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► **To cite this version:**

Evan Force, Caroline Suray, Christelle Monsempe, Chloé Danis, Gabrielle Bonfils, et al.. Diet acts on sexual behavior development in a male moth. *Insect Science*, In press, pp.13457. 10.1111/1744-7917.13457 . hal-04736663

**HAL Id: hal-04736663**

**<https://hal.science/hal-04736663v1>**

Submitted on 15 Oct 2024

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## ORIGINAL ARTICLE

## Diet acts on sexual behavior development in a male moth

Evan Force<sup>1,2</sup> , Caroline Suray<sup>1</sup>, Christelle Monsempes<sup>1</sup>, Chloé Danis<sup>1</sup>, Gabrielle Bonfils<sup>1</sup>, Stéphane Debernard<sup>2</sup> and Matthieu Dacher<sup>1,2</sup> 

<sup>1</sup>Sorbonne Université, Université Paris-Est Créteil, INRAE, CNRS, IRD, Institute for Ecology and Environmental Sciences of Paris, iEES Paris, Versailles, France and <sup>2</sup>Sorbonne Université, Université Paris-Est Créteil, INRAE, CNRS, IRD, Institute for Ecology and Environmental Sciences of Paris, iEES Paris, Paris, France

**Abstract** In many animals, drastic changes are observed during sexual maturation characterized by the reproductive system development concomitantly to the sexual behavior ontogenesis. These modifications are under the control of internal and external factors such as food. Sexual maturation requires considerable energetic investment, and diet has been shown to affect reproductive activities in many taxonomic groups, especially in insects and vertebrates. By contrast, diet effects on sexual behavior development remain largely unexplored. To elucidate this aspect, we used the male moth *Agrotis ipsilon* which undergoes sexual maturation occurring between the third and the fifth day postemergence. During this period, males are sensitive to female sex pheromones and a stereotypical sexual behavior characterized by female-oriented flight takes place. In our study, we compared (1) sex pheromone detection by electroantennography recordings and (2) behavioral response in wind tunnel assays between males fed with different diets found in nature. Compared to standard sucrose diet, males fed with sucrose, fructose, and glucose supplemented with sodium (a mineral element necessary for the locomotor activity in several moths) did not respond better to female sex pheromones but clearly exhibited an earlier behavioral response. Thus, such a diet accelerates the development of sex pheromone-mediated oriented flight, probably by facilitating the central processing of sex pheromone information in male *A. ipsilon* moths. Our results provide new information on the influence of nutritional intake on the ontogenesis of male sexual behavior in animals.

**Key words** *Agrotis ipsilon*; feeding; insect; oriented flight; sexual maturation; sex pheromone detection

Correspondence: Evan Force, Sorbonne Université, Université Paris-Est Créteil, INRAE, CNRS, IRD, Institute for Ecology and Environmental Sciences of Paris, iEES Paris, RD10-Route de Saint-Cyr, 78026 Versailles, France. Email: evan.force@inrae.fr. Matthieu Dacher, Sorbonne Université, Université Paris-Est Créteil, INRAE, CNRS, IRD, Institute for Ecology and Environmental Sciences of Paris, iEES Paris, Sorbonne Université, 4, Place de Jussieu, 75005 Paris, France. Email: matthieu.dacher@sorbonne-universite.fr

## Introduction

Drastic changes are observed during certain periods of the life cycle of many animals, such as sexual maturation in mammals (Colvin & Abdullatif, 2013) and insects (Seong *et al.*, 2023). These steps are significant transformations and are termed developmental transitions (Rewitz *et al.*, 2013; Castellano & Tena-Sempere, 2016). For a long time, the cellular and molecular mechanisms and the regulations underlying these developmental transitions have stimulated the curiosity of scientists, due to the spectacular morpho-anatomical, physiological, and behavioral modifications the feature. For instance,

sexual maturation is a developmental stage of an organism during which it acquires the ability to reproduce as a result of the development of the reproductive system and the ontogeny of sexual behaviors. These developmental processes are under the control of both internal and external factors such as food. Indeed, diet modulates the timing of mammalian puberty (Kenny & Byrne, 2018) and also regulates the display of insect sexual behaviors such as the sex pheromone attraction and the courtship in *Drosophila* females (Lebreton et al., 2015). However, in insects and other animal groups, the impact of diet on the development of sexual behaviors remains largely unexplored (Wagner & Hoback, 1999; Tolle & Wagner, 2011; Clark et al., 2015).

Over the past few decades, numerous studies performed on the male moth *Agrotis ipsilon* (Lepidoptera: Noctuidae) led to the detailed characterization of their sexual maturation, which occurs between the third and fifth day of adult life under standard laboratory conditions (i.e., males fed with sucrose only) (Gemeno & Haynes, 2000). During this period, males become sexually mature and are attracted by sex pheromones emitted by females. This increased attractiveness is tightly coupled with the development of a stereotyped sexual behavior marked by an oriented zigzag flight toward the female leading to mating (Gadenne et al., 1993; Jarriault et al., 2009). In French population, the female-emitted sex pheromones include three compounds: (Z)-7-dodecen-1-yl acetate (Z7-12:Ac), (Z)-9-tetradecen-1-yl acetate (Z9-14:Ac), and (Z)-11-hexadecen-1-yl acetate (Z11-16:Ac) in a 4 : 1 : 4 proportion respectively (Gemeno et al., 2000; Jarriault et al., 2009); other proportions have been described in populations coming from other continents (Gemeno et al., 2000; Du et al., 2015).

Detection of the female sex pheromones occurs through olfactory receptor neurons (ORNs) housed in the antennal sensilla. Moreover, it has been previously demonstrated that the antennal pheromone detection is already functional in newly emerged males (Gadenne et al., 1993). ORNs respond to odorants with a specificity that varies according to the compounds; for instance, pheromone-sensitive ORNs (Phe-ORNs) are usually very specific and respond to only one or a few similar-sex pheromone compounds (Renou et al., 1996). Such specificity of Phe-ORNs is due to the expression of specific olfactory receptors (ORs). An OR has been characterized in the antennae of male *A. ipsilon*: AipsOR3 (Gu et al., 2014), which is specific for (Z)7-12:Ac pheromonal molecule and does not recognize any volatile organic compounds (VOCs) (Vandroux et al., 2022).

The Phe-ORNs project toward the antennal lobes (ALs), the insect's primary olfactory centers that are anal-

ogous to the vertebrate olfactory bulbs. An AL is composed of structures integrating the olfactory information and termed glomeruli (Deisig et al., 2014). Glomeruli are arranged in two distinct areas: a general area dedicated to the integration of all odors and present in both females and males; and a specialized area found only in males, the macro glomerular complex (MGC), specific to the processing of pheromonal information (Deisig et al., 2014). Downward the ALs, the processed olfactory information results in an adapted behavioral response by activating descending and motor neurons.

The behavioral response of male *A. ipsilon* to female sex pheromones is characterized by a succession of distinguishable behavioral elements. First, wing activation and antennal movements precede the taking-off; then, males fly upward the airflow, following sex pheromone filaments in typical counter-turning trajectories called oriented flight (Hoffmann et al., 2020). Interestingly, the moth flight capacity is affected by their nutritional status (Shirai, 2006; He et al., 2021). Males of many Lepidoptera species display a puddling behavior to collect sodium (Smedley & Eisner, 1996; Molleman, 2010) from excrements or puddles of brackish water (Ravenscraft & Boggs, 2016). This mineral element is necessary for their locomotor activity by improving flight (Xiao et al., 2010; Ankola et al., 2021). However, puddling or other feeding behaviors of adult *A. ipsilon* have not been described in natural conditions.

Recently, we have demonstrated in male *A. ipsilon* that a diet comprising sucrose, fructose, and glucose along with sodium accelerated reproductive system maturation which was achieved 3 d after emergence rather than 5 d with a sucrose-only diet (Force et al., 2023). Owing to the importance of food intake in pheromone-induced flight in other moth species (Shirai, 2006; He et al., 2021), we hypothesize that adult diet may impact sex pheromone responsiveness in male *A. ipsilon*. To test this hypothesis, we evaluated the effect of various diets of adult males on both sex pheromone detection by electroantennography (EAG) recordings and behavioral response to female sex pheromones by wind tunnel assays. Our results demonstrated that a sodium-supplemented diet rich in sucrose, fructose, and glucose did not impact female sex pheromone detection but accelerated the ontogenesis of behavioral response in male *A. ipsilon* moths.

## Materials and methods

### *Insects and dietary regimens*

Our work was conducted on newly emerged male *Agrotis ipsilon*. They were reared in our laboratory at

INRAE, Versailles, France. Rearing was carried out according to the protocol described by Force *et al.* (2023). Every morning, 25 male adults were collected and placed in transparent boxes each containing five individuals (20 cm × 11.5 cm × 5 cm). The collection day was recorded as the first day of adult life (noted D1). The moths had *ad libitum* access to a 12% sugar solution (fed animals) or water (unfed animals) depending on the diet described below; unfed males can survive for 5 d without becoming weak.

Diet effects on sexual behavior were studied in males at 3 d (D3) or 5 d (D5) of adult life, corresponding respectively to the beginning and end of sexual maturation in standard laboratory conditions, that is, moths fed 12% sucrose (Gemeno & Haynes, 2000). From emergence, moths have been subjected to one of five different diets chosen according to results obtained by Force *et al.* (2023): water, as negative control (unfed animals); sucrose 12% (mass/mass, equivalent to 13.6% mass/volume), conventionally used for laboratory rearing; 4% sucrose + 4% fructose + 4% glucose; 1% NaCl; and 4% sucrose + 4% fructose + 4% glucose + 1% NaCl. The sugars used are the main constituents of the flower nectar, representing between 10% and 15% of the fresh mass, whether unique or in a mixture (Roy *et al.*, 2017). Moreover, for the sugar mixtures, the sugars are in equal proportions, what is often found in flower nectars. Sodium is an important mineral element involved in the reproduction of many moths (Smedley & Eisner, 1996; Molleman, 2010). For sodium, we chose a proportion equal to 1% (i.e., 0.17 mol/L), known to be attractive in other insects (Niewalda *et al.*, 2008; Pontes *et al.*, 2017; Kaushik *et al.*, 2018). All these nutrients come from Sigma-Aldrich (Saint-Quentin-Fallavier, France).

### Chemicals

We prepared a synthetic mixture of sex pheromones containing three compounds identified in natural extracts from the pheromone glands of female *A. ipsilon* (Picimbon *et al.*, 1997; Gemeno & Haynes, 1998): Z7-12:Ac, Z9-14:Ac and Z11-16:Ac in a 4 : 1 : 4 ratio, respectively (Hoffmann *et al.*, 2020); all molecules were from Pherobank (Wijk bij Duurstede, the Netherlands). This pheromone mixture has been identified as very attractive when trapping males in the field (Causse *et al.*, 1988). In addition, a sex pheromone concentration equal to 100 ng/μL is very active for electrophysiological and behavioral experiments (Hoffmann *et al.*, 2020). The sex pheromones were diluted in hexane (> 98% purity, CAS 110-54-3, Carlo-Erba, Val-de-Reuil, France). The mix-

ture was prepared a few days before the experiments and stored at −22 °C.

A plant volatile organic compound, geraniol (> 96% purity, CAS 106-24-1), has also been used. Geraniol is an odorant whose detection is independent of age in adult male *A. ipsilon* (Greiner *et al.*, 2002); a geraniol concentration equal to 10 μg/μL is best detected (Greiner *et al.*, 2002). Geraniol was diluted in paraffin oil. These two chemicals came from Sigma Aldrich (Saint-Quentin-Fallavier, France).

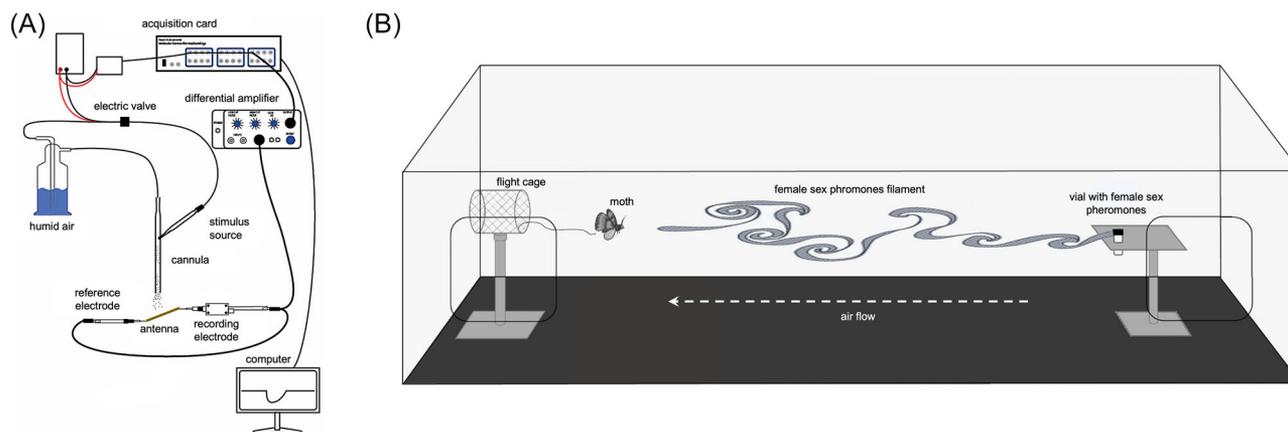
### Response to odorants in antenna electrophysiology

**Olfactory stimulation** Odorants were delivered according to the following protocol. An incoming charcoal-filtered air flow was humidified and routed through a three-way electro-valve (The Lee Company, CT, USA). After activation of the appropriate valve, the airflow was directed to a 20 cm-long glass stimulation tube (cannula) through a glass Pasteur pipette containing 1 μL of odorant on a Wattman filter paper (Fig. 1A). The opening of glass stimulation tube was on 1 cm in front of one of the moths' antennae. The antenna was stimulated with hexane, female sex pheromones (1, 10, 100, and 1000 ng), paraffin oil, and geraniol (10 μg). Stimuli lasted 500 ms and were kept 1 min apart to avoid sensory adaptation.

**Electroantennography** An antenna was cut away from the head at its base (scape). Recordings from antennae were made with glass microcapillaries containing Ag/AgCl electrodes and filled with a physiological solution (6.4 mmol/L KCl, 340 mmol/L glucose, 10 mmol/L HEPES, 12 mmol/L MgCl<sub>2</sub>, 1 mmol/L CaCl<sub>2</sub>, 12 mmol/L NaCl). The recording electrode capped the tip of the antenna after being cut at its last distal segment. The reference electrode was inserted at the base of the antenna. The recording and reference electrodes were connected to a CyberAmp 320 amplifier (Molecular Devices, CA, USA). The electrophysiological activity was sampled at 1 kHz resolution with a Digidata 1440A acquisition board (Molecular Devices). Signals were monitored on the computer screen using Clampex software (Axon pCLAMP 11, 2023) and analyzed using Clampfit software (Axon pCLAMP 11, 2023).

### Wind tunnel sex pheromone response

**Device** The study of behavioral response to female sex pheromones in male *A. ipsilon* was carried out using a Plexiglas® wind tunnel (VT Plastics, Gennevilliers, France) measuring 188.5 cm (length) × 73 cm (width)



**Fig. 1** (A) Diagram of EAG experimental device. (B) Wind tunnel diagram.

× 72 cm (height). This device had 2 side hatches. Upstream of the tunnel was a filtration chamber consisting in a particle filter and an activated carbon filter. Downstream, the wind tunnel included an extraction hood, which generated an air stream in it by evacuating the air outside the building (0.3 m/s). In addition, at the downstream end of the device, a cage was positioned (28 cm high and at 36.5 cm width) to host the moth before the start of the experiment. At the other end, a glass vial containing 1  $\mu$ L of odorant (deposited on a Wattman filter paper) was placed on a platform 26 cm above the tunnel floor and at 36.5 cm width (Fig. 1B); this vial was renewed every 4 moths (approximately every 20 min). A Teflon tube connected to the vial was connected to a stimulation system composed of solenoid valves controlled by a ValveBank (ValveBank<sup>TM</sup> 4&8 II, AutoMate Scientific, Inc., Berkeley, USA). An electronic flow meter (Digital flow switch, PFM7 Series, SMC Corporation) set the flow rate of air entering the vial at 25 mL/min. The experiments were performed at a temperature and relative humidity respectively equal to 23 °C and 50%–60%. The room was into darkness and the wind tunnel was side-lit by an array of 11 infrared projectors (5W,  $\lambda = 850$  nm). The tunnel floor was covered with a black cloth.

**Experimental procedure** Moths were moved to the experiment room and kept in the dark at least 2 h before starting experiments to allow them to acclimatize. The experiments were carried out between 13:30 and 16:00, the period of the scotophase during which the moths are most active (Barrozo *et al.*, 2010; Force *et al.*, 2024a). For each test, we placed an animal in the cage within the wind tunnel and directly started the olfactory stimulation for 3 min. Either hexane (negative control) or 100 ng female sex pheromones were tested for D1, D3, and D5 males

fed with either one of the diets for D3 and D5. The order of moths' passage was randomized, and each moth was only tested once. A 2-min delay was observed between each test.

**Data acquisition, extraction, and processing** The behavioral response of male *A. ipsilon* to female sex pheromones is characterized by several behaviors: oriented flight (flight in zigzags against the airflow and beyond half of the tunnel), full flight (oriented flight reaching a 10 cm radius around the source vial) and landing on the vial. All these behaviors were recorded visually during stimulation. The observation of different behaviors occurred with 2 wide-field infrared-sensitivity cameras (Basler Pilot Ace, piA640–210m, Tamron 4–12F/1.2 lens) placed above the wind tunnel. Moreover, during the experiments, the cameras recorded every 10 ms the position of the moth in the 3 dimensions of space. The trajectometry software TrackIT 3D 2.0 (SciTracks, Pfaffhausen, Switzerland), used along with the cameras, allowed to reconstruct the flight trajectory (three coordinates  $x$ ,  $y$ , and  $z$ ). The cage hosting the moth was the origin point ( $x = 0$ ,  $y = 0$ ,  $z = 0$ ).

For each moth, the flight trajectory file was processed using a software developed with R 4.1.2 software (R Core Team, 2021). The trajectory was manually cleaned to keep only the oriented response, that is, the flight trajectory between the flight cage and the odor source. If during a test an individual performed oriented flight several times, only the most complete behavioral response was retained (i.e., the longest flight trajectory). After this processing, the trajectory was analyzed using an automatic segmenting algorithm that works by iterations (Data S1). This algorithm allowed the flight trajectory to be divided into a succession of well-defined segments. We obtained

the three-dimension coordinates of the trajectory at all time points, and the algorithm consists in cutting it in segments as a function of a threshold. To start, a first segment (S1), connecting the first and last points of the trajectory is created. The Euclidian distances of each point to the track is computed. The point with the distance above the threshold and the most remote from the segment S1 is used to create two new segments S2 and S3. Then, the procedure is applied for segments S2 and S3 and to any new segments created until there is no more point with a distance from a segment above the threshold. Several parameters relating to the trajectories were analyzed: the number of segments (indicating the straightness of the trajectory), the flight speed as well as the angle of turn between two consecutive segments. Male moths that did not fly or did not display an oriented response were excluded from these analyses.

### Statistical analysis

Data analysis was done using R 4.1.2 (R Core Team, 2021) and RStudio 2021.09.2 software. The sample sizes are reported in the legend of each figure. The alpha risk was 0.05. Details of the codes for performing all the analysis are reported in supporting information (Data S2–S4).

For dataset acquired by EAG, dose-response curve analysis was performed using a repeated-measurement ANOVA test (lmerTest package, 3.1-3 version, lmer function) followed by a paired Student's *t*-test (stats package, 4.2.2 version, *t.test* function corrected by Holm's method for repeated comparisons) when it was significant. Analysis of antennal response for a specific dose of odorant according to age and diet of moths was carried out by the following procedure: the assumption of normal data distribution was tested by the Shapiro–Wilk test (stats package, 4.2.2 version, *shapiro.test* function), and a Box–Cox procedure (MASS package, 7.3-58.1 version, *boxcox* function) was conducted to find a transformation when the data distribution was not normal. When this was satisfied, an ANOVA (stats package, 4.2.2 version, one-way test function) was performed, and when significant, *post hoc* tests were carried out. The choice of *post hoc* test depends on the equality of variances, tested with Bartlett's test (stats package, 4.2.2 version, *Bartlett.test* function), as well as the sample size. In case of different variances, a Tamhane test (PMCMRplus package, 1.9.6 version, *tamhaneT2Test* function) was performed; for equal variances and equal sample sizes, Tukey's test (stats package, 4.2.2 version, *TukeyHSD* function) was applied. Alternatively, if the Box–Cox procedure failed to identify a suitable transformation, data were analyzed

with a Kruskal–Wallis test (stats package, 4.2.2 version, *kruskal.test* function). If it was significant, a Dunn's *post hoc* test (DescTools package, 0.99.47 version, *DunnTest* function) was performed.

For wind tunnel-oriented response behaviors, proportion comparisons were made using a generalized linear model (logistic regression, stats package, 4.2.2 version, *glm* function). In addition, an analysis of latency times for each of these behaviors was conducted with Cox regression (survival package, 3.4-0 version, *coxph* function).

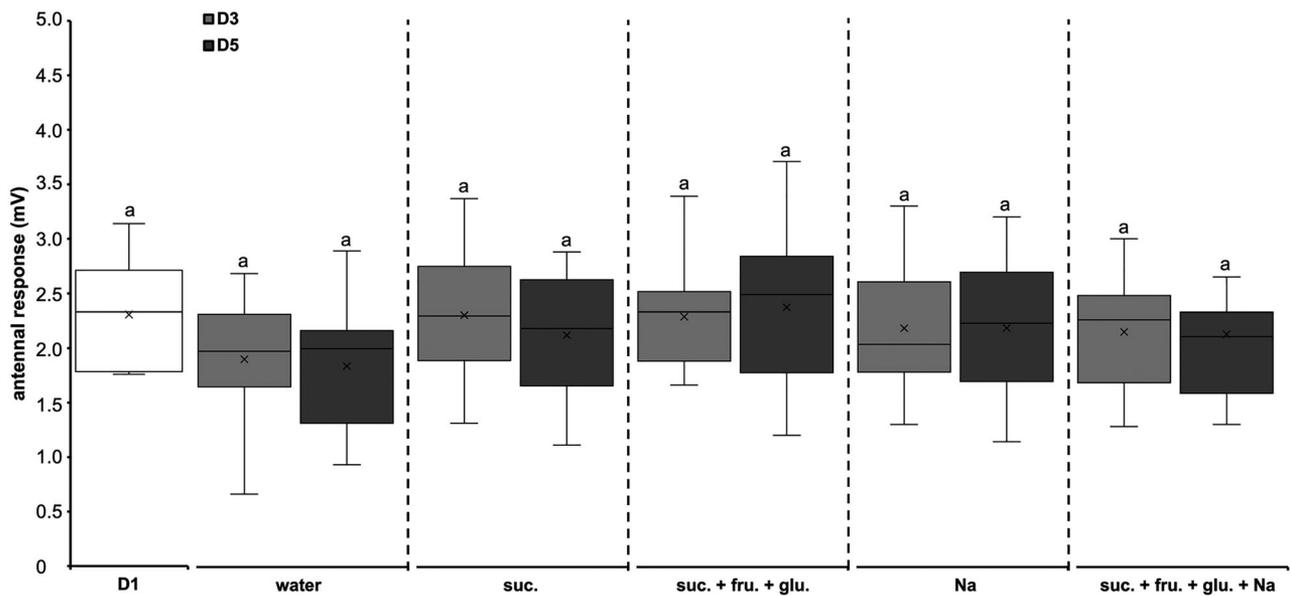
For flight trajectory analysis, the flight speed was analyzed using a mixed model (lme4 package, 1.1-31 version, *lmer* function), treating each segment as a repeated measurement within an individual. The number of segments was compared between different groups of moths using a generalized linear mixed model (quasi-poisson regression, stats package, 4.2.2 version, *glm* function). The turn angle was analyzed by a mixed Bayesian statistics model (circular regression, circular package, 0.4-95 version, *coef\_cir* function) (Cremers & Klugkist, 2018), which computes a confidence interval of the difference between the two groups. Thus, if the value 0 is outside the interval, the result is significant.

## Results

### *Sex pheromone detection is independent of diet at the antennae level*

We first assessed diet effect on the detection of geraniol. Geraniol was detected from D1 (paired Student's *t*-test,  $t_9 = 10.56$ ,  $P < 0.0001$ ) (Fig. S1). Whichever the diet, on D3 and D5, the antennal responses for geraniol were also significantly higher than paraffin oil (Tables S1 and S2) and they were identical to D1 (ANOVA test,  $F_{2,27} \leq 2.04$ ,  $P \geq 0.15$ ) (Fig. 2).

As with geraniol, 100 ng and 1 000 ng of female sex pheromones (but not 1 and 10 ng) were detected from D1 (paired Student's *t*-test,  $t_9 \geq 10.26$ ,  $P < 0.0001$ ) (Fig. S1). On D3 (paired Student's *t*-test,  $t_9 \geq 2.39$ ,  $P \leq 0.02$ ) (Table S3) and D5 (paired Student's *t*-test,  $t_9 \geq 2.96$ ,  $P \leq 0.005$ ) (Table S4), whatever the diet, 100 ng of sex pheromones was always detected compared to hexane. Moreover, for this same dose, no significant difference was observed after a comparison between diets both at D3 (ANOVA test on data transformed to the  $-0.2$  power,  $F_{4,45} = 0.14$ ,  $P = 0.967$ ) and D5 (ANOVA test on data transformed to the  $0.2$  power,  $F_{4,45} = 0.64$ ,  $P = 0.635$ ).



**Fig. 2** Antennal response after stimulation with geraniol according to age and diet. The antennal response was measured in unfed males on the first postemergence day (D1), and in males fed with various diets on the third (D3) or the fifth (D5) postemergence day. Crosses denote the mean, bars indicate the median, and boxes represent the 75% quartiles ( $n = 10$  for each group). Letters represent the statistical differences after ANOVA test ( $P < 0.05$ ).

*A diet rich in sucrose, fructose, and glucose with or without sodium induces an early behavioral response to female sex pheromones*

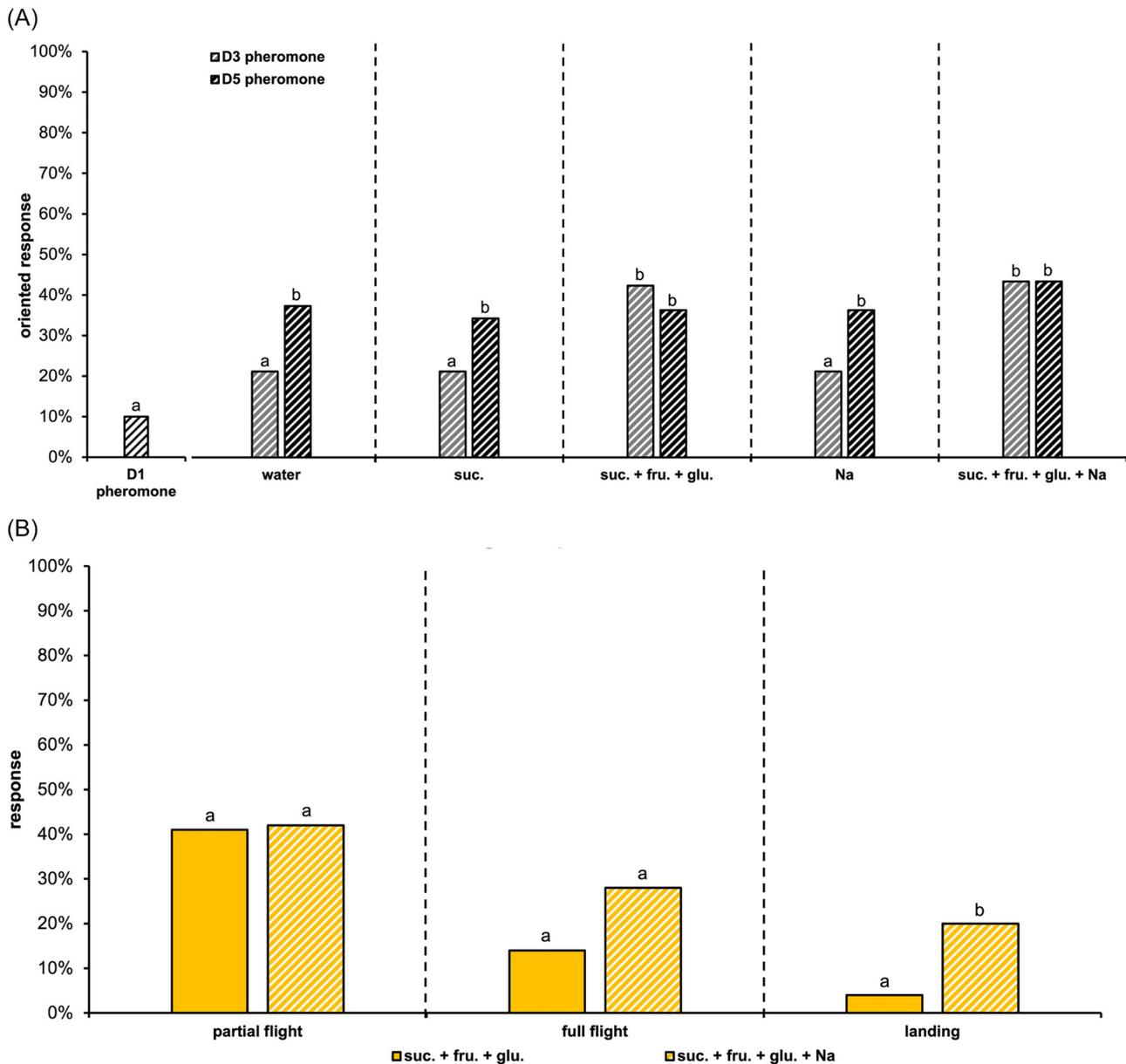
We examined the male behavioral response to the female sex pheromones according to diets during the first 5 d of adult life. Only males fed with sucrose, fructose, and glucose with or without sodium performed oriented flights from D3 (logistic regression,  $z \leq -0.63$ ,  $P \geq 0.532$ ), whereas unfed moths and moths fed with sucrose performed an oriented response at D5 (logistic regression,  $z \geq 2.68$ ,  $P \leq 0.008$ ) (Fig. 3A). Thus, D3 males fed sucrose, water, or Na alone are as reactive as D1 males, while those fed a high-sugar diet become as reactive as D5 males.

In addition, for males fed with three sugars with or without sodium, no differences were observed for partial and full flight behaviors (logistic regression,  $z \leq 1.64$ ,  $P \geq 0.100$ ), but those fed with added sodium performed more landings (logistic regression,  $z = 2.20$ ,  $P = 0.028$ ) (Fig. 3B). Regardless of the diet, approximately 96% of moths took off, half of them in 15 s (Cox regression,  $z \leq 0.85$ ,  $P \geq 0.394$ ) (Fig. S2A). Moreover, half of the moths fed with sucrose, fructose, and glucose supplemented or not with sodium performed oriented flight within 30 s (Cox regression,  $z = 0.07$ ,  $P = 0.941$ ) (Fig. S2B).

Independently of diets, D5 males displayed oriented flight (logistic regression,  $z \geq 2.97$ ,  $P \leq 0.003$ ), without any differences in behaviors (logistic regression,  $z \leq -1.04$ ,  $P \geq 0.299$ ). Additionally, approximately 98% of them took off, half in 15 s (Cox regression,  $z \leq -0.46$ ,  $P \geq 0.648$ ) (Fig. S3A). Then half of the males performed an oriented flight after an average of 50 s (Cox regression,  $z \leq 0.68$ ,  $P \geq 0.682$ ) (Fig. S3B).

*Flight parameters in response to female sex pheromones are not affected by diet*

Finally, on D3 and D5, we assessed as a function of diet several flight parameters of males performing the oriented response to female sex pheromones. D3 males fed sucrose, fructose, and glucose with or without sodium showed an identical straight trajectory (quasi-poisson glmer,  $t = 0.66$ ,  $P = 0.511$ ), a flight speed approximately equal to 70 cm/s (lmer,  $t = 1.65$ ,  $P = 0.109$ ), and turn angles around  $72^\circ$  (circular regression, [54; 68]) (Fig. 4). For D5 males, regardless of the diet, the straight trajectory was similar (quasi-poisson glmer,  $t \leq -1.89$ ,  $P \geq 0.062$ ), with a flight speed on average equal to 80 cm/s (lmer,  $t \leq 1.05$ ,  $P \geq 0.295$ ), and turn angles approximately equal to  $71^\circ$  (circular regression, [67; 108]) (Fig. 4).



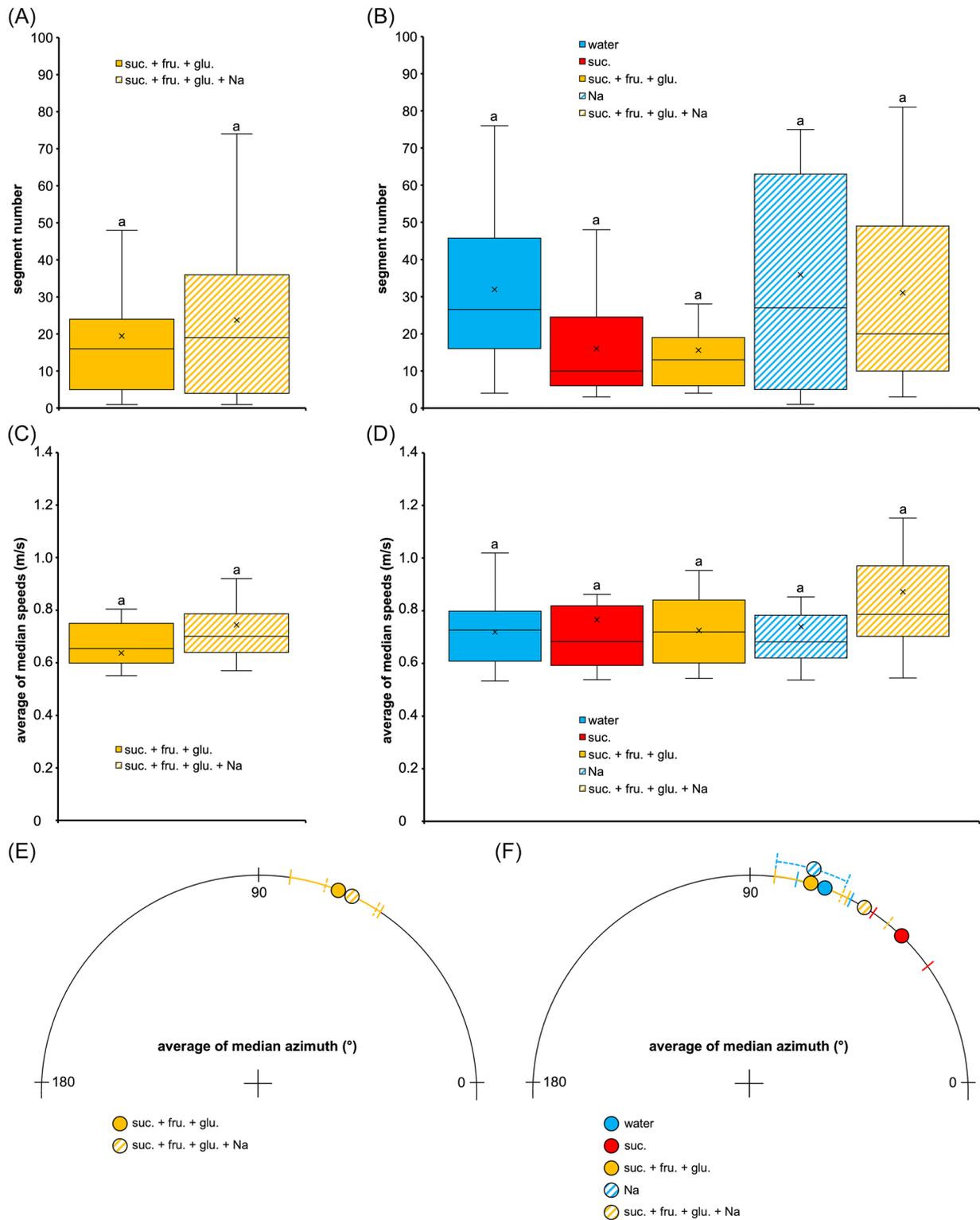
**Fig. 3** Oriented response after stimulation with 100 ng of sex pheromones according to age and diet. Values correspond to response rate. (A) Oriented response in males on the first (D1), third (D3), and fifth (D5) postemergence days. The oriented response was measured in not fed males on D1, and in males fed with various diets on D3 or D5 ( $n_{D1} = 50$ ,  $n_{water} = 50$ ,  $n_{D3/suc.} = 49$ ,  $n_{D5/suc.} = 48$ ,  $n_{suc.+fru.+glu.} = 49$ ,  $n_{D3/Na} = 50$ ,  $n_{D5/Na} = 49$ ,  $n_{D3/suc.+fru.+glu.+Na} = 50$ ,  $n_{D5/suc.+fru.+glu.+Na} = 48$ ). Letters represent the statistical differences after logistic regression ( $P < 0.05$ ). (B) Results for D3 males depending on the oriented response behaviors. Letters represent the statistical differences after logistic regression ( $P < 0.05$ ).

## Discussion

This study aimed to explore the impact of diet on the development of sexual behavior by measuring the antennal detection and the behavioral response to female sex

pheromones through the analysis of oriented flight parameters in the male *A. ipsilon*.

Our results first demonstrated that diet had no impact on the detection of geraniol (Fig. 2) and 100 ng of sex pheromones. Thus, diet does not appear to alter the



**Fig. 4** Oriented flight parameters according to diet. The oriented flight parameters were measured in males fed with various diets on the third (D3) or the fifth (D5) postemergence day ( $n_{D3/suc.+fru.+glu.} = 21$ ,  $n_{D3/suc.+fru.+glu.+Na} = 21$ ,  $n_{D5/water} = 18$ ,  $n_{D5/suc.} = 16$ ,  $n_{D5/suc.+fru.+glu.} = 18$ ,  $n_{D5/Na} = 18$ ,  $n_{D5/suc.+fru.+glu.+Na} = 21$ ). (A) Results for straightness of the trajectory in D3 males. Letters represent the statistical differences after Imer ( $P < 0.05$ ). Crosses denote the mean, bars indicate the median, and boxes represent the 75% quartiles. (B)

detection of sex pheromones at the antennal level in *A. ipsilon* males. We then observed male's behavioral response to sex pheromones according to diet. D3 water-fed males are as reactive as D1 males and become reactive from D5 (Fig. 3A). Despite the deprivation of food, this development of sexual behavior might result in the use of energy reserves accumulated during the larval stage (Plesnar-Bielak *et al.*, 2017; Angell *et al.*, 2020). Interestingly, a diet composed of sucrose led to the same results as those described in unfed males, thus suggesting sucrose is not absorbed, or not sufficiently, in the intestinal tract in *A. ipsilon*, as described in other Lepidoptera (Zibae *et al.*, 2008). By contrast, a diet composed of sucrose, fructose, and glucose promoted the development of sexual behavior between the first and third days of adult life: D3 males fed a high-sugar diet become as reactive as D5 males (Fig. 3A). It is therefore reasonable to assume that glucose and fructose are responsible for this acceleration of sexual behavioral ontogenesis by their absorption in the gut. In addition, in males fed only with sodium, the behavioral response to the sex pheromones was similar to that of unfed and sucrose-fed males; while for males fed sodium along with sucrose, fructose, and glucose, the development of sexual behavior occurred before the third day of adult life. Taken together, these data provide evidence that sodium alone does not impact the ontogenesis of male behavioral response to female sex pheromones, but that it might potentiate the stimulatory effects of a diet rich in glucose and fructose by optimizing their absorption in the intestinal tract. Otherwise, knowing that males are always able to detect 100 ng of sex pheromones independently of age and diet, the antennal detection of pheromone signal is already functional from the emergence. Consequently, it is reasonable to believe that the ontogenesis of sex pheromone-mediated oriented flight relies mainly on the maturation of the central nervous system especially within the olfactory centers involved in the processing of pheromone information.

Furthermore, D3 males fed sucrose, fructose, and glucose with sodium performed more landing behaviors (Fig. 3B). This suggests a sodium effect on the oriented flight and more particularly on the flight in proximity to the female. It has been uncovered in the moth *Helicoverpa armigera* that sodium deprivation during larval life led to a decrease in the flight speed of adults (Xiao *et al.*,

2010). Indeed, the mass of muscles involved in flight increased in larvae fed a sodium-supplemented diet, as described in the moth *Pieris rapae* (Snell-Rood *et al.*, 2014). Nevertheless, in Lepidoptera adults, the effects of sodium consumption on flight are not well understood. In our study, flight parameters (number of segments, speed, and turning angle) were not impacted by any diet (Fig. 4). This is consistent with the work of Mitra *et al.* (2016), which showed in *Battus philenor* that sodium did not affect speed, duration, distance traveled, and lift generated during the flight. However, these authors have reported that sodium was implicated in courtship behavior in the males of this species: males fed sodium took longer to circle females during courtship and tended to spend more time courting females before mating compared to males fed water. Moreover, sodium-fed males courted females with more vigor than water-fed males (Mitra *et al.*, 2016; Ankola *et al.*, 2021). Taken together with our results, these observations suggest that sodium consumption impacts the quality of male nuptial flight, which could explain the observed increase in landing behaviors near the pheromonal source, assuming male *A. ipsilon* performs courtship flight. On the other hand, several studies have reported that sugar nectar affects flight performance by increasing endurance, distance traveled and speed in some migratory species (Shirai, 2006; He *et al.*, 2021). Although we did not show any significant diet effect on the oriented flight parameters at the scale of a wind tunnel and in the specific context of reproduction, it is nevertheless possible that a sugar diet has an impact on global flight performance at a large spatial scale like during long-distance pheromone tracking or migration of the male *A. ipsilon* (Causse *et al.*, 1989).

Developmental transitions such as sexual maturation require considerable energy for organisms (Kupferberg, 1997). In Lepidoptera, this energy is provided in part by nutrients from nectar (Roy *et al.*, 2017). Additionally, some male moths practice puddling behavior from puddles of brackish water or feces for mineral element intakes such as sodium (Boggs & Dau, 2004; Molleman, 2010; Ravenscraft & Boggs, 2016; Ankola *et al.*, 2021). Sodium is involved in excretion, but also mainly in locomotion (Nicolson, 1976; Dow, 1992; Zeiske, 1992; Peyronnet *et al.*, 2000) by affecting the courtship flight performance in some species for example (Mitra *et al.*,

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Results for straightness of the trajectory in D5 males. Letters represent the statistical differences after lmer ( $P < 0.05$ ). Crosses denote the mean, bars indicate the median, and boxes represent the 75% quartiles. (C) Results for flight speed in D3 males. Letters represent the statistical differences after quasi-poisson glmer ( $P < 0.05$ ). Crosses denote the mean, bars indicate the median, and boxes represent the 75% quartiles. (D) Results for flight speed in D5 males. Letters represent the statistical differences after quasi-poisson glmer ( $P < 0.05$ ). Crosses denote the mean, bars indicate the median, and boxes represent the 75% quartiles. (E) Results for angle of turns in D3 males. Values correspond to mean  $\pm$  SEM. (F) Results for angle of turns in D5 males. Values correspond to mean  $\pm$  SEM.

2016). In addition, sodium would also have a major role in reproduction by facilitating the absorption of other nutrients in the gut (McLean & Caveney, 1993). In Lepidoptera and more broadly in insects, the assimilation of sucrose requires its hydrolysis into glucose and fructose by the activity of  $\beta$ -fructofuranosidase or invertase (EC3.2.1.26) (Eberhard *et al.*, 2007). For instance, this enzyme is present in the saliva and intestinal lumen of the butterfly *Chilo suppressalis* (Zibae *et al.*, 2008). Thus, it is also possible that male *A. ipsilon* digests sucrose through its conversion to glucose and fructose, although these sugars can also be collected directly from nectars.

In addition, it has been well described that the mesenteron, a portion of the insect intestinal tract, is the main site for nutrient assimilation (Li *et al.*, 2021), and especially glucose and fructose used in metabolism. The absorption of glucose and fructose is carried out by co-transporters with sodium located at the apical membrane of mesenteron epithelial cells (Caccia *et al.*, 2007; Bifano *et al.*, 2010; Li *et al.*, 2021). The fate of fructose in hemolymph is still unknown, although its conversion into glucose is possible. By contrast, glucose is a reducing sugar that can disrupt insect homeostasis. Thus, a low hemolymph glucose level is maintained by its conversion within fat bodies into trehalose, a nonreducing sugar (Wyatt & Kalf, 1957; Li *et al.*, 2021). Circulating trehalose is then transferred inside cells and then hydrolyzed into glucose (Zhang *et al.*, 2022). The previously mentioned catabolic pathways in energy metabolism could also operate in adult male *A. ipsilon*. In the context of the sexual behavior's development, this hypothesis is in agreement with our results obtained for males fed with sucrose, fructose, and glucose supplemented with sodium, a diet for which the quantity of glucose available, absorbed and metabolized by the organism is probably significantly higher than that provided by the hydrolysis of sucrose in the case of a sucrose-only diet for example.

Recently in several groups of insects, alongside energy and metabolic aspects related to a sugar diet, it has been shown that fasting or a variation in dietary resources leads to fluctuations in the biosynthesis of juvenile hormone (JH) (Perez-Hedo *et al.*, 2014; Watanabe & Sasaki, 2021; Bai *et al.*, 2022). Indeed glucose is the obligatory fuel for many brain activities, and it is particularly essential for the neurosecretory cells of the pars intercerebralis (Dogra & Gillott, 1971; Masumura *et al.*, 2000; Badisco *et al.*, 2013). The latter synthesizes insulin-like peptide (ILP) circulating in the hemolymph and acting on the corpora allata (CAs) (Tu *et al.*, 2005; Badisco *et al.*, 2013), a small pair of cerebral glands secreting JH. Thus, it is reasonable to hypothesize that *A. ipsilon* males fed with sucrose, fructose, and glucose with or without sodium have

a higher JH hemolymph level in comparison to both unfed males and only fed sucrose.

It has been previously revealed that the male sexual maturation of *A. ipsilon* is triggered by an increase in hemolymph levels of JH (Duportets *et al.*, 1998), a hormone known to promote the ontogenesis of sex pheromone-guided orientation flight of males by increasing the sensitivity of ALs neurons to sex pheromone signal (Gadenne & Anton, 2000; Duportets *et al.*, 2013; Bozzolan *et al.*, 2015; Gassias *et al.*, 2019; Aguilar *et al.*, 2023). Taking into consideration the above-mentioned data, we speculate that glucose-containing diets promote the development of sexual behavior through an increased JH biosynthesis which in turn facilitates the central processing of pheromone information in *A. ipsilon*. To test this hypothesis, it would be interesting to evaluate the impact of these diets on (i) the hemolymph levels of JH by reverse-phase high-performance liquid chromatography coupled with a fluorescent detector, (ii) the *in vitro* rates of JH biosynthesis of CAs by radiochemical assay, and on (iii) the activity of ALs neurons in response to sex pheromones by intracellular recordings (Duportets *et al.*, 1996, 1998). In addition, recent studies performed in *Drosophila melanogaster* demonstrated that JH is essential for the expression of male courtship behavior by regulating the sensitivity of the antennal Or47b Phe-ORN (Dweck *et al.*, 2015; Lin *et al.*, 2016). Lin *et al.* (2016) showed that this regulatory action of JH was mediated by its Methoprene-tolerant receptor through the activation of the Or47b expression. We cannot exclude the possibility that diet modulates the sensitivity of the antennal OR3 Phe-ORN in *A. ipsilon* through a peripheral JH action. Experiments are currently in progress to examine the effect of JH deprivation on both the response of OR3 Phe-ORN to sex pheromones by monosensillary recordings and the antennal expression of OR3 by real-time quantitative PCR in *Agrotis ipsilon* males fed with various sugar-rich diets.

Our study brought evidence that diet influences the orchestration of sexual maturation and especially the development of male sexual behavior in adult *A. ipsilon*. Thus, diets composed of sucrose, fructose, and glucose with or without sodium accelerate the behavioral responsiveness to female sex pheromones, probably by facilitating the central processing of pheromone information. These results are consistent with our previous work demonstrating that such diets promote the full maturation of the reproductive system promptly (Force *et al.*, 2023) with the acquisition of sexual behavior, thus optimizing reproductive fitness (Force *et al.*, 2024b). Overall, our results reinforce the hypothesis that, in nature, adult male *A. ipsilon* feeds on nectars composed of sucrose, fructose, and glucose, and practices puddling behavior for sodium intake.

Finally, our work offers new insights into the influences of nutritional intakes in the ontogenesis of male sexual behaviors in insects and might expand to other taxonomic groups, such as mammals, between which the olfactory system shares remarkable anatomical and functional similarities (Matsunami & Amrein, 2003). The next step will be to study the functional interplay between diet and the endocrine system in the development of sexual behavior.

## Acknowledgments

We thank our technical colleagues from laboratory for rearing the animals used in the experiments, and Philippe Lucas for helpful comments on the manuscript.

## Disclosure

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Data availability statement

All relevant data can be found within the article and its supporting information. Further inquiries can be directed to the corresponding authors.

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Manuscript received February 21, 2024  
 Final version received September 9, 2024  
 Accepted September 13, 2024

## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Data S1** R script for automatic segmenting algorithm.

**Data S2** R scripts for dataset acquired by EAG.

**Data S3** R scripts for wind tunnel-oriented response behaviors.

**Data S4** R scripts for flight trajectory.

**Fig. S1** Antennal response after stimulation with geraniol and sex pheromones in unfed males on the first post-emergence day.

**Fig. S2** Cumulative responses according to males fed with various diets on the third post-emergence day .

**Fig. S3** Cumulative responses according to males fed with various diets on the fifth post-emergence day .

**Table S1** Antennal response after stimulation with geraniol for males fed with various diets on the third post-emergence day .

**Table S2** Antennal response after stimulation with geraniol for males fed with various diets on the fifth post-emergence day .

**Table S3** Antennal response after stimulation with 100 ng of sex pheromones for males fed with different diets at the third post-emergence day .

**Table S4** Antennal response after stimulation with 100 ng of sex pheromones for males fed with different diets at the fifth post-emergence day .