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Methoprene-tolerant and *Krüppel homolog 1* are actors of juvenile hormone-signaling controlling the development of male sexual behavior in the moth *Agrotis ipsilon*

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Abstract

In insects, juvenile hormone (JH) is critical for the orchestration of male reproductive maturation. For instance, in the male moth, *Agrotis ipsilon*, the behavioral response and the neuronal sensitivity within the primary olfactory centers, the antennal lobes (ALs), to the female-emitted sex pheromone increase with fertility during adulthood and the coordination between these events is governed by JH. However, the molecular basis of JH action in the development of sexual behavior remains largely unknown. Here, we show that the expression of the paralogous JH receptors, *Methoprene-tolerant 1* and *2* (*Met1*, *Met2*) and of the JH-inducible transcription factor, *Krüppel homolog 1* (*Kr-h1*) within ALs raised from the third day of adult life and this dynamic is correlated with increased behavioral responsiveness to sex pheromone. Met1-, Met2- and Kr-h1-depleted sexually mature males exhibited altered sex pheromone-guided orientation flight. Moreover, injection of JH-II into young males enhanced the behavioral response to sex pheromone with increased AL *Met1*, *Met2* and *Kr-h1* mRNA levels. By contrast, JH deficiency suppressed the behavioral response to sex pheromone coupled with reduced AL *Met1*, *Met2* and *Kr-h1* mRNA levels in allatectomized old males and these inhibitions were compensated by an injection of JH-II in operated males. Our results demonstrated that JH acts through Met-Kr-h1 signaling pathway operating in ALs, to promote the pheromone information processing and consequently the display

of sexual behavior in synchronization with fertility to optimize male reproductive fitness. Thus, this study provides insights into the molecular mechanisms underlying the hormonal regulation of reproductive behavior in insects.

Keywords: *Methoprene-tolerant*, *Krüppel homolog 1*, juvenile hormone signaling, antennal lobe, reproductive maturation, sexual behavior, *Agrotis ipsilon.*

1. Introduction

In animals, sexual maturation is a period of life during which occur the ontogenesis of mating behavior and the initiation of sexual signaling as well as reproductive maturation. The orchestration of these behavioral and physiological changes needs to be tightly synchronized for reproductive success (Roff, 1993; Stearns, 1992). For instance, animals acquire the ability to deliver and respond to sexual chemosignals only when they have a high degree of fertility (Dey *et al*., 2015; Gadenne *et al*., 2016; Lin *et al*., 2016). It is well established that this reproductive synchrony is governed by the actions of endogenous signals, and especially hormonal factors, on both the genital apparatus and the nervous system (Bozzolan *et al*., 2015; Lin *et al*., 2016; Gassias *et al*., 2018; Romeo, 2003). Thus, evidence has accumulated over the last decades that the development of sexual behavior relies, at least in part, on the remodeling of neuronal networks through organizational and activational effects of hormones (Lenz *et al*., 2012; Abrieux *et al*., 2014; Lin *et al*., 2016; Romeo, 2003). Elucidation of the neuronal and molecular basis of this endocrine regulation is important for a better understanding of mechanisms by which adults acquire the behavioral responsiveness to sexual cues.

In almost all vertebrate species, gonadal steroids are known to govern the display of different components of reproductive behavior by fine-tuning the sensory responsiveness to sexually relevant signals. For instance, in socially monogamous birds, androgens play a key role in the maturation of sexual preference and are also required for the performance of courtship and copulatory behaviors by interfering with the olfactory, auditory and visual pathways when juvenile males reach their sexual maturity (Ball & Balthazart, 2004; Adkins-Regan & Leung, 2006). In cyprinid fishes, methyltestosterone and ketotestosterone have the ability to elicit courtship behavior in juvenile males by increasing their olfactory sensitivity to female-emitted pheromonal prostaglandins (Belanger *et al*., 2010). In addition, there is a growing body of evidence that in male rodents, sexual motivation, female's partner preference and coital response are controlled by modulatory actions of testosterone and estradiol on the central processing of female odors within the olfactory bulb (Bakker *et al*., 2002; Moffatt, 2003; Balthazart *et al*., 2004; Yoshikage *et al*., 2007; Henley *et al*., 2010).

In insects, sesquiterpenoid hormones, represented most prominently by juvenile hormones (JH), are produced by a pair of cephalic endocrine glands, the corpora allata. These lipophilic hormones are key regulators of post-embryonic development by repressing the onset of metamorphosis, and thus maintaining the juvenile characters of larvae (Hiruma & Kaneko, 2013; Jindra *et al*., 2013). In different groups of insects, JH was also found to be responsible for the coordination of sexual displays with reproductive maturity in adult females and males. For instance, it has been reported in the tephritid fruit flies, *Anastrepha obliqua* and *Anastrepha suspensa* that the treatment of newly emerged males with a JH synthetic analog, methoprene, induced an acceleration of sexual maturation marked by a precious development of calling behavior and sexual signaling and conjointly with pheromone release (Teal *et al*., 2000; Chacón-Benavente *et al*., 2013). In *Drosophila melanogaster*, JH-deficient females exhibited delayed onset of mating and altered sex pheromone synthesis, and the normal timing of mating was recovered by JH application (Bilen *et al*., 2013). In the noctuid moths *Pseudaletia unipuncta* and *Agrotis ipsilon*, JH was shown to enhance female calling behavior and pheromone production paralleled with oocyte maturation (Cusson *et al*., 1990; Cusson *et al*., 1994) and also to facilitate the execution of sex pheromone-guided orientation flight of male in synchrony with the growth of the accessory sex glands (ASGs) (Gadenne *et al*., 1993; Duportets *et al*., 1998; Gassias *et al*., 2021).

It is well documented that most biological actions of JH are mediated by a nuclear protein complex composed of two members of the basic helix-loop-helix (bHLH) / Per-ARNT-Sim (PAS) family of transcription factors: the JH receptor Methopren-tolerant (Met) (Jindra *et al*., 2015a) and its dimerizing partner, initially termed Taiman (Tai) in *D. melanogaster*, and also Ftz-F1-interacting steroid receptor coactivator in *A. aegypti* or steroid receptor activator in other insects (Charles *et al*., 2011; Jindra *et al*., 2015b; Li *et al*., 2011, Li *et al*., 2014; Zhang *et al*., 2011). Two paralogous *Met* genes, *Met* and *Germ cell-expressed*, have been isolated from different *Drosophila* species and two *Met* genes, *Met1* and *Met2*, were also recently characterized in several lepidopteran species including *A. ipsilon* (Gassias *et al*., 2021), *Operophtera brumata* (Derks *et al*., 2015), *Bombyx mori* (Daimon *et al*., 2015; Kayukawa & Shinoda, 2015), *Chilo suppressalis* (Miao *et al*., 2020) and *Danaus plexippus* (Zhan *et al*., 2011). In presence of JH, the Met-Tai heterodimeric complex binds to an E-box like response element in the promoter regions of JH target genes, resulting in their transcriptional regulation (Li *et al*., 2011, Li *et al*., 2014; Zhang *et al*., 2011).

Krüppel homolog 1 (*Kr-h1*), which encodes a C2H2 zinc-finger type transcription factor, has been identified as an early JH-response gene that works downstream of the Met-Tai complex (Cui *et al*., 2014; Kayukawa *et al*., 2012). In various insect species, gene knockdown experiments revealed that Met and Tai are required to mediate the stimulatory actions of JH in the maturation of the female and male reproductive systems including oogenesis, vitellogenesis, testicular development and ASG differentiation and that JH can operate either without or with the requirement of Kr-h1 (Song *et al*., 2014; Smykal *et al*., 2014; Lin *et al*., 2015; Gujar & Palli, 2016; Yue *et al*., 2018; Gassias *et al*., 2021; Holtof *et al*., 2021). By contrast, a limited number of studies have been undertaken to identify the JH-initiated molecular pathways controlling the occurrence of reproductive behavior. For example, it has been uncovered in *D. melanogaster* that Met is involved in transmitting JH signal that promotes courtship behavior in old sexually mature males through an elevation of the sensitivity of olfactory receptor neurons (ORNs) to aphrodisiac pheromone (Lin *et al*., 2016). More recently, Met and Tai were found to be actors of JH signaling pathway in the onset of male copulation behavior in the desert locust, *Shistocerca gregaria* (Holtof *et al*., 2021).

In order to further elucidate the molecular mechanisms by which JH regulates the neuronal circuits that underlie mating activity, we turned to the long-lived noctuid moth *A. ipsilon* for which JH was found to regulate the central processing of pheromone information related to the maturation of male sexual behavior (Anton & Gadenne, 1999; Gadenne & Anton, 2000). In this species, newly emerged males are sexually immature and do not behaviorally respond to the female-produced sex pheromone (Duportets *et al*., 1998; Gadenne *et al*., 1993). Three to four days after emergence, males become sexually mature and are highly attracted to the female sex pheromone during the middle of the scotophase and elicit an orientation flight towards their mating partners (Duportets *et al*., 1998; Gadenne *et al*., 1993; Gassias *et al*., 2021). This increase in behavioral responsiveness is paralleled with a volumetric growth of glomeruli belonging into the macroglomerular complex (MCG), which is devoted to the processing of sex pheromone input from ORNs housed in the trichoid sensilla of the antennae, and a higher sensitivity of neurons responding to pheromone signal within the primary olfactory centers, the antennal lobes (ALs) (Anton & Gadenne, 1999; Gadenne & Anton, 2000). It has also been shown in old sexually mature males, where the circulating level of JH is high that the removal of the corpora allata resulted in decreased behavioral response and AL neuron sensitivity to pheromone information and which were subsequently

restored if allatectomized males were injected with JH. By contrast, young sexually immature males, with a low circulating level of JH, exhibited increased behavioral and neuronal activities to sex pheromone after JH administration (Anton & Gadenne, 1999; Duportets *et al*., 1998; Gadenne & Anton, 2000).

Here, we tested the hypothesis that *Met* and *Kr-h1* are mediators of the central action of JH in the enhancement of sex pheromone-guided orientation flight of *A. ipsilon* male. We first evaluated the influence of age on the expression level of *Met1*, *Met2* and *Kr-h1* within ALs in relation with sex pheromone responsiveness. We then examined the impact of RNAi-induced knockdown of *Met1*, *Met2* or *Kr-h1* on the behavioral response to sex pheromone. Finally, we manipulated the circulating JH titer either by injection of exogenous JH or allatectomy and analyzed the effect of treatment on *Met1*, *Met2*, *Kr-h1* mRNA levels in ALs and the behavioral response to sex pheromone. Taken together, our results highlighted that *Met1*, *Met2* and *Kr-h1* are components of JH signaling pathways which function within ALs to facilitate the pheromone information processing and consequently the elicitation of sexual attraction behavior of male in *A. ipsilon*.

2. Materials and methods

2.1. Chemicals

We prepared a synthetic sex pheromone blend at 1 ng/µl containing three components identified in natural extracts of the pheromone glands of the female *A. ipsilon* (Picimbon *et al*., 1997; Gemeno & Haynes, 1998): (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) mixed at a ratio of 4:1:4. This blend was proven to be the most attractive to males in field trapping experiments and it elicits a similar behavior in a wind tunnel as natural extracts of the pheromone gland (Barrozo *et al*., 2010; Vitecek *et al*., 2013). The three components were purchased from Sigma Aldrich (Saint-Quentin Fallavier, France) and diluted in hexane (>98 % purity, Carlo-Erba, Val-de-Reuil, France). A dose of 10 ng of the sex pheromone blend was used in the experiments and this dose had previously been described as behaviorally and electrophysiologically active (Gadenne *et al*., 2001; Deisig *et al*., 2012; Gassias *et al*., 2018).

2.2. Insects and tissue collection

Adult *A. ipsilon* originated from a laboratory colony established in Bordeaux and transferred to Versailles, France. 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 & Bues, 1974). Pupae were sexed, and males and females were kept separately on 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. The males were anaesthetized with an exposure to carbon dioxide and 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 *Met1*, *Met2* and *Kr-h1*, ALs were dissected in Ringer's solution and immediately flash-frozen in Eppendorf (Montesson, France) vials kept in liquid nitrogen and stored at −80 °C until treatment. The dissected males were then euthanized by using liquid nitrogen.

2.3. Allatectomy

Removal of the corpora allata was performed according to the protocol described by Gadenne *et al.* (1993). Newly emerged males were allatectomized <3 h after emergence. Care was taken to ensure that only the corpora allata and not the corpora cardiaca were removed. Sham operations were performed by opening the head and touching, but not removing the corpora allata.

2.4. Juvenile hormone injection

At the beginning of scotophase, males (0.2 g body weight) were anaesthetized with carbon dioxide and JH-II (15 µg in 2 µl mineral oil) was injected into the lateral region of the intersegment membrane between the second and third abdominal segments by using a syringe with a 32-gauge needle (Hamilton, Reno, NV, USA). Oil injections were used as control experiments. JH-II (78 % purity) was purchased from SciTech (Prague, Czech Republic).

2.5. cDNA synthesis

Total RNA was extracted from ALs 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 U 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 cDNA was synthesized from total RNAs (1 µg) with SuperScript II Reverse Transcriptase (RT) (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 reaction volume of 20 µl. The mix was heated to 65 °C for 5 min before the enzyme was added and then incubated for 1 h at 42 °C.

2.6. Quantification of Met1, Met2 *and* Kr-h1 *expressions in ALs*

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed from RNA extraction of pooled ALs of several males and using the LightCycler 480 Real-Time PCR Detection System (Roche, Saint-Quentin Fallavier, France) according to the manufacturer's instructions. Each 12 µl reaction consisted of 6 µl Absolute Blue SYBR Green fluor (Thermo Scientific, Waltham, MA, USA), 4 µl cDNA (25 ng/µl) and 2 µl of specific primer pairs at 10 µmol l−1 for *Met1* (qMet1dir and qMet1rev), *Met2* (qMet2dir and qMet2rev) or *Kr-h1* (qKr-h1dir and qKr-h1rev) (**Table S1**). PCR conditions were 35 cycles of 95 °C for 30 s, 65 °C for 30 s, 72 °C for

30 s and the length of amplified products was 234 bp, 258 bp and 213 bp for *Met1*, *Met2* and *Kr-h1*. 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 (undiluted, 1/5, 1/25, 1/125 and 1/625) were used to construct a relative standard curve to determine the PCR efficiency 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 eight independent biological replicates using the technical platform of The Integrative Biology Institute (Sorbonne University, France). Expression levels were analyzed with LightCycler 480 software (Roche Diagnotics GmbH, Mannheim, Germany) 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 (Met1, Met2, Kr-h1) and the reference gene, the *A. ipsilon Ribosomal protein L8* (*RpL8*) 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) by using the formula: $NE = (E\ RpL8)^{Ct\ RpL8} / (E\ Met, Kr\text{-}h1)^{Ct\ Met,\ Kr\text{-}h1}$ with NE meaning normalized expression, E meaning PCR efficiency and Ct corresponding to the crossing-point values. Then, mean and standard error were calculated with the NE values for the biological replicates. The sequences of the specific primers RpL8dir and RpL8rev for *RpL8* are given in **Table S1**.

2.7. RNA interference experiments

Double-stranded RNA (dsRNA) production and injection conditions were established by following the protocols used in *A. ipsilon* for the silencing of *Met1*, *Met2* and *Kr-h1* (Gassias *et al*., 2021). The dsRNAs targeting *Met1* (Met1-1-dsRNA, 486 bp; and Met1-2-dsRNA, 528 bp), *Met2* (Met2-1-dsRNA, 480 bp; and Met2-2-dsRNA, 478 bp), *Kr-h1* (Kr-h1-1-dsRNA, 466 bp; and Kr-h1-2-dsRNA, 472 bp), and bacterial *β-galactosidase* (*LacZ*) (372 bp; control) were chosen by using the E-RNAi web application (http://e-rnai.dkfz.de/) (Arziman *et al*., 2005) and produced with a MEGAscript® T7 High Yield Transcription Kit (Ambion, Villebon-sur-Yvette, France) according

to the manufacturer's instructions. A PCR was performed on 1 µl plasmid (50 ng/µl) with specific primers for each target gene (Met1-1-T7dir/Met1-1-T7rev, Met1-2-T7dir/Met1-2-T7rev, Met2-1-T7dir/Met2-1-T7rev, Met2-2-T7dir/Met2-2-T7rev, Kr-h1-1-T7dir/Kr-h1-1-T7rev, Kr-h1-2-T7dir/Kr-h1-2-T7rev and LacZ-T7dir/LacZ-T7rev) (**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, Düren, Germany) 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 of T7 10× Buffer in a final volume of 20 µl. After spectrometry quantification, the reaction mix was incubated for 15 min at 37 °C with 2 U of Turbo DNase (Ambion, Villebon-sur-Yvette, France). The dsRNA precipitation was performed by addition of 30 µl of water treated with diethylpyrocarbonate (DEPC) and 20 µl LiCl (Sigma Aldrich, Saint-Quentin Fallavier, France), storage of the mixture at −20 °C for 2 h, and then centrifugation for 30 min at 16000 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 to 0.5 µg/µl in saline solution containing CaCl2 7.1 mM, Na2β-glycerophosphate 22 mM, MgSO4 13.5 mM, MgCl2 26.9 mM, KCl 29.5 mM, glucose 23.9 mM, pH 6.8 (Sigma Aldrich, Saint-Quentin Fallavier, France).

Day-1 males were anaesthetized with carbon dioxide and injected with 1 µg dsRNA in the lateral region of the intersegment membrane between the second and third abdominal segments. The injections were performed at the beginning of the scotophase by using a Hamilton syringe with a 32-gauge needle. On day-5, ALs were dissected for the *Met1*, *Met2* and *Kr-h1* expression analysis. Control groups were composed of non-injected, bacterial LacZ-dsRNA-injected and saline-injected males.

2.8. Behavioral experiments to sex pheromone in a wind tunnel

Behavioral tests were performed using a Plexiglass wind tunnel measuring 190 cm length × 75 cm width × 75 cm height (VT Plastics, Genevilliers, France). Both ends of the tunnel were enclosed with white synthetic fabric allowing air to pass through but preventing insect escape. Air

movement was provided by an exhaust fan at the downwind end of the tunnel sucking the air at a speed of 0.3 m/s and evacuating odorized air out of the building. The tunnel was maintained in dark except for one red visible light source allowing visual observation. Experiments were performed at 23 °C, 40 ± 10 % relative humidity, during the middle of the scotophase (between 1:00 pm and 4:00 pm) when males respond maximally to the sex pheromone with a characteristic anemotactic zigzagging flight (Barrozo *et al*., 2010). A single experimental male was introduced into the wind tunnel and placed on a 36 cm high platform in the middle of the tunnel width and 160 cm downwind from the pheromone source. After 1 min, during which the male adjusted to the airflow, the pheromone blend was delivered for 3 min. During this time, the following behavioral items were observed: take-off, partial flight (flight half way between the release site and the pheromone source), complete flight (arrival within 10 cm of the source) and landing on the pheromone source. The partial and complete flights as well as landings were considered as an orientated response towards the sex pheromone. Orientated and random flights were counted together in order to quantify the general flight activity of males and this activity was around 95 % for all behavioral tests. We also noted the delay in the orientated response of males by measuring the period of time between the onset of pheromone stimulation and the take-off. All experiments were performed double-blind to avoid partial observations. The flight tracks were recorded and analyzed using Trackit 3D 2.0 (SciTracks, Pfaffhausen, Switzerland). Two cameras (Basler Pilot, piA640–210 m with Tamron ½" 4-12F/1.2 lenses) were positioned above the tunnel at 60 cm from each other to cover the whole tunnel flight section with overlapping fields. Images from the two cameras were analyzed in real time and the x, y, and z coordinates of moth's position were extracted every 10 ms. On each experimental day, 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 stimulus 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, Silsden, United Kingdom) 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, ME, 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, Sigma Aldrich, Saint-Quentin Fallavier, France) inserted through the septum and connected with polytetrafluorethylene tubing (1.32 mm i.d., 20 cm length, Sigma Aldrich, Saint-Quentin Fallavier, France). A 10 µl sample of the pheromone blend at 1 ng/µl was deposited on a filter paper and placed inside the vial. Contaminated air was removed from the set-up by an exhaust fan after each behavioral assay.

2.9. Age-related experiments

Males of different ages (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 quantification of the behavioral response to sex pheromone. The males were then killed by using liquid nitrogen, and the ALs were dissected from frozen heads for analysis of *Met1*, *Met2* and *Kr-h1* expressions.

2.10. Statistical analysis

The means of *Met1*, *Met2* and *Kr-h1* expression levels were compared using an ANOVA or Kruskal-Wallis' test followed by a multiple comparison by their corresponding post-hoc test. $P < 0.05$ was accepted as statistically significant. For behavioral experiments, statistical differences between groups of males were evaluated using an R × C test of independence by means of a G-test and also by applying the Williams correction (Sokal & Rohlf, 1995).

2.11. Animal ethics

Adequate measures were taken to minimize pain or discomfort of animals and that the experiments were conducted in accordance with international standards on animal welfare.

3. Results

3.1. Age-related changes in the behavioral response to sex pheromone and Met1, Met2 *and* Kr-h1 *expressions in ALs*

We determined the proportion of sex pheromone-guided orientation flight in relation with the *Met1*, *Met2* and *Kr-h1* transcriptional activities in the ALs for 1- to 5-day-old males. The percentage of orientated behavioral response is only about 15 % and 12 % in 1- and 2-day-old sexually immature males and approximately 74 % and 81 % in 4- and 5-day-old sexually mature males, respectively (**Fig. 1A**). *Met1*, *Met2* and *Kr-h1* mRNAs are expressed at low level in ALs within the early days of adult life (Dunn's test, $P \leq 0.0093$) (**Fig. 1B**, **C** and **D**). Then, expression gradually increases from day-3 to day-4 (Dunn's test, $P \leq 0.0093$) and remains high at day-5 (Dunn's test, $P \geq 0.2799$) (**Fig. 1B**, **C** and **D**). The levels of *Met1*, *Met2* and *Kr-h1* mRNAs in ALs of 5-day-old sexually mature males are approximatively 2.6-, 3.6- and 3-fold higher than in 1-day-old sexually immature males, respectively (**Fig. 1B**, **C** and **D**).

3.2. Interactions between Met1, Met2 and Kr-h1 in ALs

In order to ascertain whether the expressions of *Met1*, *Met2* and *Kr-h1* in ALs are linked to each other, we evaluated the impact of injection of Met1-1-dsRNA, Met2-1-dsRNA and Kr-h1-1-dsRNA into 1-day-old males on the levels of corresponding transcripts in ALs four days after administration. *Met1* and *Met2* silencing in the ALs of 5-day-old males (**Fig. 2A** and **B**) were estimated around 74 % (Dunn's test, $P \leq 0.0001$) and 78 % (Dunn's test, $P \leq 0.0001$) respectively, and resulted in a 60 %–70 % reduction of the *Kr-h1* mRNA expression (**Fig. 2C**) (Dunn's test, $P \leq 0.0001$) whereas *Kr-h1* silencing of approximatively 65 % had no effect on the AL *Met1* and *Met2* mRNA levels as compared with control groups (Dunn's test, $P = 1.000$) (**Fig. 2A** and **B**). In addition, the expression level of AL *Met2* and *Met1* remained unchanged in Met1- (Dunn's test, P ≥ 0.02792) and Met2-depleted (Dunn's test, $P \geq 0.03563$) males respectively (**Fig. 2A** and **B**).

3.3. Effect of Met1, Met2 and Kr-h1 silencing on the behavioral response to sex pheromone

We analyzed the consequence of injection of Met1-1-dsRNA, Met2-1-dsRNA and Kr-h1-1-dsRNA into 1-day-old males on the behavioral response of male to sex pheromone four days after administration. Met1-, Met2 and Kr-h1- depleted 5-day-old males exhibited a decline in the

expression of sex pheromone-guided orientation flight with increased delay response in comparison with control groups (**Fig. 3A** and **B**). The general flight activity of Met1-, Met2 and Kr-h1-depleted males (around 85 %) was not statistically different than that of control males (around 92 %), showing that *Met1*, *Met2* and *Kr-h1* knockdown did not alter the flight ability of moths (**Fig. 3C**). In order to consolidate our RNAi results, we also tested the impact of second dsRNA designed against *Met-1*, *Met-2* or *Kr-h1* (Met1-2-dsRNA, Met2-2-dsRNA or Kr-h1-2-dsRNA) and which caused alterations in the behavioral responsiveness to sex pheromone that were similar to those induced in Met1-1-dsRNA- and Met2-1-dsRNA- and Kr-h1-1-dsRNA-treated males (**Fig. S1**).

3.4. Effect of JH on the behavioral response to sex pheromone and the AL Met1, Met2 and Kr-h1 expressions

In order to reveal a potential relationship between *Met1*, *Met2* and *Kr-h1* and the JH action, we manipulated the circulating JH titer in sexually immature and mature males either by injection of exogenous JH or allatectomy and then observed the impact of treatment on the behavioral response to sex pheromone and the AL *Met1*, *Met2* and *Kr-h1* transcriptional activities. Administration of JH-II into newly emerged males led to an increase in the sex pheromone-triggered orientation flight with decreased delay response (**Fig. 4A** and **B**) that is paralleled with an elevation of the AL *Met1*, *Met2* and *Kr-h1* mRNA expression levels in comparison with control groups (oil-injected and non-injected 2-day-old males) (Tamhane and Tukey's tests, $P \leq 0.0001$) (**Fig. 4C**, **D** and **E**). By contrast, the ablation of corpora allata of newly emerged males resulted in altered behavioral responsiveness to sex pheromone (**Fig. 5A**) coupled with reduced AL *Met1*, *Met2* and *Kr-h1* mRNA levels five days after allatectomy as compared with group controls (non-operated and sham-operated 5-day-old males) (Dunn and Tamhane's test, $P \leq 0.0001$) (**Fig. 5B**, **C** and **D**). We demonstrated that the injection of JH-II, the following day of allatectomy, raised both the behavioral responsiveness to sex pheromone (**Fig. 5A**) and the AL *Met1*, *Met2* and *Kr-h1* mRNA expressions in allatectomized 5-day-old males, reaching almost the levels of non-operated and sham-operated 5-day-old males (Dunn and Tamhane's test, $P \geq 0.0001$) (**Fig. 5B**, **C** and **D**). We also verified that injection of oil into allatectomized males had no effect on the sex pheromone-

triggered flight behavior (**Fig. 5A**) and the AL *Met1*, *Met2* and *Kr-h1* expressions (Dunn and Tamhane's test, $P = 1.0000$) (**Fig. 5B**, **C** and **D**).

4. Discussion

The purpose of our work was to explore the molecular function of *Met* and *Kr-h1* as mediators of the central action of JH in the acquisition of male sexual behavior by using the long-lived moth *A. ipsilon* as a model organism for which the responsiveness to sex pheromone signal are known to be age- and JH-dependent. Our results showed that the amounts of *Met1*, *Met2* and *Kr-h1* transcripts were higher in the ALs of sexually mature old males as compared with those of sexually immature young males. This revealed the existence of an age-related upregulation of the transcriptional activities of *Met1*, *Met2* and *Kr-h1* in the ALs and which coincides with the age-related increase in the neuronal response to pheromone information and the emergence of sex pheromone-guided orientation flight in old males. Similarly, studies performed in the honey bee *Apis mellifera* indicated that an elevated expression of *Kr-h1* is associated to the brain remodeling required for the ontogenesis of adult behaviors within the community of workers (Shpigler *et al.*, 2010). In the mice *Mus musculus*, a high transcriptional activation of the *Krüppel-like factor 7* was found to be indispensable for the regulation of neuronal processes, including neurite outgrowth, axon guidance and neurogenesis, in the development and the adult plasticity of the olfactory pathways (Laub *et al.*, 2005). Moreover, in the terrestrial mollusk *Limax valentianus*, a Krüppel-like factor has been detected in the protocerebrum, the locus of odor information processing, where its expression was induced by neuronal activity and sensory input (Fukunaga *et al.*, 2006).

We observed that the administration of dsRNAs targeting various regions of *Met1*, *Met2* and *Kr-h1* resulted in an approximatively 65 %–78 % reduction in mRNA expression levels in the ALs, and led to similar defective phenotypes of the behavioral response to sex pheromone in dsRNA-treated males. These results strengthened our RNAi experiments even though we could not verify the decline of the corresponding proteins in the ALs of Met1-, Met2- and Kr-h1-deficient males because the antibodies against *Agrotis* Met1, Met2 and Kr-h1 proteins are not available. *Met1*, *Met2* and *Kr-h1* silencing caused alterations in sex pheromone-triggered orientation flight that manifested by a lower percentage of responding males and an increased response delay. We also found that *Met1* and *Met2* knockdown provoked a decrease in the AL *Kr-h1* expression whereas *Kr-h1* knockdown had no impact on the AL *Met1* and *Met2* expressions. Taken together, our RNAi data provided functional evidence that in *A. ipsilon*, *Met1*, *Met2* and *Kr-h1* are involved in the maturation of male sexual behavior and that *Kr-h1* works downstream of *Met1* and *Met2*. In

agreement with this finding, it has been demonstrated in *D. melanogaster* that RNAi-mediated depletion of *Met* disrupted the execution of male copulatory behavior (Wilson *et al*., 2003; Lin *et al*., 2016) and more recently, it has been revealed that the loss of *Met* function by RNAi resulted in an impaired mating behavior in *S. gregaria* males (Holtof *et al*., 2021).

It has been previously reported in *A. ipsilon* that JH-deprived old males exhibited altered sex pheromone-guided orientation flight (Duportets *et al*., 1998; Gadenne & Anton, 2000) as observed in *Met1*-, *Met2*- and *Kr-h1*-depleted males. Based on this observation, it was conceivable to assume that *Met1*, *Met2* and *Kr-h1* are linked to JH action in the modulation of male sexual activity. In order to reveal this relationship, we manipulated the circulating JH titer in males of different ages and examined the resulting changes on their orientated response to sex pheromone and the AL *Met1*, *Met2* and *Kr-h1* expression levels. The injection of JH into young males enhanced the sex pheromone behavioral response with increased AL *Met1*, *Met2* and *Kr-h1* transcriptional activities. By contrast, JH deficiency inhibited the sex pheromone behavioral response coupled with reduced AL *Met1*, *Met2* and *Kr-h1* mRNA amounts in allatectomized old males and these suppressive effects were partly rescued by a combined injection of JH in operated males. These data highlighted that in *A. ipsilon*, JH operates through the induction of the Met1, Met2 and Kr-h1-dependent signaling pathways that take places in ALs, to facilitate the central processing of pheromone information and thereby, contributing to the onset of sexual attraction behavior in sexually mature old males. In concordance with this result, it is well documented in rodents that testicular hormones are critical for the accomplishment of male reproductive behaviors by governing a reshaping of neuronal circuits and especially within the olfactory bulb glomeruli, and thus affecting the perception of female urinary pheromones (Moffatt, 2003; Yoshikage *et al*., 2007; Henley *et al*., 2010). In future studies, it would be useful to prove that *Met1*, *Met2* and *Kr-h1* are mediators of enhancing effects of JH on the sex pheromone-driven responses of AL neurons, and to achieve this, we will evaluate the impact of RNAi-induced *Met1*, *Met2* and *Kr-h1* silencing on the neuronal activity by intracellular recordings within the *A. ipsilon* MCG glomeruli that are specialized in the integration of pheromone signal. We cannot rule the possibility that the JH-Met-Kr-h1 signaling pathway might be functional in other areas of the brain that the primary olfactory neuropiles and especially within second-order olfactory centers including the lateral horn and the mushroom bodies which are known to display a high degree of anatomical and physiological plasticity in the

context of chemosensory experience and the behavioral development (Menzel & Manz, 2005; Okada *et al*., 2007; Reinhard & Claudianos, 2012). In line with this speculation, *Met-1*, *Met-2* and *Kr-h1* transcripts were detected in the *A. ipsilon* brain after the excision of ALs (P. Aguilar, unpublished data). In addition, in situ hybridization analysis on the brain of honey bee workers demonstrated that changes in synaptic organization of mushroom bodies were accompanied by increased *Kr-h1* expression during the transition to foraging tasks (Hewes, 2008; Shpigler *et al*., 2010). Experiments are in progress to establish in situ the mapping of *Met1*, *Met2* and *Kr-h1* expressions in the entire brain of *A. ipsilon* adult.

Previous works indicated that the antennal sensory system of *A. ipsilon* is fully functional in newly emerged immature males and that the electrophysiological response of the ORNs of trichoid sensilla to the female sex pheromone is age- and JH-independent (Gadenne *et al*., 1993). Such a finding is supported by the observation that *Met1*, *Met2* and *Kr-h1* are expressed in the antennae at extremely low levels throughout adulthood (P. Aguilar, unpublished data). Thus, the initiation of male sexual behavior by JH appears mainly driven by modulatory actions on the central olfactory pathways in *A. ipsilon*. By contrast, it has been uncovered in *D. melanogaster* that JH confers the ability to engage in the courtship behavior by increasing the sensitivity of antennal ORNs to stimulatory pheromones in reproductively mature males and that Met is required in transducing the JH signal (Lin *et al*., 2016). This is consistent with other studies conducted on cyprinid fishes showing that androgens have direct actions on the ORNs to enhance the detection of female pheromonal prostaglandins and the expression of courtship behaviors (nuzzling and quivering) in juvenile males (Belanger *et al*., 2010).

It is well established that JH plays a key role in the coordination between reproductive maturity and sexual activity (Cusson *et al*., 1994; Teal *et al*., 2000; Bilen *et al*., 2013; Holtof *et al*., 2021). In *A. ipsilon* males, JH was found to fulfill this function through simultaneous actions on both the ALs and the ASGs (Gadenne *et al*., 1993; Duportets *et al*., 1998; Anton & Gadenne, 1999; Gassias *et al*., 2021). In addition, we have recently reported that *Met1*, *Met2* and *Kr-h1* act as transducers of JH signal that stimulates the growth and the secretory activity of the ASGs, and especially the synthesis of fluid seminal proteins (Gassias *et al*., 2021) and which are necessary for spermatophore production and nourishment, activation, and capacitation of spermatozoa until

fertilization (Avila *et al*., 2011). Consistent with the above-mentioned data and the obtained results in this study, it seems reasonable to propose that the acquisition of responsiveness to sex pheromone and the maturation of the ASGs are conjointly under the influence of the Met-Kr-h1-dependent signaling pathways and that the synchronization of these physiological events relies in part on the concomitant activation of these JH-initiated cascades in the ALs and the ASGs in response to the elevation of circulating JH levels throughout the adulthood of *A. ipsilon* male. This assumption is reinforced by the observation that age-dependent increases in the AL *Met1*, *Met2* and *Kr-h1* expressions are timely correlated with those measured in the ASGs (Gassias *et al*., 2021). Interestingly, our study highlighted overlapping functions of Met1 and Met2 in JH-regulated male sexual development of *A. ipsilon* as reported in JH-mediated female reproductive maturation of *C. suppressalis* (Miao *et al*., 2020).

In mammals and insects, there is a growing body of evidence that hormonal factors interact with biogenic amines and this functional interplay has been particularly described in the context of male reproduction. For example, testosterone is known to be essential for the display of male reproductive behavior by enhancing the processing of sexually relevant stimuli and this action has been shown to be driven through alterations in the synthesis, release and receptor expression for neurotransmitters such as dopamine (DA) and serotonin (Hull *et al*., 1999, Hull *et al*., 2004). JH was found to be a critical signal in the emergence of DA-mediated mating flight of *A. mellifera* drones by increasing simultaneously the expression levels of DA and D1-type dopamine receptor (Dop1) in the brain (Akasaka *et al*., 2010; Sasaki *et al*., 2012). In addition, we have previously revealed in *A. ipsilon* male that age-related changes in neuronal and behavioral responses to sex pheromone are sustained by DA-Dop1 signaling pathway that operates in ALs through cAMP-dependent mechanisms (Gassias *et al*., 2019). In order to explore the existence of a regulation of the dopaminergic system by JH in *A. ipsilon*, experiments are underway to evaluate the impact of JH administration in young males and of JH deprivation in old males on the amounts of DA, *Dop1* expression and cAMP in ALs.

5. Conclusions

Our study provides molecular insights into how the insect gonadotropic hormone, JH, utilizes Met-Kr-h1-mediated signaling pathways to act conjointly on the olfactory centers and the reproductive apparatus in order to coordinate the onset of male sexual behavior with the acquisition of fertility, and thus maximizing reproductive fitness. A further step in the understanding at the molecular level of this JH-regulated reproductive synchrony is to undertake the identification of the Kr-h1 target genes of the peripheral and central JH signaling pathways. Our findings also offers potential applications for agricultural pest control. The larvae of most moth species have negative consequences on agricultural food production as major crop pests. The crucial role of the JH-Met-Kr-h1 signaling cascade in the control of the physiology reproductive of the male moths projects Met and Kr-h1 as potential molecular targets for the development of pest control strategies.

Declaration of competing interest

The authors disclose no potential conflicts of interest.

References

Abrieux, A., Duportets, L., Debernard, S., Gadenne, C., 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: 312, https://doi: 10.3389/fnbeh.2014.00312.

Adkins-Regan, E., Leung, C.H., 2006. Sex steroids modulate changes in social and sexual preference during juvenile development in zebra finches. 50, 772-778. https://10.1016/j.yhbeh.2006.07.003.

Akasaka, S., Sasaki, K., Harano, K., 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.

Anton, S., 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.

Avila, F.W., Sirot, L.K., LaFlamme, B.A., Rubinstein, C.D., Wolfner, M.F., 2011. Insect seminal fluid proteins: identification and function. Annu. Rev. Entomol. 56, 21-40. https://doi: 10.1146/annurev-ento-120709-144823.

Bakker, J., Honda, S., Harada, N., Balthazart, J., 2002. Sexual partner preference requires a functional aromatase (cyp19) gene in male mice. Horm. Behav. 42, 158-171. https://doi: 10.1006/hbeh.2002.1805.

Ball, G.F., Balthazart, J., 2004. Hormonal regulation of brain circuits mediating male sexual behavior in birds. Physiol. Behav. 83, 329-346. https://doi: 10.1016/j.physbeh.2004.08.020.

Balthazart, J., Baillien, M., Cornil, C.A., Ball, G.F., 2004. Preoptic aromatase modulates male sexual behavior: slow and fast mechanisms of action. Physiol. Behav. 83, 247-270. https:// doi: 10.1016/j.physbeh.2004.08.025.

Barrozo, R.B., Gadenne, C., Anton, S., 2010. Switching attraction to inhibition: mating-induced reversed role of sex pheromone in an insect. J. Exp. Biol. 213, 2933-2939.

Belanger, R.M., Pachkowski, M.D., Stacey, N.E., 2010. Methyltestosterone-induced changes in electro-olfactogram responses and courtship behaviors of cyprinids. Chem. Senses. 35, 65-74. https://doi: 10.1093/chemse/bjp085.

Bilen, J., Atallah, J., Azanchi, R., Levine, J.D., Riddiford, L.M. 2013. Regulation of onset of female mating and sex pheromone production by juvenile hormone in Drosophila melanogaster. Proc. Natl. Acad. Sci. U S A. 110, 18321-18326. https://doi: 10.1073/pnas.1318119110.

Bozzolan, F., Duportets, L., Limousin, D., Wycke, M.A., Demondion, E., François, A., Abrieux, A., Debernard, S., 2015. Synaptotagmin I, a molecular target for steroid hormone signaling controlling the maturation of sexual behavior in an insect. FEBS J. 282, 1432-1444. https://doi: 10.1111/febs.13231.

Chacón-Benavente, R., López-Guillen, G., Hernández, E., Rojas, J.C., Malo, E.A. 2013. Juvenile hormone analog enhances calling behavior, mating success, and quantity of volatiles released by Anastrepha obliqua (Diptera: Tephritidae). Environ. Entomol. 42, 262-269. https://doi: 10.1603/EN12045.

Charles, J.P., Iwema, T., Epa, V.C., Takaki, K., Rynes, J., Jindra, M., 2011. Ligand-binding properties of a juvenile hormone receptor, methoprene-tolerant. Proc. Natl. Acad. Sci. U S A. 108, 21128-21133. https://doi: 10.1073/pnas.1116123109.

Cui, Y., Sui, Y., Xu, J., Zhu, F., Palli, S.R., 2014. Juvenile hormone regulates Aedes aegypti Krüppel homolog 1 through a conserved E box motif. Insect Biochem. Mol. Biol. 52, 23-32. https://doi: 10.1016/j.ibmb.2014.05.009.

Cusson, M., McNeil, J.N., Tobe, S.S. 1990. In vitro biosynthesis of juvenile hormone by corpora allata of Pseudaletia unipuncta virgin females as a function of age, environmental conditions, calling behaviour and ovarian development. J. Insect. Physiol. 36, 139-146.

Cusson, M., Tobe, S.S., McNeil, J.N. 1994. Juvenile hormones: their role in the regulation of the pheromonal communication system of the armyworm moth, Pseudaletia unipuncta. Arch. Insect Biochem. and Physiol. 25, 329-345.

Deisig, N., Kropf, J., Vitecek, S., Pevergne, D., Rouyar, A., Sandoz, J.C, Lucas, P., Gadenne, C., Anton, S., Barrozo, R., 2012. Differential interactions of sex pheromone and plant odour in the olfactory pathway of a male moth. PLoS One. 7, https://doi: 10.1371/journal.pone.0033159.

Derks, M. F., Smit, S., Salis, L., Schijlen, E., Bossers, A., Mateman, C., Pijl A. S., De Ridder D., Groenen M.A., Visser M. E., Megens H-J., 2015. The genome of winter moth (Operophtera brumata) a genomic perspective on sexual dimorphism and phenology. Genome. Biol. Evol. 7, 2321-2332. https://doi: 10.1093/gbe/evv145.

Dey, D., Chamero, P., Pru, J.K., Chien, M.S., Ibarra-Soria, X., Spencer, K.R., Logan, D.W, Matsunami, H., Peluso, J.J., Stowers, L., 2015. Cyclic regulation of sensory perception by a female hormone alters behavior. Cell. 161, 1334-1344. https://doi: 10.1016/j.cell.2015.04.052.

Daimon, T., Uchibori, M., Nakao, H., Sezutsu, H., Shinoda, T., 2015. Knockout silkworms reveal a dispensable role for juvenile hormones in holometabolous life cycle. Proc. Natl. Acad. Sci. U S A. 112, 4226-4235. https://doi.org/10.1073/pnas.1506645112.

Duportets, L., Dufour, M.C., Couillaud, F., 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.

Fukunaga, S., Matsuo, R., Hoshino, S., Kirino, Y., 2006. Novel Krüppel-like factor is induced by neuronal activity and by sensory input in the central nervous system of the terrestrial slug, Limax valentianus. J. Neurobiol. 66, 169-181.

Gadenne, C., 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., Anton, S., 2016. Plasticity in Insect Olfaction: To Smell or Not to Smell?. Annu. Rev. Entomol. 61, 317-333.

Gadenne, C., Dufour, M.C., 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.

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

Gassias, E., Durand, N., Demondion, E., Bourgeois, T., Aguilar, P., Bozzolan, F., Debernard S., 2019. A critical role for Dop1-mediated dopaminergic signaling in the plasticity of behavioral and neuronal responses to sex pheromone in a moth. J. Exp. Biol. 222, http://doi: 10.1242/jeb.211979.

Gassias, E., Durand, N., Demondion, E., Bourgeois, T., Bozzolan, F., Debernard, S., 2018. The insect HR38 nuclear receptor, a member of the NR4A subfamily, is a synchronizer of reproductive activity in a moth. FEBS J. 285, 4019-4040. https://doi: 10.1111/febs.14648.

Gassias, E., Maria, A., Couzi, P., Demondion, E., Durand, N., Bozzolan, F., Aguilar, P., Debernard, S., 2021. Involvement of Methoprene-tolerant and Krüppel homolog 1 in juvenile hormone-signaling regulating the maturation of male accessory glands in the moth Agrotis ipsilon. Insect Biochem. Mol. Biol. 132, https://doi: 10.1016/j.ibmb.2021.103566.

Gemeno, C., 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.

Gujar, H., Palli, S.R., 2016. Juvenile hormone regulation of female reproduction in the common bed bug, Cimex lectularius. Sci. Rep. 6, 35546. https://doi: 10.1038/srep35546.

Henley, C.L., Nunez, A.A., Clemens, L.G., 2010. Exogenous androgen during development alters adult partner preference and mating behavior in gonadally intact male rats. Horm. Behav. 57, 488-495. https://doi: 10.1016/j.yhbeh.2010.02.007.

Hewes, R.S., 2008. The buzz on fly neuronal remodeling. Trends Endocrinol. Metab. 19, 317-323. https://doi: 10.1016/j.tem.2008.07.008.

Hiruma, K., Kaneko, Y., 2013. Hormonal regulation of insect metamorphosis with special reference to juvenile hormone biosynthesis. Curr. Top. Dev. Biol. 103, 73-100. https://doi: 10.1016/B978-0-12-385979-2.00003-4.

Holtof, M., Van Lommel, J., Gijbels, M., Dekempeneer, E., Nicolai, B., Vanden Broeck, J., Marchal, E., 2021. Crucial role of juvenile hormone receptor components Methoprene-Tolerant and Taiman in sexual maturation of adult male desert locusts. Biomolecules. 11, 244. https://doi: 10.3390/biom11020244.

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. https://doi: 10.1016/s0166-4328(99)00086-8.

Hull, E.M., Muschamp, J.W., Sato, S., 2004. Dopamine and serotonin: influences on male sexual behavior. Physiol. Behav. 83, 291-307. https://doi: 10.1016/j.physbeh.2004.08.018.

Jindra, M., Bellés, X., Shinoda, T., 2015b. Molecular basis of juvenile hormone signaling. Curr. Opin. Insect Sci. 11, 39-46. https://doi: 10.1016/j.cois.2015.08.004.

Jindra, M., Palli, S.R., Riddiford, L.M., 2013. The juvenile hormone signaling pathway in insect development. Annu. Rev. Entomol. 58, 181-204. https://doi: 10.1146/annurev-ento-120811-153700.

Jindra, M., Uhlirova, M., Charles, J.P., Smykal, V., Hill, R.J., 2015a. Genetic evidence for function of the bHLH-PAS protein Gce/Met as a juvenile hormone receptor. PLoS Genet. 11, e1005394. https://doi: 10.1371/journal.pgen.1005394.

Kayukawa, T., Minakuchi, C., Namiki, T., Togawa, T., Yoshiyama, M., Kamimura, M., Mita, K., Imanishi, S., Kiuchi, M., Ishikawa, Y., Shinoda, T., 2012. Transcriptional regulation of juvenile hormone-mediated induction of Krüppel homolog 1, a repressor of insect metamorphosis. Proc. Natl. Acad. Sci. U S A. 109, 11729-11734. https://doi: 10.1073/pnas.1204951109.

Kayukawa, T., Shinoda, T., 2015. Functional characterization of two paralogous JH receptors, methoprene tolerant 1 and 2, in the silkworm, Bombyx mori (Lepidoptera: Bombycidae). Jpn. Soc. Appl. Entomol. Zool. 50, 383-391. https:// doi: 10.1007/s13355-015-0345-8.

Laub, F., Lei, L., Sumiyoshi, H., Kajimura, D., Dragomir, C., Smaldone, S., Puche, A.C., Petros, T.J., Mason, C., Parada, L.F., Ramirez, F., 2005. Transcription factor KLF7 is important for neuronal morphogenesis in selected regions of the nervous system. Mol. Cell. Biol. 25, 5699-5711. https://doi: 10.1128/MCB.25.13.5699-5711.

Lenz, K.M., Nugent, B.M., McCarthy, M.M., 2012. Sexual differentiation of the rodent brain: dogma and beyond. Front. Neurosci. 21, https://doi.org/10.3389/fnins.2012.00026.

Li, M., Liu, P., Wiley, J.D., Ojani, R., Bevan, D.R., Li, J., Zhu, J., 2014. A steroid receptor coactivator acts as the DNA-binding partner of the methoprene-tolerant protein in regulating juvenile hormone response genes. Mol. Cell. Endocrinol. 394, 47-58. https://doi: 10.1016/j.mce.2014.06.021.

Li, M., Mead, E.A., Zhu, J., 2011. Heterodimer of two bHLH-PAS proteins mediates juvenile hormone-induced gene expression. Proc. Natl. Acad. Sci. U S A. 108, 638-43. https://doi: 10.1073/pnas.1013914108.

Lin, H.H., Cao, D.S., Sethi, S., Zeng, Z., Chin, J.S.R., Chakraborty, T.S., Shepherd, A.K., Nguyen, C.A., Yew, J.Y., Su, C.Y., Wang, J.W., 2016. Hormonal modulation of pheromone detection enhances male courtship success. Neuron. 90, 1272-1285. https://doi: 10.1016/j.neuron.2016.05.004.

Lin, X., Yao, Y., Wang, B., 2015. Methoprene-tolerant (Met) and Krüppel-homologue 1 (Kr-h1) are required for ovariole development and egg maturation in the brown plant hopper. Sci. Rep. 5, 18064. https://doi: 10.1038/srep18064.

Menzel, R., Manz, G., 2005. Neural plasticity of mushroom body-extrinsic neurons in the honeybee brain. J. Exp. Biol. 208, 4317-4332. https ://doi: 10.1242/jeb.01908.

Miao, L., Zhang, N., Jiang, H., Dong, F., Yang, X., Xu, X., Qian, K., Meng, X., Wang, J., 2020. Involvement of two paralogous Methoprene-Tolerant genes in the regulation of vitellogenin and vitellogenin receptor expression in the rice stem borer, Chilo suppressalis. Front. Genet .10, https://doi: 10.3389/fgene.2020.00609.

Moffatt, C.A., 2003. Steroid hormone modulation of olfactory processing in the context of socio-sexual behaviors in rodents and humans. Brain Res. Rev. 43, 192-206. https:// doi: 10.1016/s0165-0173(03)00208.

Okada, R., Rybak, J., Manz, G., Menzel, R., 2007. Learning-related plasticity in PE1 and other mushroom body-extrinsic neurons in the honeybee brain. J. Neurosci. 27, 11736-11747. https://doi: 10.1523/JNEUROSCI.2216-07.

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., 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., Bues, R., 1974. Elevage de plusieurs espèces de lépidoptères sur milieu artificiel simplifié. Ann. Zool. Ecol. Anim. 2, 79-91.

Reinhard, J., Claudianos, C., 2012. Molecular Insights into honey bee brain plasticity, in: Galizia, C.G., Eisenhardt, D., Giurfa, M., (Eds), Honeybee neurobiology and behavior, Springer, Chap. 5.3, pp. 359-372, https://doi:10.1007/978-94-007-2099-227.

Roff, D.A., 1993.The evolution of life histories: theory and analysis, Springer, New York.

Stearns, S.C., 1992. The evolution of life histories, Oxford University Press.

Romeo, R.D., 2003. Puberty: a period of both organizational and activational effects of steroid hormones on neurobehavioural development. J. Neuroendocrinol. 15, 1185-1192. https://doi: 10.1111/j.1365-2826.2003.01106.

Sasaki, K., Akasaka, S., Mezawa, R., Shimada, K., 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.

Shpigler, H., Patch, H.M., Cohen, M., Fan, Y., Grozinger, C.M., Bloch, G., 2010. The transcription factor Krüppel homolog 1 is linked to hormone mediated social organization in bees, BMC Evol. Biol. 10, 120. https://doi: 10.1186/1471-2148-10-120.

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

Smykal, V., Bajgar, A., Provaznik, J., Fexova, S., Buricova, M., Takaki, K., Hodkova, M., Jindra, M., Dolezel, D., 2014. Juvenile hormone signaling during reproduction and development of the linden bug, Pyrrhocoris apterus. Insect Biochem. Mol. Biol. 45, 69-76. https://doi: 10.1016/j.ibmb.2013.12.003.

Sokal, R.R. and Rohlf, F.J., 1995. Biometry: The principles and practice of statistics in biological research, third ed. Freeman, W.H., New York, pp. 315.

Song, J., Wu, Z., Wang, Z., Deng, S., Zhou, S., 2014. Krüppel homolog 1 mediates juvenile hormone action to promote vitellogenesis and oocyte maturation in the migratory locust. Insect Biochem. Mol. Biol. 52, 94-101. https://doi: 10.1016/j.ibmb.2014.07.001.

Teal, P.E., Gomez-Simuta, Y., Proveaux, A.T., 2000. Mating experience and juvenile hormone enhance sexual signaling and mating in male Caribbean fruit flies. Proc. Natl. Acad. Sci. U S A. 97, 3708-3712. https://doi: 10.1073/pnas.97.7.3708.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3, research0034.0031.

Vitecek, S., Maria, A., Blais, C., Duportets, L., Gaertner, C., Dufour, M.C., Siaussat, D., Debernard, S., Gadenne, C., 2013. Is the rapid post-mating inhibition of pheromone response triggered by ecdysteroids or other factors from the sex accessory glands in the male moth Agrotis ipsilon? Horm. Behav. 63, https://doi: 10.1016/j.yhbeh.2013.03.010.

Yoshikage, M., Toshiaki, I., Seiichi, K., Nobuo, K., Nishimura, M., 2007. Sex steroids modulate the signals from volatile female odors in the accessory olfactory bulb of male mice. Neurosci. Lett. 413, 5-11. https://doi: 10.1016/j.neulet.2006.11.025.

Yue, Y., Yang, R.L., Wang, W.P., Zhou, Q.H., Chen, E.H., Yuan, G.R., Wang, J.J., Dou, W., 2018. Involvement of Met and Kr-h1 in JH-Mediated reproduction of female Bactrocera dorsalis (Hendel). Front. Physiol. 9, 482. https://doi: 10.3389/fphys.2018.00482.

Zhan, S., Merlin, C., Boore, J. L., Reppert, S. M., 2011. The monarch butterfly genome yields insights into long-distance migration. Cell 147, 1171-1185. https://doi: 10.1016/j.cell.2011.09.052.

Zhang, Z., Xu, J., Sheng, Z., Sui, Y., Palli, S.R., 2011. Steroid receptor co-activator is required for juvenile hormone signal transduction through a bHLH-PAS transcription factor, methoprene tolerant. J. Biol. Chem. 286, 8437-8447. https://doi: 10.1074/jbc.M110.191684.

Figures

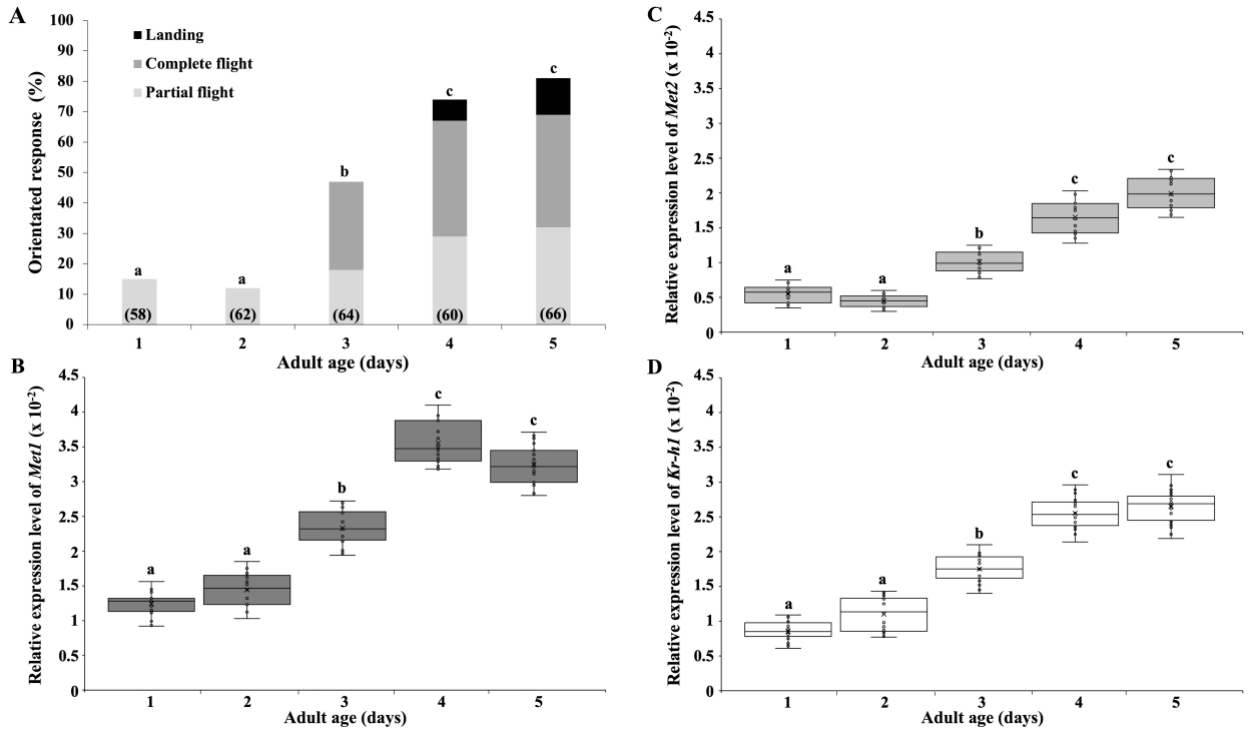
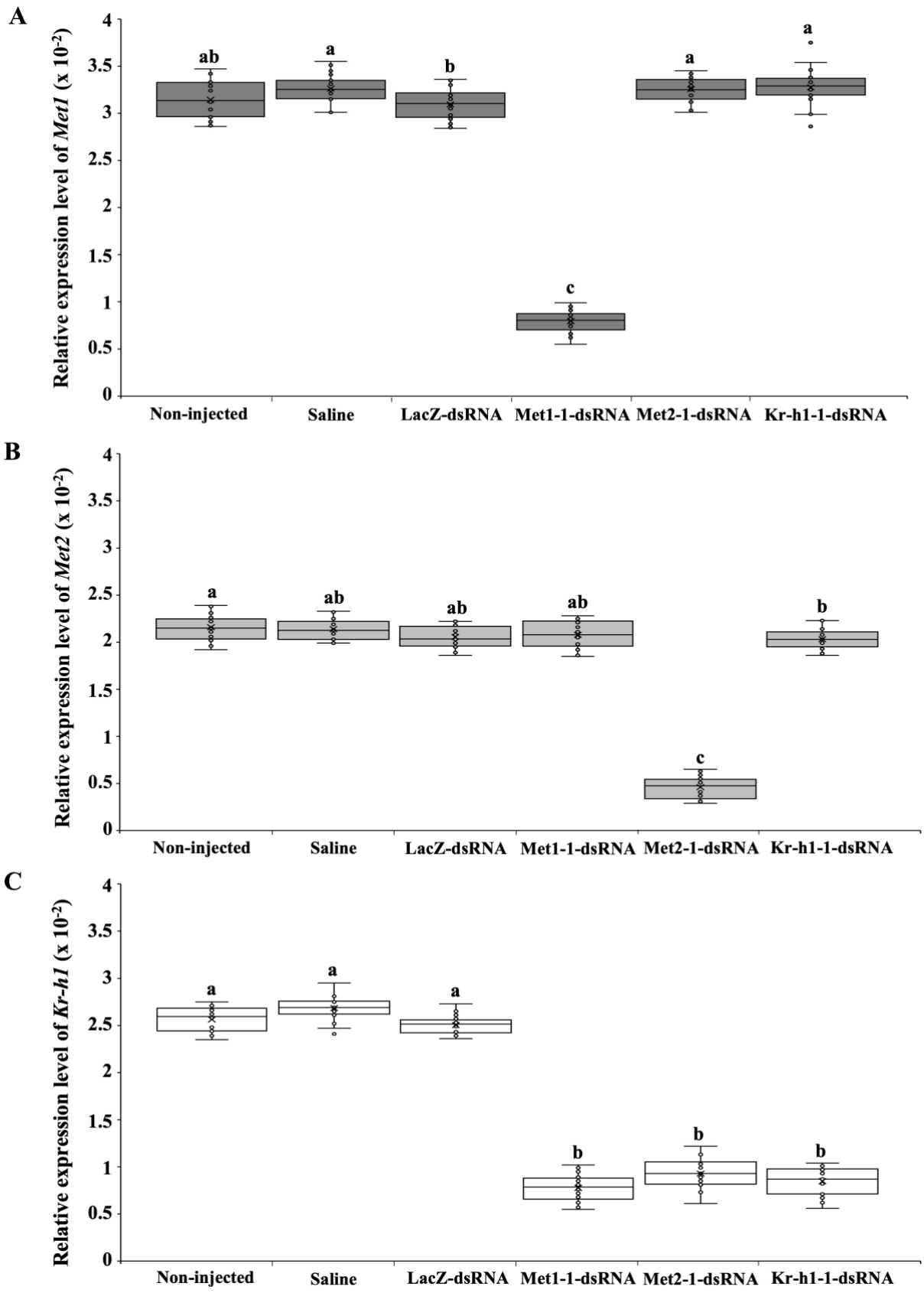
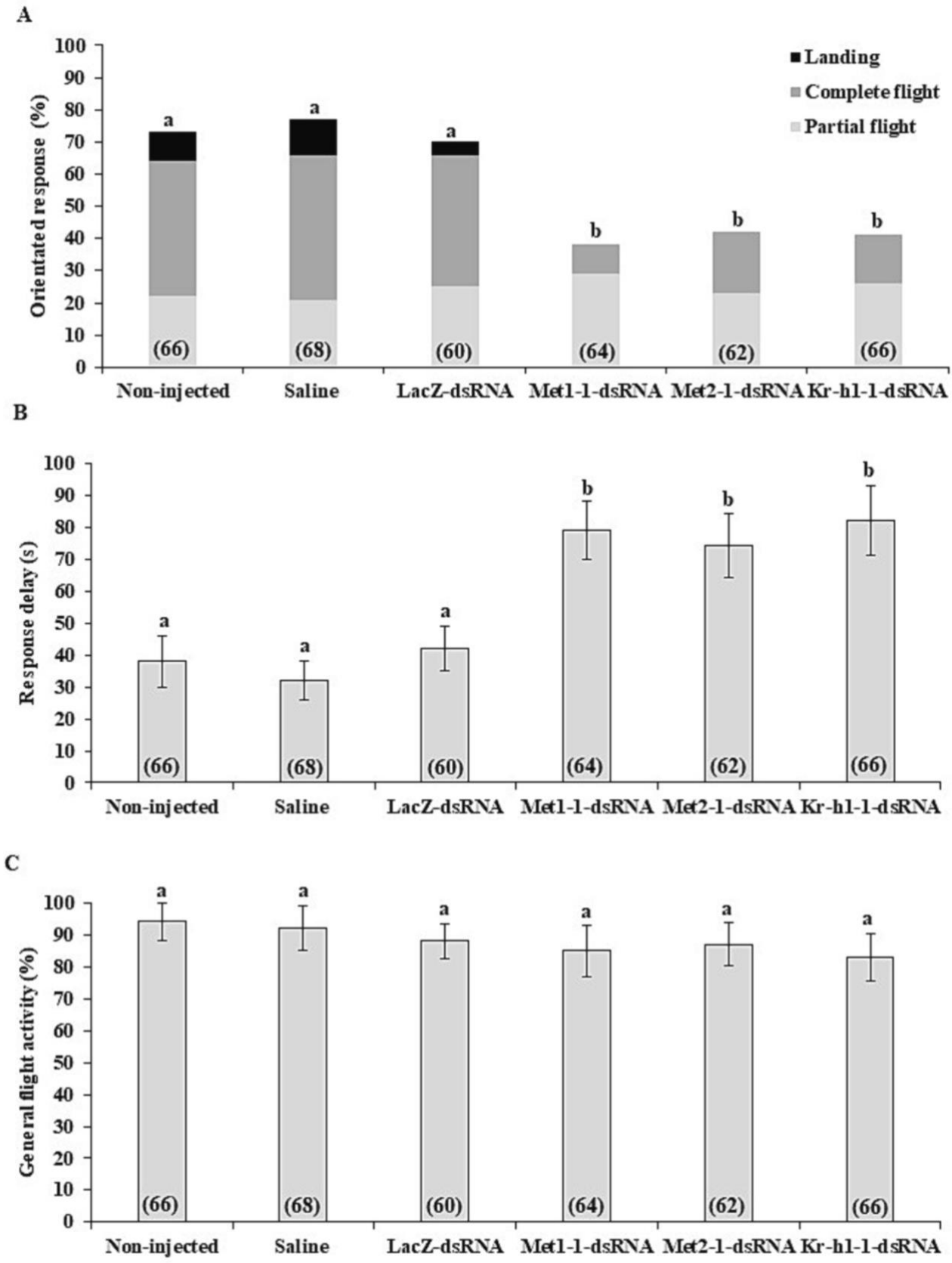


Figure 1. Effect of age on the behavioral response to sex pheromone and the level of AL *Met1*, *Met2* and *Kr-h1* expressions in *A. ipsilon* males. The percentage of sex pheromone-guided orientation flight in the wind tunnel experiments (**A**) and the amounts of *Met1* (**B**), *Met2* (**C**) and *Kr-h1* (**D**) mRNA in ALs were evaluated for 1- to 5-day-old males. Each qRT-PCR was run in three technical replicates and eight independent biological replicates. For the behavioral tests, numbers within bars indicate the number of tested males; the different letters indicate a statistically significant difference (G-test; $P < 0.05$). For the AL *Met1*, *Met2* and *Kr-h1* mRNA levels, the bars represent the value distribution and the different letters indicate a statistically significant difference (Dunn's test; $P < 0.05$).


A
Relative expression level of Met1 (x 10-2)
4
3.5
3
2.5
2
1.5
1
0.5
0
ab
a
b
c
a
a
Non-injected
Saline
LacZ-dsRNA
Met1-1-dsRNA
Met2-1-dsRNA
Kr-h1-1-dsRNA
B
Relative expression level of Met2 (x 10-2)
a
ab
ab
ab
c
b
Non-injected
Saline
LacZ-dsRNA
Met1-1-dsRNA
Met2-1-dsRNA
Kr-h1-1-dsRNA
C
Relative expression level of Kr-h1 (x 10-2)
a
a
a
b
b
b
Non-injected
Saline
LacZ-dsRNA
Met1-1-dsRNA
Met2-1-dsRNA
Kr-h1-1-dsRNA

731

Figure 2. Interactions between *Met1*, *Met2* and *Kr-h1* in ALs. One-day-old males received an injection of saline solution, bacterial LacZ-dsRNA, Met1-1-dsRNA, Met2-1-dsRNA or Kr-h1-1-dsRNA, or no injection (non-injected). For each treatment and four days after injection, the relative expressions of *Met1* (**A**), *Met2* (**B**) and *Kr-h1* (**C**) were quantified in ALs. Each qRT-PCR was run in three technical replicates and eight independent biological replicates. The bars represent the value distribution and the different letters indicate a statistically significant difference (Dunn's test; $P < 0.05$).


A
Orientated response (%)
Landing
Complete flight
Partial flight
a
a
a
b
b
b
(66)
(68)
(60)
(64)
(62)
(66)
Non-injected
Saline
LacZ-dsRNA
Met1-1-dsRNA
Met2-1-dsRNA
Kr-h1-1-dsRNA
B
Response delay (s)
a
a
a
b
b
b
(66)
(68)
(60)
(64)
(62)
(66)
Non-injected
Saline
LacZ-dsRNA
Met1-1-dsRNA
Met2-1-dsRNA
Kr-h1-1-dsRNA
C
General flight activity (%)
a
a
a
a
a
a
(66)
(68)
(60)
(64)
(62)
(66)
Non-injected
Saline
LacZ-dsRNA
Met1-1-dsRNA
Met2-1-dsRNA
Kr-h1-1-dsRNA

739

Figure 3. Effect of *Met1*, *Met2* and *Kr-h1* silencing on the behavioral response of 5-day-old sexually mature males to sex pheromone. One-day-old males received an injection of saline solution, bacterial LacZ-dsRNA, Met1-1-dsRNA, Met2-1-dsRNA or Kr-h1-1-dsRNA, or no injection (non-injected). For each treatment and four days after injection, the percentage of sex pheromone-triggered orientation flight (**A**), the response delay (**B**) and the general flight activity (**C**) were determined in the wind tunnel experiments. Numbers within bars indicate the number of tested males; the different letters indicate a statistically significant difference (G-test; $P < 0.05$).

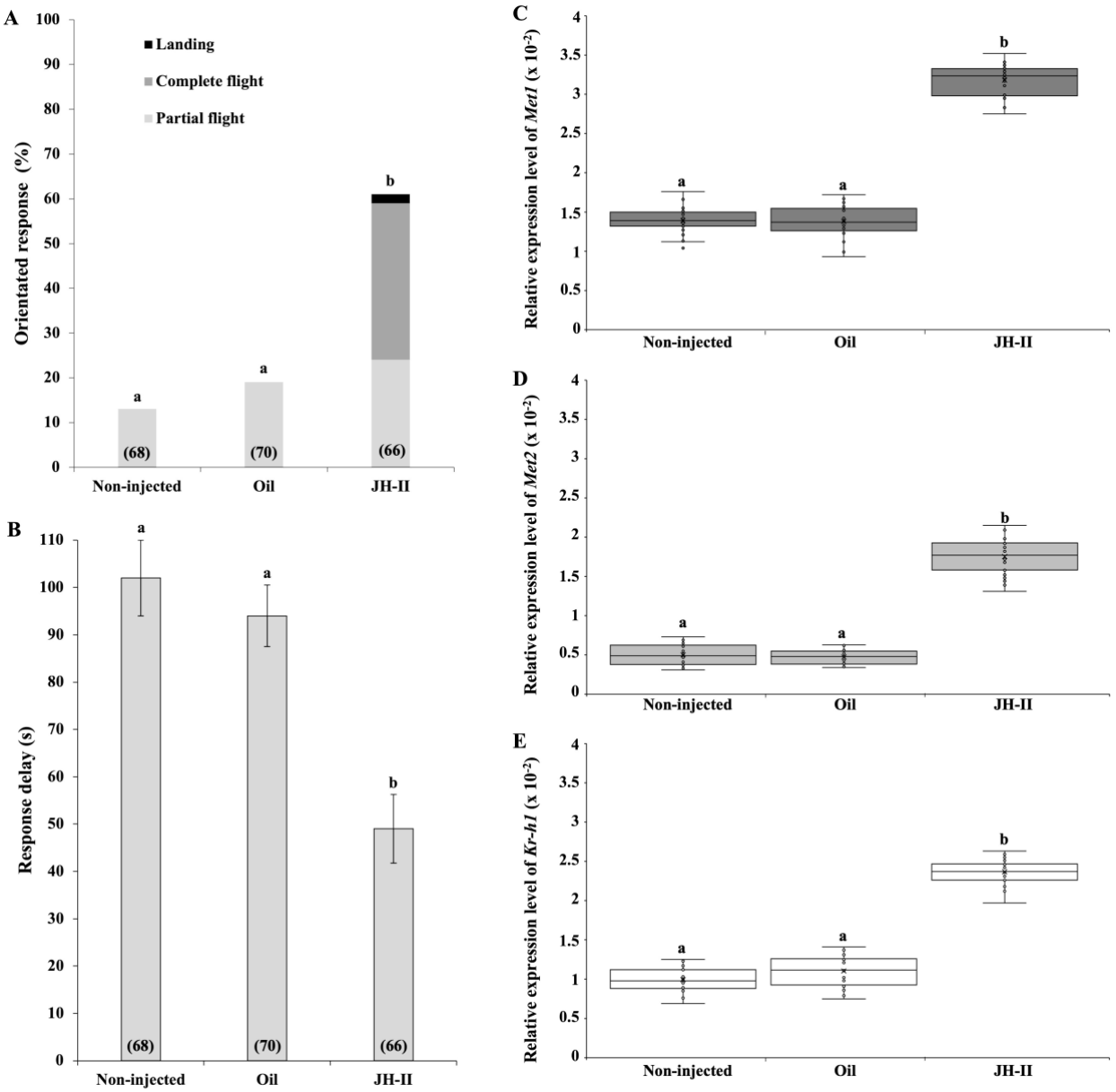


Figure 4. Effect of JH-II injection on the behavioral response to sex pheromone and the AL *Met1*, *Met2* and *Kr-h1* expressions in 2-day-old sexually immature males. Newly emerged males (day-0) received an injection of JH-II, or mineral oil or no injection (non-injected) and the percentage of sex pheromone-mediated orientation flight (**A**), the response delay (**B**) and the relative expressions of *Met1* (**C**), *Met2* (**D**) and *Kr-h1* (**E**) in ALs were determined two days after injection. Each qRT-PCR was run in three technical replicates and eight independent biological replicates. For the behavioral tests, numbers within bars indicate the number of tested males; the different letters indicate a statistically significant difference (G-test; $P < 0.05$). For the AL *Met1*, *Met2* and *Kr-h1* mRNA levels, the bars represent the value distribution and the different letters indicate a

statistically significant difference (Tukey's tests for *Met1* and *Kr-h1* relative expression levels, Tamhane's test for *Met2* relative expression level; $P < 0.05$).

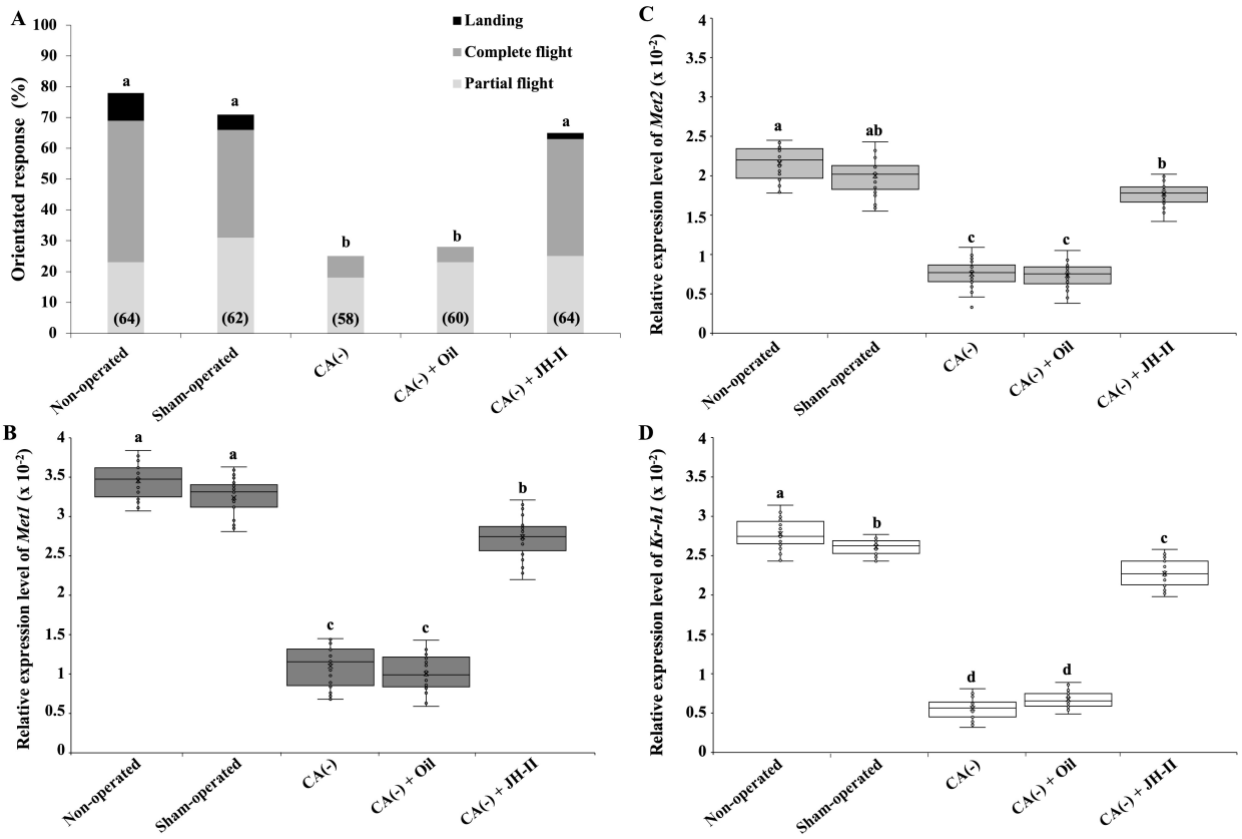


Figure 5. Effect of allatectomy and JH-II injection on the behavioral response of 5-day-old sexually mature males to sex pheromone. Allatectomy (CA-) was performed on the day of emergence (day-0) and injections was carried out on the next day (day-1) and the percentage of sex pheromone-guided orientation flight (**A**) and the relative expressions of *Met1* (**B**), *Met2* (**C**) and *Kr-h1* (**D**) in ALs were determined at day-5. Controls groups were composed of oil-injected, non-operated and sham-operated males. Each qRT-PCR was run in three technical replicates and eight independent biological replicates. For the behavioral tests, numbers within bars indicate the number of tested males; the different letters indicate a statistically significant difference (G-test; $P < 0.05$). For the AL *Met1*, *Met2* and *Kr-h1* mRNA levels, the bars represent the value distribution and the different letters indicate a statistically significant difference (Dunn's test for *Met1* and *Met2* relative expression levels, Tamhane's test for *Kr-h1* relative expression level; $P < 0.05$).

Supplementary Table

Primer name	Sequence
qMet1dir	5’-TCGCAACGCAAAGTTGATAAG-3’
qMet1rev	5’ -TAGATCTATATGACAGTATCC-3’
qMet2dir	5’-TCTGCAGCACCCGCCATGTCGCTG-3’
qMet2rev	5’-AGGACTAAACTGCTGCGGTGATG-3’
qKr-h1dir	5’-ACTAAAGAACGGCCGTACCGA-3’
qKr-h1rev	5’-CCCTTTTCCACAGCCGGGCGC-3’
RpL8dir	5’-CCAGTTTGTCTACTGCGGCAA-3’
RpL8rev	5’-GCTTAACCCTAGTACGCTTGGCA-3’
LacZ T7dir	5’-taatacgactcactatagggATGACCATGATTACGCCAAGC-3’
LacZ T7rev	5’-taatacgactcactatagggCCATTCGCCATTCAGGCTGCG-3’
Met1-1-T7dir	5’-taatacgactcactatagggAGTGCGCATCTGGGAATGCACTCT-3’
Met1-1-T7rev	5’-taatacgactcactatagggCTTTTCTTGAACGTTGGGTGATAC-3’
Met2-1-T7dir	5’-taatacgactcactatagggGACTCATCACCGCAGCAGTTTAGTCC-3’
Met2-1-T7rev	5’-taatacgactcactatagggGCATGGATTCTCGGAGCGGGCGCGCG-3’
Kr-h1-1-T7dir	5’-taatacgactcactatagggATAGGTGAGGAGGAACGAGCTCAT-3’
Kr-h1-1-T7rev	5’-taatacgactcactatagggCAGTTGTTTCGAGCAGGTGAACCC-3’
Met1-2-T7dir	5’-taatacgactcactatagggGAAGTAACTGGGACGTCAGCCTTT-3
Met1-2-T7rev	5’-taatacgactcactatagggATCACAGTCCACCTTTCTCCTCTT-3’
Met2-2-T7dir	5’-taatacgactcactatagggTCCTGTGGAAACTGTCGACATGAAC-3’
Met2-2-T7rev	5’-taatacgactcactatagggCATTTCGAAATCTTCGAACTCGCTTG-3’
Kr-h1-2-T7dir	5’-taatacgactcactatagggTGTGACATATGTCTCCGAGATTTC-3’
Kr-h1-2-T7rev	5’-taatacgactcactatagggGGCAGCTGGGGCAAATTGTATCAC-3’

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

Supplementary Figure

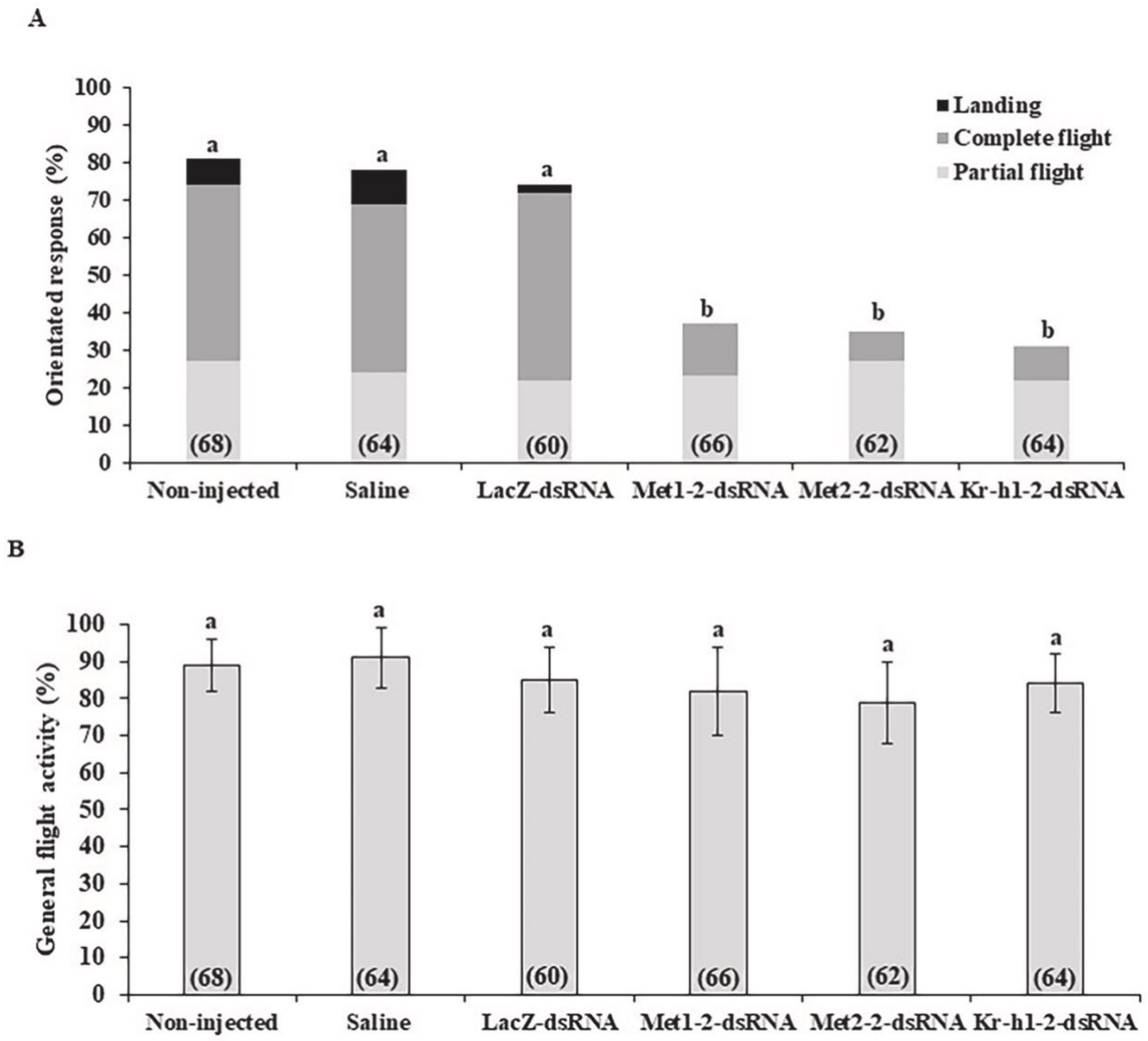


Figure S1. Effect of Met1-2-dsRNA, Met2-2-dsRNA and Kr-h1-2-dsRNA on the behavioral response to sex pheromone in 5-day-old sexually mature males. One-day-old males received an injection of saline solution, bacterial LacZ-dsRNA, Met1-2-dsRNA, Met2-2-dsRNA or Kr-h1-2-dsRNA, or no injection (non-injected) and the percentage of orientation flight towards to sex pheromone (**A**) and of general flight activity (**B**) were determined four days after injection. Numbers within bars indicate the number of tested males; the different letters indicate a statistically significant difference (G-test; $P < 0.05$).