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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

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

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

## 33 **1. Introduction**

34 In animals, sexual maturation is a period of life during which occur the ontogenesis of mating  
35 behavior and the initiation of sexual signaling as well as reproductive maturation. The orchestration  
36 of these behavioral and physiological changes needs to be tightly synchronized for reproductive  
37 success (Roff, 1993; Stearns, 1992). For instance, animals acquire the ability to deliver and respond  
38 to sexual chemosignals only when they have a high degree of fertility (Dey *et al.*, 2015; Gadenne  
39 *et al.*, 2016; Lin *et al.*, 2016). It is well established that this reproductive synchrony is governed by  
40 the actions of endogenous signals, and especially hormonal factors, on both the genital apparatus  
41 and the nervous system (Bozzolan *et al.*, 2015; Lin *et al.*, 2016; Gassias *et al.*, 2018; Romeo, 2003).  
42 Thus, evidence has accumulated over the last decades that the development of sexual behavior  
43 relies, at least in part, on the remodeling of neuronal networks through organizational and  
44 activational effects of hormones (Lenz *et al.*, 2012; Abrieux *et al.*, 2014; Lin *et al.*, 2016; Romeo,  
45 2003). Elucidation of the neuronal and molecular basis of this endocrine regulation is important for  
46 a better understanding of mechanisms by which adults acquire the behavioral responsiveness to  
47 sexual cues.

48  
49 In almost all vertebrate species, gonadal steroids are known to govern the display of different  
50 components of reproductive behavior by fine-tuning the sensory responsiveness to sexually  
51 relevant signals. For instance, in socially monogamous birds, androgens play a key role in the  
52 maturation of sexual preference and are also required for the performance of courtship and  
53 copulatory behaviors by interfering with the olfactory, auditory and visual pathways when juvenile  
54 males reach their sexual maturity (Ball & Balthazart, 2004; Adkins-Regan & Leung, 2006). In  
55 cyprinid fishes, methyltestosterone and ketotestosterone have the ability to elicit courtship behavior  
56 in juvenile males by increasing their olfactory sensitivity to female-emitted pheromonal  
57 prostaglandins (Belanger *et al.*, 2010). In addition, there is a growing body of evidence that in male  
58 rodents, sexual motivation, female's partner preference and coital response are controlled by  
59 modulatory actions of testosterone and estradiol on the central processing of female odors within  
60 the olfactory bulb (Bakker *et al.*, 2002; Moffatt, 2003; Balthazart *et al.*, 2004; Yoshikage *et al.*,  
61 2007; Henley *et al.*, 2010).

62

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

80  
81 It is well documented that most biological actions of JH are mediated by a nuclear protein  
82 complex composed of two members of the basic helix-loop-helix (bHLH) / Per-ARNT-Sim (PAS)  
83 family of transcription factors: the JH receptor Methopren-tolerant (Met) (Jindra *et al.*, 2015a) and  
84 its dimerizing partner, initially termed Taiman (Tai) in *D. melanogaster*, and also Ftz-F1-  
85 interacting steroid receptor coactivator in *A. aegypti* or steroid receptor activator in other insects  
86 (Charles *et al.*, 2011; Jindra *et al.*, 2015b; Li *et al.*, 2011, Li *et al.*, 2014; Zhang *et al.*, 2011). Two  
87 paralogous *Met* genes, *Met* and *Germ cell-expressed*, have been isolated from different *Drosophila*  
88 species and two *Met* genes, *Met1* and *Met2*, were also recently characterized in several lepidopteran  
89 species including *A. ipsilon* (Gassias *et al.*, 2021), *Operophtera brumata* (Derks *et al.*, 2015),  
90 *Bombyx mori* (Daimon *et al.*, 2015; Kayukawa & Shinoda, 2015), *Chilo suppressalis* (Miao *et al.*,  
91 2020) and *Danaus plexippus* (Zhan *et al.*, 2011). In presence of JH, the Met-Tai heterodimeric  
92 complex binds to an E-box like response element in the promoter regions of JH target genes,  
93 resulting in their transcriptional regulation (Li *et al.*, 2011, Li *et al.*, 2014; Zhang *et al.*, 2011).

94 *Krüppel homolog 1 (Kr-h1)*, which encodes a C2H2 zinc-finger type transcription factor, has been  
95 identified as an early JH-response gene that works downstream of the Met-Tai complex (Cui *et al.*,  
96 2014; Kayukawa *et al.*, 2012). In various insect species, gene knockdown experiments revealed  
97 that Met and Tai are required to mediate the stimulatory actions of JH in the maturation of the  
98 female and male reproductive systems including oogenesis, vitellogenesis, testicular development  
99 and ASG differentiation and that JH can operate either without or with the requirement of Kr-h1  
100 (Song *et al.*, 2014; Smykal *et al.*, 2014; Lin *et al.*, 2015; Gujar & Palli, 2016; Yue *et al.*, 2018;  
101 Gassias *et al.*, 2021; Holtof *et al.*, 2021). By contrast, a limited number of studies have been  
102 undertaken to identify the JH-initiated molecular pathways controlling the occurrence of  
103 reproductive behavior. For example, it has been uncovered in *D. melanogaster* that Met is involved  
104 in transmitting JH signal that promotes courtship behavior in old sexually mature males through an  
105 elevation of the sensitivity of olfactory receptor neurons (ORNs) to aphrodisiac pheromone (Lin *et al.*,  
106 2016). More recently, Met and Tai were found to be actors of JH signaling pathway in the onset  
107 of male copulation behavior in the desert locust, *Shistocerca gregaria* (Holtof *et al.*, 2021).

108  
109 In order to further elucidate the molecular mechanisms by which JH regulates the neuronal  
110 circuits that underlie mating activity, we turned to the long-lived noctuid moth *A. ipsilon* for which  
111 JH was found to regulate the central processing of pheromone information related to the maturation  
112 of male sexual behavior (Anton & Gadenne, 1999; Gadenne & Anton, 2000). In this species, newly  
113 emerged males are sexually immature and do not behaviorally respond to the female-produced sex  
114 pheromone (Duportets *et al.*, 1998; Gadenne *et al.*, 1993). Three to four days after emergence,  
115 males become sexually mature and are highly attracted to the female sex pheromone during the  
116 middle of the scotophase and elicit an orientation flight towards their mating partners (Duportets  
117 *et al.*, 1998; Gadenne *et al.*, 1993; Gassias *et al.*, 2021). This increase in behavioral responsiveness  
118 is paralleled with a volumetric growth of glomeruli belonging into the macroglomerular complex  
119 (MCG), which is devoted to the processing of sex pheromone input from ORNs housed in the  
120 trichoid sensilla of the antennae, and a higher sensitivity of neurons responding to pheromone  
121 signal within the primary olfactory centers, the antennal lobes (ALs) (Anton & Gadenne, 1999;  
122 Gadenne & Anton, 2000). It has also been shown in old sexually mature males, where the  
123 circulating level of JH is high that the removal of the corpora allata resulted in decreased behavioral  
124 response and AL neuron sensitivity to pheromone information and which were subsequently

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

129  
130 Here, we tested the hypothesis that *Met* and *Kr-h1* are mediators of the central action of JH  
131 in the enhancement of sex pheromone-guided orientation flight of *A. ipsilon* male. We first  
132 evaluated the influence of age on the expression level of *Met1*, *Met2* and *Kr-h1* within ALs in  
133 relation with sex pheromone responsiveness. We then examined the impact of RNAi-induced  
134 knockdown of *Met1*, *Met2* or *Kr-h1* on the behavioral response to sex pheromone. Finally, we  
135 manipulated the circulating JH titer either by injection of exogenous JH or allatectomy and  
136 analyzed the effect of treatment on *Met1*, *Met2*, *Kr-h1* mRNA levels in ALs and the behavioral  
137 response to sex pheromone. Taken together, our results highlighted that *Met1*, *Met2* and *Kr-h1* are  
138 components of JH signaling pathways which function within ALs to facilitate the pheromone  
139 information processing and consequently the elicitation of sexual attraction behavior of male in *A.*  
140 *ippsilon*.

## 141 2. Materials and methods

### 142 2.1. Chemicals

143 We prepared a synthetic sex pheromone blend at 1 ng/μl containing three components  
144 identified in natural extracts of the pheromone glands of the female *A. ipsilon* (Picimbon *et al.*,  
145 1997; Gemeno & Haynes, 1998): (Z)-7-dodecen-1-yl acetate (Z7-12:OAc), (Z)-9-tetradecen-1-yl  
146 acetate (Z9-14:OAc) and (Z)-11-hexadecen-1-yl acetate (Z11-16:OAc) mixed at a ratio of 4:1:4.  
147 This blend was proven to be the most attractive to males in field trapping experiments and it elicits  
148 a similar behavior in a wind tunnel as natural extracts of the pheromone gland (Barrozo *et al.*, 2010;  
149 Vitecek *et al.*, 2013). The three components were purchased from Sigma Aldrich (Saint-Quentin  
150 Fallavier, France) and diluted in hexane (>98 % purity, Carlo-Erba, Val-de-Reuil, France). A dose  
151 of 10 ng of the sex pheromone blend was used in the experiments and this dose had previously  
152 been described as behaviorally and electrophysiologically active (Gadenne *et al.*, 2001; Deisig *et*  
153 *al.*, 2012; Gassias *et al.*, 2018).

### 154 2.2. Insects and tissue collection

155 Adult *A. ipsilon* originated from a laboratory colony established in Bordeaux and  
156 transferred to Versailles, France. The colony is based on field catches in southern France and wild  
157 insects are introduced each spring. Insects were reared on an artificial diet in individual cups until  
158 pupation (Poitout & Bues, 1974). Pupae were sexed, and males and females were kept separately  
159 on an inversed light/dark cycle (16 h light:8 h dark photoperiod, with the scotophase starting at  
160 10:00 am) at 22 °C. Newly emerged adults were removed from the hatching containers every day  
161 and were given access to a 20 % sucrose solution *ad libitum*. The day of emergence was considered  
162 as day-0. The males were anaesthetized with an exposure to carbon dioxide and tissue dissections  
163 were performed in the middle of the scotophase (between 1:00 pm and 4:00 pm) when males  
164 respond maximally to the sex pheromone (Barrozo *et al.*, 2010). For the expression profiles of  
165 *Met1*, *Met2* and *Kr-h1*, ALs were dissected in Ringer's solution and immediately flash-frozen in  
166 Eppendorf (Montesson, France) vials kept in liquid nitrogen and stored at -80 °C until treatment.  
167 The dissected males were then euthanized by using liquid nitrogen.

### 168 2.3. Allatectomy

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

#### 173 2.4. Juvenile hormone injection

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

#### 179 2.5. cDNA synthesis

180 Total RNA was extracted from ALs with TRIzol reagent (TRI Reagent®, Euromedex,  
181 Souffelweyersheim, France) according to the manufacturer's instructions, and were quantified by  
182 spectrophotometry at 260 nm. DNase treatment was performed with 2 U of TURBO™ DNase 1  
183 (Ambion, Villebon-sur-Yvette, France) for 30 min at 37 °C followed by a 10-min inactivation at  
184 75 °C. After DNase treatment, single-stranded cDNA was synthesized from total RNAs (1 µg) with  
185 SuperScript II Reverse Transcriptase (RT) (Invitrogen, Carlsbad, CA, USA) according to the  
186 manufacturer's instructions. The reaction contained a deoxynucleotide triphosphate (dNTP) mix,  
187 RNase OUT, Oligo (dT) primer and sterile water to a final reaction volume of 20 µl. The mix was  
188 heated to 65 °C for 5 min before the enzyme was added and then incubated for 1 h at 42 °C.

#### 189 2.6. Quantification of *Met1*, *Met2* and *Kr-h1* expressions in ALs

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

197 30 s and the length of amplified products was 234 bp, 258 bp and 213 bp for *Met1*, *Met2* and *Kr-*  
198 *h1*. Fluorescence measurements over a 55–95 °C melting curve confirmed the presence of a single  
199 specific peak and the absence of primer-dimer peaks for the used primer pairs. Each run included  
200 a negative control (water) and a fivefold dilution series of pooled cDNA (from all conditions). The  
201 fivefold dilution series (undiluted, 1/5, 1/25, 1/125 and 1/625) were used to construct a relative  
202 standard curve to determine the PCR efficiency and for further quantification analysis. In all  
203 experiments, the primers gave amplification efficiencies of 90–100 %. Each reaction was run in  
204 three technical replicates with eight independent biological replicates using the technical platform  
205 of The Integrative Biology Institute (Sorbonne University, France). Expression levels were  
206 analyzed with LightCycler 480 software (Roche Diagnostics GmbH, Mannheim, Germany) and the  
207 GENORM Visual Basic application for Microsoft EXCEL (Issy-les-Moulineaux, France) as  
208 described by [Vandesompele et al. \(2002\)](#). The cycle threshold (Ct) values were determined for the  
209 candidate gene (*Met1*, *Met2*, *Kr-h1*) and the reference gene, the *A. ipsilon Ribosomal protein L8*  
210 (*RpL8*) gene (accession number JX975720.1), which exhibited the most stable expression levels  
211 amongst other tested control genes (*Ribosomal protein L13*, *Glyceraldehyde-3-phosphate*  
212 *dehydrogenase* and  $\beta$ -*actin*). The average Ct value of each triplicate reaction was used to normalize  
213 the candidate gene expression level to the geometric mean of the *RpL8* level in the Q-GENE  
214 software ([Simon, 2003](#)) by using the formula:  $NE = (E_{RpL8})^{Ct_{RpL8}} / (E_{Met, Kr-h1})^{Ct_{Met, Kr-h1}}$  with  
215 NE meaning normalized expression, E meaning PCR efficiency and Ct corresponding to the  
216 crossing-point values. Then, mean and standard error were calculated with the NE values for the  
217 biological replicates. The sequences of the specific primers *RpL8dir* and *RpL8rev* for *RpL8* are  
218 given in **Table S1**.

## 219 2.7. RNA interference experiments

220 Double-stranded RNA (dsRNA) production and injection conditions were established by  
221 following the protocols used in *A. ipsilon* for the silencing of *Met1*, *Met2* and *Kr-h1* ([Gassias et al.,](#)  
222 [2021](#)). The dsRNAs targeting *Met1* (*Met1-1*-dsRNA, 486 bp; and *Met1-2*-dsRNA, 528 bp), *Met2*  
223 (*Met2-1*-dsRNA, 480 bp; and *Met2-2*-dsRNA, 478 bp), *Kr-h1* (*Kr-h1-1*-dsRNA, 466 bp; and *Kr-*  
224 *h1-2*-dsRNA, 472 bp), and bacterial  $\beta$ -*galactosidase* (*LacZ*) (372 bp; control) were chosen by using  
225 the E-RNAi web application (<http://e-rnai.dkfz.de/>) ([Arziman et al., 2005](#)) and produced with a  
226 MEGAscript® T7 High Yield Transcription Kit (Ambion, Villebon-sur-Yvette, France) according

227 to the manufacturer's instructions. A PCR was performed on 1 µl plasmid (50 ng/µl) with specific  
228 primers for each target gene (Met1-1-T7dir/Met1-1-T7rev, Met1-2-T7dir/Met1-2-T7rev, Met2-1-  
229 T7dir/Met2-1-T7rev, Met2-2-T7dir/Met2-2-T7rev, Kr-h1-1-T7dir/Kr-h1-1-T7rev, Kr-h1-2-  
230 T7dir/Kr-h1-2-T7rev and LacZ-T7dir/LacZ-T7rev) (**Table S1**) under the following program: 35  
231 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min. PCR products were purified with a  
232 Nucleospin® extract II kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) and quantified  
233 by spectrometry. The transcription reaction was then performed overnight at 37 °C on 1 µg of PCR  
234 product in a reaction mix containing 2 µl of each nucleotide, 2 µl of T7 RNA polymerase enzyme  
235 and 2 µl of T7 10× Buffer in a final volume of 20 µl. After spectrometry quantification, the reaction  
236 mix was incubated for 15 min at 37 °C with 2 U of Turbo DNase (Ambion, Villebon-sur-Yvette,  
237 France). The dsRNA precipitation was performed by addition of 30 µl of water treated with  
238 diethylpyrocarbonate (DEPC) and 20 µl LiCl (Sigma Aldrich, Saint-Quentin Fallavier, France),  
239 storage of the mixture at -20 °C for 2 h, and then centrifugation for 30 min at 16000 g. The pellet  
240 was washed with 1 ml 75 % ethanol, centrifuged and dried before elution in 11 µl DEPC water.  
241 Samples were denatured at 95 °C for 5 min followed by a rehybridization step of 1 h 30 min at  
242 room temperature. The dsRNA integrity was then checked by loading on an agarose gel. Before  
243 injection, dsRNA was diluted to 0.5 µg/µl in saline solution containing CaCl<sub>2</sub> 7.1 mM, Na<sub>2</sub>β-  
244 glycerophosphate 22 mM, MgSO<sub>4</sub> 13.5 mM, MgCl<sub>2</sub> 26.9 mM, KCl 29.5 mM, glucose 23.9 mM,  
245 pH 6.8 (Sigma Aldrich, Saint-Quentin Fallavier, France).

246

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

## 253 2.8. Behavioral experiments to sex pheromone in a wind tunnel

254 Behavioral tests were performed using a Plexiglass wind tunnel measuring 190 cm length  
255 × 75 cm width × 75 cm height (VT Plastics, Genevilliers, France). Both ends of the tunnel were  
256 enclosed with white synthetic fabric allowing air to pass through but preventing insect escape. Air

257 movement was provided by an exhaust fan at the downwind end of the tunnel sucking the air at a  
258 speed of 0.3 m/s and evacuating odorized air out of the building. The tunnel was maintained in  
259 dark except for one red visible light source allowing visual observation. Experiments were  
260 performed at 23 °C,  $40 \pm 10$  % relative humidity, during the middle of the scotophase (between  
261 1:00 pm and 4:00 pm) when males respond maximally to the sex pheromone with a characteristic  
262 anemotactic zigzagging flight (Barrozo *et al.*, 2010). A single experimental male was introduced  
263 into the wind tunnel and placed on a 36 cm high platform in the middle of the tunnel width and 160  
264 cm downwind from the pheromone source. After 1 min, during which the male adjusted to the  
265 airflow, the pheromone blend was delivered for 3 min. During this time, the following behavioral  
266 items were observed: take-off, partial flight (flight half way between the release site and the  
267 pheromone source), complete flight (arrival within 10 cm of the source) and landing on the  
268 pheromone source. The partial and complete flights as well as landings were considered as an  
269 orientated response towards the sex pheromone. Orientated and random flights were counted  
270 together in order to quantify the general flight activity of males and this activity was around 95 %  
271 for all behavioral tests. We also noted the delay in the orientated response of males by measuring  
272 the period of time between the onset of pheromone stimulation and the take-off. All experiments  
273 were performed double-blind to avoid partial observations. The flight tracks were recorded and  
274 analyzed using Trackit 3D 2.0 (SciTracks, Pfaffhausen, Switzerland). Two cameras (Basler Pilot,  
275 piA640–210 m with Tamron ½” 4-12F/1.2 lenses) were positioned above the tunnel at 60 cm from  
276 each other to cover the whole tunnel flight section with overlapping fields. Images from the two  
277 cameras were analyzed in real time and the x, y, and z coordinates of moth's position were extracted  
278 every 10 ms. On each experimental day, different groups of males were tested including at least  
279 one group of males that were expected to show a high response level to avoid experimental bias.

280  
281 The pheromone stimulus was delivered with a programmable olfactometer adapted from  
282 Party *et al.* (2009). Air from the building was charcoal-filtered and re-humidified, then divided into  
283 several equal flows ( $300 \pm 10$  ml/min) using a Y connector (model 1/8” P514, Upchurch Scientific,  
284 Silsden, United Kingdom) and a 5-port manifold (model P-115, Upchurch Scientific Silsden,  
285 United Kingdom). Each flow was connected to a miniature electrovalve (model LHDA1233115H,  
286 the Lee Company, Westbrook, ME, USA) driven by a Valve-Bank programmer (AutoMate  
287 Scientific, Berkeley, CA, USA). Activating the appropriate valve directed the flow to a 4-ml glass

288 vial containing the pheromone source and closed by a septum cork. The inlet and outlet of the  
289 source pheromone were made of two hypodermic needles (18-g size, Sigma Aldrich, Saint-Quentin  
290 Fallavier, France) inserted through the septum and connected with polytetrafluorethylene tubing  
291 (1.32 mm i.d., 20 cm length, Sigma Aldrich, Saint-Quentin Fallavier, France). A 10  $\mu$ l sample of  
292 the pheromone blend at 1 ng/ $\mu$ l was deposited on a filter paper and placed inside the vial.  
293 Contaminated air was removed from the set-up by an exhaust fan after each behavioral assay.

#### 294 *2.9. Age-related experiments*

295 Males of different ages (1, 2, 3, 4 and 5 days) were collected in the mid-scotophase and  
296 transferred to cylindrical plastic containers in a dark box, then transported from the rearing room  
297 to the wind tunnel room for quantification of the behavioral response to sex pheromone. The males  
298 were then killed by using liquid nitrogen, and the ALs were dissected from frozen heads for analysis  
299 of *Met1*, *Met2* and *Kr-h1* expressions.

#### 300 *2.10. Statistical analysis*

301 The means of *Met1*, *Met2* and *Kr-h1* expression levels were compared using an ANOVA  
302 or Kruskal-Wallis' test followed by a multiple comparison by their corresponding post-hoc test.  $P$   
303  $< 0.05$  was accepted as statistically significant. For behavioral experiments, statistical differences  
304 between groups of males were evaluated using an  $R \times C$  test of independence by means of a G-test  
305 and also by applying the Williams correction (Sokal & Rohlf, 1995).

#### 306 *2.11. Animal ethics*

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

### 309 3. Results

#### 310 3.1. Age-related changes in the behavioral response to sex pheromone and *Met1*, *Met2* and *Kr-h1* 311 expressions in ALs

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

#### 322 3.2. Interactions between *Met1*, *Met2* and *Kr-h1* in ALs

323 In order to ascertain whether the expressions of *Met1*, *Met2* and *Kr-h1* in ALs are linked to  
324 each other, we evaluated the impact of injection of *Met1*-1-dsRNA, *Met2*-1-dsRNA and *Kr-h1*-1-  
325 dsRNA into 1-day-old males on the levels of corresponding transcripts in ALs four days after  
326 administration. *Met1* and *Met2* silencing in the ALs of 5-day-old males (**Fig. 2A and B**) were  
327 estimated around 74 % (Dunn's test,  $P \leq 0.0001$ ) and 78 % (Dunn's test,  $P \leq 0.0001$ ) respectively,  
328 and resulted in a 60 %–70 % reduction of the *Kr-h1* mRNA expression (**Fig. 2C**) (Dunn's test,  $P \leq$   
329 0.0001) whereas *Kr-h1* silencing of approximately 65 % had no effect on the AL *Met1* and *Met2*  
330 mRNA levels as compared with control groups (Dunn's test,  $P = 1.000$ ) (**Fig. 2A and B**). In  
331 addition, the expression level of AL *Met2* and *Met1* remained unchanged in *Met1*- (Dunn's test,  $P$   
332  $\geq 0.02792$ ) and *Met2*-depleted (Dunn's test,  $P \geq 0.03563$ ) males respectively (**Fig. 2A and B**).

#### 333 3.3. Effect of *Met1*, *Met2* and *Kr-h1* silencing on the behavioral response to sex pheromone

334 We analyzed the consequence of injection of *Met1*-1-dsRNA, *Met2*-1-dsRNA and *Kr-h1*-  
335 1-dsRNA into 1-day-old males on the behavioral response of male to sex pheromone four days  
336 after administration. *Met1*-, *Met2* and *Kr-h1*- depleted 5-day-old males exhibited a decline in the

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

346  
347 *3.4. Effect of JH on the behavioral response to sex pheromone and the AL Met1, Met2 and Kr-h1*  
348 *expressions*

349  
350 In order to reveal a potential relationship between *Met1*, *Met2* and *Kr-h1* and the JH action,  
351 we manipulated the circulating JH titer in sexually immature and mature males either by injection  
352 of exogenous JH or allatectomy and then observed the impact of treatment on the behavioral  
353 response to sex pheromone and the AL *Met1*, *Met2* and *Kr-h1* transcriptional activities.  
354 Administration of JH-II into newly emerged males led to an increase in the sex pheromone-  
355 triggered orientation flight with decreased delay response (**Fig. 4A and B**) that is paralleled with  
356 an elevation of the AL *Met1*, *Met2* and *Kr-h1* mRNA expression levels in comparison with control  
357 groups (oil-injected and non-injected 2-day-old males) (Tamhane and Tukey's tests,  $P \leq 0.0001$ )  
358 (**Fig. 4C, D and E**). By contrast, the ablation of corpora allata of newly emerged males resulted in  
359 altered behavioral responsiveness to sex pheromone (**Fig. 5A**) coupled with reduced AL *Met1*,  
360 *Met2* and *Kr-h1* mRNA levels five days after allatectomy as compared with group controls (non-  
361 operated and sham-operated 5-day-old males) (Dunn and Tamhane's test,  $P \leq 0.0001$ ) (**Fig. 5B, C**  
362 and **D**). We demonstrated that the injection of JH-II, the following day of allatectomy, raised both  
363 the behavioral responsiveness to sex pheromone (**Fig. 5A**) and the AL *Met1*, *Met2* and *Kr-h1*  
364 mRNA expressions in allatectomized 5-day-old males, reaching almost the levels of non-operated  
365 and sham-operated 5-day-old males (Dunn and Tamhane's test,  $P \geq 0.0001$ ) (**Fig. 5B, C and D**).  
366 We also verified that injection of oil into allatectomized males had no effect on the sex pheromone-

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

369

## 370 4. Discussion

371 The purpose of our work was to explore the molecular function of *Met* and *Kr-h1* as  
372 mediators of the central action of JH in the acquisition of male sexual behavior by using the long-  
373 lived moth *A. ipsilon* as a model organism for which the responsiveness to sex pheromone signal  
374 are known to be age- and JH-dependent. Our results showed that the amounts of *Met1*, *Met2* and  
375 *Kr-h1* transcripts were higher in the ALs of sexually mature old males as compared with those of  
376 sexually immature young males. This revealed the existence of an age-related upregulation of the  
377 transcriptional activities of *Met1*, *Met2* and *Kr-h1* in the ALs and which coincides with the age-  
378 related increase in the neuronal response to pheromone information and the emergence of sex  
379 pheromone-guided orientation flight in old males. Similarly, studies performed in the honey bee  
380 *Apis mellifera* indicated that an elevated expression of *Kr-h1* is associated to the brain remodeling  
381 required for the ontogenesis of adult behaviors within the community of workers (Shpigler *et al.*,  
382 2010). In the mice *Mus musculus*, a high transcriptional activation of the *Krüppel-like factor 7* was  
383 found to be indispensable for the regulation of neuronal processes, including neurite outgrowth,  
384 axon guidance and neurogenesis, in the development and the adult plasticity of the olfactory  
385 pathways (Laub *et al.*, 2005). Moreover, in the terrestrial mollusk *Limax valentianus*, a Krüppel-  
386 like factor has been detected in the protocerebrum, the locus of odor information processing, where  
387 its expression was induced by neuronal activity and sensory input (Fukunaga *et al.*, 2006).

388  
389 We observed that the administration of dsRNAs targeting various regions of *Met1*, *Met2* and  
390 *Kr-h1* resulted in an approximately 65 %–78 % reduction in mRNA expression levels in the ALs,  
391 and led to similar defective phenotypes of the behavioral response to sex pheromone in dsRNA-  
392 treated males. These results strengthened our RNAi experiments even though we could not verify  
393 the decline of the corresponding proteins in the ALs of *Met1*-, *Met2*- and *Kr-h1*-deficient males  
394 because the antibodies against *Agrotis* *Met1*, *Met2* and *Kr-h1* proteins are not available. *Met1*,  
395 *Met2* and *Kr-h1* silencing caused alterations in sex pheromone-triggered orientation flight that  
396 manifested by a lower percentage of responding males and an increased response delay. We also  
397 found that *Met1* and *Met2* knockdown provoked a decrease in the AL *Kr-h1* expression whereas  
398 *Kr-h1* knockdown had no impact on the AL *Met1* and *Met2* expressions. Taken together, our RNAi  
399 data provided functional evidence that in *A. ipsilon*, *Met1*, *Met2* and *Kr-h1* are involved in the  
400 maturation of male sexual behavior and that *Kr-h1* works downstream of *Met1* and *Met2*. In

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

405  
406 It has been previously reported in *A. ipsilon* that JH-deprived old males exhibited altered sex  
407 pheromone-guided orientation flight (Duportets *et al.*, 1998; Gadenne & Anton, 2000) as observed  
408 in *Met1*-, *Met2*- and *Kr-h1*-depleted males. Based on this observation, it was conceivable to assume  
409 that *Met1*, *Met2* and *Kr-h1* are linked to JH action in the modulation of male sexual activity. In  
410 order to reveal this relationship, we manipulated the circulating JH titer in males of different ages  
411 and examined the resulting changes on their orientated response to sex pheromone and the AL  
412 *Met1*, *Met2* and *Kr-h1* expression levels. The injection of JH into young males enhanced the sex  
413 pheromone behavioral response with increased AL *Met1*, *Met2* and *Kr-h1* transcriptional activities.  
414 By contrast, JH deficiency inhibited the sex pheromone behavioral response coupled with reduced  
415 AL *Met1*, *Met2* and *Kr-h1* mRNA amounts in allatectomized old males and these suppressive  
416 effects were partly rescued by a combined injection of JH in operated males. These data highlighted  
417 that in *A. ipsilon*, JH operates through the induction of the *Met1*, *Met2* and *Kr-h1*-dependent  
418 signaling pathways that take places in ALs, to facilitate the central processing of pheromone  
419 information and thereby, contributing to the onset of sexual attraction behavior in sexually mature  
420 old males. In concordance with this result, it is well documented in rodents that testicular hormones  
421 are critical for the accomplishment of male reproductive behaviors by governing a reshaping of  
422 neuronal circuits and especially within the olfactory bulb glomeruli, and thus affecting the  
423 perception of female urinary pheromones (Moffatt, 2003; Yoshikage *et al.*, 2007; Henley *et al.*,  
424 2010). In future studies, it would be useful to prove that *Met1*, *Met2* and *Kr-h1* are mediators of  
425 enhancing effects of JH on the sex pheromone-driven responses of AL neurons, and to achieve this,  
426 we will evaluate the impact of RNAi-induced *Met1*, *Met2* and *Kr-h1* silencing on the neuronal  
427 activity by intracellular recordings within the *A. ipsilon* MCG glomeruli that are specialized in the  
428 integration of pheromone signal. We cannot rule the possibility that the JH-Met-Kr-h1 signaling  
429 pathway might be functional in other areas of the brain that the primary olfactory neuropiles and  
430 especially within second-order olfactory centers including the lateral horn and the mushroom  
431 bodies which are known to display a high degree of anatomical and physiological plasticity in the

432 context of chemosensory experience and the behavioral development (Menzel & Manz, 2005;  
433 Okada *et al.*, 2007; Reinhard & Claudianos, 2012). In line with this speculation, *Met-1*, *Met-2* and  
434 *Kr-h1* transcripts were detected in the *A. ipsilon* brain after the excision of ALs (P. Aguilar,  
435 unpublished data). In addition, in situ hybridization analysis on the brain of honey bee workers  
436 demonstrated that changes in synaptic organization of mushroom bodies were accompanied by  
437 increased *Kr-h1* expression during the transition to foraging tasks (Hewes, 2008; Shpigler *et al.*,  
438 2010). Experiments are in progress to establish in situ the mapping of *Met1*, *Met2* and *Kr-h1*  
439 expressions in the entire brain of *A. ipsilon* adult.

440  
441 Previous works indicated that the antennal sensory system of *A. ipsilon* is fully functional in  
442 newly emerged immature males and that the electrophysiological response of the ORNs of trichoid  
443 sensilla to the female sex pheromone is age- and JH-independent (Gadenne *et al.*, 1993). Such a  
444 finding is supported by the observation that *Met1*, *Met2* and *Kr-h1* are expressed in the antennae at  
445 extremely low levels throughout adulthood (P. Aguilar, unpublished data). Thus, the initiation of  
446 male sexual behavior by JH appears mainly driven by modulatory actions on the central olfactory  
447 pathways in *A. ipsilon*. By contrast, it has been uncovered in *D. melanogaster* that JH confers the  
448 ability to engage in the courtship behavior by increasing the sensitivity of antennal ORNs to  
449 stimulatory pheromones in reproductively mature males and that Met is required in transducing the  
450 JH signal (Lin *et al.*, 2016). This is consistent with other studies conducted on cyprinid fishes  
451 showing that androgens have direct actions on the ORNs to enhance the detection of female  
452 pheromonal prostaglandins and the expression of courtship behaviors (nuzzling and quivering) in  
453 juvenile males (Belanger *et al.*, 2010).

454  
455 It is well established that JH plays a key role in the coordination between reproductive  
456 maturity and sexual activity (Cusson *et al.*, 1994; Teal *et al.*, 2000; Bilen *et al.*, 2013; Holtof *et al.*,  
457 2021). In *A. ipsilon* males, JH was found to fulfill this function through simultaneous actions on  
458 both the ALs and the ASGs (Gadenne *et al.*, 1993; Duportets *et al.*, 1998; Anton & Gadenne, 1999;  
459 Gassias *et al.*, 2021). In addition, we have recently reported that *Met1*, *Met2* and *Kr-h1* act as  
460 transducers of JH signal that stimulates the growth and the secretory activity of the ASGs, and  
461 especially the synthesis of fluid seminal proteins (Gassias *et al.*, 2021) and which are necessary for  
462 spermatophore production and nourishment, activation, and capacitation of spermatozoa until

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

474  
475 In mammals and insects, there is a growing body of evidence that hormonal factors interact  
476 with biogenic amines and this functional interplay has been particularly described in the context of  
477 male reproduction. For example, testosterone is known to be essential for the display of male  
478 reproductive behavior by enhancing the processing of sexually relevant stimuli and this action has  
479 been shown to be driven through alterations in the synthesis, release and receptor expression for  
480 neurotransmitters such as dopamine (DA) and serotonin (Hull *et al.*, 1999, Hull *et al.*, 2004). JH  
481 was found to be a critical signal in the emergence of DA-mediated mating flight of *A. mellifera*  
482 drones by increasing simultaneously the expression levels of DA and D1-type dopamine receptor  
483 (*Dop1*) in the brain (Akasaka *et al.*, 2010; Sasaki *et al.*, 2012). In addition, we have previously  
484 revealed in *A. ipsilon* male that age-related changes in neuronal and behavioral responses to sex  
485 pheromone are sustained by DA-*Dop1* signaling pathway that operates in ALs through cAMP-  
486 dependent mechanisms (Gassias *et al.*, 2019). In order to explore the existence of a regulation of  
487 the dopaminergic system by JH in *A. ipsilon*, experiments are underway to evaluate the impact of  
488 JH administration in young males and of JH deprivation in old males on the amounts of DA, *Dop1*  
489 expression and cAMP in ALs.

## 490 **5. Conclusions**

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

501 **Declaration of competing interest**

502       The authors disclose no potential conflicts of interest.

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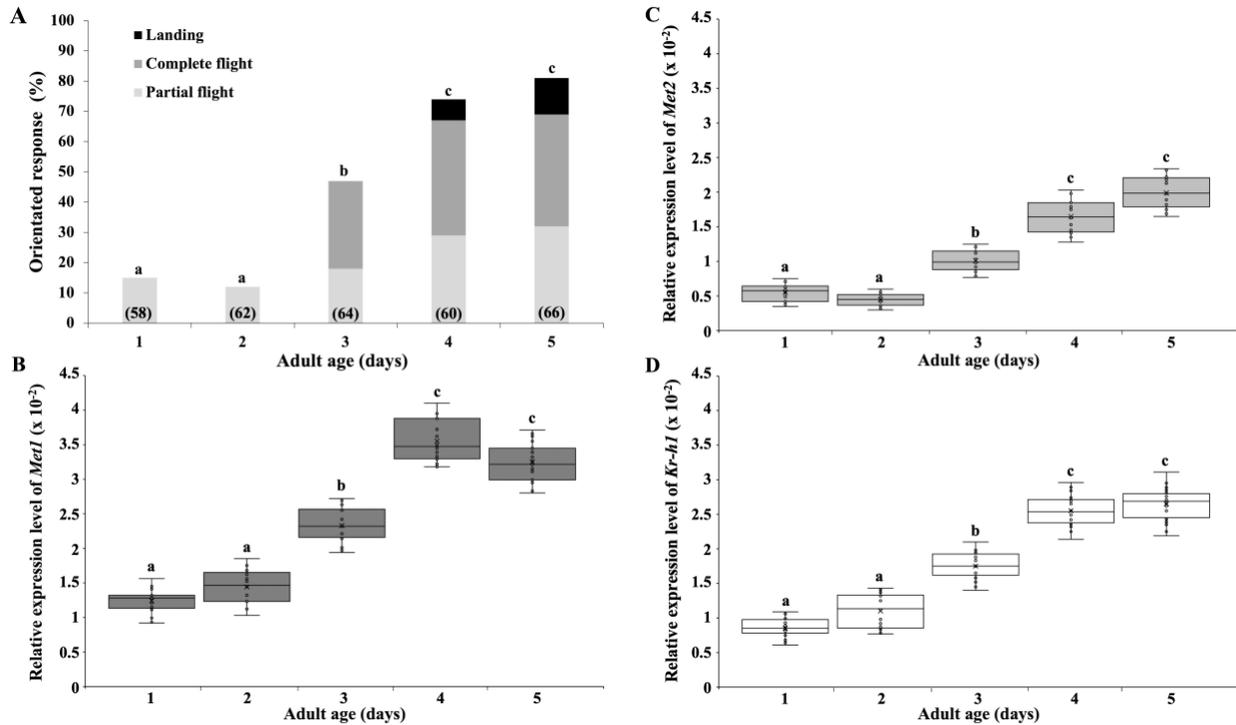
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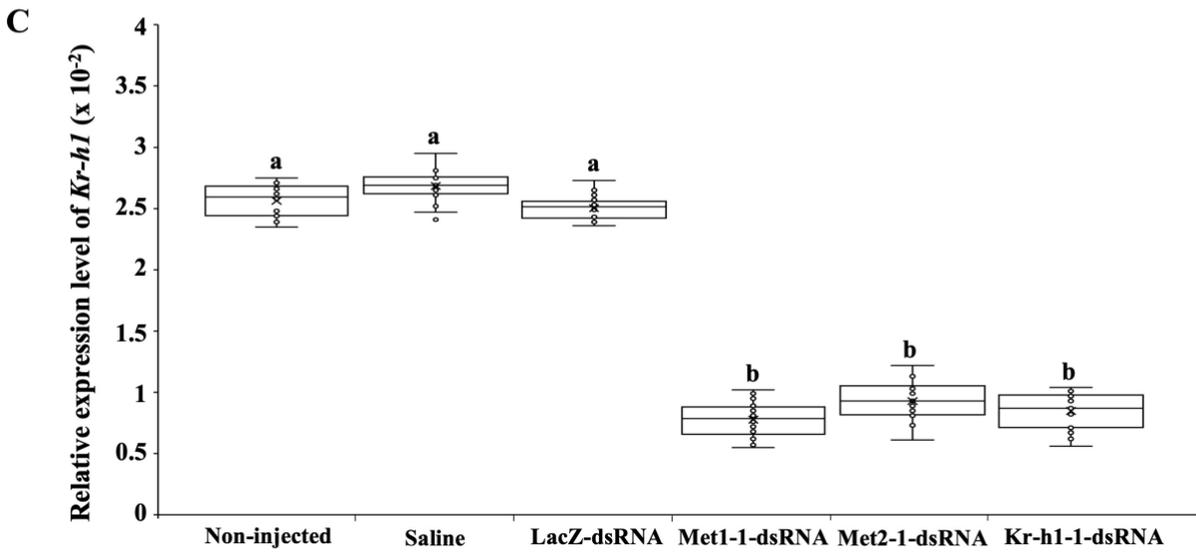
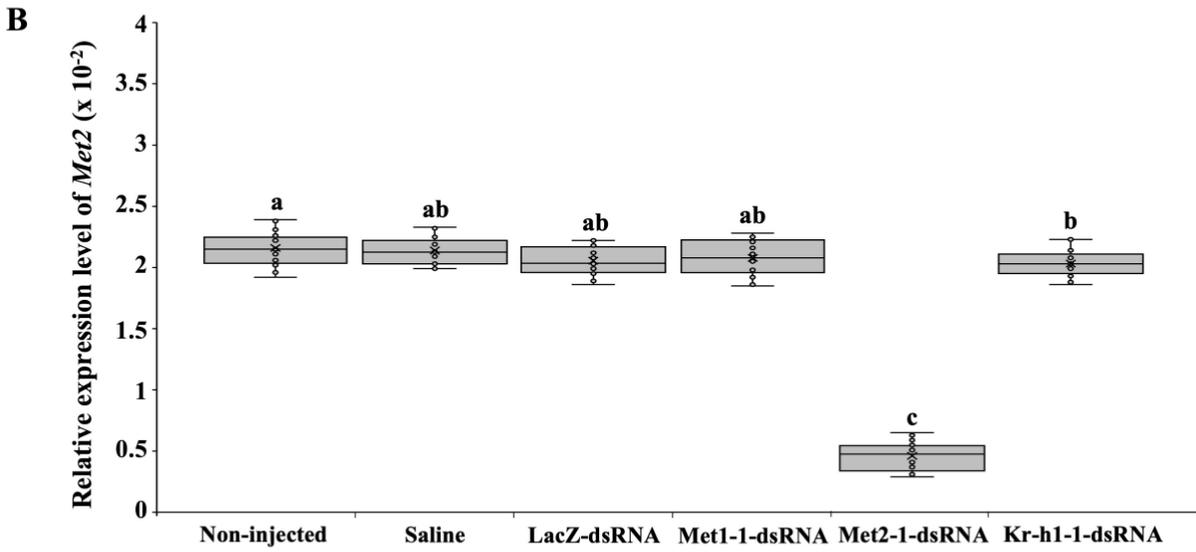
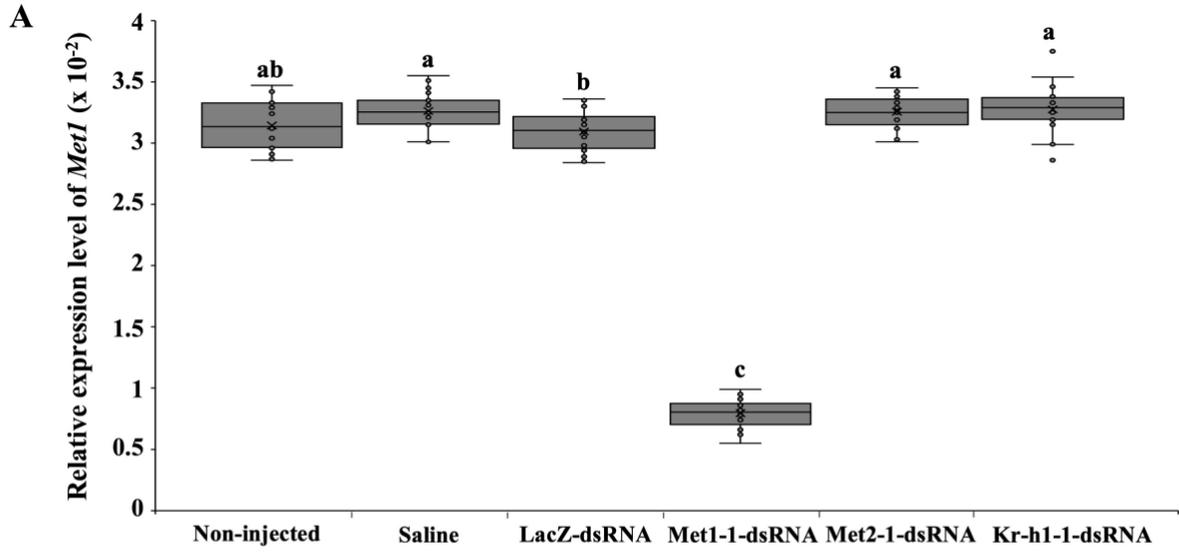
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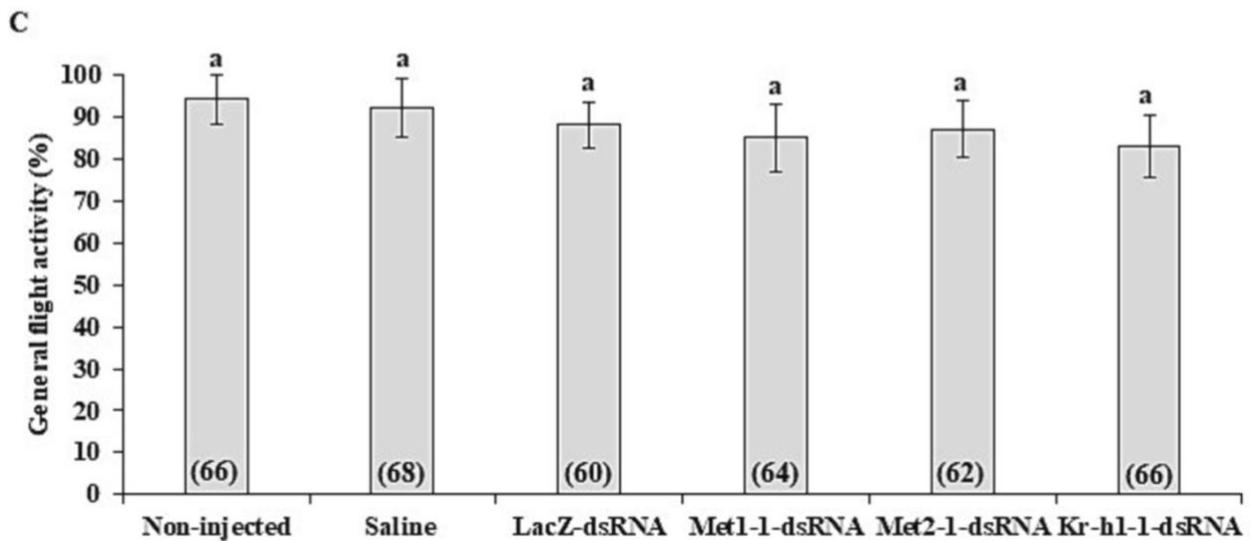
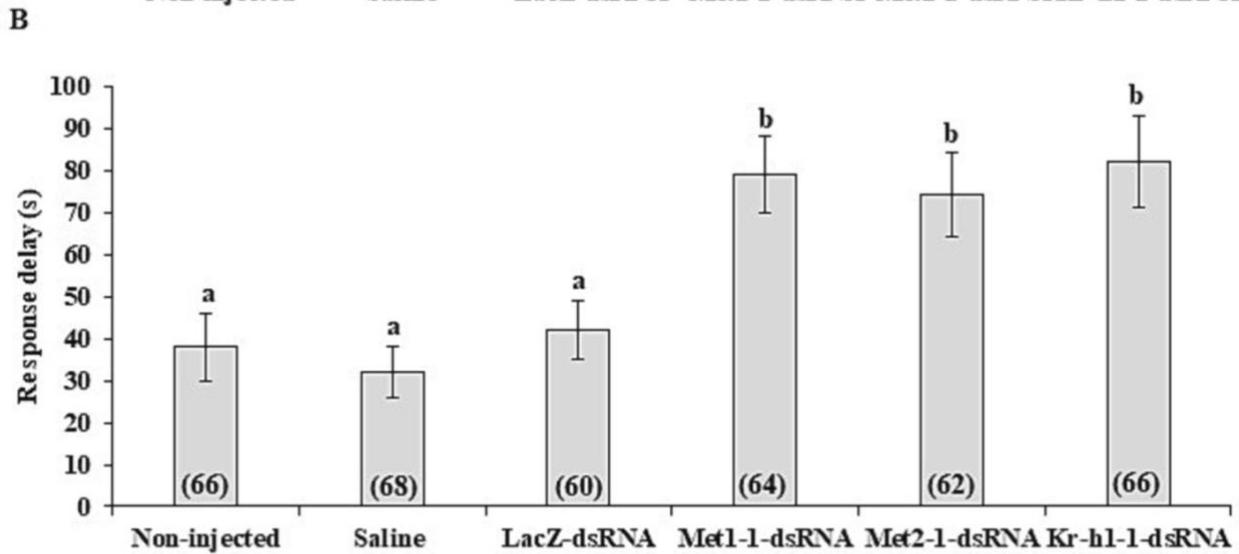
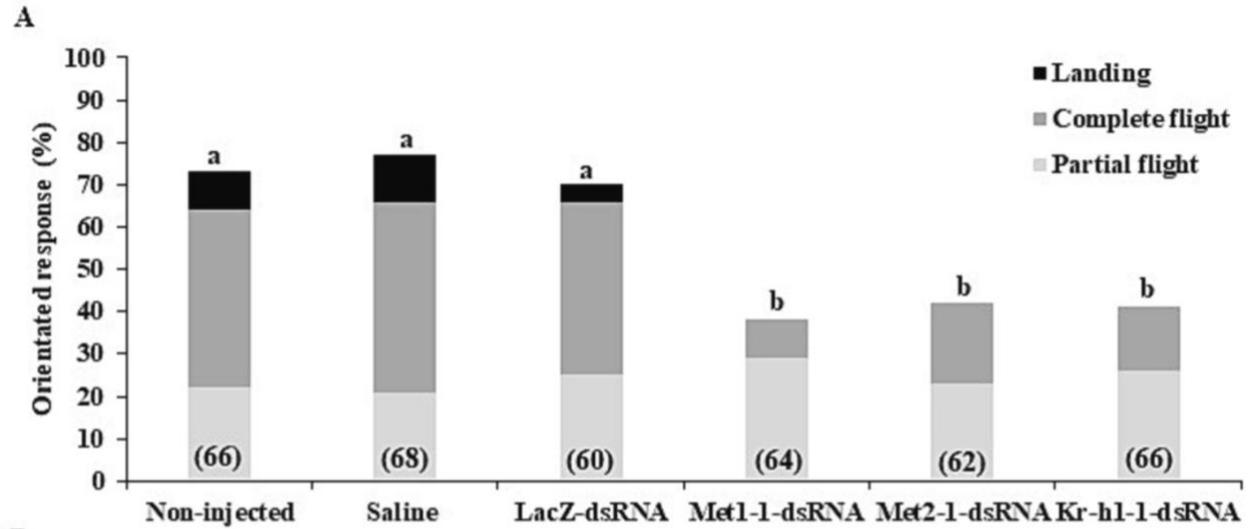
720 **Figures**



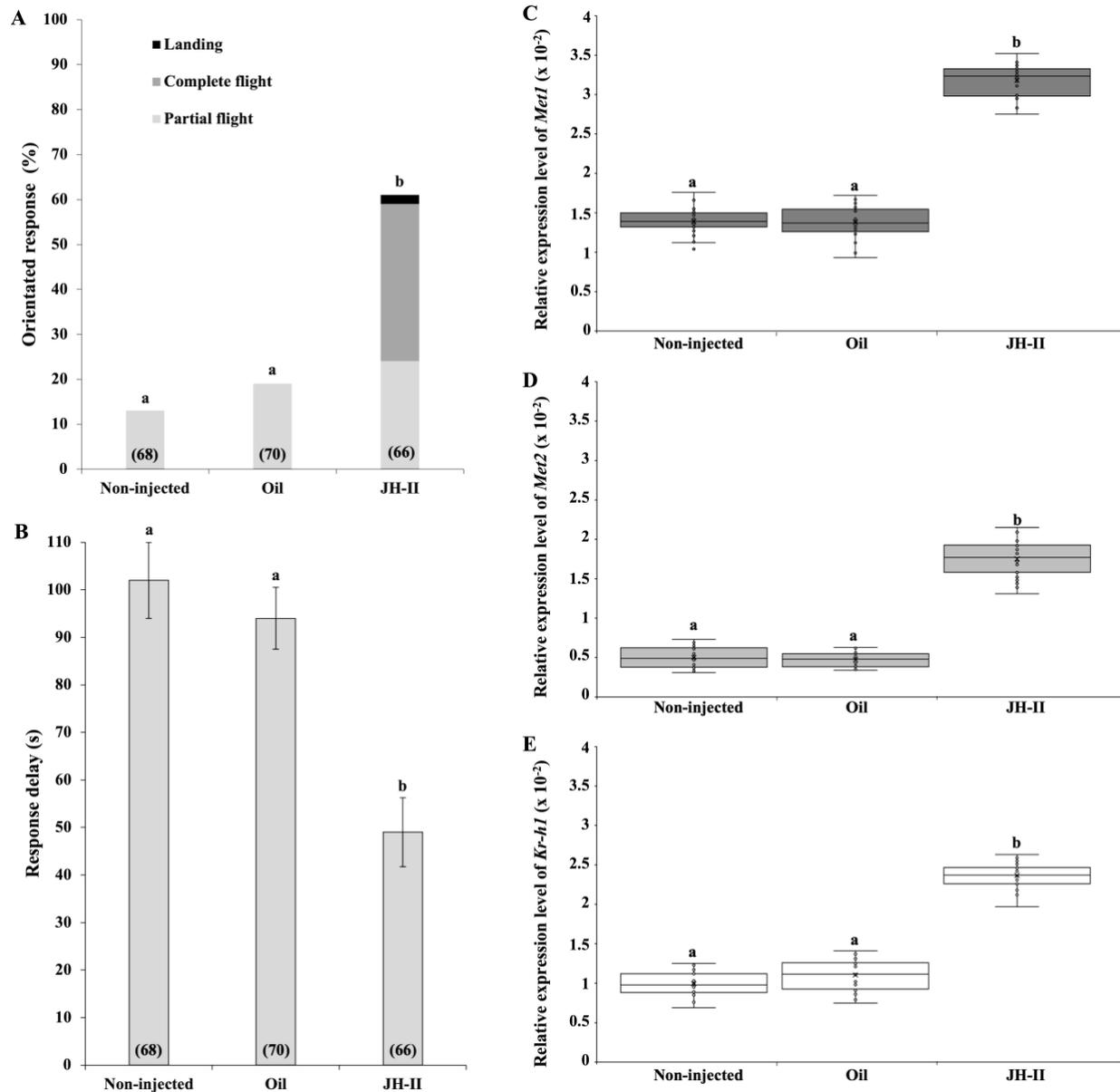
721  
 722 **Figure 1.** Effect of age on the behavioral response to sex pheromone and the level of AL *Met1*,  
 723 *Met2* and *Kr-h1* expressions in *A. ipsilon* males. The percentage of sex pheromone-guided  
 724 orientation flight in the wind tunnel experiments (A) and the amounts of *Met1* (B), *Met2* (C) and  
 725 *Kr-h1* (D) mRNA in ALs were evaluated for 1- to 5-day-old males. Each qRT-PCR was run in  
 726 three technical replicates and eight independent biological replicates. For the behavioral tests,  
 727 numbers within bars indicate the number of tested males; the different letters indicate a statistically  
 728 significant difference (G-test;  $P < 0.05$ ). For the AL *Met1*, *Met2* and *Kr-h1* mRNA levels, the bars  
 729 represent the value distribution and the different letters indicate a statistically significant difference  
 730 (Dunn's test;  $P < 0.05$ ).



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

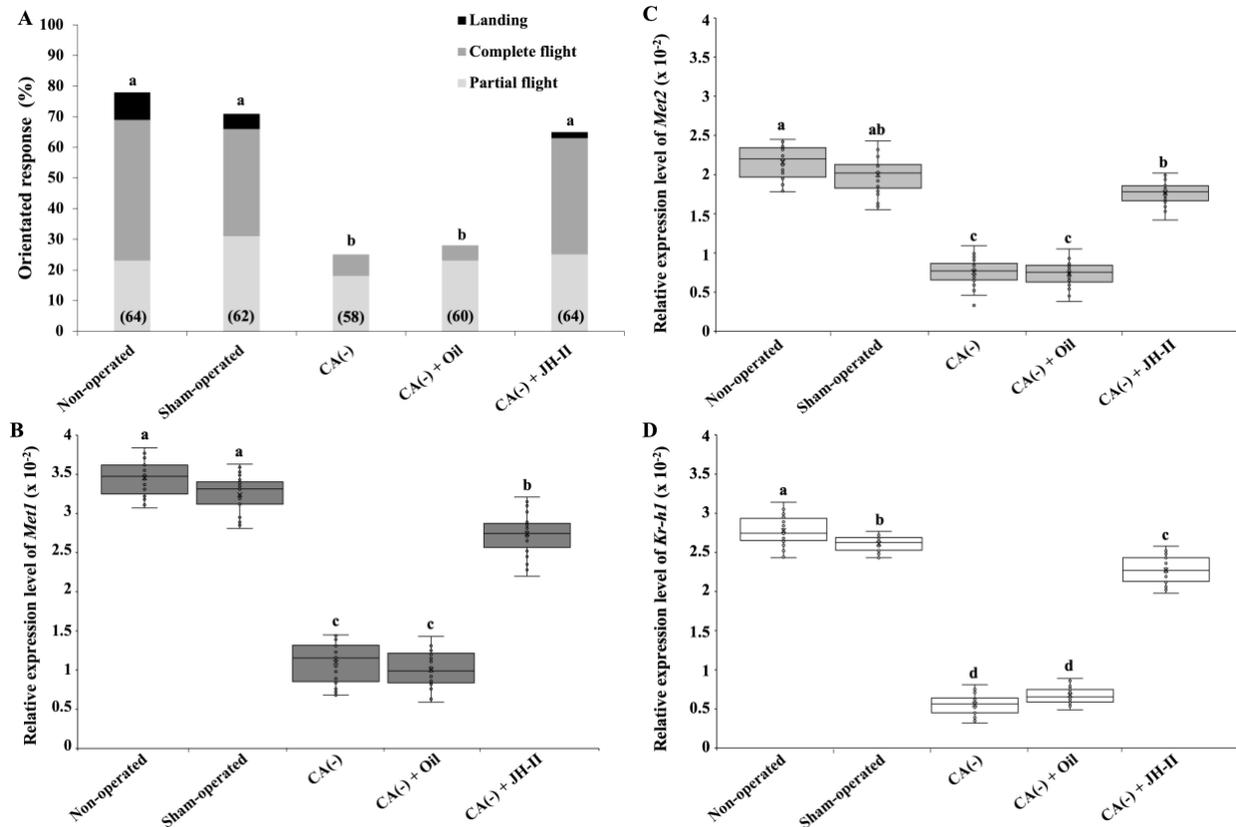


740 **Figure 3.** Effect of *Met1*, *Met2* and *Kr-h1* silencing on the behavioral response of 5-day-old  
741 sexually mature males to sex pheromone. One-day-old males received an injection of saline  
742 solution, bacterial LacZ-dsRNA, Met1-1-dsRNA, Met2-1-dsRNA or Kr-h1-1-dsRNA, or no  
743 injection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



747  
 748 **Figure 4.** Effect of JH-II injection on the behavioral response to sex pheromone and the AL *Met1*,  
 749 *Met2* and *Kr-h1* expressions in 2-day-old sexually immature males. Newly emerged males (day-0)  
 750 received an injection of JH-II, or mineral oil or no injection (non-injected) and the percentage of  
 751 sex pheromone-mediated orientation flight (A), the response delay (B) and the relative expressions  
 752 of *Met1* (C), *Met2* (D) and *Kr-h1* (E) in ALs were determined two days after injection. Each qRT-  
 753 PCR was run in three technical replicates and eight independent biological replicates. For the  
 754 behavioral tests, numbers within bars indicate the number of tested males; the different letters  
 755 indicate a statistically significant difference (G-test;  $P < 0.05$ ). For the AL *Met1*, *Met2* and *Kr-h1*  
 756 mRNA levels, the bars represent the value distribution and the different letters indicate a

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



759  
 760 **Figure 5.** Effect of allatectomy and JH-II injection on the behavioral response of 5-day-old  
 761 sexually mature males to sex pheromone. Allatectomy (CA-) was performed on the day of  
 762 emergence (day-0) and injections was carried out on the next day (day-1) and the percentage of sex  
 763 pheromone-guided orientation flight (A) and the relative expressions of *Met1* (B), *Met2* (C) and  
 764 *Kr-h1* (D) in ALs were determined at day-5. Controls groups were composed of oil-injected, non-  
 765 operated and sham-operated males. Each qRT-PCR was run in three technical replicates and eight  
 766 independent biological replicates. For the behavioral tests, numbers within bars indicate the number  
 767 of tested males; the different letters indicate a statistically significant difference (G-test;  $P < 0.05$ ).  
 768 For the AL *Met1*, *Met2* and *Kr-h1* mRNA levels, the bars represent the value distribution and the  
 769 different letters indicate a statistically significant difference (Dunn's test for *Met1* and *Met2* relative  
 770 expression levels, Tamhane's test for *Kr-h1* relative expression level;  $P < 0.05$ ).

771 **Supplementary Table**

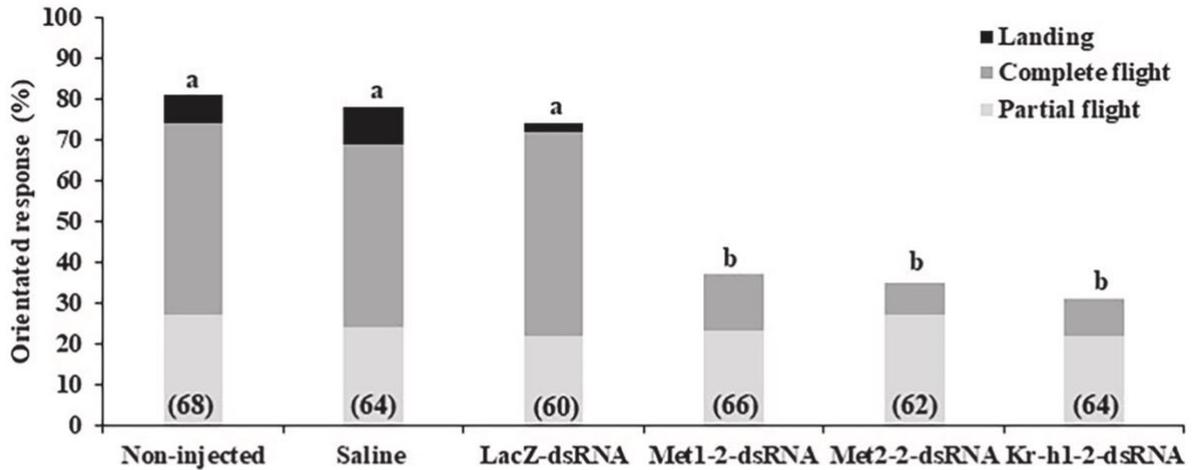
772

773	Primer name	Sequence
774	qMet1dir	5'-TCGCAACGCAAAGTTGATAAG-3'
775	qMet1rev	5'-TAGATCTATATGACAGTATCC-3'
776	qMet2dir	5'-TCTGCAGCACCCGCCATGTTCGCTG-3'
777	qMet2rev	5'-AGGACTAAACTGCTGCGGTGATG-3'
778	qKr-h1dir	5'-ACTAAAGAACGGCCGTACCGA-3'
779	qKr-h1rev	5'-CCCTTTTCCACAGCCGGGCGC-3'
780	RpL8dir	5'-CCAGTTTGTCTACTGCGGCAA-3'
781	RpL8rev	5'-GCTTAACCCTAGTACGCTTGGCA-3'
782	LacZ T7dir	5'-taatacgactcactatagggATGACCATGATTACGCCAAGC-3'
783	LacZ T7rev	5'-taatacgactcactatagggCCATTTCGCCATTTCAGGCTGCG-3'
784	Met1-1-T7dir	5'-taatacgactcactatagggAGTGCGCATCTGGGAATGCACTCT-3'
785	Met1-1-T7rev	5'-taatacgactcactatagggCTTTTCTTGAACGTTGGGTGATAC-3'
786	Met2-1-T7dir	5'-taatacgactcactatagggGACTCATCACCGCAGCAGTTTAGTCC-3'
787	Met2-1-T7rev	5'-taatacgactcactatagggGCATGGATTCTCGGAGCGGGCGCGCG-3'
788	Kr-h1-1-T7dir	5'-taatacgactcactatagggATAGGTGAGGAGGAACGAGCTCAT-3'
789	Kr-h1-1-T7rev	5'-taatacgactcactatagggCAGTTGTTTCGAGCAGGTGAACCC-3'
790	Met1-2-T7dir	5'-taatacgactcactatagggGAAGTAACTGGGACGTCAGCCTTT-3'
791	Met1-2-T7rev	5'-taatacgactcactatagggATCACAGTCCACCTTTCTCCTCTT-3'
792	Met2-2-T7dir	5'-taatacgactcactatagggTCCTGTGGAACTGTTCGACATGAAC-3'
793	Met2-2-T7rev	5'-taatacgactcactatagggCATTTCGAAATCTTCGAACTCGCTTG-3'
794	Kr-h1-2-T7dir	5'-taatacgactcactatagggTGTGACATATGTCTCCGAGATTTTC-3'
795	Kr-h1-2-T7rev	5'-taatacgactcactatagggGGCAGCTGGGGCAAATTGTATCAC-3'

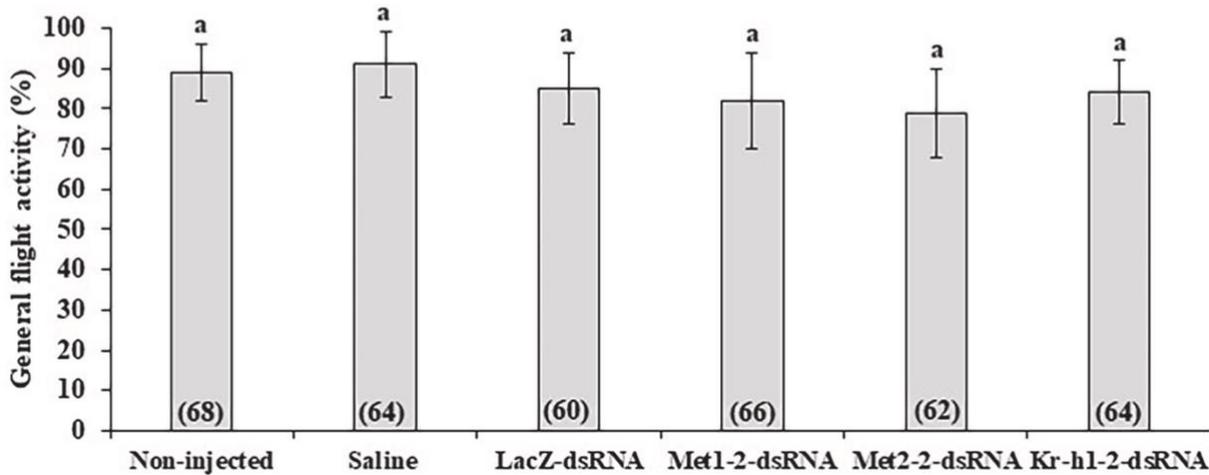
796 **Table S1.** List of the primers used in the study.

797 **Supplementary Figure**

A



B



798  
 799 **Figure S1.** Effect of Met1-2-dsRNA, Met2-2-dsRNA and Kr-h1-2-dsRNA on the behavioral  
 800 response to sex pheromone in 5-day-old sexually mature males. One-day-old males received an  
 801 injection of saline solution, bacterial LacZ-dsRNA, Met1-2-dsRNA, Met2-2-dsRNA or Kr-h1-2-  
 802 dsRNA, or no injection (non-injected) and the percentage of orientation flight towards to sex  
 803 pheromone (A) and of general flight activity (B) were determined four days after injection.  
 804 Numbers within bars indicate the number of tested males; the different letters indicate a statistically  
 805 significant difference (G-test;  $P < 0.05$ ).