 1439-1440.

Sokal, R.R. and Rohlf, F.J. (1995). Biometry: The principles and practice of statistics in biological research, pp. 315. Freeman WH, editor, NY.

Sotnikova, T.D. and Gainetdinov, R.R. (2009). Octopamine and other monoamines in invertebrates. *Encyclopedia of Neuroscience*, [https://doi: org/10.1016/B978-008045046-9.01158-X](https://doi.org/10.1016/B978-008045046-9.01158-X).

Srivastava, D.P., Yu, E.J., Kennedy, K., Chatwin, H., Reale, V., Hamon, M., Smith, T. and Evans, P.D. (2005). Rapid, nongenomic responses to ecdysteroids and catecholamines mediated by a novel *Drosophila* G-protein-coupled receptor. *J. Neurosci.* **25**, 6145-6155.

Strader, C.D., Candelore, M.R., Hill, W.S., Sigal, I.S. and Dixon, R.A. (1989). Identification of two serine residues involved in agonist activation of the beta-adrenergic receptor. *J. Biol. Chem.* **264**, 13572-13578.

Strader, C.D., Fong, T.M., Graziano, M.P. and Tota, M.R. (1995). The family of G-protein-coupled receptors. *FASEB J.* **9**, 745-54.

Strader, C.D., Sigal, I.S., Candelore, M.R., Rands, E., Hill, W.S. and Dixon, R.A. (1988). Conserved aspartic acid residues 79 and 113 of the beta-adrenergic receptor have different roles in receptor function. *J. Biol. Chem.* **25**, 10267-10271.

Sugamori, K.S., Demchyshyn, L.L., McConkey, F., Forte, M.A. and Niznik, H.B. (1995). A primordial dopamine D1-like adenylyl cyclase-linked receptor from *Drosophila melanogaster* displaying poor affinity for benzazepines. *FEBS Lett.* **362**, 131-138.

Undieh, A.S. (2010). Pharmacology of signaling induced by dopamine D(1)-like receptor activation. *Pharmacol. Ther.* **128**, [https://doi: 10.1016/j.pharmthera.2010.05.003](https://doi.org/10.1016/j.pharmthera.2010.05.003).

Unoki, S., Matsumoto, Y. and Mizunami, M. (2005). Participation of octopaminergic reward system and dopaminergic punishment system in insect olfactory learning revealed by pharmacological study. *Eur. J. Neurosci.* **22**, 1409-1416.

Verlinden, H. (2018). Dopamine signalling in locusts and other insects. *Insect Biochem. Mol. Biol.* **97**, [https://doi: 10.1016/j.ibmb.2018.04.005](https://doi.org/10.1016/j.ibmb.2018.04.005).

Vinauger, C., Lahondère, C., Wolff, G.H., Locke, L.T., Liaw, J.E., Parrish, J.Z., Akbari, O.S., Dickinson, M.H. and Riffell, J.A. (2018). Modulation of host learning in *Aedes aegypti* mosquitoes. *Curr. Biol.* **28**, [https://doi: 10.1016/j.cub.2017.12.015](https://doi.org/10.1016/j.cub.2017.12.015).

Watanabe, T., Sadamoto, H. and Aonuma, H. (2013). Molecular basis of the dopaminergic system in the cricket *Gryllus bimaculatus*. *Invert. Neurosci.* **13**, [https://doi: 10.1007/s10158-013-0153-1](https://doi.org/10.1007/s10158-013-0153-1).

Wicker-Thomas, C. and Hamann, M. (2008). Interaction of dopamine, female pheromones, locomotion and sex behavior in *Drosophila melanogaster*. *J. Insect Physiol.* **54**, [https://doi: 10.1016/j.jinsphys.2008.08.005](https://doi.org/10.1016/j.jinsphys.2008.08.005).

Xu, G., Wu, S.F., Gu, G.X., Teng, Z.W., Ye, G.Y. and Huang, J. (2017). Pharmacological characterization of dopamine receptors in the rice striped stem borer, *Chilo suppressalis*. *Insect Biochem. Mol. Biol.* **83**, [https://doi: 10.1016/j.ibmb.2017.03.004](https://doi.org/10.1016/j.ibmb.2017.03.004).

Yamamoto, K. and Vernier, P. (2011). The evolution of dopamine systems in chordates. *Front. Neuroanat.* **29**, [https://doi: 10.3389/fnana.2011.00021](https://doi.org/10.3389/fnana.2011.00021).

Yang, J., Ye, M., Tian, C., Yang, M., Wang, Y. and Shu, Y. (2013). Dopaminergic modulation of axonal potassium channels and action potential waveform in pyramidal neurons of prefrontal cortex. *J. Physiol.* **591**, [https://doi: 10.1113/jphysiol.2013.251058](https://doi.org/10.1113/jphysiol.2013.251058).

Yue, E.L., Cleland, T.A., Pavlis, M. and Linster, C. (2004). Opposing effects of D₁ and D₂ receptor activation on odor discrimination learning. *Behav. Neurosci.* **118**, doi.org/10.1037/0735-7044.118.1.184.

Zizzo, M.G., Mulè, F., Mastropaolo, M. and Serio, R. (2010). D1 receptors play a major role in the dopamine modulation of mouse ileum contractility. *Pharmacol. Res.* **61**, [https://doi: 10.1016/j.phrs.2010.01.015](https://doi.org/10.1016/j.phrs.2010.01.015).

Figures

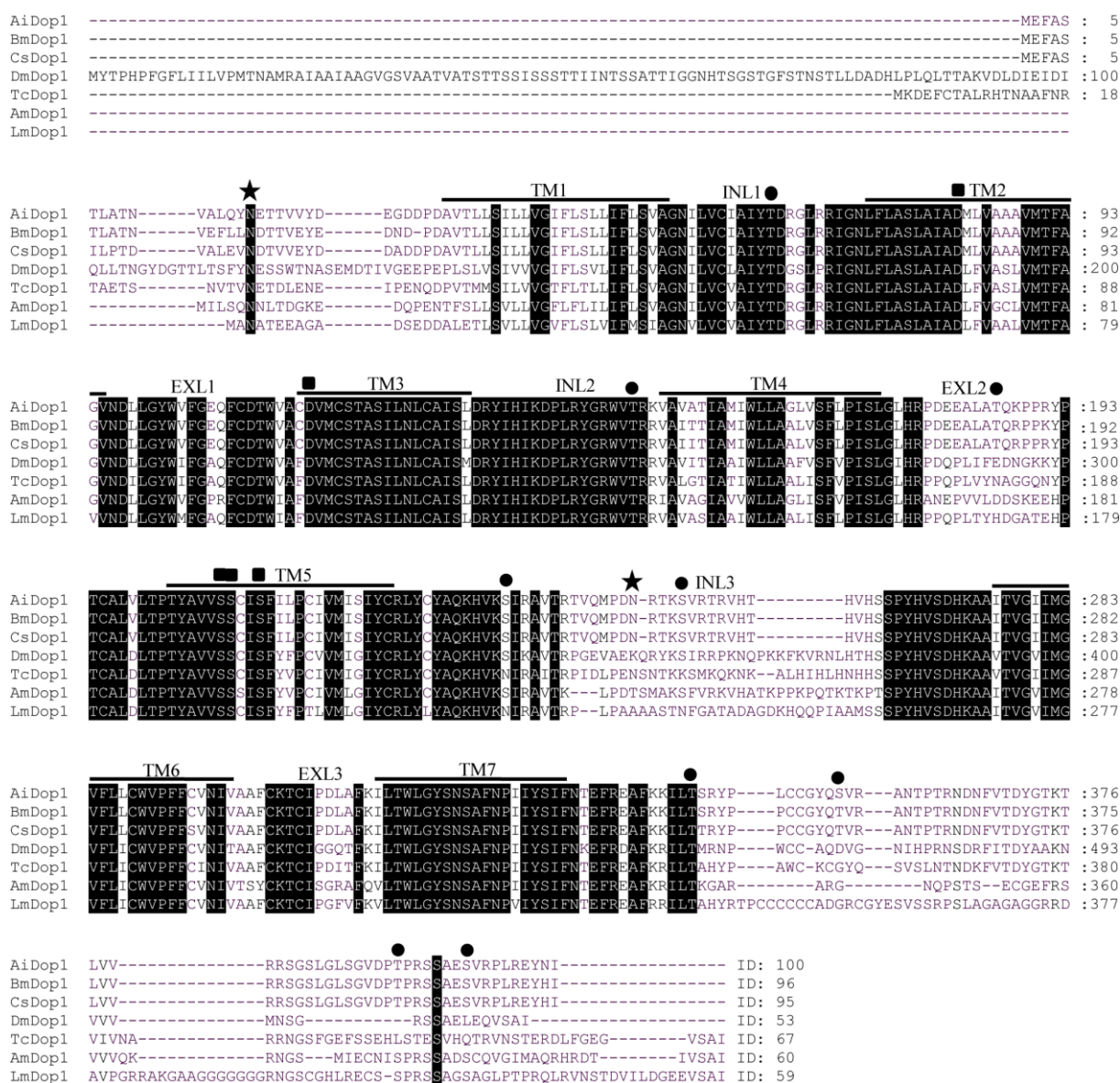


Figure 1. Analysis and comparison of the protein sequence of *A. ipsilon* Dop1 with other insect D1-like dopamine receptors. The deduced amino acid sequence of AiDop1 was aligned with sequences of orthologous receptors from *B. mori* (BmDop1), *C. suppressalis* (CsDop1), *D. melanogaster* (DmDop1), *T. castaneum* (TcDop1), *A. mellifera* (AmDop1) and *L. migratoria* (LmDop1). GenBank accession numbers of the proteins used are given in Materials and methods. The amino acid position is indicated in the right and dashes represent gaps introduced to maximize alignment scores. Amino acid residues conserved between all sequences are printed in white letters on a black background. The transmembrane domains (TM1-TM7) are delineated by bold black lines and the intracellular and extracellular loops are

indicated by the symbols (INL) and (EXL), respectively. Amino acid residues belonging to the binding domain of DA are indicated by filled squares. Potential *N*-linked glycosylation sites and potential phosphorylation sites by PKC are labelled by filled asterisks and filled circles, respectively. The value at the end of each sequence represents the percent identity (ID) with AiDop1.

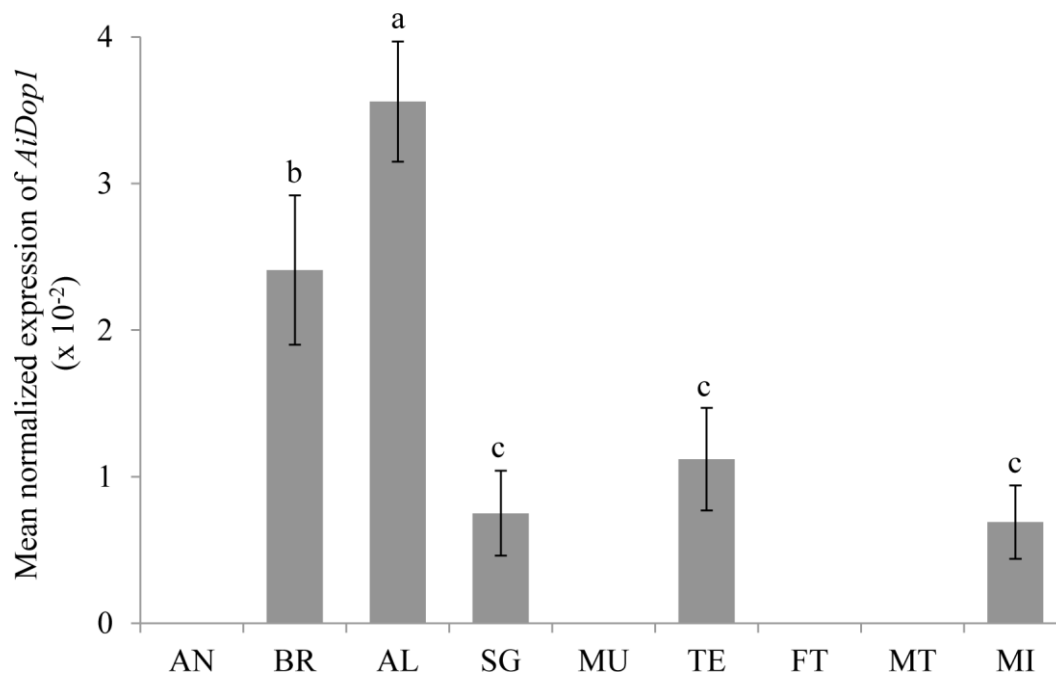


Figure 2. Tissue expression pattern of *AiDop1* in 5-day-old *A. ipsilon* males. Total RNAs from antennae (AN), brains without ALs (BR), antennal lobes (AL), sex accessory glands (SG), thoracic muscles (MU), testes (TE), fat bodies (FT), malpighian tubules (MT) and midguts (MI) were reverse transcribed to cDNAs for *AiDop1* expression analysis by qPCR. Each real time qPCR was run in three technical replicates with six independent biological replicates. Error bars represent standard errors, and those with the same letter are not significantly different (analysis of variance; Tukey's test; $P < 0.05$).

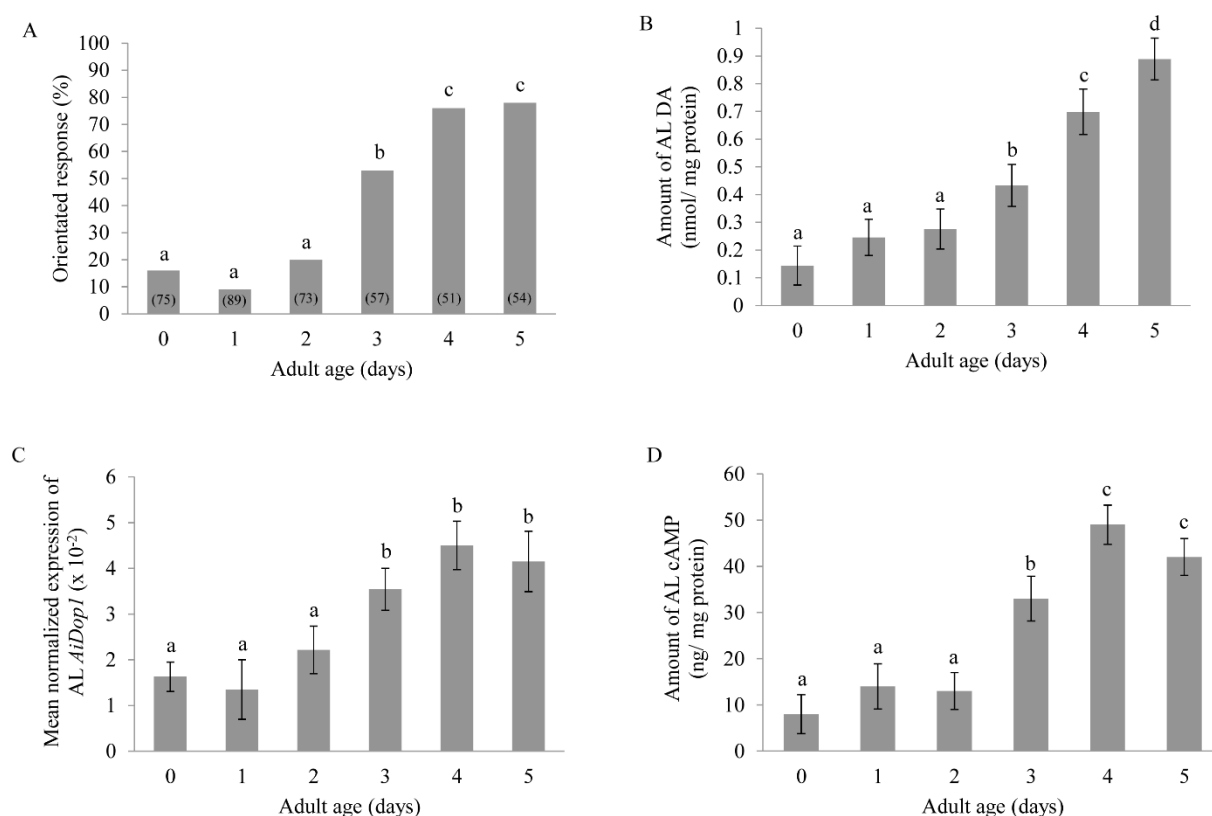


Figure 3. Age-related plasticity in the sex pheromone response and the amounts of AL DA, *AiDop1* transcript and cAMP in *A. ipsilon* males. The behavioral response to sex pheromone and the levels of DA, *AiDop1* transcript and cAMP in ALs were evaluated for 0- to 5-day old males. **(A)** Percentages of orientated responses of males. **(B)** DA amounts. **(C)** Relative expression of *AiDop1*. **(D)** cAMP amounts. Each real time qPCR was run in three technical replicates with six independent biological replicates. The DA and cAMP amounts were quantified from 10 ALs for each experimental group with 10 biological replicates. For the DA, *AiDop1* mRNA and cAMP levels, the bars represent means \pm SD and those with the same letter are not significantly different (analysis of variance; Tukey's test; $P < 0.05$). For the behavioral tests, the numbers in parentheses indicate the numbers of tested males and the columns with the same letter are not significantly different (G-test; $P < 0.05$).

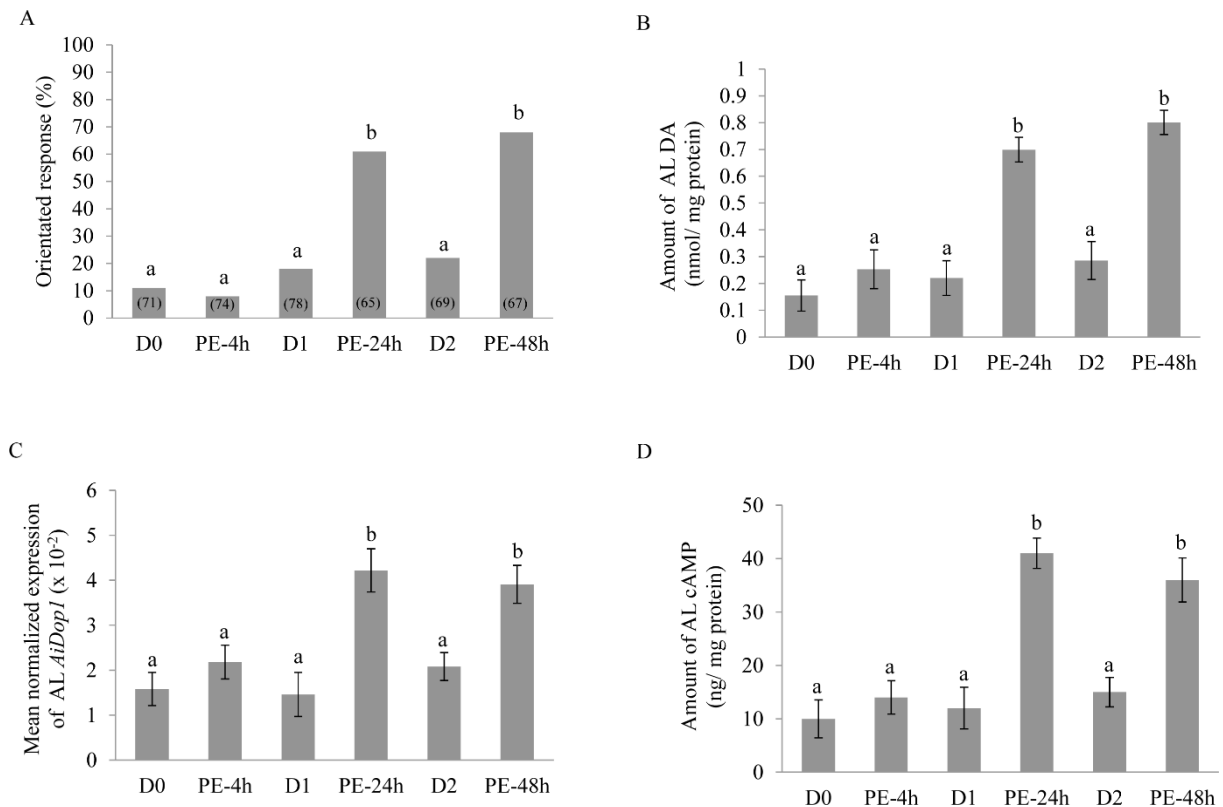


Figure 4. Pre-exposure-related plasticity in the sex pheromone response and the amounts of AL DA, *AiDop1* transcript and cAMP in *A. epsilon* males. Newly emerged males (day-0) were pre-exposed for 3 min to the blend pheromone. The behavioral response to sex pheromone and the levels of DA, *AiDop1* transcript and cAMP in ALs were determined 4h, 24h and 48h later after pre-exposure (PE). **(A)** Percentages of orientated responses of males. **(B)** DA amounts. **(C)** Relative expression of *AiDop1*. **(D)** cAMP amounts. Unexperienced 0-day-, 1-day- and 2-day-old males (D0, D1 and D2) were used as controls. Each real time qPCR was run in three technical replicates with seven independent biological replicates. The DA and cAMP amounts were quantified from 10 ALs for each experimental group with 10 biological replicates. For the DA, *AiDop1* mRNA and cAMP levels, the bars represent means \pm SD and those with the same letter are not significantly different (analysis of variance; Tukey's test; $P < 0.05$). For the behavioral tests, the numbers in parentheses indicate the numbers of tested males and the columns with the same letter are not significantly different (G-test; $P < 0.05$).

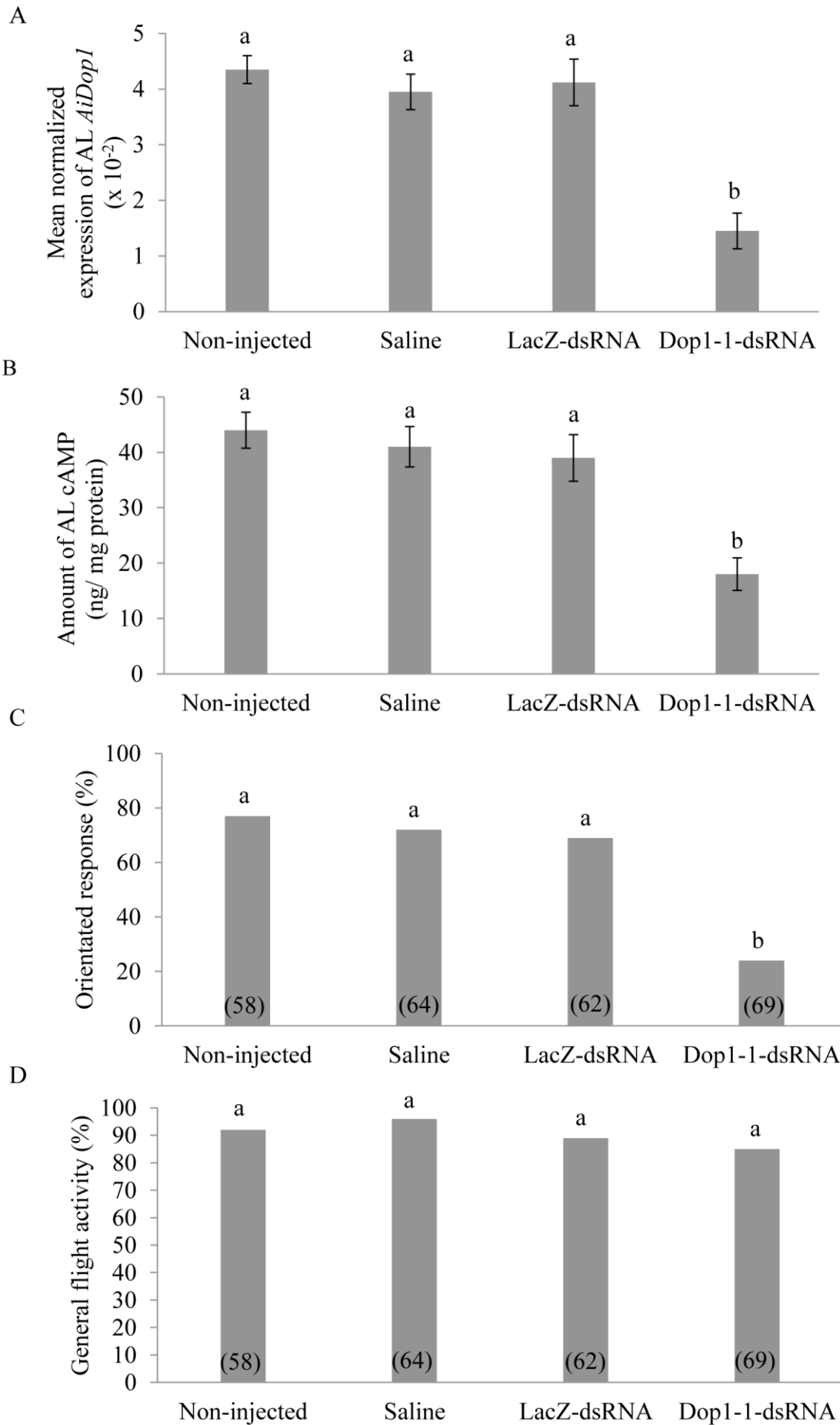


Figure 5. Efficiency and effect of double-stranded RNA (dsRNA)-mediated *AiDop1* silencing on both the amount of AL cAMP and the behavioral response to sex pheromone in *A. ipsilon* males. Two-day-old males received an injection of saline solution, bacterial *Beta-galactosidase* (LacZ)-dsRNA or Dop1-1-dsRNA, or no injection (non-injected). For each treatment and three days after injection, the relative expression of *AiDop1* mRNA (**A**) and the amount of cAMP (**B**) were quantified in ALs, and the percentages of orientated responses (**C**) and of general flight activity (**D**) were determined in the wind tunnel experiments. Each real time qPCR was run in three technical replicates with five independent biological replicates. The cAMP amounts were quantified from 10 ALs for each experimental group with 10 biological replicates. For the *AiDop1* mRNA and cAMP levels, the bars represent means \pm SD and those with different letters are significantly different (analysis of variance; Tukey's test; $P < 0.05$). For the behavioral tests, the numbers in parentheses indicate the numbers of tested males and the columns with the same letter are not significantly different (G-test; $P < 0.05$).

Supplementary information

TATCGGTCGTCATGTCGTCGTAATGCATGCGTCGATTGGCTGCAACATATTGCGGCTTTGCCTGATTGCGTAACAAATGCCCGTG
CTTGTCGCCGGTGAAATGCGCTTGCGTACCATTTGCGGCATGCGTCACGTGCAATCCCAATTTCCTAATGCGGTAATGCGTACT

```

1 - ATGGAGTTCGCCTCC ACCTTAGCTACCAAT GTGGCGCTTCAGTAC AATGAGACGACGGTG GTGTACGACGAGGGA
1 - M E F A S T L A T N V A L Q Y N E T T V V Y D E G

76 - GATGACCCGGATGCT GTCACGTTGCTCTCC ATACTGCTTGTCGGT ATATTCTGTCTATTG CTGATATTCCTCAGC
26 - D D P D A V T L L S I L L V G I F L S L L I F L S

151 - GTAGCAGGAAACATA CTGGTCTGCATCGCG ATATACACGGACCGA GGGTTGCGACGCATC GGGAACCTGTTCTCG
51 - V A G N I L V C I A I Y T D R G L R R I G N L F L

226 - GCATCGTTGGCCATC GCTGACATGCTGGTA GCTGCTGCGGTCATG ACCTTTGCAGGAGTC AATGACCTGCTTGA
76 - A S L A I A D M L V A A A V M T F A G V N D L L G

301 - TACTGGGTATTTCGGC GAGCAGTTCTGCGAC ACCTGGGTGGCGTGT GACGTCATGTGCTCA ACCGCTCCATACTC
101 - Y W V F G E Q F C D T W V A C D V M C S T A S I L

376 - AACCTGTGCGCTATC TCGCTCGACAGATAC ATTACATCAAAGAC CCTTTGAGGTACGGT CGCTGGGTGACCCGC
126 - N L C A I S L D R Y I H I K D P L R Y G R W V T R

451 - AAGTGGGCGGTAGCC ACAATAGCCATGATC TGCTGTAGCAGGC CTGGTCAGTTTCTTG CCCATCTCGCTAGG
151 - K V A V A T I A M I W L L A G L V S F L P I S L G

526 - CTTACAGGCCTGAT GAAGAAGCCCTGGCC ACACAGAAGCCCCCG AGATACCCACGTGC GCGTTGGTCTGACG
176 - L H R P D E E A L A T Q K P P R Y P T C A L V L T

601 - CCGACGTACGCGGTC GTCTCCAGCTGTATA TCGTTTACTACTACCG TGTATTGTTATGATT AGTATATACTGCAGA
201 - P T Y A V V S S C I S F I L P C I V M I S I Y C R

676 - CTATACTGCTACGCT CAAAAACACGTCAAG TCAATCCGGGCCGTC ACCCGAACGGTTCAA ATGCCGGACAACCGG
226 - L Y C Y A Q K H V K S I R A V T R T V Q M P D N R

751 - ACGAAGTCCGTCCGG ACTCGAGTGCACACC CACGTGCACTCGTCA CCGTACCACGTCTCC GATCACAAGGCGGCC
251 - T K S V R T R V H T H V H S S P Y H V S D H K A A

826 - ATTACTGTGGGTATC ATCATGGGAGTGTTC CTCTTTGCTGGGTG CCCTTCTTCTGTGTG AACATCGTCGCTGCG
276 - I T V G I I M G V F L L C W V P F F C V N I V A A

901 - TTTTGTAAACGTGT ATACCGGATTTGGCA TTCAAAATCCTGACG TGGCTTGGCTACTCA AACTCCGCGTTCAAC
301 - F C K T C I P D L A F K I L T W L G Y S N S A F N

976 - CCTATCATATACTCA ATATTCAACACAGAG TTCCGGGAAGCCTTC AAGAAGATCCTAACG TCCAGGTATCTCTA
326 - P I I Y S I F N T E F R E A F K K I L T S R Y P L

1051 - TGCTGCGGATACCAA AGCGTCAGAGCCAAC ACACCAACACGAAAC GACAACTTTGTCACT GACTACGGGACTAAA
351 - C C G Y Q S V R A N T P T R N D N F V T D Y G T K

1126 - ACCTTGGTGGTGC GA TAGCGGGTCCCTT GGCTCTCAGGAGTG GACCCCACTCCAAG TCATCGGCAGAGTCC
376 - T L V V R R S G S L G L S G V D P T P R S S A E S

1201 - GTACGCCCACTCAGA GAGTACAACATT
401 - V R P L R E Y N I

```

TAATTGTCGATGCGTGGCCTGAATGCGTCGAATGTCGTACGTGCGTACGTTGCGCCGTGCAATTGCGTCGATGCGTATTGCCCGTGC
CAATGTCGATGCGTCTGTCGTAATCTCATGCGTTGCGAGTCGAGTCGTCGTTGCGGTCGTCGACTGTCGTCGTAATGTCGTCG
CCGTGAATGCTCTGTCGTAATGTCGTAATGTCGTAATGTCGTAATGTCGTAATGTCGTAATGTCGTAATGTCGTAATGTCGTAATGTCG
TGCTATGCGCATGCGTCGTAATGTCGTAATGTCGTAATGTCGTAATGTCGTAATGTCGTAATGTCGTAATGTCGTAATGTCGTAATGTCG
TGCTAAACTGCTGTCGTAATGTCGTAATGTCGTAATGTCGTAATGTCGTAATGTCGTAATGTCGTAATGTCGTAATGTCGTAATGTCG
CACAAGTCG
CAAGTCG
GT**AATAA**AGTTGACTGCGTTGCGAGACATGCGTACTAGTCGATGCGCAATCTTCATCGTCGTCGAGAATGCTGAATCGTAGTCGGA
CTCTATCGAAGCGAAAAA

Figure S1. Nucleotide and deduced amino acid sequences of *A. ipsilon* Dop1. Nucleotide (upper line) and amino acid (lower line) numbers are given on the left. The polyadenylation signal (AATAAA) in the 3'-UTR is in bold type.

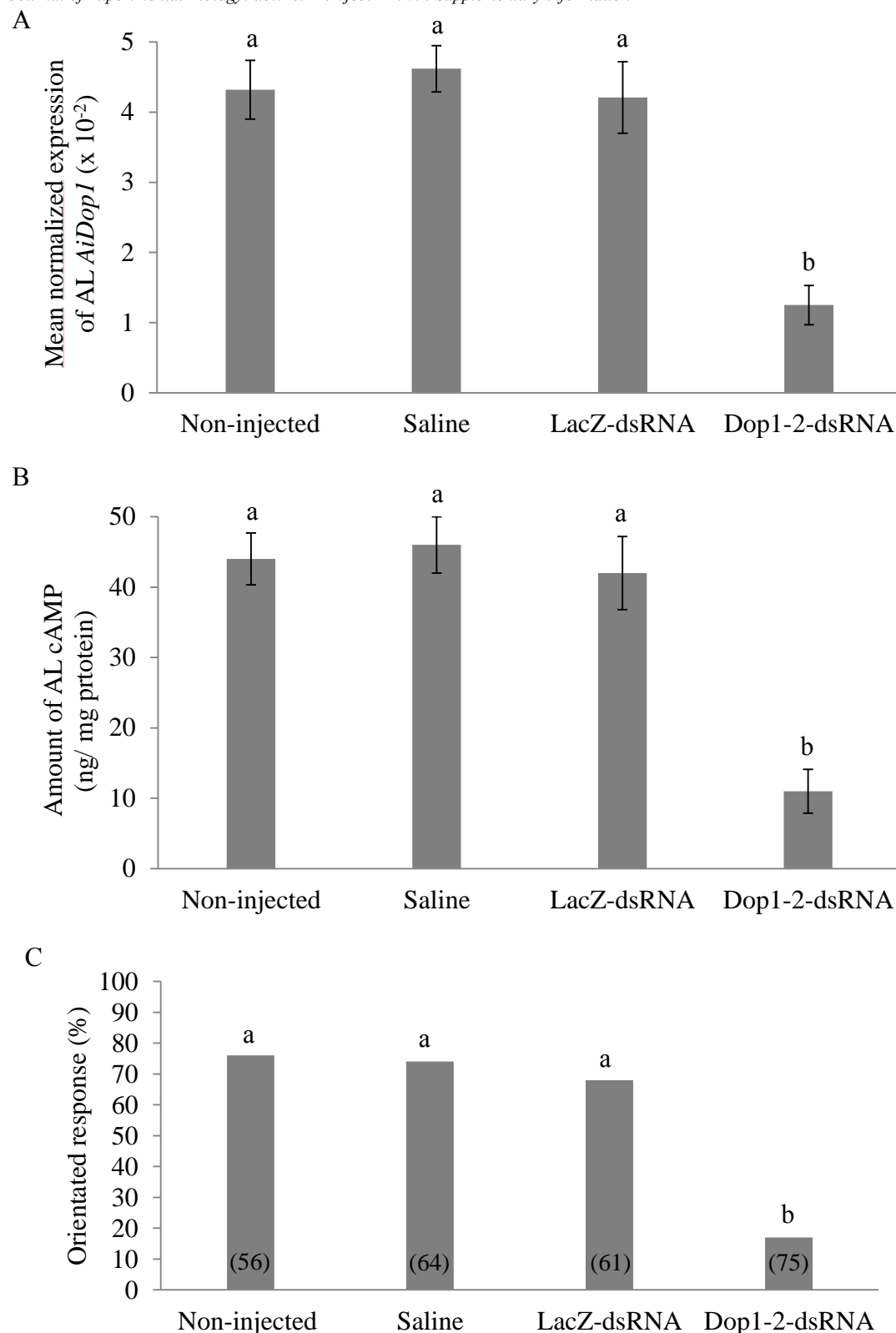


Figure S2. Efficiency and effect of Dop1-2-dsRNA on both the amount of AL cAMP and the sex pheromone behavioral response in *A. ipsilon* males. Two-day-old males received an injection of saline solution, bacterial Beta-galactosidase (LacZ)-dsRNA or Dop1-2-dsRNA, or no injection (non-injected). For each treatment and three days after injection, the relative expression of *AiDop1* mRNA (A) and the amount of cAMP (B) were quantified in ALs, and the percentages of orientated responses were determined in the wind tunnel experiments (C). Each real time qPCR was run in three technical replicates with five independent biological replicates. The cAMP amounts were quantified from 10 ALs for each experimental group with 10 biological replicates. For the *AiDop1* mRNA and cAMP levels, the bars represent means \pm SD and those with different letters are significantly different (analysis of variance; Tukey's test; $P < 0.05$). For the behavioral tests, the numbers in parentheses indicate the numbers of tested males and the columns with the same letter are not significantly different (G-test; $P < 0.05$).

Table S1. List of the primers used in the study.

Primer name	Sequence
Dop1Gdir1	5'-CTGATATTYCTCAGYGTAGCA-3'
Dop1Grev1	5'-GTTGAATATCGAGTAKATRAT-3'
Dop15'-RACE	5'-CGCGATGCAGACCAGTATGTT-3'
Dop13'-RACE	5'-TCAAACCTCCGCGTTCAACCCT-3'
qDop1dir	5'-GGGCTTCACAGGCCTGATGAAGAA-3'
qDop1rev	5'-CATTTGAACCGTTCGGGTGACGGC-3'
RpL8dir	5'-CCAGTTTGTCTACTGCGGCAA-3'
RpL8rev	5'-GCTTAACCCTAGTACGCTTGGCA-3'
LacZ T7 dir	5'-taatacgactcactatagggATGACCATGATTACGCCAAGC-3'
LacZ T7 rev	5'-taatacgactcactatagggCCATTCGCCATTCAGGCTGCG-3'
Dop1-1 T7 dir	5'-taatacgactcactatagggTCAGTACAATGAGACGACGGTGG-3'
Dop1-1 T7 rev	5'-taatacgactcactatagggTGATGTGAATGTATCTGAGCGAG-3'
Dop1-2 T7 dir	5'-taatacgactcactatagggTACTGCAGACTATACTGCTACGCTC-3'
Dop1-2 T7 rev	5'-taatacgactcactatagggGGCTCTGACGCTTTGGTATCCGCAGC