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ORIGINAL ARTICLE

Insights on the nutritional ecology of a nocturnal pollinating insect

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Abstract Nutritional ecology examines the environmental effects on nutritional needs, food intake and foraging behaviors, and the use of nutrients ingested by animals. Adults of many insects' species feed on nectars rich in sugars allowing them to match the nutritional needs necessary for reproduction. Among insects, Lepidoptera are often considered opportunistic foragers that visit a wide variety of available flowers, although with some preferences. While nutritional ecology of diurnal Lepidoptera is beginning to be explored, very little work focuses on nocturnal species because they are complicated to study in the wild. To address this, we used new laboratory approaches to study feeding behaviors (number and duration of visits to artificial flowers, food preferences associated with the texture and odors of the flowers) as well as gustatory detection by antennae (proboscis extension reflex) in the male crop pest moth *Agrotis ipsilon*. We showed that (i) food responsiveness is age-dependent and increases mainly with sugar quantity and marginally with sugar quality, (ii) diet quality impacts feeding behaviors in the first days of adulthood, and (iii) male moths choose their food through floral cues. Taken together, these data allow to define this species as a generalist forager with a preference for flowers with sugary nectars rich in sucrose, fructose, and glucose. Our results thus provide considerable information on the close links between food sources and nutritional ecology in this species, which is important for guiding future studies on their behavioral ecology, population dynamics, as well as for population monitoring and for regional pest management.

Key words *Agrotis ipsilon*; feeding dynamics; food preferences; food responsiveness; Lepidoptera; nectar

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Introduction

Nutritional ecology is a rapidly expanding scientific domain. It examines the effects of the environment on nutritional needs, search and food intake behaviors, as well as the use of nutrients ingested by animals (Raubenheimer *et al.*, 2009). Covering numerous biological scales and exploring their interactions, nutritional ecology is particularly interested in feeding behaviors linked to the nutritional resources (Eckhardt *et al.*, 2014) through field (Machovsky-Capuska *et al.*, 2018) and laboratory (Rowe *et al.*, 2018) studies. Although this field is very active,

few studies have focused on the nutritional ecology of insect models.

Among insects, moths represent 90% of all described Lepidoptera, and they are a highly diverse group considered to be the main contributors to nocturnal pollination (MacGregor *et al.*, 2015). They are mainly active during the night (Saunders, 2002; Lazzari & Insausti, 2008; Groot, 2014), and display foraging and food consumption controlled by the circadian clock (Kim & Hong, 2015; Suszczynska *et al.*, 2017; Zhang *et al.*, 2021; Force *et al.*, 2023b). Often, Lepidoptera adults are anthophilous and have a proboscis allowing them to consume flower nectar, which composition can be roughly divided in either sucrose, fructose, and glucose or a mixture of these three sugars (Pacini *et al.*, 2003; Roy *et al.*, 2017). Many pollinators, including some moths, prefer sucrose solutions to those containing exclusively fructose or glucose (Hostachy *et al.*, 2019; Zhang *et al.*, 2023), and their antennae are more sensitive to sucrose than to glucose or fructose (Hostachy *et al.*, 2019; Zhang *et al.*, 2023). Thus, increased sensitivity for sucrose would reflect a feeding preference, which could manifest as a higher number of sucrose-rich flower visitations in the wild.

Lepidoptera are often considered opportunistic foragers that visit a wide variety of available flowers (Bąkowski *et al.*, 2010). Foraging depends in part on the depth of the corolla (linked to the effort to reach nectar) and the clustering of flowers. Moreover, the length of proboscis limits the range of flowers from which nectar can be extracted (Corbet, 2000). There is a very strong correlation between the length of corolla tube and the sucrose concentration of floral nectar (Tiedge & Lohaus, 2017): indeed, flowers with short corollas produce nectars rich in glucose and fructose with little sucrose (which make them less prone to evaporation) (Baker & Baker, 1983). Besides, increased proportion of sucrose in flowers with long corollas (which are more resistant to evaporation) also leads to a lower viscosity of the nectar; if nectar viscosity is too high, it will prevent pollinators from extracting it, ultimately making them avoiding such flowers (Jürgens, 2004). Thus, by considering the availability of the nutritional resource as the first attractant, when a Lepidoptera finds suitable floral nectar, it is reasonable to assume that it will continue to visit the flowers that produce it. Goulson *et al.* (1997) found that adults of *Thymelicus flavus* preferred certain floral nectar and constantly visited these flowers. Such a foraging strategy appears advantageous to the Lepidoptera: consistency of flower visits reduces flower handling time and thus increases the time available for other important activities, such as reproduction.

However, in a natural habitat where nectar diversity exists (in terms of energy supply), the ability of Lepidoptera to demonstrate flexibility in their food preferences is adaptive value allowing them to efficiently choose the most rewarding flowers in their current environment. Therefore, learning to quickly recognize reliable nutritional resources and to avoid flowers that do not produce nectar (or available nectar) or energy-poor nectar seems to be very advantageous, particularly in terms of lifespan and reproduction (Rusterholz & Erhardt, 2000; Force *et al.*, 2023a, 2024a, 2024b). Lepidoptera may associate physical attractants to locate floral rewards, for example, shape and texture of the flower (Wester & Lunau, 2017), as well as visual and chemical cues at longer distances. Often, moths and more broadly Lepidoptera use both flower scent and visual displays as attractants to locate their host flowers (Raguso & Willis, 2005; Stöckl & Kelber, 2019).

Studies of foraging and food preferences in diurnal insects generally employ flowers observation in the hope of recording visits. Such a method is biased toward the flower chosen for observation and is extremely difficult to apply for nocturnal insects (MacGregor *et al.*, 2015). Several studies employed the analysis of pollen found on nocturnal Lepidoptera collected from the wild to identify the flower with which they interact (Chang *et al.*, 2018), as in *Agrotis ipsilon* (Liu *et al.*, 2016). While these works give preliminary information about foraging, it is not sufficient to understand the nutritional ecology of these species. Using new experimental approaches, laboratory work focusing on feeding behaviors (number and duration of artificial flower visits, and food preferences associated with texture and odors of flowers) as well as on gustatory detection by antennae (proboscis extension reflex, PER) provides valid information for nutritional ecology (and its potential plasticity) of adult nocturnal insect. Through these experimental approaches carried out on male *A. ipsilon*, a cosmopolitan nocturnal pest of many crops for which diet is known to finely modulate its reproduction, we first showed using PER that food responsiveness was age-dependent and varied with sugar quantity and marginally with sugar quality of nectar. Then, thanks to automated feeding cages specially designed to study moth feeding behaviors over a long period, we demonstrated that diet quality impacted the feeding behaviors early in adult life, by affecting duration of foraging and floral visit, as well as the number of flower visits. Finally, through behavioral experiments based on olfactometry and the measurement of locomotor activity, we observed that male moths marginally chose nectar with various sugars thanks to floral odors but clearly

discriminated the presence of sugar in the nectar with floral texture. Our results provide information on the close links between food sources and nutritional ecology in an adult nocturnal insect, allowing us to define male *A. ipsilon* as a generalist nectar forager with, however, a preference for flowers with sugary nectars and more specifically rich in sucrose, fructose and glucose.

Materials and methods

Insects and dietary regimens

Our work was carried out on male *A. ipsilon* (Lepidoptera: Noctuidae) after their emergence. This insect is native to the south of France. The animals are raised in our laboratory at INRAE in Versailles, France. Rearing was carried out according to the protocol described by Force *et al.* (2023a). During the morning, among the emerged male moths, we collected several individuals that had been placed either in breeding boxes (20 cm × 11.5 cm × 5 cm, each containing 5 moths), in automated feeding cages (each welcoming a single moth), or in glass tubes in LAM25 (1 male per tube) according to experiments described below. The collection day was considered the first day of postemergence life (noted D1). Experiments were conducted during the first 5 d of adult life (i.e., from D1 to D5), without any mortality since fasting moths survive at least 7 d.

Just after the emergence, D1 males were provided with osmosed water or different diets according to experiments: osmosed water with sucrose only (noted suc.) or sucrose, fructose and glucose (noted suc. + fru. + glu.). We focused on these three sugars as they are the major constituents of flower nectar, the main food of adult moths, with typically 10%–15% of the fresh mass for each sugar, possibly up to 30% for sucrose (Roy *et al.*, 2017). We excluded fructose and glucose-only diets because it has been showed in males *A. ipsilon* that the antenna gustatory responsiveness to these sugars is at a very low level compared to sucrose alone or the mixture of sucrose + fructose + glucose. Thus, males would not consume fructose and glucose alone (Hostachy *et al.*, 2019). Moreover, the nectar sucrose-to-glucose/fructose ratio was observed to be higher in certain night flowering plants (Tiedge & Lohaus, 2017). Knowing that *A. ipsilon* is a nocturnal insect, it is possible that males consume nectars in which sucrose is the dominant sugar and that its presence is crucial for flower nectar intake. All sugars come from Sigma-Aldrich (Saint-Quentin-Fallavier, France).

Proboscis extension reflex (PER) to study food responsiveness

Newly emerged adults were collected during the morning and grouped by 5 in breeding boxes (20 cm × 11.5 cm × 5 cm) with *ad libitum* access to osmosed water and stored in oven at controlled conditions (23 °C, 65% relative humidity, 16 L : 8 D photoperiod). Experiments were conducted under dim red light and started in the middle of the scotophase, when moths are the most active (Force *et al.*, 2023b), under a temperature of 23 °C and 50%–60% relative humidity. Experiments were performed on day 1 (D1), day 3 (D3) and day 5 (D5) postemergence. On the morning of the experiments, animals were tethered in plastic tubes made from 1 mL pipette cones cut to fit a moth, so only the head (with antennae and proboscis) protruded. Their position was further assured by inserting a small ball of absorbing paper or cotton behind them in the tube and fixing the top of their body to the tube using adhesive tape. Legs were blocked by tape or inside the tube. Once restrained, moths were left to acclimatize in the experimental room until the beginning of the experiment.

Assessing sugar responsiveness consisted in presenting solutions of increasing concentration with a 10-min interval between each presentation. A solution presentation consisted in touching both antennae of the moth with a wooden toothpick imbibed with the sugar solution for 1–4 s. A response was recorded if the moth released a PER, that is, extended its proboscis beyond its position at the onset of the stimulation (the initial position varies from one animal to the other, but the occurrence of the proboscis extension can be observed unambiguously). Moths were not fed, and great care was taken to avoid touching the proboscis with the toothpick coming from behind the head. We defined PER rate as the proportion of animals releasing a PER upon presentation of sugar solution on the antennae.

Sugar solution concentrations were 0% (i.e., osmosed water), 0.1%, 0.3%, 1%, 3%, 10%, and 30% (weight/weight in osmosed water, for example, 30% is 30 g of sugar in 70 mL of osmosed water). These concentrations correspond to 0.1%, 0.4%, 1.1%, 4.3%, 11.1%, and 42.9% weight/volume, respectively. After the presentations of all sugar solutions, another presentation of the 0% solution was made after wise to monitor the occurrence of sensitization, that is, the fact moths might start to respond to weak stimuli such as 0% solution just because they are excited by previous sugar presentations. This set of concentrations is inspired by previous studies in moths (Hostachy *et al.*, 2019) and corresponds to a logarithmic increase in the stimulus intensity (i.e., around -1 , -0.5 ,

0, 0.5, 1, 1.5), consistently with Weber's law in sensory physiology (Shafir & Yehonatan, 2014). Furthermore, sucrose is the reference sugar used in PER experiments in various insects (Scheiner *et al.*, 2013). However, it is well known that nectar can also include fructose and glucose in different proportions. Thus, we measured responsiveness for mixtures between these three sugars. The same concentrations were used with sugars in equal amounts (i.e., a 3% sucrose/fructose/glucose solution contains 1% sucrose, 1% fructose and 1% glucose weight/weight).

Automated feeding cages to study feeding behavior and hemolymph volume quantification to estimate dynamic food consumption

Feeding dynamic monitoring of male *A. ipsilon* was carried out using automated feeding cages, already used for a previous study on feeding behaviors in adults of this same species (Force *et al.*, 2023b). Each of the cages was equipped with a passive feeder enclosed in a transparent plastic circular box 5 cm in diameter and 2.5 cm in height, large enough to avoid constraining moth locomotion (wing deployment and on-site flight possible). The passive feeder consisted of a 30 mm diameter polyamide disc pierced in its center with an orifice (6 mm in diameter and 12 mm long), and a beveled tunnel 10 mm wide at the outside and 6 mm wide in the center. It was covered with a transparent plastic sheet. In addition, in the center of the tunnel, an infrared motion sensor was set and triggered as soon as the moth entered the artificial flower (for device pictures, see Force *et al.*, 2023b). This device did not allow food consumption to be quantified. However, the entrance to the feeder was used as a proxy for food consumption. Most nectarivorous Lepidoptera are opportunistic flower visitors and probe flowers of different sizes and shapes (Gilbert & Singer, 1975; Krenn, 2010). *A. ipsilon* has a short proboscis, the length of which is approximately half the total length of the insect. The morphology of its proboscis seems adapted to feeding from short-corolla flowers as suggested by studies in other moths (Krenn, 2010). Thus, the shape of the passive feeder imitating a flower with a short corolla seems well suited to accessing food in the center of the device, probably encouraging *A. ipsilon* to enter the artificial flower. These new devices were validated to measure specifically feeding behavior (number and duration of flower visits) without bias from global locomotor activity of moths (Force *et al.*, 2023b).

We had five automated cages operating in parallel, which were in a chamber at controlled conditions (23 °C, 50%–60% relative humidity, 16 L : 8 D photoperiod).

Each of the cages was occupied by a single individual. The cages were used for feeding dynamic monitoring, by measuring specifically the number of visits as a function of time as well as the duration of each visit, with the infrared sensor inside the artificial flower. While the actual consumption is not directly measured, we assumed these parameters are correlated to it. These experiments were performed on moths during the first 5 d of adult life (from D1 to D5). Moths had *ad libitum* access to osmosed water or one of the following diets: sucrose 12%; or 4% sucrose with 4% fructose and 4% glucose. The sugars represent 12% of the fresh mass, whether for a single sugar or a mixture of several sugars, mimicking the majority of flower nectars foraged by adult Lepidoptera (Pacini *et al.*, 2003; Roy *et al.*, 2017).

Data were recorded as text on a micro-SD card. The management of the experimental process was carried out automatically by a microcontroller (P8×32A) equipped with a clock making it possible to annotate each event with a date/time. At the end of an experimental session, data were sent to a computer with a USB wire using Termites RS232 terminal software.

While the food intake is not directly measured by automated feeding cages, we assessed the hemolymph volume (fairly constant throughout life but possibly increased by food intake, particularly by water composing the nectar) as an indicator of dynamic food consumption. Measurements of hemolymph volume were carried out in adult males aged 1, 3, and 5 d postemergence, having *ad libitum* access to water or 12% sucrose. To collect the hemolymph, the top of the moth's head was cut and the entire animal was placed in a 0.25 mL Eppendorf tube with the pellet previously cut (head of the moth was oriented toward the open pellet). This tube was then placed in a 1.5 mL Eppendorf tube and then centrifuged at 1 rcf for 3 min. At the end of the centrifugation, the volume of the hemolymph recovered in the base of the 1.5 mL Eppendorf tube was measured.

Locomotor activity monitor (LAM) system and Y-tube olfactometer to study feeding preferences

The assessment of feeding preferences was carried out using two experimental designs. The first performed with Locomotor Activity Monitors (Model LAM25, Trikinetics Inc., MA, USA) was based on discrimination of diet with a texture; it is known that physical floral traits (size, shape, texture) are parameters involved in insect foraging (Riffell & Alarcón, 2013). Then, a second experimental device employing a Y-tube olfactometer was used to evaluate feeding preferences between different sugary

diets in association with a flower odor; insects including moths can associate a floral odor with nectar (Cunningham *et al.*, 2004).

The LAM25 system consisted of a data collection computer, power interface unit PSIU9, activity monitors, tubes with dipped cotton on the ends, and an incubator (23 °C, 50%–60% relative humidity, 16 L : 8 D photoperiod). LAM25 and Power Interface Unit PSIU9 come from Trikinetics Inc. (MA, USA). LAM25 monitoring unit has 32 independent channels (25 mm in diameter), into which polymeric methyl methacrylate (PMMA) sample tubes with an inner diameter of 2.1 cm and a length of 10 cm fit. The PPMA tubes positioned in the first LAM25 are linked with another PPMA tubes in a second LAM25 by perforated Plexiglas tubes (ensuring ventilation) of the same diameter and a length of 5 cm; and formed recording tubes occupied by a single individual. The ends of recording tubes were obstructed by cotton dipped with different nutrient solutions (Fig. 6A). In addition, at each end and 1 cm from the cotton dipped with nutrient solution, the walls of the recording tube are covered for 2 cm with sandpaper (rough area) or with paper (smooth area). This experimental device was used for feeding preference monitoring linked to the texture, by measuring specifically the locomotor activity in proximity to cotton soaked in nutrient solution. While the food consumption is not directly measured, we assumed this parameter is correlated to it. Thus, the locomotor activity close to cotton soaked in nutrient solution was used as a proxy for food consumption. The moth locomotor activity in each tube was measured using infrared beams and sensors. The experiments were performed on naïve moths just emerging during the first 5 d of adult life (from D1 to D5), and in each tube, a pair of nutrient solutions (available *ad libitum*) were given to determine dietary preferences: (1) A cotton soaked with a 12% sucrose solution, and the other was dipped with a 12% sucrose (control group with dietary regimen used in standard rearing condition); (2) A cotton soaked with water, and the other was dipped with a 12% sucrose solution; (3) A cotton soaked with 12% sucrose solution, and the other was dipped with 4% sucrose + 4% fructose + 4% glucose;

LAM25 monitors were connected to the computer with the PSIU9 power interface unit. The DAMSystem3 (MA, USA) data collection program was configured to collect 1-min bin data from each locomotor activity monitor and save the data on a hard drive.

Experiments of feeding preferences regarding floral odors were performed with a Y-tube olfactometer. The olfactometer consisted of a 2.1 cm inner diameter glass Y-tube, the main segment was 13 cm long, and each of the two arms was 9.5 cm long. Charcoal-purified air was

delivered into each arm (flow rate of 0.5 L/min) and was stabilized by a flowmeter (Key Instruments, PA, USA) to ensure identical incoming air flow in each arm. All experiments were performed during the scotophase (between 1:00 p.m. and 3:30 p.m.) under a red light in a room maintained at 23 °C with 50%–60% relative humidity. The experimental set-up was first tested in 3-d-old (D3), starved and naïve male moth *A. ipsilon* with the following controls: (i) a configuration expected to induce no moth choice with paraffin oil in each arm; (ii) in one arm 100 µg of *cis*-3-hexenyl acetate ($\geq 95\%$ purity, CAS 3681-71-8), a floral volatile organic compounds (VOC) detected by the male moth *A. ipsilon* (Rouyar *et al.*, 2015) and paraffin oil in the other arm; (iii) 100 µg of heptanal ($\geq 95\%$ purity, CAS 111-71-7), another floral VOC detected by *A. ipsilon* male (Rouyar *et al.*, 2015) versus paraffin oil; (iv) 100 µg of *cis*-3-hexenyl acetate in one arm and 100 µg of heptanal in other arm. Floral odorants were diluted in paraffin oil at a concentration equal to 100 µg/µL; all chemicals came from Sigma Aldrich (Saint-Quentin-Fallavier, France). 1 µL of odorant or paraffin oil was loaded on filter paper. A paper with paraffin oil was placed in one arm and a second filter paper with the floral odorant dilution in the other arm. Moreover, 1 adult moth was placed at the end of the main olfactometer segment and its behavior was observed during 1 min. We considered that the moth made a choice when it entered an arm. Each moth was tested only once. To avoid any bias during experiments, the olfactometer was flipped from side to side after 3 tests. Furthermore, after 6 tests, the olfactometer was washed with TFD4 detergent (Franklab, Montigny-le-Bretonneux, France) diluted to 3% for 30 min, then rinsed with distilled water.

Following previous controls, we assessed the sugary feeding preferences regarding floral odors for D3 male *A. ipsilon*, which had been reared, from emergence, inside a box (40 cm × 20 cm × 5 cm). This rearing device contained 12 males and was placed under controlled conditions (23 °C, 50%–60% relative humidity, 16 L : 8 D photoperiod). On one short side of the box, a filter paper with 1000 µg of *cis*-3-hexenyl acetate was deposited and encircled by (i) water (mimicking nonsugar nectar) or (ii) a 12% sucrose solution available *ad libitum*. On the other short side, a filter paper with 1000 µg of heptanal was deposited, encircled by (i) a 12% sucrose solution or (ii) a sugary solution composed of sucrose 4% + fructose 4% + glucose 4% also available *ad libitum*. After 2 d of rearing during which associative learning occurred between sugar solution and floral odor, each D3 male was tested in a Y-tube olfactometer as described above with a filter paper with 100 µg of *cis*-3-hexenyl acetate in one arm and a second paper with 100 µg of heptanal in the other arm.

Data processing and analysis

Statistical data analysis was performed using R 4.1.2 (R Core Team, 2021) with RStudio 2021.09.2. The sample sizes are reported in the legend of each figure, * $P < 0.05$.

For each sugar concentration in a dose-response plot, PER rates were compared with a mixed-effects logistic regression on repeated measurements (generalized linear mixed-effects model, lme4 package, 1.1-31 version). Moreover, for certain sugar concentrations, PER rates were compared using logistic regression (generalized linear model, lme4 package, 1.1-31 version) to assess the effect of diet quality.

For data acquired from automated feeding cages (assessing the feeding behavior), analysis of the day-to-day visit number within the same cohort was performed by Friedman's tests (stats package, 4.2.2 version) followed by Nemenyi's *post hoc* tests (DescTools package, 0.99.47 version). Comparisons between cohorts were performed using Wilcoxon's sum of rank tests. Fisher-Snedecor's test (stats package, 4.2.2 version) was used to compare the variance of visit durations inside the artificial flower. In addition, we carried out an analysis of the distributions of visit durations by using a Poisson mixed-model (lme4 package, 1.1-31 version). The durations were binned as previously determined by Force *et al.* (2023b): equal to 1 s, between 2 and 20 s, between 21 and 70 s, and finally more than 71 s up to 200 s (duration from which we considered that the moth was in a state of rest). Furthermore, Cosinor model (DiscoRhythm package, 1.20.0 version) (Carlucci *et al.*, 2019) was used out to analyze the circadian rhythm (period, phase, and amplitude) of the daily visits of the artificial flower for each cohort.

For the hemolymph volume data, the assumption of normal data distribution was tested by the Shapiro-Wilk test (stats package, 4.2.2 version). When this was satisfied, an ANOVA (stats package, 4.2.2 version) was carried out, and when it was significant, a Tukey's test (stats package, 4.2.2 version) was conducted. Alternatively, nonnormal data were analyzed with a Kruskal-Wallis' test (stats package, 4.2.2 version) followed, if it was significant, by a Dunn's *post hoc* test (DescTools package, 0.99.47 version).

For data acquired from LAM25, analysis of locomotor activity close to nutrient solutions (considered as a proxy of food preferences) according to days and for each pair of dietary regimens was performed by a logistic regression on repeated measurements (generalized linear mixed-effects model, lmerTest package, 3.1-3 version). A logistic regression (generalized linear model, lme4 pack-

age, 1.1-31 version) was used to analyze the behavioral data acquired from Y-tube olfactometer.

Ethics note

French legislation for animal welfare is based on that of the European Union (directive 2010/63/EU) according to which no invertebrate other than cephalopods benefit from ethical protection. However, all the experiments were carried out with care, limiting stressful situations for the animals as much as possible.

Results

Food responsiveness depends on age and increases mainly with sugar quantity and marginally with sugar quality

Food responsiveness to sugary diets is age-dependent We assessed food responsiveness by recording the occurrence of PER after antennal stimulation (following Hostachy *et al.*, 2019) in *A. ipsilon* males at 1 (D1), 3 (D3), and 5 d (D5) of adult life (Fig. 1). We touched their antennae with either one of artificial nectar solutions representing the various floral nectars possibly encountered in nature. The response threshold (i.e., concentration for which PER rate is significantly higher than for water alone) decreased with age and was linked to sucrose quantity. For D1 males, the response threshold was equal to 1% for sucrose (mixed-effect logistic regression, $z = 2.04$, $P = 0.041$) and 3% for sugar mixture (1% sucrose + 1% fructose + 1% glucose) (mixed-effect logistic regression, $z = 2.57$, $P = 0.009$). For D3 males, these thresholds were reduced to reach respectively 0.3% (mixed-effect logistic regression, $z = 2.76$, $P = 0.006$), and 1% (about 0.3% for each sugar) (mixed-effect logistic regression, $z = 3.33$, $P < 0.001$); then for D5 males, the response threshold remained equal to 0.3% for sucrose (mixed-effect logistic regression, $z = 1.88$, $P = 0.049$), while it decreased further to reach 0.3% for sugar mixture (0.1% sucrose + 0.1% fructose + 0.1% glucose) (mixed-effect logistic regression, $z = 2.29$, $P = 0.022$).

Although spaced out over time, the succession of stimulations by sugar solutions at increasing concentrations can be accompanied by a nonassociative learning phenomenon: sensitization. This is why we also measured the PER after a final stimulation with water at the end of each series of experiments for males aged 1, 3, and 5 d post-emergence. A nonassociative learning phenomenon was observed for males stimulated with sucrose (Fig. S1A)

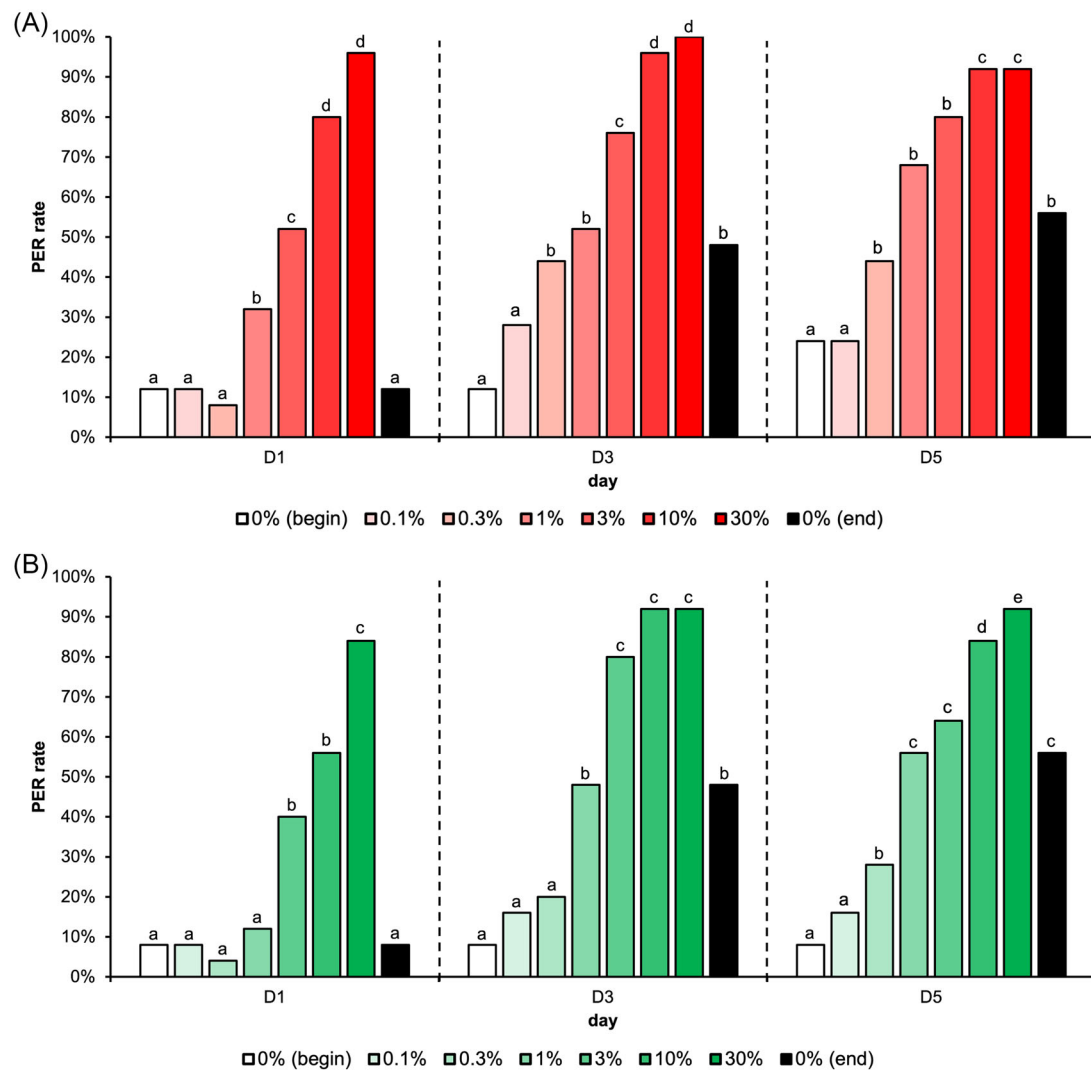


Fig. 1 Effect of sugar concentration on food responsiveness. Proboscis extension reflex (PER) rates were measured on the first (D1), the third (D3), or the fifth (D5) day of adult life ($n = 25$ for each cohort). Letters represent the statistical differences after a logistic regression. For each tested day and regardless the nutrient solutions, the plots present the dose-responses for sucrose concentrations (red bars) (A), for mixture of sucrose + fructose + glucose concentrations (green bars) (B).

and sucrose + fructose + glucose from the third day of adult life (Fig. S1B) (mixed-effect logistic regression, $z \geq 2.19$, $P \leq 0.028$).

Food responsiveness to sugary diets seems to vary according to their quality After examining the effects of the sugar quantity in nectars for males aged 1, 3 and 5 d of adult life, we studied the impact of sugar quality (i.e., sugar composition) on food responsiveness in the previous data. Here, we only considered nectars containing 1% or 3% sucrose, that is, sucrose either alone or in a mixture with fructose + glucose (when animals release a

PER often enough). No statistical difference in response was observed after stimulation of the moths with 1% sucrose or 1% sucrose + 1% fructose + 1% glucose (logistic regression, $z \leq 0.96$, $P \geq 0.337$) (Fig. 2A), except on the third day postemergence where response was significantly increased for the sugar mixture (logistic regression, $z = 2.23$, $P = 0.026$). Similarly, food responsiveness did not vary after the stimulation of moths with sugar solutions concentrated at 3% sucrose or approximately 3% sucrose + 3% fructose + 3% glucose, that is, the 10% sugar solution (logistic regression, $z \leq 1.58$, $P \geq 0.115$) (Fig. 2B). However, although the sugar quality of nectar

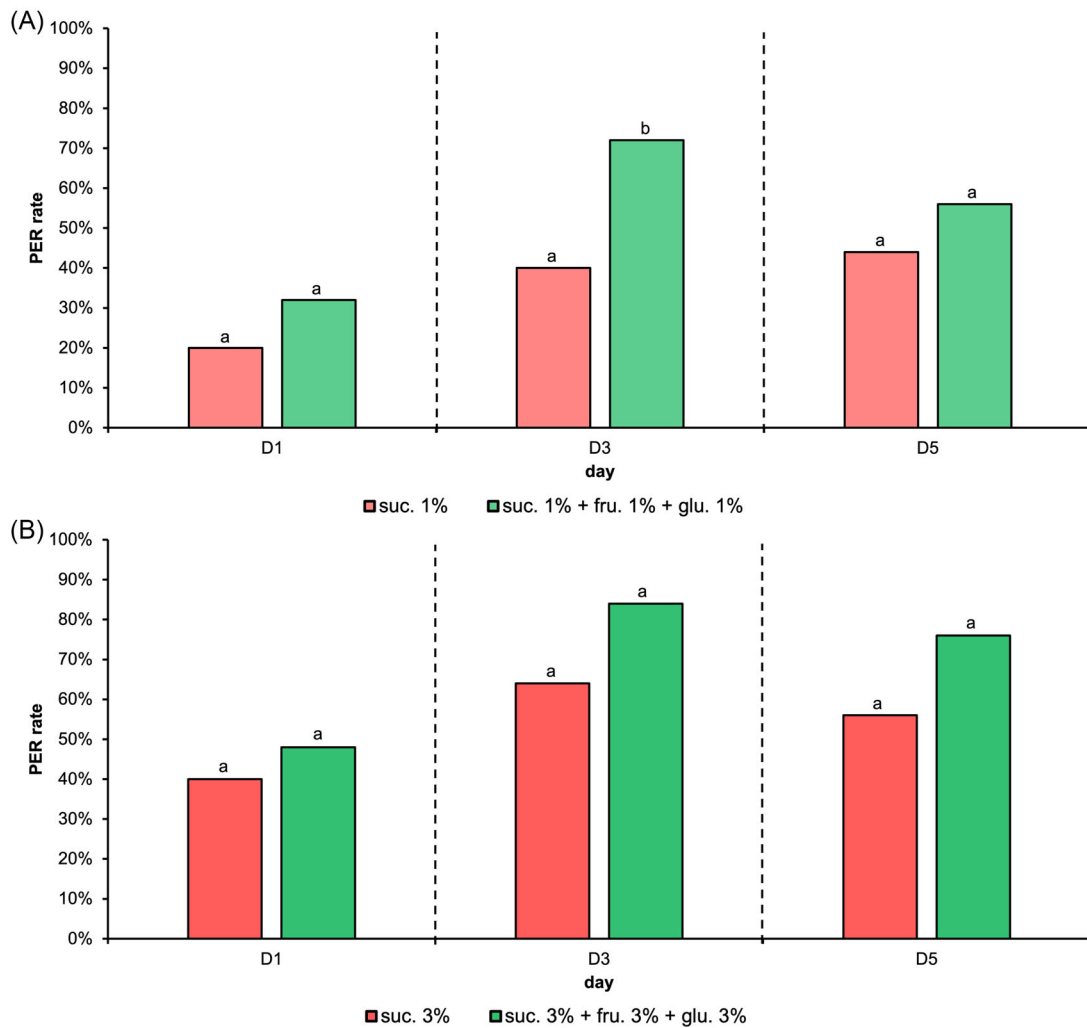


Fig. 2 Effect of sugar quality on the proboscis extension reflex (PER) in male *Agrotis ipsilon*. The PER rates were measured on the first (D1), the third (D3), or the fifth (D5) day of adult life ($n = 25$ for each cohort, same data as in Figure 1). (A) For each tested day, the plots present the PER rates for a sugar solution either rich in 1% sucrose (red bar), either in mixture of 1% sucrose + 1% fructose + 1% glucose (green bar). (B) For each tested day, the plots present the PER rates for a sugar solution either rich in 3% sucrose (red bar), either in mixture of 3% sucrose + 3% fructose + 3% glucose (green bar). Letters represent the statistical differences after a logistic regression.

did not globally impact food responsiveness, the presence of fructose and glucose increased the gustatory responsiveness by average 10% to 20%.

Diet quality impacts the feeding behaviors in the first days of adulthood

Diet impacts the flower visit duration Feeding dynamic monitoring of male *A. ipsilon* was carried out using automated feeding cages, already used in a previous study on feeding behaviors in this species (Force *et al.*, 2023b).

We first compared the distribution of visit durations as well as the global visit duration over the 3 d postemergence according to diets available in the artificial flower (Fig. 3A, B). Moths having access to a sugary diet frequently visited the flower between 1 and 2 s (Poisson mixed-model, $\chi^2 = 9.04$, $P = 0.011$), while males having access to only nonsugar nectar (water) stayed more frequently between 70 and 200 s inside the flower (Poisson mixed-model, $\chi^2 = 3.86$, $P = 0.041$) (Fig. 3A). Similarly, a comparison of global durations indicated that males with access to nonsugar nectar visited flowers longer than those able to collect a sugary diet (Fisher-Snedecor's test,

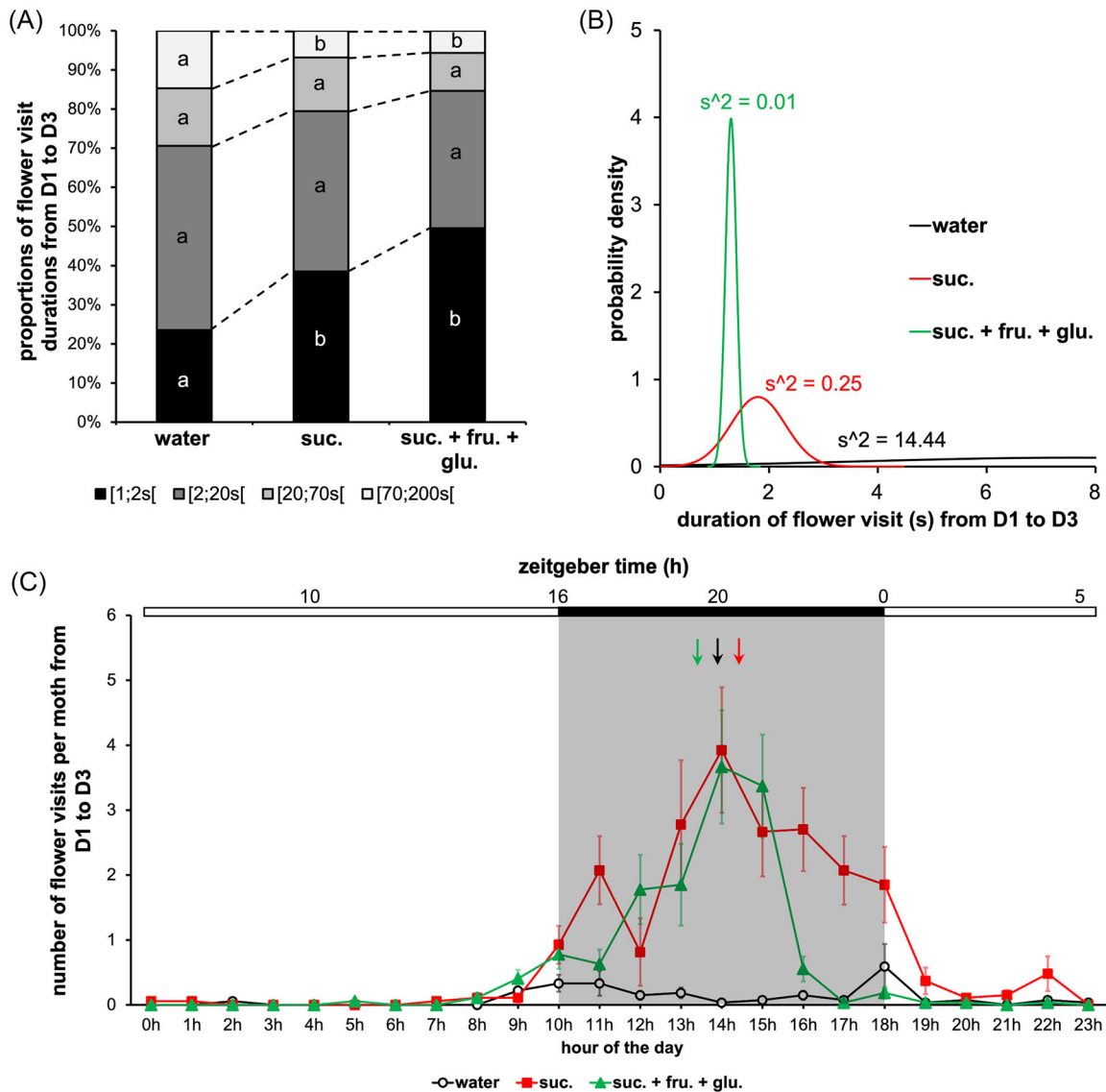


Fig. 3 Feeding behavior is regulated by the quality of nectar available. The experiment was carried out over 3 d from emergence with a photoperiod 16 L : 8 D ($n = 9$ for each cohort). (A) Distribution of flower visit durations (data from D1 and D3 are pooled) for males fed with a diet consisting in either water (nonsugar nectar), 12% sucrose, or 4% sucrose + 4% fructose + 4% glucose. Letters indicate the statistical differences after a Poisson mixed-model. (B) Duration of flower visit from D1 to D3 according to the cohorts water (nonsugar nectar), 12% sucrose, and 4% sucrose + 4% fructose + 4% glucose (black line, water; red line, sucrose; green line, mixture of three sugars). Curves represent normal laws fitted to the data (s^2 is the variance). A Fisher-Snedecor's test was performed to analyze the variance of data. (C) Mean visit number per moth for the cohorts water (nonsugar nectar), 12% sucrose, 4% sucrose + 4% fructose + 4% glucose, according to the time of day, the 3 d of experimentation are cumulated (black line, water; black arrow, phase for water; red line, sucrose; red arrow, phase for sucrose; green line, mixture of three sugars; green arrow, phase for mixture of three sugars). Values correspond to the mean \pm SEM. Cosinor model was performed to analyze the circadian rhythms.

$F_{5,7} = 45.15$, $P < 0.001$) (Fig. 3B). More surprisingly, moths having access to a nectar composed of sucrose also visited flowers longer than those able to collect the sugar mixture (Fisher-Snedecor's test, $F_{7,7} = 45.38$, $P < 0.001$) (Fig. 3B).

Flower visit occurs during scotophase and follows a diet-dependent circadian rhythm We then focused on the number of flower visits during the day (Fig. 3C). Regardless of diet, we found that feeding behavior followed a circadian rhythm (period approximately equal to

24 h, Cosinor model, $P < 0.008$) with visits limited to scotophase, and a phase between 1 and 3 p.m. (Cosinor model, $P < 0.008$). Although the period and phase of the rhythm were not affected, the amplitude (i.e., the duration of the foraging period during a day) was significantly impacted by the diet available in the flower (Fig. 3C). Indeed, the rhythm of moths having access to sucrose had a high amplitude approximately equal to 12 h; whereas those able to access sucrose + fructose + glucose had an amplitude in average equal to 9 h (Cosinor model, $P < 0.001$). Then, the amplitude of the rhythm was very low (equal to 1 h) for moths with access to water (Cosinor model, $P = 0.008$).

Feeding dynamics during adult life depend on diets

While the previous analyses were carried out over the first 3 d of adult life, the comparison of feeding dynamics according to the diet (on the same animals) was carried out up to the 5 d postemergence (Fig. 4A–C). We observed that moths having access to nonsugar nectar (water) or nectar rich in sucrose performed constant daily visits over the 5 d of experimentation (Friedman's test, $\chi^2 \leq 2.08$, $P \geq 0.721$) (Fig. 4D). By contrast, moths fed a diet composed of sucrose + fructose + glucose showed a decrease number of visits from the third day of adult life (Friedman's test, $\chi^2 = 9.55$, $P = 0.048$). For this reason, the previous analyses of duration and circadian occurrences of flower visits were restricted to the first 3 d. A comparison between the three cohorts reported that the flower visit number was identical on the first day of adult life (Wilcoxon's test, $W \leq 59.50$, $P \geq 0.087$) (Fig. 4D). In addition, moths with nonsugar nectar visited the flower less on the second day postemergence compared to those with access to a sugary diet (Wilcoxon's test, $W = 72.00$, $P = 0.006$); although it was not statistically significant, this trend was also noticeable for the third (Wilcoxon's test, $W = 60.50$, $P = 0.079$) and the fourth day of experiment (Wilcoxon's test, $W = 60.00$, $P = 0.084$). Furthermore, on the fifth day of adult life, only the moths having access to sucrose showed a much higher flower visit number than those of the moths having access to nonsugar nectar or nectar rich in three sugars (Wilcoxon's test, $W \geq 66.00$, $P \leq 0.026$) (Fig. 4D).

As entering the feeder was used as a proxy for food consumption, we estimated the consumption dynamic according to diet by measuring the hemolymph volume (fairly constant throughout life but possibly increased by food intake, particularly by water composing the nectar) (Fig. S2). The hemolymph volume for adult males fed with the sucrose diet increased between D1 and D3 (Tukey's test, $P = 0.007$) and remained constant after D3 (Tukey's test, $P = 0.998$), which was consistent with

previous results showing a very significant number of flower visits from the first day of adult life. Nevertheless, D3 males fed water had a hemolymph volume similar to D1 and D5 males (Dunn's test, $P \geq 0.257$), although D1 and D5 males differed (Dunn's test, $P = 0.017$). This reflected a slower increase in the volume of hemolymph over the 5 d postemergence, probably resulting from the low frequency of floral visits as described above.

Male moths choose their food through floral cues

Male moths appear to choose nectar composed of various sugars regarding floral odors The assessment of feeding preferences was carried out using two experimental designs. A first assay performed with Locomotor Activity Monitors (LAM) was based on discrimination of diet according to an associated flower odor: we studied the possible association between nectar and floral odors in feeding choices in 3-d-old male *A. ipsilon* (Fig. 5). Although *cis*-3-hexenyl-acetate and heptanal are detected by male *A. ipsilon* moths (Rouyar *et al.*, 2015), these two floral odors did not cause any attraction or repulsion of animals (logistic regression, $z \leq -0.36$, $P \geq 0.718$) (Fig. 5A–D). However, after training moths in the presence of these neutral odors with different nectars, neither odor clearly induced an attractive response. Indeed, moths trained with either *cis*-3-hexenyl-acetate associated to nonsugar nectar (water) or heptanal associated to 12% sucrose nectar didn't preferentially chose odor after exposure (logistic regression, $z = -0.80$, $P = 0.427$) (Fig. 5E). Nevertheless, although this was not statistically significant, moths trained with *cis*-3-hexenyl-acetate associated to 12% sucrose nectar and heptanal to 4% sucrose + 4% fructose + 4% glucose nectar seemed to preferentially choose heptanal after exposure to both odors (6% for *cis*-3-hexenyl-acetate against 28% for heptanal) (logistic regression, $z = -1.63$, $P = 0.104$) (Fig. 5F).

Male moths discriminate the presence of sugar in nectar with floral texture Finally, we were interested in the possible discrimination of nectar by flower texture during the first 5 d of the adult life of male *A. ipsilon* (Fig. 6). Although moths are neither influenced by their spatial environment (Fig. 6B) nor preferentially attracted by a texture (logistic regression, $t_{139} = 0.70$, $P = 0.483$) (Fig. 6C), they are nevertheless capable of discriminating texture in the presence of sugars in the nectar: moths subjected to water and a nectar composed of 12% sucrose have preferentially chosen the sugary solution during the first and second days of adult life (logistic regression, t_{135}

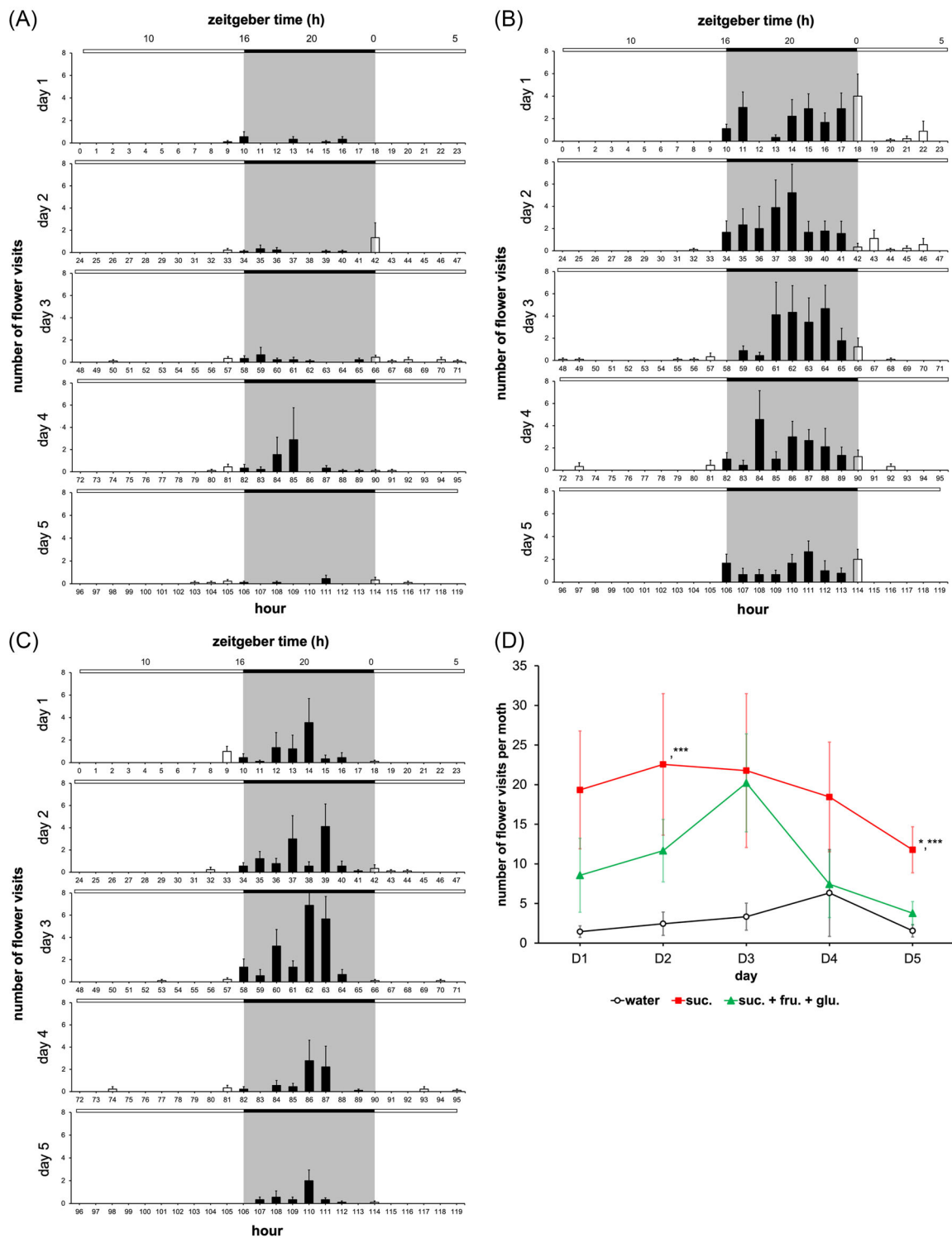


Fig. 4 Feeding dynamic in male *A. ipsilon* according to diet in the artificial flower. The experiment was carried out over 5 d from emergence ($n = 9$ for each cohort, values correspond to the mean \pm SEM). Diagram plots for water (nonsugar nectar) (A), 12% sucrose (B), for 4% sucrose + 4% fructose + 4% glucose (C) solution available during the 5 d of experimentation (full bar, flower visit during scotophase; empty bar, flower visit during photophase). (D) Mean visit number per moth for water (nonsugar nectar), 12% sucrose and mixture of 4% sucrose + 4% fructose + 4% glucose cohorts depending on the days (black line, water; red line, sucrose; green line,

mixture of three sugars). A Nemenyi's test was performed to compare the visit number within a same cohort. A Wilcoxon's test ($*P < 0.05$; $***P < 0.001$) was performed to compare the three cohorts.

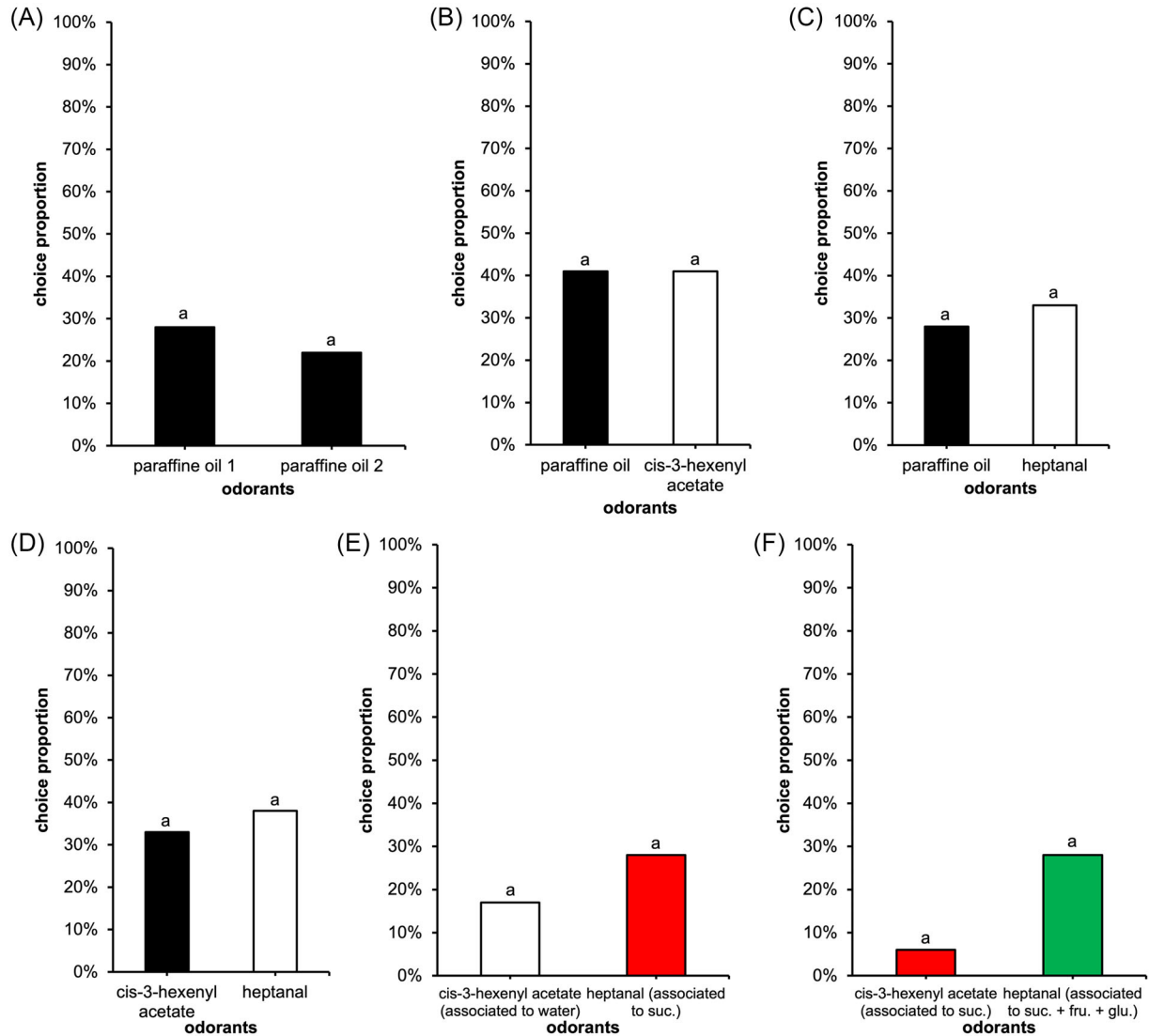


Fig. 5 Feeding preferences in association to a floral odor in male moth *A. ipsilon*. The experiment was carried out on the third day of adult life in naïve moths (A–D) or in trained moths (E and F) ($n = 18$ for each cohort). Letters indicate the statistical differences after a logistic regression. (A) Choice proportion with paraffin oil in each arm to dissociate any bias linked to the experimental environment (logistic regression, $z = -0.38$, $P = 0.701$). (B) Choice proportion between paraffin oil and *cis*-3-hexenyl-acetate. (C) Choice proportion between paraffin oil and heptanal. (D) Choice proportion between *cis*-3-hexenyl-acetate and heptanal. (E) Choice proportion between *cis*-3-hexenyl-acetate and heptanal after association of water (nonsugar nectar) with *cis*-3-hexenyl-acetate and 12% sucrose with heptanal during the first 2 d of adult life. (F) Choice proportion between *cis*-3-hexenyl-acetate and heptanal after association of 12% sucrose with *cis*-3-hexenyl-acetate and 4% sucrose + 4% fructose + 4% glucose with heptanal during the first 2 d of adult life.

$= 3.07$, $P = 0.003$; textures groups were pooled as they were not different) (Fig. 6D). However, moths did not discriminate sugar quality of the nectar with floral texture (logistic regression, $t_{139} = -1.08$, $P = 0.282$) (Fig. 6E).

Discussion

This study aimed to characterize the nutritional ecology of a nocturnal pollinating insect, namely male *A. ipsilon*

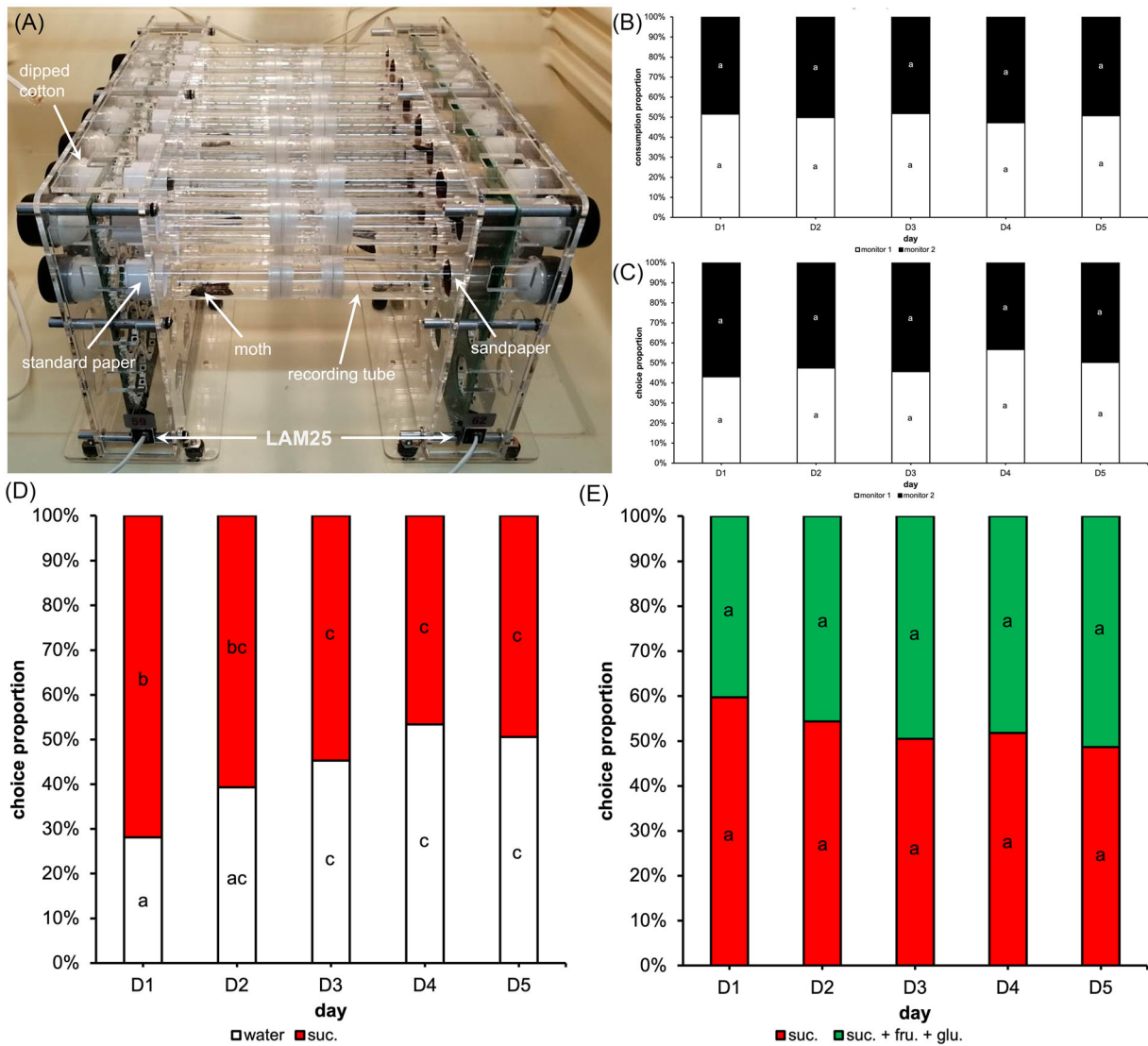


Fig. 6 Feeding preferences in association to texture in male *A. ipsilon*. The experiment was carried out over 5 d from emergence with a photoperiod 16 L: 8 D ($n = 16$ for each cohort). Letters indicate the statistical differences after a logistic regression on repeated measurements. (A) Photographs in side view of the LAM25 devices for automatic measurement of feeding preferences in association with texture in males *A. ipsilon*. (B) Feeding preferences between sucrose 12% (monitor 1) and sucrose 12% (monitor 2) to dissociate any bias linked to the experimental environment (recording tubes were without textures). (C) Feeding preferences between sucrose 12% (monitor 1) and sucrose 12% (monitor 2) to dissociate any bias linked to the texture. (D) Feeding preferences between water (nonsugar nectar) and 12% sucrose solution. (E) Feeding preferences between 12% sucrose and 4% sucrose + 4% fructose + 4% glucose solutions.

(for which diet is known to finely modulate its reproduction), by examining the food responsiveness with proboscis extension reflex (PER), by assessing feeding behaviors with automated feeding cages as well as food preferences with locomotor activity monitor system and Y-tube olfactometer.

Detection of sugars present in floral nectar is crucial for adult Lepidoptera. Indeed, these nutrients are known to affect their life span as well as their reproduction (Rusterholz & Erhardt, 2000; Force *et al.*, 2023a, 2024a, 2024b). The total proportion of sugars in flower nectar generally varies between 10%–15% of the fresh mass, and can

sometimes reach 30% for sucrose (Pacini *et al.*, 2003; Roy *et al.*, 2017). Many nectar-consuming insect species actively move their antennae not only to smell odors but also to detect sugar in flower nectar. In adult male *A. ipsilon*, antenna stimulation with nectar provokes the PER, a behavior causing food intake. Our behavioral assays measuring PER rate demonstrated that food responsiveness was dependent on age as well as nectar, that is, the quantity of sugars, and to a lesser extent, the sugar quality (Figs. 1 and 2). Indeed, we noticed that the gustatory response threshold to sugars decreased with age and was linked to the sucrose quantity: with increasing age, moths significantly detected nectars with very low amounts of sucrose (Fig. 1). Then, for nectars low in sugar (less than 15%) known to be more easily consumed by moths (Kim *et al.*, 2011; Coates *et al.*, 2023), and composed of the same sucrose quantity, the presence of fructose and glucose provoked on average 10% to 20% more gustatory responses compared to sucrose-only nectar (Fig. 2). Taken together, these first data indicate that the male *A. ipsilon* consume sugary nectars, with higher consumption of nectars rich in sucrose, fructose and glucose.

Sugar detection is carried out using gustatory sensilla (GSs) mainly distributed on antennae as well as on mouthparts (Dahanukar *et al.*, 2005). Gustatory receptors (GRs), expressed within gustatory neurons located in GSs, participate in the detection of sugars (Freeman *et al.*, 2014). Generally, GRs involved in sugar sensing respond to a wide range of sugars. It has been observed that several types of GRs can detect a single type of sugar and that conversely, several types of sugar can activate a single type of GRs (Dahanukar *et al.*, 2007; Slone *et al.*, 2007). Furthermore, in Noctuidae group, which includes *A. ipsilon*, feeding behaviors are closely linked to sugar detection (Popescu *et al.*, 2013; Tang *et al.*, 2014). The initiation of food intake is a process often induced by a combination of sugars. In *Manduca sexta* larvae, solution composed of sucrose and glucose activates GRs causing the induction of feeding behavior (Glendinning *et al.*, 2007). However, it is not uncommon that sucrose only triggers feeding behavior as in *Bombyx mori* larvae (Sasaki & Asaoka, 2006). Recent studies have shown that certain GRs are specific for fructose in *B. mori* (Morinaga *et al.*, 2022), and in *Helicoverpa armigera* (Jiang *et al.*, 2015). In the latter, for a series of increasing fructose concentrations, it was demonstrated that GSs activation rate was positively correlated with the PER (Jiang *et al.*, 2015). Additionally, a recent study on male *A. ipsilon* related that sucrose elicited more gustatory responses than fructose and that glucose response was much lower than those of the two other sugars (Hostachy *et al.*, 2019). However, replacing half the sucrose or half the fructose

with glucose did not reduce gustatory response, while it increased for the mixture of sucrose and fructose, thus suggesting a synergy between these two sugars (Hostachy *et al.*, 2019). Hostachy *et al.* (2019) also noted that glucose did not have a synergistic effect in the sucrose + fructose + glucose mixture. Moreover in insects, several studies have reported long- and short-term changes in gustatory sensitivity, particularly linked to morphological changes in GSs number (Bernays & Chapman, 1998). However, few studies have examined the variation in GR expression as a function of age. It was shown in moth *Mythimna separata* that the expression of a sugar GR was higher in 1-d-old compared to 5-d-old males (He *et al.*, 2017). Taken together, these data suggest that antennae of male *A. ipsilon* possess sensilla sensitive to sucrose, fructose and a lesser extent to glucose, thus reinforcing our previous results according to which the male *A. ipsilon* is a moth consuming sugary nectars, with a higher consumption for nectars rich in various sugars including fructose and glucose. Furthermore, for an increasing concentration of sugars, the increase in gustatory response is probably linked to an increasing activation rate of sugar-sensitive GSs. To verify this, it would be interesting to study the electrophysiological response of antennae depending on sugar quality (sugar alone or in mixture) and sugar quantity of nectar. Age-dependent variations in sugar sensitivity in male *A. ipsilon* could be explained by a variation in number of sugar-sensitive GSs and/or by a modulation of sugar GRs expression. As was previously done for olfactory receptors in *A. ipsilon* (Gu *et al.*, 2014), antenna transcriptomic analysis at different stages of adult life could be carried out to characterize the sugar GRs and to study their expression profile.

Nectarivorous insects can distinguish flowers offering nectars with different nutritional properties (Raguso & Willis, 2005; Stöckl & Kelber, 2019) and can adapt their foraging behavior accordingly (number and duration of flower visits, duration of the foraging period, volume of nectar consumed). Indeed, during the first days of adult life, males *A. ipsilon* explore flowers composed of non-sugary nectar for a longer time than those with sugary nectars (Fig. 3A, B). Furthermore, beyond the presence of sugar in nectar, nectar quality also influences the visit duration. Moths visiting flowers with nectars rich in various sugars (sucrose, fructose and glucose) visit the flower very quickly compared to those exploring flowers with sucrose-only nectar (Fig. 3B). According to our previous interpretations, these results are consistent and reinforce the previous conclusion stating that the male *A. ipsilon* consume sugary nectars and in particular those composed of sucrose, fructose and glucose. Then, it is reasonable to think that visit duration is linked to the detection of

sugary nectar. Moths that do not detect sugars in nectar would spend more time exploring the flower, in search of nutritional resources. In the case of sugary nectars, the presence of various sugars, notably sucrose and fructose known to have a synergistic effect on antennal detection in male *A. ipsilon* (Hostachy *et al.*, 2019), would quickly lead to food intake thus limiting the time spent in the flower. Furthermore, in male *A. ipsilon*, foraging is strictly limited to scotophase and follows a circadian rhythm (Fig. 4A–C) (Force *et al.*, 2023b). Although nectar affects neither the period nor the phase of the rhythm, foraging duration (i.e., the amplitude of the rhythm) is directly linked to nectar composition of visited flowers: moths visiting flowers with nectar rich in various sugars spend less time foraging compared to those foraging from flowers with sucrose-only nectar (Fig. 3C). Here again these results support the previous insight that male *A. ipsilon* forage flowers with nectar rich in various sugars. Furthermore, knowing that nutritional needs can be positively correlated with duration of foraging in some insects (Norberg, 2021), we can hypothesize that nectar with sucrose, fructose and glucose would be energetically richer than a nectar composed of sucrose alone, and could modulate the physiology of male moth.

At a broader temporal resolution, during the first 5 d of adult life, male *A. ipsilon* moths constantly visit flowers with sucrose-rich nectar; on the other hand, for those having access to flowers with nectar composed of various sugars (sucrose, fructose, glucose), the visit number begins to decrease from the third postemergence day and reaches its minimum on the fifth day of life adult (Fig. 4D). Moths generally feed on nectar providing the energy necessary for the development of reproductive system as well as the ontogeny of sexual behaviors (Liu *et al.*, 2017; He *et al.*, 2022). Numerous studies have revealed the importance of sugary nectars as food sources for improving physical condition and increasing the lifespan, reproduction and flight performance of adults (Lee & Heimpel, 2008; He *et al.*, 2021; Force *et al.*, 2023a). In male *A. ipsilon*, nectars rich in various sugars drastically influence reproductive activities by accelerating sexual maturation (Force *et al.*, 2023a, 2024b) and increasing reproductive success (Force *et al.*, 2024a). Indeed, males fed with sucrose, fructose and glucose are sexually mature on the third postemergence day while those fed with sucrose reach sexual maturity on the fifth day of adult life (Force *et al.*, 2023a, 2024b). Taken together, these data suggest that during the first days of adult life, feeding dynamics of male *A. ipsilon* would be closely linked to its physiological state, especially to its reproductive state. To verify this hypothesis, it would be interesting to analyze the feeding dynamics in newly emerged male *A. ip-*

silon after an injection of trehalose (the majority sugar circulating in the insect hemolymph and used in cellular metabolism following to hydrolysis into glucose) (Mayack *et al.*, 2020) and also after an injection of juvenile hormone known to initiate and regulate male sexual maturation in this species (Aguilar *et al.*, 2023). In short, our previous conclusion that male *A. ipsilon* forages on flowers with nectar composed of sucrose, fructose and glucose (i.e., assumed to be energetically rich) is further supported in view of the close link between feeding dynamics and physiological state.

Optimal foraging theory predicts that when given a choice, animals will prefer foods with a high net return (MacArthur & Pianka, 1966). However, if a nutritional resource becomes scarce, or a preferred food resource becomes difficult to obtain, foods of lower energetical quality may be consumed (Aigner & Scott, 2002). Indeed, *A. ipsilon* males with exclusive access to flowers with nonsugar nectar (considered very low in energy) constantly visit these flowers, although the floral visit frequency is considerably less than for moths with exclusive access to flowers with sugary nectar (Fig. 4D). In our experiments, regardless of quality, nectar was distributed in morphologically identical artificial flowers. Taken together, these results suggest that moths learn that entering the flower provides an energetically profitable nutrient resource, similar to operant conditioning with nectar as a reinforcer (Staddon & Cerutti, 2003). For many insects, it is very beneficial to learn to find reliable resources and to avoid flowers that do not produce nectar or energetically poor nectar, which can deprive them of a profitable meal. Since the primary attractants of a flower (nectar and pollen) are often hidden, the localization of floral rewards generally occurs through secondary attractants (e.g., color, odor, shape and/or texture) (Wester & Lunau, 2017). Additionally, many adult Lepidoptera can learn to associate colors, odors, shapes and/or textures with a food reward (Raguso & Willis, 2005; Stöckl & Kelber, 2019). In situations where it is difficult to rely on visual cues, odors are of considerable importance, particularly in nocturnal pollinators (Raguso & Willis, 2005). Our study shows that males of the nocturnal moth *A. ipsilon* are probably capable of associating a floral odor with a nectar (Fig. 5). Indeed, moths appear to choose odors associated to nectar rich in various sugars (sucrose, fructose, glucose) (Fig. 5F), thus considerably supporting our previous results demonstrating that the male *A. ipsilon* is a moth foraging flowers with nectar composed of various sugars. Moreover, associations between floral odors and nectars have been noted in *H. armigera* (Cunningham *et al.*, 2006). This would allow Lepidoptera to identify and approach energetically profitable nutritional

resources. Although little studied in Lepidoptera, behavioral and cognitive mechanisms that govern flower choice are well-known in honeybees (Sherry & Strang, 2015). This information provides a basis for understanding the mechanisms controlling floral nectar preference in other pollinators such as moths. Floral nectar preference in honeybees is partly controlled by odors; but another sensory modality, linked to the petal texture, plays an important role in the flower choice (Whitney *et al.*, 2009). Surprisingly, as in honeybees, *A. ipsilon* males discriminate nectars with floral texture (Fig. 6), and more particularly sugary nectar from those with nonsugar nectar (Fig. 6D). Thus, in male *A. ipsilon*, in the context of searching for flower nectar, associative learning would offer a considerable biological advantage as in other nocturnal pollinating insects (Cunningham *et al.*, 2004).

Finally, flowers and insects co-evolved and formed a relationship considered mutually beneficial. The abundance and composition of flowering plant species are fundamental aspects of insect pollinator biology, and both can change throughout their lifespan. Thus, the ability to distinguish, learn, and move from one flower to another in the face of a changing environment is of crucial importance. Furthermore, many nocturnal insects of the Noctuidae family carry out seasonal migrations. Although they are often involved in agricultural and forestry damage, they participate in pollination over large geographic regions (Chapman *et al.*, 2015). *A. ipsilon* is a species of crop pest moth known to migrate over very long distances (Causse *et al.*, 1989). Regarding its ecology, it is very likely that *A. ipsilon* is a generalist pollinator species. Indeed, using DNA barcodes, pollen morphology and distribution area of plants, a study identified the host plant used by *A. ipsilon* adults: they fed on the nectar of a very wide range of plants, mainly dicotyledonous Angiosperms (Liu *et al.*, 2016). While this work gives preliminary information about foraging, it is not sufficient to understand the nutritional ecology of this species. Here, using new experimental approaches, our study made it possible to characterize feeding behaviors and their variation depending on consumed nectars in male *A. ipsilon*, thus, allowing to determine the nutritional ecology of this nocturnal pollinating insect as a generalist species with, however, a preference for flower with sugary nectars and more particularly rich in sucrose, fructose, and glucose.

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Disclosure

The authors declare no competing or financial interests.

Data availability statement

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

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Supporting Information

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

Fig. S1 Sensitization to sugary solutions in male *Agrotis ipsilon*.

Fig. S2 Hemolymph volume of male *A. ipsilon* according to water (nonsugar nectar) or 12% sucrose diet.