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Evan Force, Philippe Couzi, Matthieu Dacher, Stéphane Debernard. Diet impacts the reproductive system's maturation in the male moth *Agrotis ipsilon* (Noctuidae, Lepidoptera). *Journal of Insect Physiology*, 2023, 148, pp.104532. 10.1016/j.jinsphys.2023.104532 . hal-04139739

HAL Id: hal-04139739

<https://hal.science/hal-04139739>

Submitted on 12 Sep 2023

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Diet Impacts the Reproductive System's Maturation in the Male Moth *Agrotis ipsilon* (Noctuidae, Lepidoptera)

Published in *Journal of Insect Physiology*, 148:104532; on June 21, 2023

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Short running title: Diet and Sexual Maturation in *A. ipsilon*

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Abstract

In male moth *Agrotis ipsilon*, sexual maturation occurs between the third and the fifth day of adult life and is characterized by the development of the reproductive organs such as testes and accessory sex glands. Since sexual maturation requires considerable energy investment, we hypothesized that diet would be an essential regulatory factor in this developmental process. Indeed, the links between the male diet and reproductive physiology have not been described as in females. To test the previous hypothesis, we offered male moths diets corresponding to different flower nectars found in nature, and measured morphological and functional changes in the testes and accessory sex glands. In comparison to a diet composed of sucrose only, males fed with a diet composed of diverse sugars, including glucose, supplemented with sodium led to an earlier increase in the length and the protein content of accessory sex glands, as well as a reduction of the testicular volume accompanied by an acceleration of the sperm bundle transfer from the testes to the duplex. These results show that these specific diets accelerate the maturation of the reproductive system in male moth *Agrotis ipsilon*.

Keywords: *Agrotis ipsilon*, insect, feeding, sexual maturation, accessory sex glands, proteins, testes, sperm bundles.

28 **1. Introduction**

29 In holometabolous insects, metamorphosis is a spectacular developmental transition marked
30 by a major organogenic remodeling combining histolysis and histogenesis (Truman & Riddiford,
31 2002) and observable during the transition from the larval stage to the imago stage (Nishimura,
32 2020; Yamanaka *et al.*, 2013). Often, in parallel to the metamorphosis, it occurs sexual maturation
33 during which the sexually immature organism acquires the ability to reproduce through
34 morphological and functional changes in the reproductive system.

35
36 It is well-known that internal factors, such as hormones and biogenic amines, are involved in
37 the regulation of developmental processes (Yamanaka *et al.*, 2013). Furthermore, developmental
38 processes such as sexual maturation require considerable energy (Kupferberg, 1997). Thus, food is
39 a very studied external factor in some orders of insects. In aphids and male bees, it has been
40 demonstrated that food intake influences the juvenile hormone (JH) and/or dopamine levels (Bai
41 *et al.*, 2022; Watanabe & Sasaki, 2021). In females Lepidoptera, the diet was found to increase
42 longevity, fecundity, and gamete production, especially with flower nectars containing sugars or
43 sodium (Boggs & Dau, 2004; Geister *et al.*, 2008; Marchioro & Foerster, 2013; O'Brien *et al.*,
44 2004). Thus, these results revealed a link between feeding and reproduction. Surprisingly, there are
45 no data showing interactions between these two physiological functions in male Lepidoptera,
46 notably in the context of sexual maturation.

47
48 During the sexual maturation of male moths, the reproductive system is subjected to morpho-
49 anatomical and functional modifications (Hiroyoshi *et al.*, 2021; Moors *et al.*, 2009). The testes,
50 which produces the sperm, decrease in volume (Klein *et al.*, 2021) and the sperm bundles are
51 transferred from the testes to the duplex where is formed the spermatophore that is then transferred
52 to the female during copulation (Baker *et al.*, 2003; Fernandez & Cruz-Landim, 2005; Muzzi *et al.*,
53 2020). Conjointly, the accessory sex glands grow in length (Baker *et al.*, 2003), change color
54 (Fernandez & Cruz-Landim, 2005; Muzzi *et al.*, 2020), and produce proteins (but also
55 carbohydrates and lipids) (Southon *et al.*, 2020) that are necessary for the formation of the
56 spermatophore and also for the sperm survival during their storage within the duplex and their
57 transit in the female genital duct (Fernandez & Cruz-Landim, 2005; Muzzi *et al.*, 2020).

58

59 In male moth *Agrotis ipsilon* (Noctuidae, Lepidoptera), sexual maturation takes place after
60 the metamorphosis between the third and the fifth post-emergence day in the standard rearing
61 conditions (Gemeno & Haynes, 2000) and is triggered by hormonal changes (Gadenne & Anton,
62 2000; Bozzolan *et al.*, 2015; Duportets *et al.*, 2013). For instance, a rise in the circulating level of
63 JH promotes the growth and the activity of accessory sex glands (Gassias *et al.*, 2021), and induces
64 the development of the olfactory centers located in the brain at the origin of the perception of the
65 female sex pheromone (Gadenne & Anton, 2000). All of these changes reflect the acquisition of
66 sexual maturity in male *A. ipsilon*.

67
68 However, the diet of the imago *A. ipsilon* remains unknown. As this insect has several
69 generations per year, its food probably comes from diverse nutritional resources. It is well
70 documented that most adult moth species feed from flower nectars with variable compositions.
71 Generally, this nutrient solution contains water, solubilized carbohydrates such as sucrose, glucose,
72 fructose, and amino acids readily available to the organism (Broadhead & Raguso, 2021; Nepi *et*
73 *al.*, 2012; Roy *et al.*, 2017). Among carbohydrates, sucrose is the major sugar circulating within
74 plants and is generally the primary component of many flower nectars (Nicolson & Thornburg,
75 2007). Glucose alone or glucose with fructose is also present in some flower nectars (Nicolson &
76 Thornburg, 2007; Roy *et al.*, 2017). Another feeding behavior has been described in males
77 Lepidoptera that consume excrements or puddles of brackish water (Ravenscraft & Boggs, 2016).
78 This puddling behavior does not provide energy-rich elements but rather certain mineral elements,
79 such as sodium, that are essential for the homeostasis of the organism (Ankola *et al.*, 2021; Boggs
80 & Dau, 2004; Molleman, 2010). The sodium collected was found to be necessary for their
81 locomotor activity by improving flight (Ankola *et al.*, 2021; Xiao *et al.*, 2010), as well as in their
82 reproduction notably by promoting spermatogenesis and sperm mobility (Mitra *et al.*, 2016;
83 Molleman, 2010; Smedley & Eisner, 1996).

84
85 The objective of our study was to evaluate the potential interactions between feeding and
86 reproductive organs during sexual maturation in male *A. ipsilon*. Thus, we examined the impact of
87 different diets on the growth and the activity of accessory sex glands, the testicular volume as well
88 as the sperm bundle number within testes and duplex during the first five days of adult life. Our

89 results demonstrated that a diet rich in diverse sugars supplemented with sodium promoted the
90 development of the reproductive system in male moth *A. ipsilon*.

91 2. Materials and Methods

92 2.1. Insects and Dietary Treatments

93 Our research focused on adult males *Agrotis ipsilon* (Noctuidae, Lepidoptera). This insect
94 is native to the south of France. The animals are raised in our laboratory at INRAE, Versailles,
95 France. Larvae are stored in collective boxes and then individually with the same artificial food
96 until the metamorphosis (Poitout & Buès, 1974). Males and females are separated during
97 metamorphosis: the pupae have a sexual dimorphism that allows to differentiate them. The pupae
98 are then kept in two different air-conditioned rooms (22 °C, 50-60% relative humidity) and
99 subjected to an inverted photoperiod (8h of darkness and 16h of light, light at 6 pm). Each morning,
100 among the emerged moths, we recovered and placed 40 insects in 8 boxes (20 x 11.5 x 5 cm) each
101 containing 5 males. The harvest day was considered as the first post-emergence day (noted D1).
102 The moths had access *ad libitum* to a 12% sweet solution (fed males, see below) or water (fasting
103 males) depending on treatments. The males can fast for 5 days before getting weak. The fed males
104 gained weight while the fasting males lost weight (data not shown).

105
106 Just after the emergence, D1 males were subjected to different diets, and the effects were
107 examined in males on the third day (D3) and fifth day (D5) post-emergence. These days
108 correspond, respectively, to the beginning and the end of sexual maturation in the standard rearing
109 conditions (Gemeno & Haynes, 2000). Another condition, noted water, is negative control (fasting
110 males). The other seven solutions contained osmosed water in addition to:

- 111 • Sucrose only at 12% (mass/mass or 13.6% mass/volume) (noted suc.). This diet is
112 conventionally used in our laboratory for rearing;
- 113 • 6% sucrose with 6% glucose (noted suc. + glu.);
- 114 • 4% sucrose with 4% fructose, and 4% glucose (noted suc. + fru. + glu.);
- 115 • 1% NaCl (noted Na);
- 116 • 12% sucrose, and 1% NaCl (noted suc. + Na);
- 117 • 6% sucrose with 6% glucose, and 1% NaCl (noted suc. + glu. + Na);
- 118 • 4% sucrose with 4% fructose, 4% glucose, and 1% NaCl (noted suc. + fru. + glu. + Na).

119 These carbohydrates are the major constituents of the flower nectar, main food source for adult
120 Lepidoptera, with generally 10-15% of the fresh mass for a single sugar or a mixture of three sugars

121 (Roy *et al.*, 2017). All reagents (sugars, NaCl, etc.) come from Sigma-Aldrich (Saint-Quentin-
122 Fallavier, France).

123 2.2. Dissection

124 Biometric experiments were carried out on diverse organs (accessory sex glands, testes, and
125 duplex) of the male reproductive system (**Fig. 1**). Dissections consisted of a dorsal opening of the
126 abdomen and were performed in Ringer solution (6.13% glucose, 0.24% HEPES, 0.11% MgCl₂,
127 0.07% NaCl, 0.05% KCl, 0.01% CaCl₂) under a Leica S9D stereo microscope. Following
128 dissections, the testes and the duplex were quickly analyzed while the accessory sex glands were
129 collected and placed in 1.5 mL centrifugation tubes immediately bathed in liquid nitrogen after
130 measuring their length, then stored at -80 °C for subsequent experiments (protein extraction and
131 quantification).

132 2.3. Testicular Volume and Sperm Bundle Counting

133 To determine the testicular volume, visual measurements of length (L), width (l), and height
134 (h) of excised testes were made using a microscope slide grid (1 mm x 1 mm) under a Leica S9D
135 stereo microscope. The testes were assumed to be ellipsoid, so their volume was calculated from
136 the ellipsoid volume formula: $V = (4/3 \times \pi \times L \times l \times h)/8$. Illustrative photographs were acquired
137 by a Leica MC170 HD digital camera mounted on a Leica S9D stereo microscope. After excision,
138 the duplex and the testes were dilacerated in 100 µL of Ringer solution, thus allowing the release
139 of sperm bundles that were stained by adding 5 µL of methylene blue. The counting of sperm
140 bundles was then made from 5 µL of solution deposited in a cup of the Fluoslid counting cell
141 under an Orthoplan photonic microscope (Leitz, Germany). Any filamentous structure with fine
142 extensions was considered to be a sperm bundle (Konagaya *et al.*, 2020; Perveen, 2008).

143 2.4. Accessory Sex Gland Length

144 The length of excised accessory sex glands was visually measured using a microscope slide
145 grid (1 mm x 1 mm). Illustrative photographs were acquired by a Leica MC170 HD digital camera
146 mounted on a Leica S9D stereo microscope.

147 2.5. Accessory Sex Gland Protein Quantification

148 The excised and dissected accessory sex glands were immersed in 400 μ L of Tris 20 mM
149 pH 7.4 Carl Roth buffer (Karlsruhe, Germany) and homogenized for 20 seconds with a Polytron
150 PT 10-35 GT homogenizer. After sonication, the samples were centrifuged at 10,000 RPM for 10
151 minutes (Eppendorf Centrifuge 5804 R) at 4 °C and the resulting supernatant was collected. The
152 protein concentration was then estimated using the BiCinchoninic acid Assay (BCA), method with
153 the Bovine Serum Albumin concentration ranging from 1 to 40 μ g protein standard range. The
154 quantification of each sample was made from 10 μ L of protein extract mixed with 10 μ L of Tris
155 buffer 20 mM pH 7.4 Carl Roth (Karlsruhe, Germany) and 1 mL of reagent. The reagent consisted
156 of 20 μ L CuSO₄ 4% and 0.98 mL BCA solution. The samples were heated at 37 °C for 30 minutes
157 and the absorbance was then read at 562 nm with a spectrophotometer (Eppendorf BioPhotometer
158 6131). Each data point for the protein extraction was obtained from three pairs of accessory sex
159 glands stored in a 1.5 mL centrifuge tube.

160 *2.6. Data Processing and Analysis*

161 Data statistical analysis was performed using data processing software R 4.1.2 ([R Core](#)
162 [Team, 2021](#)) and RStudio 2021.09.2+382. The sample size ranges from 15 to 24 males in each
163 condition (the exact sample size is mentioned in the legend of each figure). The alpha risk was
164 0.05.

165
166 For each dataset, the assumption of normal data distribution was tested by the Shapiro-
167 Wilk test, and a Box-Cox procedure was conducted to find a transformation when data distribution
168 was not normal (it is indicated in the legend of each figure). When this was satisfied, an ANOVA
169 was carried out, and when it was significant, post-hoc tests were conducted. The choice of the post-
170 hoc test depends on the equality of the variances, tested with Bartlett's test, as well as on the sample
171 size. In case of different variances, a Tamhane's test was performed; for equal variances and equal
172 sample sizes, a Tukey's test was applied, otherwise a Scheffe's test was used. Alternatively, if the
173 Box-Cox procedure failed to identify an appropriate transformation, non-normal data were
174 analyzed with a Kruskal-Wallis' test. If it was significant, Dunn's post-hoc test was performed (the
175 statistical test used for each of the datasets is indicated in the legend of the figures).

176

177 After comparison between D1, D3, and D5 for each diet using ANOVA or Kruskal-Wallis
178 tests, and in case of significant differences, we only reported the corresponding post-hoc tests for
179 the sake of conciseness.

180 *2.7. Ethics Note*

181 The French legislation for animal welfare is modeled on the European Union (Directive
182 2010/63/EU) according to which no invertebrate other than cephalopods enjoy ethical protection.
183 However, all manipulations were carried out with care, minimizing stress situations for the animals.

184 3. Results

185 3.1. *The length of accessory sex glands is raised by a diet composed of diverse sugars supplemented* 186 *with sodium*

187 We first evaluated the impact of diet on the growth of accessory sex glands by measuring
188 their length (**Fig. 2**). Regardless of tested diets, the length of accessory sex glands of D3 and D5
189 males is greater than those of D1 males (Tamhane's test for water, $p \leq 0.0001$; Dunn's test for suc.
190 + Na, $p \leq 0.0001$; and Tukey's test for other diets, $p \leq 0.0014$), whereas it is not significantly
191 different between the D3 and D5 males (Tamhane's test for water, $p = 0.5326$; Dunn's test for suc.
192 + Na, $p = 1.0000$; and Tukey's test for other diets, $p \geq 0.0569$) (**Fig. 2A**). Thus, the development
193 of accessory sex glands takes place during the first three post-emergence days. In D3 males, the
194 length of accessory sex glands is increased for a diet containing sucrose + glucose + sodium, and
195 the three sugars with sodium (average 3.6 ± 0.1 cm) as compared to the conditions water, sodium,
196 and sucrose alone (approximately 3.2 ± 0.1 cm) (Dunn's test, $p \leq 0.0374$) (**Fig. 2B**). For D5 males,
197 the accessory sex glands for the diet containing the three sugars supplemented with sodium are
198 longer (approximately 4.0 ± 0.1 cm) than those other conditions (average 3.7 ± 0.1 cm at most)
199 (Tukey's test, $p \leq 0.0001$), but sucrose + glucose + sodium is not different that sucrose + glucose
200 + fructose + sodium (**Fig. 2C**). Accessory sex gland photographs show the increase in length in D3
201 and D5 males fed with the three sugars supplemented with sodium (**Fig. 2G-I**) compared to D3
202 and D5 fasting males (**Fig. 2D-F**).

204 3.2. *The protein content of accessory sex glands is increased by a diet composed of diverse sugars* 205 *with sodium*

207 We then examined the effect of diet on the activity of accessory sex glands by quantifying
208 their protein content (**Fig. 3**). Regardless of treatments, the protein amount of accessory sex glands
209 of D3 and D5 males is higher than those of D1 males (Dunn's test, $p \leq 0.0004$) (**Fig. 3A**). In D3
210 males, the protein amount is highest for the treatments rich in sucrose + glucose + sodium, and the
211 three sugars with or without sodium (approximately 390 ± 8 μg , against 340 ± 9 μg on average for
212 the other treatments) (Dunn's test, $p \leq 0.0464$) (**Fig. 3B**). For D5 males, the diet composed of the

213 three sugars with sodium significantly raises the protein amount (average $450 \pm 10 \mu\text{g}$) as compared
214 to the conditions water, sodium, sucrose alone, and the three sugars (approximately $380 \pm 7 \mu\text{g}$)
215 (Dunn's test, $p \leq 0.0276$) (**Fig. 3C**). The increase in protein content within accessory sex glands is
216 correlated with a color change from white structures for the sexually immature stage to reddish
217 structures for the sexually mature stage. This color variation occurs between the first and the third
218 post-emergence days in males fed with the three sugars supplemented with sodium (**Fig. 2G-I**)
219 whereas it appears between the third and the fifth post-emergence days in fasting males (**Fig. 2D-**
220 **F**).

221 *3.3. A diet composed of diverse sugars supplemented with sodium accelerates the decrease in* 222 *testicular volume during the first three days of adult life*

223 We also analyzed the consequences of diet on the development of testes by measuring their
224 volume (**Fig. 4**). For all tested diets, the testicular volume of D3 and D5 males is lower than those
225 of D1 males (Tamhane's test for suc. + glu., $p \leq 0.0003$; Dunn's test for suc., suc. + glu. + Na, and
226 suc. + fru. + glu. + Na, $p \leq 0.0279$; and Scheffe's test for other diets, $p \leq 0.0368$). For the conditions
227 water, sodium, sucrose alone, and sucrose + glucose, the volume of testes on the third day after the
228 emergence is approximately $900 \pm 48 \text{ nL}$ and is on average between $1,100 \pm 51 \text{ nL}$ and 720 ± 39
229 nL for respectively the first day (Scheffe's test for water and Na, $p \leq 0.0368$; Dunn's test for suc.,
230 $p \leq 0.0279$; and Tamhane's test for suc. + glu., $p \leq 0.0003$) and the fifth day (Scheffe's test for
231 water and Na, $p \leq 0.0251$; Dunn's test for suc., $p \leq 0.0279$; and Tamhane's test for suc. + glu., $p \leq$
232 0.0219). For the other four diets: suc. + fru. + glu., suc. + Na, suc. + glu. + Na, and suc. + fru. +
233 glu. + Na, the testicular volume on the third day is only approximately $640 \pm 45 \text{ nL}$ and remains
234 unchanged on the fifth day (Scheffe's test for suc. + fru. + glu. and suc. + Na, $p \geq 0.9328$; Dunn's
235 test for suc. + glu. + Na and suc. + fru. + glu. + Na, $p \geq 0.2192$) (**Fig. 4A**). Thus, these results reveal
236 an acceleration of the decline of testicular volume for these latter diets. In D3 males, this
237 acceleration of the reduction of testicular volume is more pronounced for males fed with sucrose +
238 glucose + sodium, and the three sugars with sodium (average $650 \pm 40 \text{ nL}$, against $850 \pm 46 \text{ nL}$ for
239 the other diets) (Dunn's test, $p \leq 0.0248$) (**Fig. 4B**). For D5 males, no significant difference in the
240 testicular volume (approximately $700 \pm 45 \text{ nL}$) is observed among all diets (ANOVA's test, $F_{7,50.97}$
241 $= 0.7010$, $p = 0.6710$) (**Fig. 4C**). Testes photographs illustrate the greatest reduction of testicular

242 volume in D3 and D5 males fed with sucrose + fructose + glucose + sodium (**Fig. 4G-I**) in
243 comparison to D3 and D5 fasting males (**Fig. 4D-F**).

244
245 *3.4. A diet rich in diverse sugars along with sodium promotes the sperm bundle transfer from the*
246 *testes to the duplex during the first three post-emergence days*

247
248 Finally, we studied the effect of diet on the transfer of sperm bundles by counting their
249 number within the testes and the duplex (**Fig. 5**). Whatever the tested diets, the sperm bundle
250 number in testes is lower in the D3 and D5 males compared to D1 males (Tukey's test for suc. +
251 Na, $p \leq 0.0001$; Dunn's test for water, suc., and suc. + fru. + glu., $p \leq 0.0019$; and Tamhane's test
252 for other diets, $p \leq 0.0001$) (**Fig. 5A**). Therefore, the sperm bundles gradually leave the testes during
253 the first five post-emergence days. For the conditions water, sodium, sucrose alone, and sucrose +
254 glucose, the number of sperm bundles on the third day is on average $2,800 \pm 176$, and is
255 approximately between $3,950 \pm 170$ and $1,800 \pm 127$ for respectively the first day (Dunn's test, p
256 ≤ 0.0019) and the fifth day (Dunn's test, $p \leq 0.0020$). By contrast, for other conditions sucrose +
257 sodium, sucrose + glucose + sodium, and sucrose + fructose + glucose with or without sodium, the
258 sperm bundle number within the testes reaches its minimum of approximately $2,450 \pm 187$ on the
259 third day and is on average identical on the fifth day (Tukey's test for suc. + Na, $p = 0.0972$; Dunn's
260 test for suc. + fru. + glu., $p = 1.0000$; Tamhane's test for other diets, $p \geq 0.2400$) (**Fig. 5A**). Thus,
261 these results demonstrate an acceleration of the transfer of sperm bundles from testes for these
262 latter diets. In D3 males, this accelerated transfer is accentuated for males fed with the three sugars
263 with sodium (approximately $2,100 \pm 117$) compared to the conditions water, sodium, sucrose with
264 or without sodium, and sucrose + glucose (average $2,700 \pm 199$ at least) (Tukey's test, $p \leq 0.0124$)
265 (**Fig. 5B**). For D5 males, the number of testicular sperm bundles is equivalent for all conditions
266 (approximately $2,100 \pm 171$), except for the water condition (average 1,600) (Tamhane's test, $p \geq$
267 0.0480) (**Fig. 5C**).

268
269 Concerning the sperm bundles in the duplex (**Fig. 6**), **Figure 6A** shows that whatever the
270 diet, their number is significantly lower in the D1 males compared to D3 and D5 males (Dunn's
271 test, $p \leq 0.0041$) but it is identical between D3 and D5 males (Dunn's test, $p \geq 0.0850$). Thus, the
272 arrival of sperm bundles within the duplex occurs mainly before the third post-emergence day. In

273 D3 males, a higher accumulation of sperm bundles in the duplex is observed for males fed with
274 diverse sugars with sodium (average 200 ± 19) as compared to treatments water, sodium, and
275 sucrose alone (approximately 100 ± 19) (Tukey's test, $p \leq 0.0249$) (**Fig. 6B**). On the other hand,
276 for D5 males, the number of sperm bundles in the duplex is significantly similar for all diets
277 (average 180 ± 32) (Kruskal-Wallis' test, $H = 9.5211$, $df = 7$, $p = 0.2174$) (**Fig. 6C**). As a result,
278 the sperm bundle transfer from the testes to the duplex is accelerated by a diet rich in diverse sugars
279 supplemented with sodium during the first three days of the adult life.

280 **4. Discussion**

281 This study aimed to explore the impact of the diet on the maturation of the male reproductive
282 system of moths by examining the length and the content protein of accessory sex glands, as well
283 as the volume of testes and the transfer of sperm bundles from the testes to the duplex in *A. ipsilon*.

284
285 Our results demonstrated that a diet composed exclusively of water induced a weak
286 development of the reproductive system. However, it occurs during the first five days of the adult
287 life and probably results from a lack of nutrients. In fasting males, it is likely that the maturation
288 of reproductive organs is primarily achieved through the utilization of energy reserves accumulated
289 during the larval stage (Angell *et al.*, 2020; Plesnar-Bielak *et al.*, 2017). We also noticed that a diet
290 composed only of 12% of sucrose leads to effects on the reproductive organs that were relatively
291 similar to those described in fasting males. Such a result suggests that sucrose is not absorbed or
292 maybe not enough in the intestinal tract in the moths as previously mentioned in other butterflies
293 (Zibae *et al.*, 2008). By contrast, a diet consisting of sucrose supplemented with glucose was found
294 to promote the differentiation of the reproductive system. This activating effect was more effective
295 with a diet rich in sucrose, glucose, and fructose. It seems reasonable to assume the existence of
296 the assimilation of glucose and fructose in male *A. ipsilon*. It has been proved in *Drosophila* that a
297 diet rich in glucose provoked the growth of fat bodies through an accumulation of glycogen
298 (Nishimura, 2020; Wyatt & Kalf, 1957). We also noticed in *A. ipsilon* that the males fed with a diet
299 containing at least glucose exhibited an expansion of fat bodies (data not shown), thus supporting
300 the hypothesis of the use of this sugar in this moth species.

301
302 We also observed in males only fed with sodium or supplemented with 12% of sucrose that
303 the orchestration of the reproductive system's maturation was identical to that of fasting males,
304 whereas an accelerated development was provoked by adding sodium to sucrose and glucose with
305 or without fructose. Taken together, these data provided evidence that sodium alone has no impact
306 on the differentiation of the reproductive system but that this mineral can potentiate the stimulatory
307 effects of a diet composed of glucose and fructose, probably by optimizing the absorption of these
308 sugars. To strengthen these hypotheses, it would be interesting in future experiments to quantify
309 the circulating amounts of sugars in the hemolymph according to tested diets.

310

311 Diets containing 12% of glucose with or without sodium were not included in our study.
312 Indeed, it has been recently demonstrated in males *A. ipsilon* that the gustatory responsiveness to
313 glucose, measured by the proboscis extension response, is at a very low level, thus meaning they
314 would not consume this sugar alone (Hostachy *et al.*, 2019). Interestingly, Hostachy *et al.* (2019)
315 also revealed that the supplementation of glucose to various sugar mixtures did not modify the
316 gustatory response of males. Additionally, the nectar sucrose-to-glucose/fructose ratio was found
317 to be higher in plants that bloom at night (Tiedge & Lohaus, 2017). Given that *A. ipsilon* is a
318 nocturnal moth, it appears likely that males diet mainly nectar in which sucrose is the dominant
319 sugar and its presence is indispensable for flower nectar consumption.

320
321 The developmental transitions such as metamorphosis and sexual maturation require
322 considerable energy for the organisms (Kupferberg, 1997). In Lepidoptera, this energy provides by
323 nutrients taken in the flower nectar (Roy *et al.*, 2017). In addition, some male moths practice the
324 puddling behavior that permits the ingestion of mineral elements, such as sodium, from excrements
325 or puddles of brackish water (Ankola *et al.*, 2021; Boggs & Dau, 2004; Molleman, 2010 ;
326 Ravenscraft & Boggs, 2016). As many nectarivores, it has been discovered in insects that to be
327 assimilated, the sucrose must be hydrolyzed into its monosaccharide components, glucose and
328 fructose, by the activity of the β -fructofuranosidase (EC3.2.1.26) also named invertase (Eberhard
329 *et al.*, 2007). This enzyme is present in the saliva and the intestinal lumen, and more specifically
330 in the mesenteron in the moth *Chilo suppressalis* (Zibae *et al.*, 2008). Thus, it is reasonable to
331 believe that the male *A. ipsilon* has the capacity to digest sucrose through its conversion into
332 glucose and fructose, although these sugars can also be directly collected from flower nectars.

333
334 In insects, it is well described that the mesenteron is the primary site of the assimilation of
335 nutrients (Li *et al.*, 2021) and that glucose is the main nutrient used in energy metabolism. The
336 absorption of glucose and fructose through the epithelium of the mesenteron is achieved by the
337 same transporters named sodium-glucose co-transporter (SGLT) and glucose-specific transporter
338 (GLUT). These ones are located, respectively, on the apical and basal membrane of epithelial cells
339 (Bifano *et al.*, 2010; Caccia *et al.*, 2007; Li *et al.*, 2021). Thus, SGLT and GLUT transporters allow
340 the transfer of glucose and fructose into the hemolymph through the presence of sodium (Bifano *et*
341 *al.*, 2010). The fate of fructose in the hemolymph remains unknown yet, although it could be

342 converted to glucose in hemolymph. Glucose, that is a reducing sugar, has osmotic properties that
343 can disrupt the homeostasis of insects. In order to maintain low levels of glucose in the hemolymph,
344 this sugar is converted into a non-reductive sugar, trehalose, in fat bodies (Li *et al.*, 2021; Wyatt &
345 Kalf, 1957). The circulating trehalose is delivered to cells to be hydrolyzed into glucose by the
346 activity of membrane-bound and cytosolic forms of trehalases (Zhang *et al.*, 2022). These catabolic
347 pathways of glucose and trehalose in energy metabolism might operate in male *A. ipsilon*. This
348 speculation is in concordance with the promoting effects of a diet composed of sucrose, fructose,
349 and glucose along with sodium on the reproductive system's maturation. In addition, the genes
350 coding for the SGLT and GLUT co-transporters as well as the membrane and cytosolic trehalases
351 have been identified in several insects. After a comparative analysis of the Lepidoptera genome
352 with that of *A. ipsilon* (NCBI database: accession number GCA-004193855.1), we succeeded in
353 finding these sequences, thus reinforcing our hypothesis regarding the assimilation of glucose and
354 utilization of trehalose in *A. ipsilon*. In future experiments, it will be instructive to study the level
355 expression of such genes and verify their roles by evaluating the impact of inhibitors of SGLT and
356 GULT transporters and trehalases on the development of the reproductive system according to diet.

357
358 It is known that sodium is a crucial mineral element for the moths because it intervenes in
359 locomotor function by improving flight (Ankola *et al.*, 2021; Boggs & Dau, 2004; Molleman, 2010)
360 and plays a key role in reproductive function by promoting spermatogenesis and sperm mobility
361 (Mitra *et al.*, 2016; Molleman, 2010; Smedley & Eisner, 1996). Moreover, seminal vesicles as well
362 as conducting channels such as *vas deferens* are consisted of a thin layer of muscle cells ensuring
363 the progression of sperm (Spiegel *et al.*, 2013). Knowing that the activity of muscle cells requiring
364 considerable energy, a diet composed of glucose enriched with sodium might improve the transfer
365 of sperm bundles within the reproductive system, as suggested by the stimulatory effects of a diet
366 rich in sucrose, fructose, and glucose along with sodium in male *A. ipsilon*.

367
368 Over the last decade, evidence has accumulated in male *A. ipsilon* demonstrating that sexual
369 maturation is triggered by an elevation of circulating level of JH due to a raise of biosynthetic
370 activity of *corpora allata*. This hormone conjointly promotes the display of sex pheromone-guided
371 orientation flight by increasing the sensitivity of neurons responding to sex pheromone signal
372 within the antennal lobes, and the growth and the secretory activity of accessory sex glands

373 (Aguilar *et al.*, 2023; Bozzolan *et al.*, 2015; Duportets *et al.*, 2013; Gadenne & Anton, 2000;
374 Gassias *et al.*, 2021). Besides the energetic and metabolic aspects related to diet, it has been
375 highlighted in several insect groups that food intake or deprivation of nutrients engendered
376 fluctuations of JH biosynthesis (Bai *et al.*, 2022; Perez-Hedo *et al.*, 2014; Watanabe & Sasaki,
377 2021). Glucose is the long-established obligatory fuel for various activities of the brain, in
378 particular by enabling the functioning of neurosecretory cells of the *pars intercerebralis* (Badisco
379 *et al.*, 2013; Dogra & Gillott, 1971; Masumura *et al.*, 2000). This latter produced insulin-like
380 peptides (ILPs) that are distributed within the body through the hemolymph and notably act on the
381 *corpora allata* (Badisco *et al.*, 2013; Tu *et al.*, 2005). Taking into consideration the data mentioned
382 above, we hypothesize that the influence of diet, on the development of the reproductive system,
383 and probably also on the ontogenesis of sexual behavior, is in part mediated by ILPs and JH in
384 male *A. ipsilon*. We suggest that glucose-containing diets would promote ontogeny of this sexual
385 behavior *via* increased JH biosynthesis. To consolidate the existence of this hypothetical scenario,
386 preliminary experiments are in progress to quantify hemolymphatic levels of JH by using reverse-
387 phase high-performance liquid chromatography coupled to a fluorescent detector on the one hand,
388 and to evaluate the male behavioral responsiveness to female sex-pheromone in a wind tunnel
389 according to diets on the other hand.

390 **5. Conclusion**

391 Our results brought to light that diet is closely linked to the maturation of the male
392 reproductive system during adulthood of the moth *A. ipsilon*. This developmental process is
393 promoted with diets rich in diverse sugars, comprising glucose, along with sodium. Thus, these
394 specific diets increase in length and protein content of accessory sex glands and accelerate the
395 decrease in testicular volume and the transfer of sperm bundles from the testes to the duplex. In
396 addition, our data support the hypothesis that, in nature, adult male *A. ipsilon* feeds on flower
397 nectars rich in sucrose, glucose, and fructose, and practices the puddling behavior for a sodium
398 intake as described in other moth species (Smedley & Eisner, 1996; Tiedge & Lohaus, 2017).
399 Therefore, our study provides new insights into how nutritional signals influence sexual maturation
400 in male moths such as *Agrotis ipsilon*: in nature, males could thus mate as early as the third day of
401 adult life. A further step is to investigate the functional interactions between the diet and the
402 endocrine system, more especially with the JH, in the acquisition of fertility in synchronism with
403 mating behavior.

404 **Author Contributions**

405 EF, MD, and SD designed the experiments. EF and PC performed the experiments. EF and
406 MD prepared the figures and analyzed the data. EF wrote the first draft of the manuscript. All
407 authors contributed to manuscript revision, read and approved the submitted version.

408 **Disclosure**

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

411 **Acknowledgments**

412 We thank our technical colleagues from team Neuro-ethology of Olfaction in iEES Paris, and
413 more specifically Caroline Suray, for rearing the animals used in the experiments; Nicolas Durand
414 for the comparative analysis of genome of *Agrotis ipsilon*, as well as Abhishek Chatterjee for
415 helpful comments on the manuscript.

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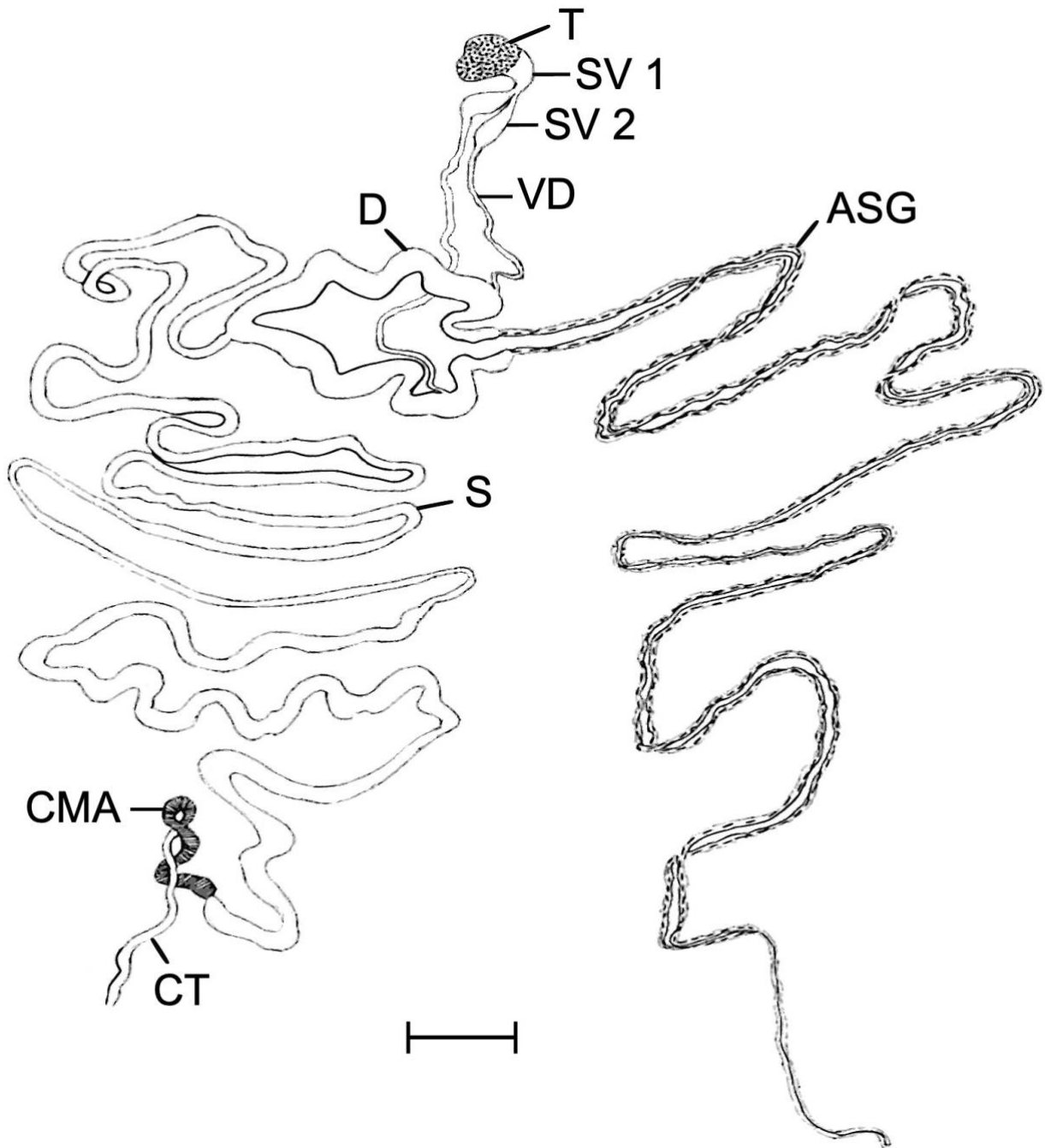
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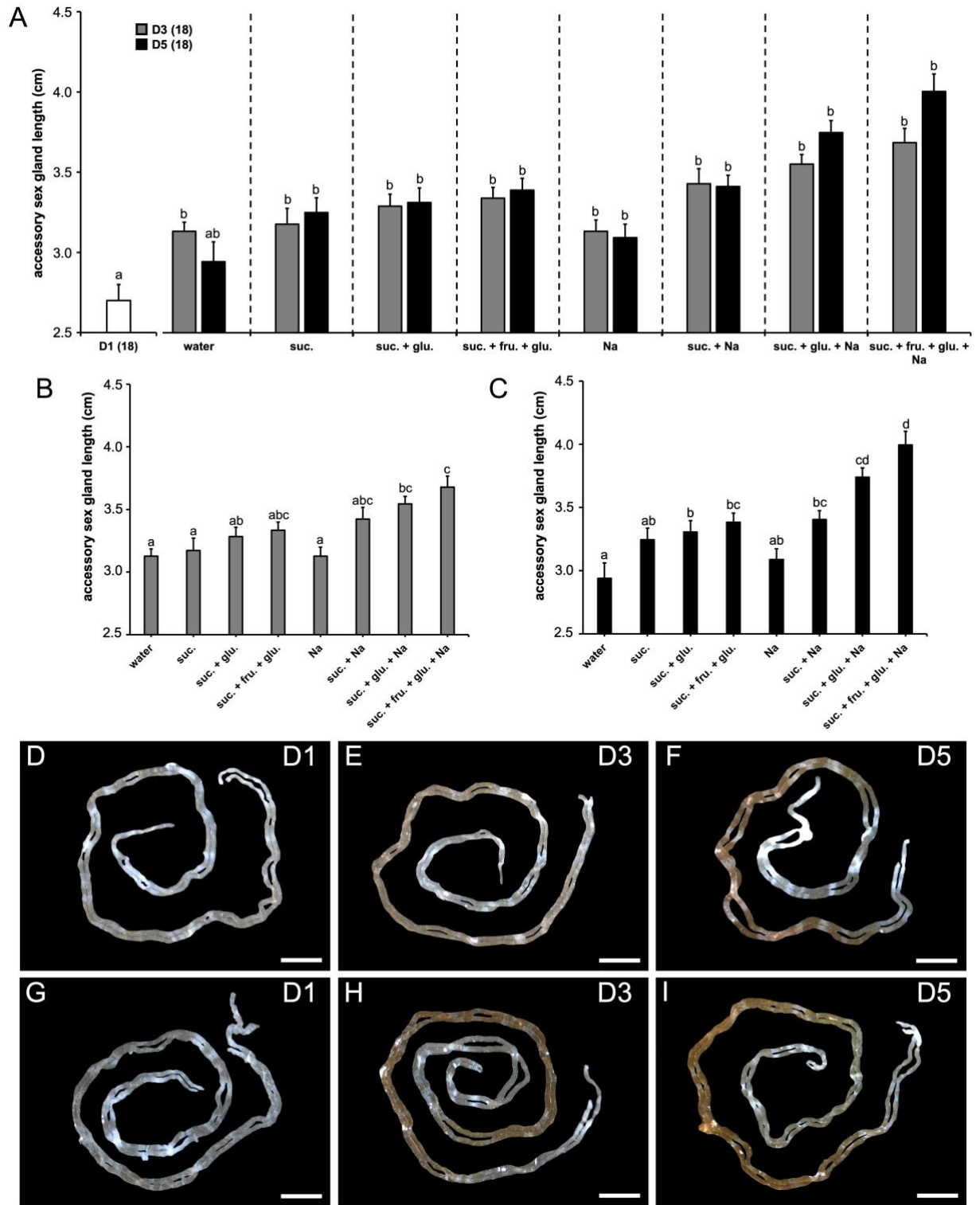
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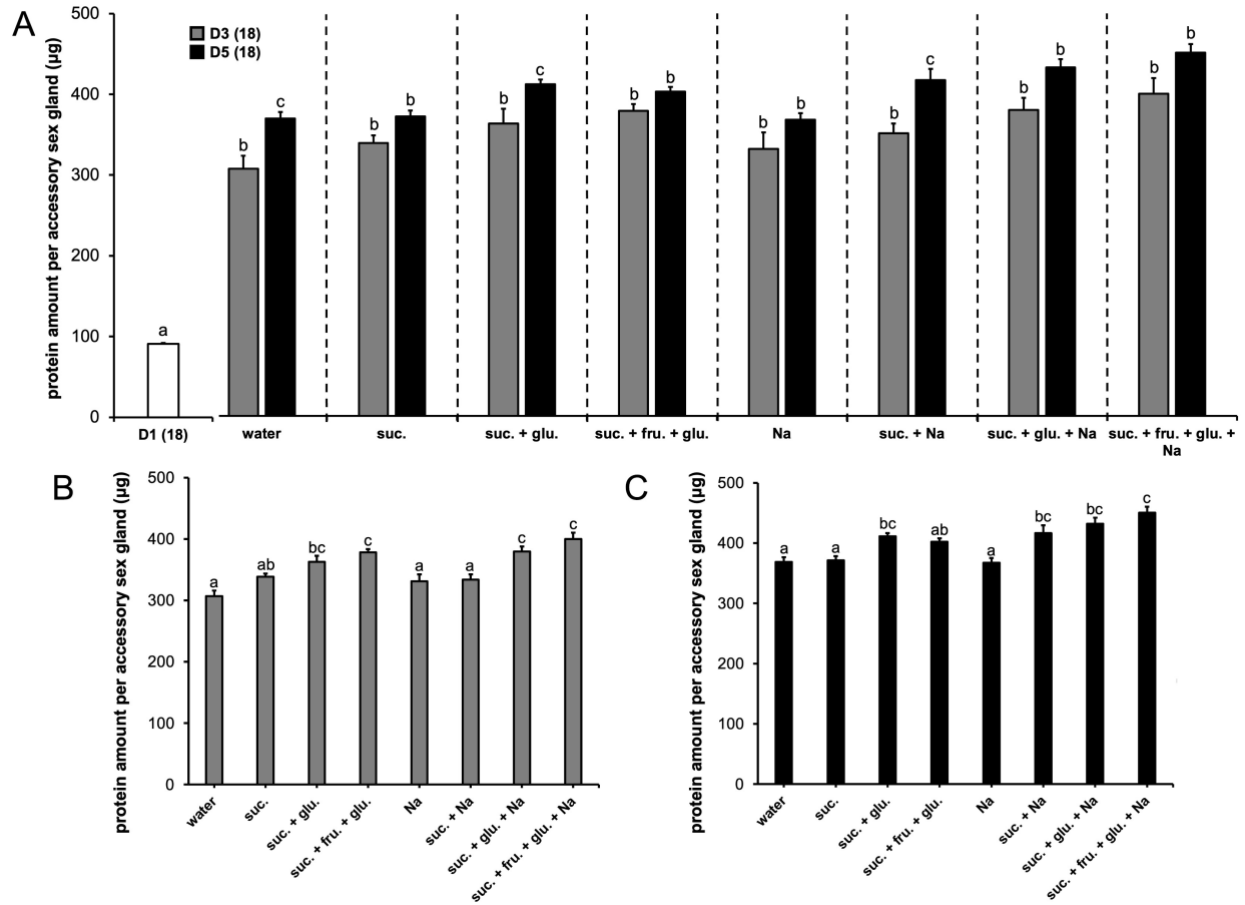


563
564 **Figure 1.** Diagram of the reproductive system in adult male moth *Agrotis ipsilon* (redrawn from
565 [Sekhon, 2017](#)). ASG: accessory sex gland; CMA: constrictor muscle area; CT: cuticular tube; D:
566 duplex; S: simplex; T: testes; SV 1: seminal vesicle 1; SV 2: seminal vesicle 2; VD: vas deferens.
567 Scale bar = 2 mm.

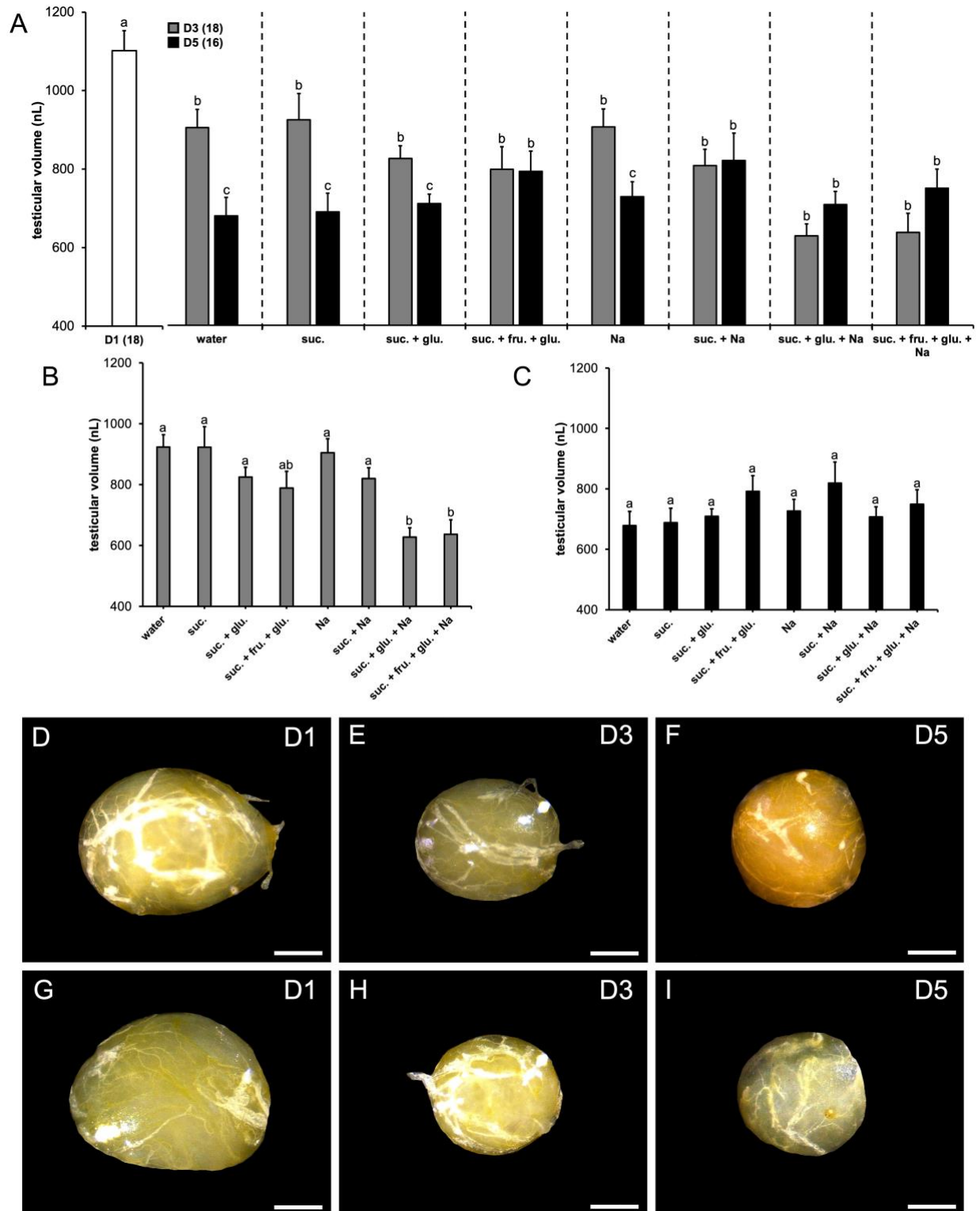


568
 569 **Figure 2.** Accessory sex gland length according to diet. The length of sex accessory glands was
 570 measured in not fed males on the first post-emergence day (D1) (white), and in males fed with
 571 various diets on the third (D3) (grey) or the fifth (D5) (black) post-emergence day. Values

572 correspond to mean \pm SEM (n = 18 for each diet). **(A)** Results for D1, D3, and D5 males. Letters
573 represent the statistical differences after Tamhane's test for water (data transformed to power 2);
574 after Dunn's test for suc. + Na; and after Tukey's test for other diets with data transformation
575 (elevated to power 0.8) for suc. ($p < 0.05$). **(B)** Same data as in (A) for D3 males. Letters represent
576 the statistical differences after Dunn's test ($p < 0.05$). **(C)** Same data as in (A) for D5 males. Letters
577 represent the statistical differences after Tukey's test (data transformed to power 1.5) ($p < 0.05$).
578 Morphology of accessory sex glands in males fed with water **(D, E, F)** and fed with sucrose +
579 fructose + glucose + Na **(G, H, I)** at D1, D3, and D5. Scale bar = 25 μ m.



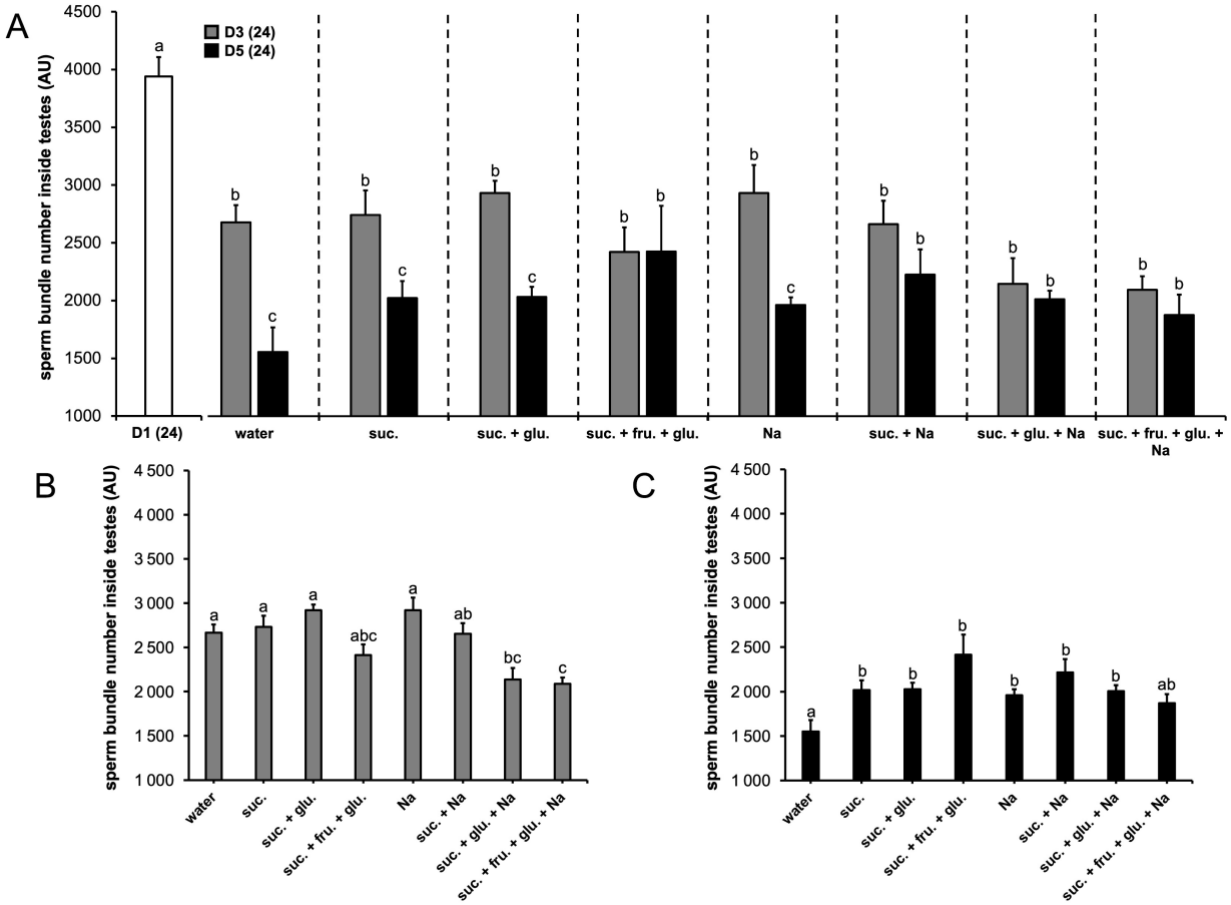
580
 581 **Figure 3.** Accessory sex gland protein content according to diet. The protein amount of accessory
 582 sex glands was quantified in not fed males on the first post-emergence day (D1) (white), and in
 583 males fed with various diets on the third (D3) (grey) or the fifth (D5) (black) post-emergence day.
 584 Values correspond to mean \pm SEM ($n = 18$ for each diet). Letters represent the statistical differences
 585 after Dunn's test ($p < 0.05$). (A) Results for D1, D3, and D5 males. (B) Same data as in (A) for D3
 586 males. (C) Same data as in (A) for D5 males.



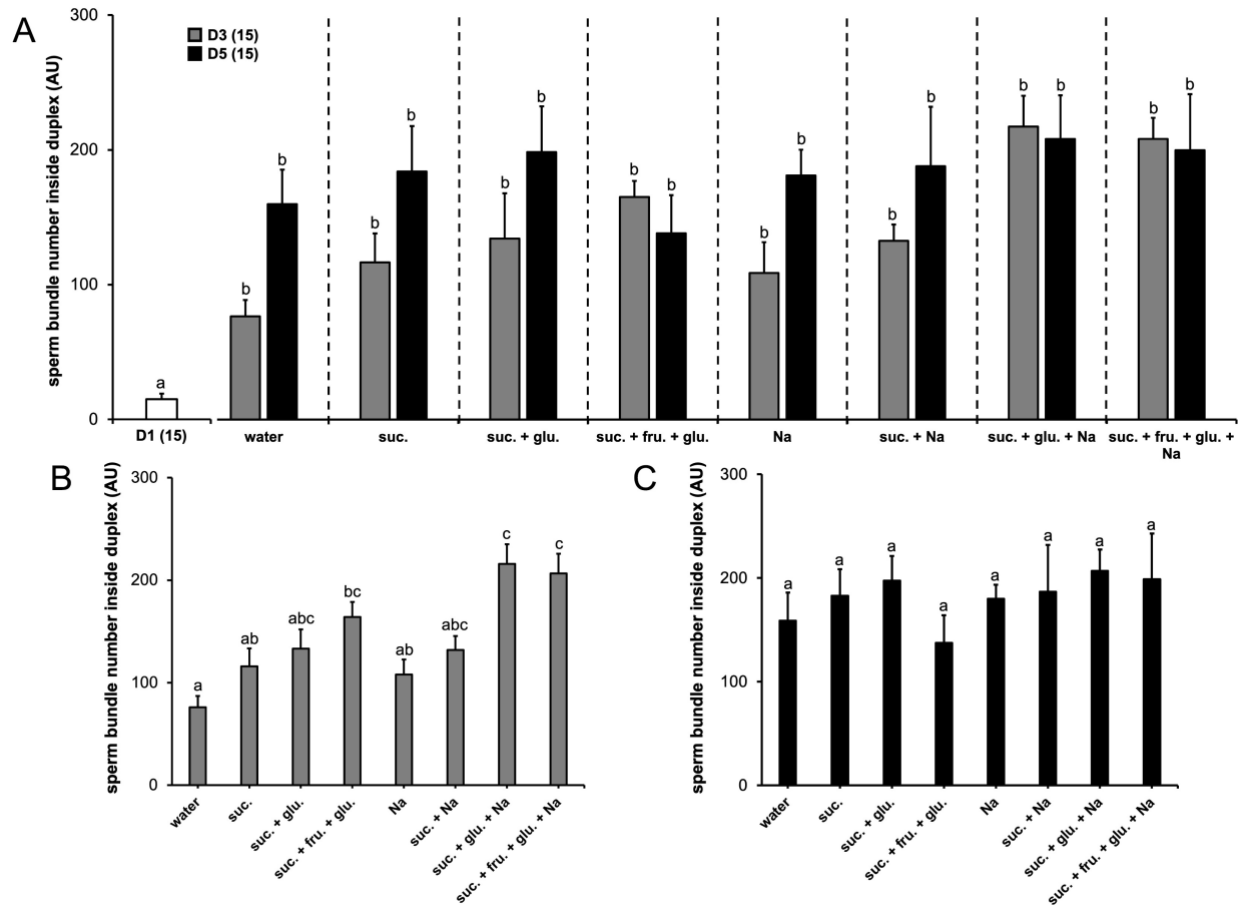
587
 588 **Figure 4.** Testicular volume according to diet. The volume of testes was calculated in not fed males
 589 on the first post-emergence day (D1) (white), and in males fed with various diets on the third (D3)
 590 (grey) or the fifth (D5) (black) post-emergence day. Values correspond to mean \pm SEM ($n_{D3} = 18$)

591 for each diet; $n_{D5} = 16$ for each diet). **(A)** Results for D1, D3, and D5 males. Letters represent the
592 statistical differences after Scheffe's test for water, Na, suc. + fru. + glu. (data transformed to power
593 0.4) and suc. + Na (data transformed at power 0.1); after Tamhane's test for suc. + glu.; and after
594 Dunn' test for other diets ($p < 0.05$). **(B)** Same data as in (A) for D3 males. Letters represent the
595 statistical differences after Dunn's test ($p < 0.05$). **(C)** Same data as in (A) for D5 males. Letters
596 represent the statistical differences after an ANOVA's test (data transformed to power 0.1) ($p <$
597 0.05).

598 Morphology of testes in males fed with water **(D, E, F)** and fed with sucrose + fructose + glucose
599 + Na **(G, H, I)** at D1, D3, and D5. Scale bar = 500 μm .



600
 601 **Figure 5.** Sperm bundle number inside testes according to diet. The number of sperm bundles in
 602 the testes was counted in not fed males on the first post-emergence day (D1) (white), and in males
 603 fed with various diets on the third (D3) (grey) or the fifth (D5) (black) post-emergence day. Values
 604 correspond to mean \pm SEM ($n = 24$ for each diet). (A) Results for D1, D3, and D5 males. Letters
 605 represent the statistical differences after Dunn's test for water, suc., and suc. + fru. + glu.; after
 606 Tukey's test for suc. + Na and after Tamhane's test for other diets ($p < 0.05$). (B) Same data as in
 607 (A) for D3 males. Letters represent the statistical differences after Dunn's test ($p < 0.05$). (C) Same
 608 data as in (A) for D5 males. Letters represent the statistical differences after Tamhane's test (data
 609 transformed to power 1.4) ($p < 0.05$).



610
 611 **Figure 6.** Sperm bundle number within the duplex according to diet. The number of sperm bundles
 612 in the duplex was counted in not fed males on the first post-emergence day (D1) (white), and in
 613 males fed with various diets on the third (D3) (grey) or the fifth (D5) (black) post-emergence day.
 614 Values correspond to mean \pm SEM ($n = 15$ for each diet). **(A)** Results for D1, D3, and D5 males.
 615 Letters represent the statistical differences after Dunn's test ($p < 0.05$). **(B)** Same data as in (A) for
 616 D3 males. Letters represent the statistical differences after Dunn's test ($p < 0.05$). **(C)** Same data
 617 as in (A) for D5 males. Letters represent the statistical differences after Kruskal-Wallis' test ($p <$
 618 0.05).