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Diet influence on male sexual maturation through interplay between insulin signaling and juvenile hormone in insects

Evan Force^{a,*}, Claudia Alvarez^b, Annabelle Fuentes^a, Annick Maria^a, Françoise Bozzolan^a, Stéphane Debernard^{a,**}

^a Sorbonne Université, Université Paris-Est Créteil, INRAE, CNRS, IRD, Institute for Ecology and Environmental Sciences of Paris, France

^b University of Madrid, Institute of Biology, Spain

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ABSTRACT

In animals, sexual maturation coincides with the development of sexual behaviors and reproductive system. These developmental events are influenced by diet and governed by endocrine signals. Here, for the first time in insects, we explored functional links between nutrition and juvenile hormone (JH) in the male reproductive physiology through the insulin signaling pathway (ISP) acting as a transducer of nutritional signals. We turned to the male moth *Agrotis ipsilon* for which sexual maturation, including accessory sex glands (ASGs) development concomitantly with antennal lobes (ALs) maturation for female sex pheromone processing and display of sexual behavior, is known to be JH- and diet-dependent. Indeed, a diet rich in sugars with sodium was previously shown to accelerate sexual maturation, which was achieved from the third day of adult life. In this study, we demonstrated that such a diet raised i) the expression of JH signaling actors (Methoprene-tolerant, Taiman, and Krüppel homolog 1) in ALs and ASGs, ii) the biosynthesis and circulating levels of JH, and iii) the expression of both *insulin receptor (InR)* and *insulin-like peptides (ILPs)* in *corpora allata* (CAs) and brain respectively. Insulin injection raised JH biosynthesis following increased *HMG-CoA reductase* expression in CAs; opposite effects were induced in InR-deficient males. Thus, we highlighted that promoting effects of a diet composed of sugars with sodium on male sexual maturation results from an early induction of ISP causing an increase in JH biosynthesis followed by a potentiation of JH actions on the development of ASGs and ALs in *A. ipsilon*.

1. Introduction

In animals, sexual maturation is a period of life during which the initiation of sexual signaling, the ontogeny of sexual behaviors, and the maturation of the reproductive system occur. These behavioral and physiological changes need to be tightly synchronized to optimize reproductive success and require considerable energy obtained by reallocation of stored energy-rich compounds and/or nutrients acquired from the diet (Dey et al., 2015; Gadenne et al., 2016; Lin et al., 2016; Roff, 1993; Stearns, 1992). It is well documented that this orchestration of sexual maturation is in part under the influence of the endocrine system through i) neuropeptides involved in the evolutionary conserved insulin signaling pathway (ISP), which functions as transducer of nutritional signals, and ii) lipophilic hormones, including steroids and sesquiterpenoid hormones, which act on reproductive system conjointly to nervous centers via organizational and activating effects (Bozzolan

et al., 2015; Gassias et al., 2019; Lin et al., 2016; Romeo, 2003). Over the past decade, accumulated evidence pointed to functional links between these chemical messengers, and the mechanisms underlying this complex network controlling sexual maturation are so far from being fully deciphered.

In insects, the juvenile hormones (JHs), a family of sesquiterpenoids, are produced by a pair of cephalic endocrine glands: the *corpora allata* (CAs) (Tobe and Stay, 1985); which are connected to the brain and closely associated to other neuroendocrine glands, the *corpora cardiaca* (CCs). JHs are synthesized through the mevalonate (MVA) pathway, an ancient metabolic pathway present in all three domains of life including bacteria, archaea, and eucaryotes (Lombard and Moreira, 2011). The first steps of the MVA pathway involve the condensation of three acetyl-CoA units to mevalonate via the activity of acetoacetyl-CoA thiolase, HMG-CoA synthase, and HMG-CoA reductase (HMGR) (Bellés et al., 2005; Noriega, 2014). By analogy with vertebrates, HMGR is the

* Corresponding author. IEES Paris, Sorbonne Université, 4, Place de Jussieu, 75005, Paris, France.

** Corresponding author. IEES Paris, Sorbonne Université, 4, Place de Jussieu, 75005, Paris, France.

E-mail addresses: evan.force@sorbonne-universite.fr (E. Force), stephane.debernard@sorbonne-universite.fr (S. Debernard).

rate-limiting enzyme regulating the MVA pathway in insects (Feyereisen et al., 1981). In addition to their critical roles in larval development and metamorphosis, JHs are also well known to coordinate sexual displays with reproductive maturity in both adult females and males. For example, it has been reported in flies *Anastrepha obliqua* and *Anastrepha suspensa* that treatment of newly emerged males with Methoprene, a JH synthetic analog, induced an acceleration of sexual maturation marked by early development of sexual behavior and sexual signaling (Chacón-Benavente et al., 2013; Teal et al., 2000). In the fruit fly, *Drosophila melanogaster*, JH-deficient females exhibited delayed mating and impaired synthesis of sex pheromones (Bilen et al., 2013). In the noctuid moths *Pseudaletia unipuncta* and *Agrotis ipsilon*, JH was found to promote female calling behavior and sex pheromone synthesis paralleled with oocyte maturation (Cusson et al., 1990, 1994), and to facilitate the execution of sex pheromone-guided flight of male following to the development of the primary olfactory centers, the antennal lobes (ALs), in synchrony with maturation of the accessory sex glands (ASGs) secreting seminal proteins for spermatophore formation as well as nourishment, activation, and capacitation of spermatozoa (Duportets et al., 1998; Gadenne et al., 1993; Gassias et al., 2021).

Most of JH biological actions are mediated by a nuclear protein complex composed of two members of the basic helix-loop-helix/Per-ARNT-Sim family of transcription factors: the JH receptor Methoprene-tolerant (Met) (Jindra et al., 2015b) and its dimerizing partner, termed Taiman (Tai) (Charles et al., 2011; Jindra et al., 2015a; Li et al., 2011, 2014; Zhang et al., 2011). In the presence of JH, the Met-Tai heterodimeric complex binds to an E-box response element or an E-box like motif in the promoter regions of JH target genes, thereby resulting in their transcriptional regulation (Li et al., 2011, 2014; Zhang et al., 2011). *Krüppel homolog 1* (*Kr-h1*), which encodes a C₂H₂ zinc finger-like transcription factor, was identified as an early JH response gene that functions downstream of the Met-Tai complex (Cui et al., 2014; Kayukawa et al., 2012). In various insect species, gene knockout experiments revealed that Met and Tai are actors of the JH signaling pathway in the development of female and male reproductive systems, including oogenesis, vitellogenesis, testicular development, and ASGs maturation (Gassias et al., 2021; Gujar and Palli, 2016; Smykal et al., 2014; Song et al., 2014), and also in the occurrence of male sexual behaviors (Aguilar et al., 2023; Holtorf et al., 2021; Lin et al., 2016).

Nowadays, it is well known that developmental processes such as sexual maturation require considerable energy (Kupferberg, 1997). For instance, it has been revealed in female and male Lepidoptera that specific adult diets have pronounced effects on fertility, egg composition, gamete production, and sexual behavior development (Boggs and Dau, 2004; Geister et al., 2008; Marchioro and Foerster, 2013; O'Brien et al., 2004). As in other metazoans, the insect ISP acts as a sensor of the organism's nutritional status by regulating key physiological processes such as development, growth, and reproduction, through the binding of insulin-like peptides (ILPs, also termed insulin-related peptides or IRPs) to their tyrosine kinase transmembrane insulin receptors (InRs) (Howell and Manning, 2011; Lin and Smagghe, 2019; Siddle, 2012). After nutrient intake, insect ILPs are mainly synthesized and released from the central nervous system (Mizoguchi and Okamoto, 2013), particularly by the brain medial neurosecretory cells (MNSCs) (Okamoto and Yamana, 2015). Moreover, insects possess varying numbers of ILPs, some of which are functionally similar to vertebrate insulin (Brogiolo et al., 2001). For example, two ILPs were found in the migratory locust, *Locusta migratoria* (Veenstra et al., 2021), eight ILPs were reported in *D. melanogaster* (Nässel et al., 2013) and *A. aegypti* (Riehle et al., 2006), and three ILPs were isolated in *A. ipsilon* (Diesner et al., 2018). By contrast, only one InR has been identified in dipterans and lepidopterans (Pan et al., 2022; Tatar et al., 2001), and two in hymenopterans and hemipterans (de Azevedo and Hartfelder, 2008; Ding et al., 2017; Guo et al., 2016; Lu and Pietrantonio, 2011).

During the past decade, the function of ISP in insect reproductive physiology has been extensively investigated in females. Experiments

performed in several species brought in evidence that the ISP activates vitellogenesis and oocyte maturation in response to a positive nutritional state (Marquez et al., 2011). Indeed, in the female *Blattella germanica*, the RNAi-mediated silencing of *InR* resulted in decreased *vitellogenin* expression and altered ovarian development (Abrisqueta et al., 2014); in the female *A. aegypti*, *ILP3* was shown to enhance egg maturation through its binding to *InR* after a blood feeding (Brown et al., 2008). Furthermore, several studies carried out on females demonstrated that ISP can act alone but also *via* connections with JH signaling. For instance, in the bug *Rhodnius prolixus*, it has been recently revealed that ISP promotes the CAs' activities through upregulation of the expression of JH biosynthetic enzymes (Leyria et al., 2022). Then, in *D. melanogaster*, the inactivation of *InR* expression in CAs led to a reduction in the *HMGR* transcriptional activity (Belgacem and Martin, 2007), causing JH-deficient phenotypes (Tu et al., 2005).

By contrast, only a few studies have revealed the regulatory effects of ISP on spermatogenesis and testicular development in *D. melanogaster* (Azuma et al., 2021; Dreyer and Shingleton, 2019), and to date, there are no available data on relationships involving nutrition, ISP, and JH in the context of reproduction of male insects. In order to reveal the existence of such a tightly link, we turned to the male moth *Agrotis ipsilon*, a model organism for which many aspects of sexual maturation (including ontogeny of sexual behavior through the AL development, as well as ASG maturation) are known to be JH- and diet-dependent. Just after emergence, males are sexually immature and their ASGs have a low protein content (Force et al., 2023a; Gassias et al., 2021). In addition, these young males are not capable of responding to sex pheromones emitted by females (Aguilar et al., 2023; Duportets et al., 1998; Gadenne et al., 1993). Five days after emergence, males reach full sexual maturity: ASGs are well-developed and exhibit an elevated protein synthesis (Force et al., 2023a; Gassias et al., 2021). These old males are highly attracted to female sex pheromones and display an oriented flight toward females (Aguilar et al., 2023; Duportets et al., 1998; Gadenne et al., 1993). This increase in behavioral responsiveness is due to the higher sensitivity of AL neurons responding to the pheromone signal (Anton and Gadenne, 1999; Gadenne and Anton, 2000). Furthermore, in male moths, CAs produce different types of JH acids; one of the most predominant forms is the JH-II acid which is converted into JH-II by the activity of juvenile hormone acid O-methyltransferase (JHAMT) in ASGs and then released into hemolymph (Guo et al., 2021; Shirk et al., 1983). JH-II levels in hemolymph of old sexually mature males were found to be higher than those of young sexually immature males. Such a difference was partly due to an increase in JH-II acid biosynthesis by CAs with aging (Duportets et al., 1998). In addition, JH-II acts simultaneously on both ASGs and ALs through the activation of the Met-Tai-Kr-h1 pathway to promote the synthesis of seminal proteins and the central processing of sex pheromone signal (Aguilar et al., 2023; Gassias et al., 2021). Furthermore, recent studies showed that a diet composed of sucrose, fructose, and glucose supplemented with sodium accelerates sexual maturation, which was achieved from the third day of adult life (Force et al., 2023a, 2024b).

In the present study, we explored the potential connections between nutritional signals, ISP, and JH signaling in the context of male sexual maturation in *A. ipsilon*. According to age, we first examined the diet impact on JH signaling by evaluating the expression levels of *Met1*, *Tai*, and *Kr-h1* in ALs and ASGs. We then quantified the JH-II hemolymph levels and the *in vitro* JH-II acid biosynthesis by CAs in correlation to the *HMGR* expression in CAs. Besides, we assessed the diet effects on ISP by quantifying the transcriptional activities of *InR* in CAs and also three ILPs (*ILP1*, *ILP2*, and *IPL4*) in the brain. We finally investigated the effects of injection of exogenous insulin or RNAi-mediated silencing of *InR* on the JH-II acid biosynthesis and the amounts of *HMGR* mRNA in CAs. Taken together, our results highlighted that promoting effects of a diet composed of sucrose, fructose, glucose plus sodium on male sexual maturation results from an early induction of ISP, which increases the JH-II acid biosynthesis causing the potentiation of JH-II actions on the

development of ASGs and ALs in *A. ipsilon*.

2. Materials and methods

2.1. Insects

This study focused on adult males *Agrotis ipsilon* (Lepidoptera: Noctuidae). This insect is native to the South of France. The animals were raised in our laboratories at Sorbonne Université. The larvae were stored in collective boxes with the same artificial food until metamorphosis (Poitout and Buès, 1974). Males and females were separated during metamorphosis thanks to the sexual dimorphism of the chrysalis which allows them to be differentiated. The nymphs were then kept in two different air-conditioned rooms (22 °C, 50–60% relative humidity) and subjected to a reverse photoperiod (8 h of darkness and 16 h of light, light starting at 6 p.m.). Each morning, among the emerged moths, we collected and placed several insects in boxes (20 x 11.5 x 5 cm) each containing 6 males at most. The harvest day was considered the first post-emergence day (noted D1). The moths had *ad libitum* access to a sugar solution or to water depending on the treatments. In addition, males can fast for at least 5 days without becoming weak.

2.2. Regimens

Immediately after emergence, D1 males were subjected to different diets. The effects of diet on their sexual maturation were examined at three (D3) and five (D5) days after emergence. These days correspond respectively to the beginning and the end of sexual maturation under standard rearing conditions. In our study, we used two solutions: a first containing osmosis water and a second containing osmosis water to which were added 4% sucrose with 4% fructose, 4% glucose, and 1% NaCl (noted suc. + fru. + glu. + Na). These sugars are the major constituents of flower nectar, the main food source of adult Lepidoptera, with generally 10–15% of the fresh mass for a single sugar or a mixture of three sugars (Roy et al., 2017). Here, the different sugars are in equal proportions, as in most flower nectars. Sodium is an important mineral element involved in the reproduction of many moths (Molleman, 2010; Smedley and Eisner, 1996). We chose a proportion equal to 1% (*i.e.*, 0.17 M), known to be attractive in other insects (Kaushik et al., 2018; Niewalda et al., 2008; Pontes et al., 2017). All nutrients (sugars and NaCl, purity >99%) came from Sigma-Aldrich (Saint-Quentin-Fallavier, France).

2.3. Collection of tissues

Brain, antennal lobes (ALs), *corpora allata* (CAs), and accessory sex glands (ASGs) were collected during dissections carried out in the middle of scotophase (between 1:00 p.m. and 4:00 p.m.) when males were most active (Barrozo et al., 2010; Force et al., 2023b), under a Leica S9D stereomicroscope. More specifically, the collection of accessory sexual glands was carried out in Ringer's solution (6.13% glucose, 0.24% HEPES, 0.11% MgCl₂, 0.07% NaCl, 0.05 % KCl, 0.01% CaCl₂). All collected tissues were immediately bathed in TRIzol (TRI Reagent®, Euromedex, Souffelweyersheim, France) in Eppendorf tubes (Montesson, France), and then stored at –80 °C.

2.4. Chemicals

Juvenile hormone-II (JH-II) was purchased from SciTech (Prague, Czech Republic). JH-II acid was obtained by an enzymatic procedure using pig liver carboxylesterase (Sigma-Aldrich, Saint-Quentin-Fallavier, France), in which three enzyme units converted 1 µmol of JH-II into JH-II acid in 1 h at 40 °C in a phosphate buffer (50 mmol/L, pH 7.4). The JH-II acid formed was subsequently purified by reverse phase high performance liquid chromatography.

2.5. *In vitro* biosynthesis of JH-II acid and *in vivo* insulin injection

CAs were dissected and incubated for 4 h at 28 °C in a salinized vial containing 150 µL of tissue culture medium M-199 (Thermo Fisher Scientific, Illkirch, France) supplemented with Ficoll (20 mg/mL) and (E,E)-farnesoic acid (200 µM) (Sigma Aldrich, Saint-Quentin Fallavier, France). Each incubation was carried out with three pairs of CAs. At the end of incubation, 500 µL of ethyl acetate was added to the sample which was vortexed for 1 min and spun for 5 min at 4 °C and 2000 g. The organic phase containing JH acid was transferred to a new vial, and then dried under nitrogen flow and stored at –80 °C. The dried extract was re-suspended in 50 µL of acetonitrile and vortexed for 1 min. JH-II acid was quantified by reverse phase high performance liquid chromatography (RP-HPLC).

Furthermore, an experiment consisting of *in vitro* quantification of JH-II acid produced by CAs was carried out on adult D3 males after a post-emergence abdominal injection of 2 µL of 0.25 µg/µL of porcine insulin (Sigma Aldrich, Saint-Quentin Fallavier, France) diluted in saline solution (CaCl₂ 7.1 mM, Na₂β-glycerophosphate 22 mM, MgSO₄ 13.5 mM, MgCl₂ 26.9 mM, KCl 29.5 mM, glucose 23.9 mM, pH 6.8) or 2 µL of saline solution (control). The injections were done in the lateral region of the intersegment membrane between the second and third abdominal segments and they were performed at the beginning of the scotophase by using a Hamilton syringe with a 32-gauge needle. Only water-fed males were examined because their circulating JH-II levels were low. Additionally, insects are able to respond to insulin by rapidly activating insulin signaling (Leyria et al., 2020).

2.6. Hemolymph collection and JH-II extraction

The collection of hemolymphs was carried out in *A. ipsilon* males aged 1-, 3- and 5-days post-emergence. The top of the moth's head was cut off and the individual was then placed in a 0.25 mL Eppendorf tube with a cut base (only the head of the animal came out of the tube at the level of the cut base). This tube was then positioned in a 1.5 mL Eppendorf tube, and centrifuged at 1 rcf for 3 min. Each sample contained 50 µL of hemolymph (from several males) mixed with 100 µL of pure ethanol (Carl Roth, Germany). Then, 200 µL of pure hexane was added to the solution and thoroughly vortexed again. The mixture was centrifuged at 10 000 g for 10 min at 4 °C and the supernatant containing JH was transferred into a new vial and dried under nitrogen flow and stored at –80 °C. The dried extract was re-suspended in 50 µL of acetonitrile and vortexed for 1 min and JH-II was quantified by RP-HPLC.

2.7. JH-II acid and JH-II quantifications

JH-II acid and JH-II titers were determined by RP-HPLC using the HPLC system (Beckman System Gold HPLC, Palo Alto, CA, USA) with model 166 ultraviolet detector (Beckmann, Palo Alto, CA, USA). The column was Kromasil® C18 reverse-phase HPLC column (4.6 x 250 mm, 5 µm particles) and was eluted using a linear gradient from 5% to 80% acetonitrile in 5 mmol/L HEPES adjusted to pH 6.2 over 20 min at a flow rate of 1 mL/min with UV detection monitored at 245 nm. The quantification of JH-II and JH-II acid was computed by comparison of the peak area with external quantitative standards using the Beckman Gold system software.

2.8. Synthesis of complementary DNA (cDNA)

Total RNA was extracted from pooled tissues of several males with TRIzol reagent (TRI Reagent®, Euromedex, Souffelweyersheim, France) according to the manufacturer's instructions and was quantified using UV/Visible Spectrophotometer (NanoPhotometer® NP80, IMPLLEN, Munich, Germany). DNase treatment was carried out with RNase-free recombinant DNase I (Roche Diagnostics GmbH, Mannheim,

Germany) for 30 min at 37 °C followed by 10 min inactivation at 70 °C. The reaction also contained RNase OUT (Invitrogen, Carlsbad, CA, USA). After DNase treatment, single-stranded cDNA was synthesized from 5 µg total RNA with SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The reaction contained a mixture of deoxynucleotide triphosphates (dNTPs), Oligo(dT) primers, and sterile water for a final volume of 20 µL. The mixture was heated at 42 °C for 50 min and then incubated at 70 °C for 15 min.

2.9. Quantification of gene expression

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was carried out from the extraction of total RNA from different tissues (brain, ALs, CAs, and ASGs) and using the LightCycler 480 Real-Time PCR Detection System (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Each 10 µL reaction included 5 µL of Absolute Blue SYBR green (Thermo Scientific, Waltham, MA, USA), 4 µL of cDNA (12.5 ng/µL), and 1 µL of 10 µmol/L specific primer pairs for *Met1* (qMet1dir and qMet1rev), *Kr-h1* (qKrh1dir and qKrh1rev), *Tai* (qTaidir and qTairev), *HMGR* (qHMGRdir and qHMGRrev), *ILP1* (qILP1dir and qILP1rev), *ILP2* (qILP2dir and qILP2rev), or *ILP4* (qILP4dir and qILP4rev) (Supplementary Table S1). The PCR conditions were 50 cycles of 95 °C for 10 s, 65 °C for 15 s, 72 °C for 15 s and the length of the amplified products was 223 bp for *Met1*, 190 bp for *Kr-h1*, 202 bp for *Tai*, 200 bp for *HMGR*, 207 bp for *ILP1*, 206 bp for *ILP2*, 200 bp for *ILP4*, and 200 bp for *InR*.

Fluorescence measurements on a melting curve between 55 and 95 °C confirmed the presence of a single specific peak and the absence of primer-dimer peaks for the primer pairs used. Each run included a negative control (water) and a series of five-fold dilutions of pooled cDNA (from all conditions). The five-fold dilution series (undiluted, 1/5, 1/25, 1/125, and 1/625) were used to construct a relative standard curve to determine PCR efficiency and for further quantification. In all experiments, the primers gave amplification efficiencies of 95–100%. Each reaction was carried out in three technical replicates with nine independent biological replicates using the technical platform of the Institute of Integrative Biology (Sorbonne Université, France). Expression levels were analyzed with LightCycler 480 software (Roche Diagnostics GmbH, Mannheim, Germany) and the GENORM Visual Basic application for Microsoft EXCEL (Issy-les-Moulineaux, France) as described by Vandesompele et al. (2002) (Vandesompele et al., 2002). Cycle threshold (Ct) values were determined for each candidate gene as well as for the reference gene encoding ribosomal protein L8 (*RpL8*) of *Agrotis ipsilon* (accession number JX975720.1). This gene had the most stable expression levels among other reference genes tested (*ribosomal protein L13*, *ribosomal protein S3*, *glyceraldehyde-3-phosphate dehydrogenase*, *α-tubulin*, and *β-actin*). The average Ct value of each triplicate was used to normalize the expression level of the candidate gene to the geometric mean of the *RpL8* level in the Q-GENE software (Simon, 2003) using the formula:

$$NE = (E_{RpL8})^{Ct_{RpL8}} / (E_{\text{candidate gene}})^{Ct_{\text{candidate gene}}}$$

with NE meaning normalized expression, E meaning PCR efficiency, and Ct corresponding to the crossover point values. Then, the mean and standard error were calculated from the NE values for the biological replicates. The sequences of the specific primers for *RpL8* (qRpL8dir and qRpL8rev) are presented in Supplementary Table S1, the length of the amplified products was 200 pb.

2.10. RNA interference experiments

Double-stranded RNA (dsRNA) production and injection conditions were established by following the protocols used in *A. ipsilon* (Aguilar et al., 2023) for the silencing of *InR*. The dsRNAs targeting *InR*

(*InR-1*-dsRNA, 492 bp; and *InR-2*-dsRNA, 512 bp) and bacterial β-galactosidase (*LacZ*-dsRNA, 372 bp; control) were chosen by using the E-RNAi web application (<http://e-rnai.dkfz.de/>) (Arziman et al., 2005) and produced with a MEGAscript® T7 High Yield Transcription Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. A PCR was performed on 1 µL plasmid (50 ng/µL) with specific primers for each target gene (*InR-1*-T7dir/*InR-1*-T7rev, *InR-2*-T7dir/*InR-2*-T7rev, *LacZ*-T7dir/*LacZ*-T7rev) (Supplementary Table S2) under the following program: 35 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min. PCR products were purified with a Nucleospin® extract II kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) and quantified using UV/Visible Spectrophotometer (NanoPhotometer® NP80, IMPLLEN, Munich, Germany). The transcription reaction was then performed overnight at 37 °C on 1 µg of PCR product in a reaction mix containing 2 µL of each nucleotide, 2 µL of T7 RNA polymerase enzyme, and 2 µL of T7 10X Buffer in a final volume of 20 µL. After spectrometry quantification, the reaction mix was incubated for 15 min at 37 °C with 2 U of Turbo DNase (Invitrogen, Carlsbad, CA, USA). The dsRNA precipitation was performed by addition of 30 µL of water treated with diethylpyrocarbonate (DEPC) and 20 µL LiCl (Sigma Aldrich, Saint-Quentin Fallavier, France), storage of the mixture at –20 °C for 2 h, and then centrifugation for 30 min at 16 000 g. The pellet was washed with 1 mL 75% ethanol, centrifuged, and dried before elution in 11 µL DEPC water. Samples were denatured at 95 °C for 5 min followed by a rehybridization step of 1 h 30 min at room temperature. The dsRNA integrity was then checked by loading on an agarose gel. Before injection, dsRNA was diluted to 0.5 µg/µL in saline solution.

Newly emerged males were anesthetized with carbon dioxide and injected with 1 µg dsRNA in the lateral region of the intersegment membrane between the second and third abdominal segments. The injections were performed at the beginning of the scotophase by using a Hamilton syringe with a 32-gauge needle. On D3 males, CAs were dissected for the *InR* and *HMGR* expression analysis. The control group was composed of bacterial *LacZ*-dsRNA-injected males. Concomitantly to the quantification of the expression level of the target genes, a measurement of the *in vitro* biosynthesis of JH-II acid and the circulating JH-II levels was carried out according to the protocol described above.

2.11. Statistical analysis

Data analysis was done using R 4.1.2 (R Core Team (2021); RStudio 2021.09.2 software. The sample sizes are reported in the legend of each figure. The alpha risk was 0.05.

For each dataset, the assumption of normal data distribution was tested by the Shapiro-Wilk test. When this was satisfied, an ANOVA was carried out, and when it was significant, post-hoc test was conducted: a Student's t-test (for comparison between two groups) or a Tukey's test (for multiple comparison). Alternatively, non-normal data were analyzed with a Kruskal-Wallis' test. If it was significant, a Wilcoxon's test (for comparison between two groups) or a Dunn's post-hoc test (for multiple comparison) was performed.

2.12. Ethics note

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

3. Results

3.1. Various sugars plus sodium potentialize the biological actions of JH on ALs and ASGs

We assessed the expression levels of actors involved in JH signaling

in ALs and ASGs for 1- (D1), 3- (D3), and 5-day-old (D5) males fed with various sugars and sodium or with water (Fig. 1). For the JH receptor *Met1*, both in ALs (Fig. 1A) and ASGs (Fig. 1B), males fed various sugars and sodium strongly expressed *Met1* with a maximum reached from D3 (Tukey's test, $p < 0.001$). By contrast for water-fed males, the expression level of *Met1* increased gradually over the first five days of adult life (Tukey's test, $p < 0.001$). At D3 and D5, a diet rich in sugars and sodium led to approximately twofold the AL and ASG *Met1* expression compared to water-fed males (Student t-test, $t \geq -12.36$, $df \leq 16.00$, $p < 0.001$).

For *Tai*, the partner of *Met1*, its expression level in ALs also significantly increased between D1 and D3 (Dunn's test, $p = 0.003$) and remained constant until D5 for males fed with sugars and sodium (Dunn's test, $p = 0.907$) (Fig. 1C). While a similar profile was observed for water-fed males (Dunn's test, $p \leq 0.001$), the AL *Tai* expression level was much lower than that of males fed with various sugars and sodium (Wilcoxon's test, $W = 0.00$, $p < 0.001$). Furthermore, in ASGs (Fig. 1D), for males fed water, the expression level of *Tai* increased significantly only at D5 (Tukey's test, $p < 0.001$), whereas for males fed sugars and sodium, *Tai*

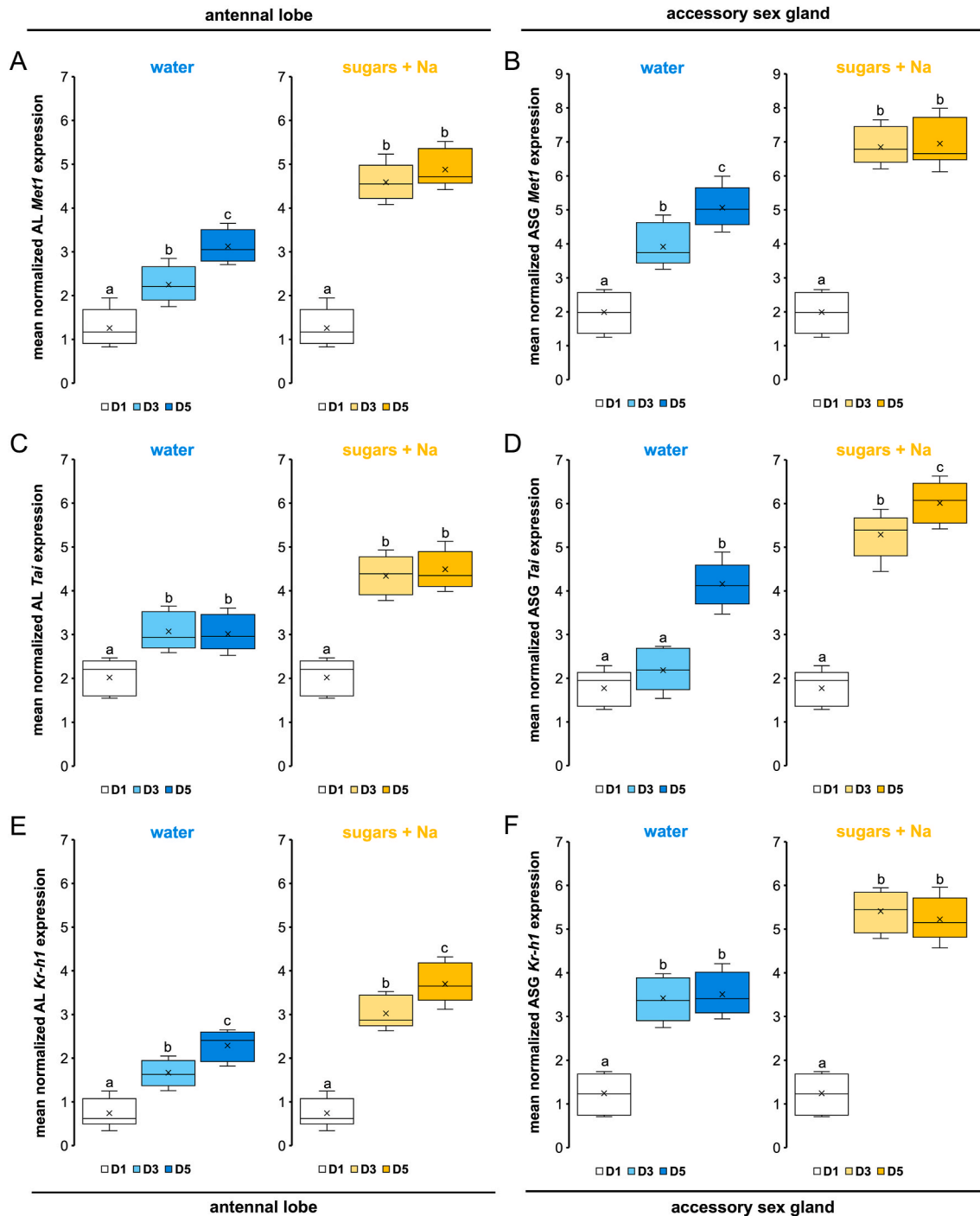


Fig. 1. Effect of diet on the expression level of *Met1*, *Tai*, and *Kr-h1* in the antennal lobe (AL) and accessory sex gland (ASG) of *Agrotis ipsilon* males. The amounts of ALs and ASGs *Met1* (A & B), *Tai* (C & D), and *Kr-h1* (E & F) mRNA were evaluated for 1- (D1), 3- (D3), and 5-day-old (D5) males fed with water or with sugars plus sodium. Each qPCR was run in 3 technical replicates and 9 independent biological replicates. The bars represent the value distribution, crosses denote the mean, and the letters indicate a statistically significant difference (Tukey's or Dunn's test, $p < 0.05$).

expression increased steadily with age to reach its maximum at D5 (Tukey's test, $p < 0.001$). Moreover, at both D3 and D5, the ASG *Tai* expression was almost two times higher than that of water-fed males (Student t-test, $t \geq -13.66$, $df \leq 15.94$, $p < 0.001$). Then in ALs, regardless of diet, the expression level of *Kr-h1* constantly increased with age (Tukey's test, $p < 0.001$) (Fig. 1E). However, at D3 and D5, males fed with sugars and sodium expressed *Kr-h1* in ALs almost twice as much as those fed with water (Student t-test, $t \geq -8.93$, $df \leq 15.52$, $p < 0.001$). In ASGs, *Kr-h1* expression level was considerably increased between D1 and D3 to reach a maximum until D5 whatever the diet (Tukey's test, $p < 0.001$) (Fig. 1F). By contrast, compared to water-fed males, *Kr-h1* was more highly expressed in ASGs at D3 and D5 in males fed with sugars and sodium (Student t-test, $t \geq -8.85$, $df \leq 15.99$, $p < 0.001$).

3.2. Diet rich in sugars with sodium drastically increases the circulating JH-II and the JH-II acid biosynthesis through an upregulation of HMGR in CAs

We then compared the circulating levels of JH-II for 1-, 3- and 5-day-old males fed with water or with sugars and sodium (Fig. 2A). While the circulating JH-II levels increased constantly from D1 to D5 for males fed water to reach 990 ± 35 fmol/ μ L at D5 (Tukey's test, $p \leq 0.022$), males fed with sugars and sodium reached a circulating JH-II level approximately equal to 1900 ± 100 fmol/ μ L from D3 (Tukey's test, $p \leq 0.021$). Furthermore, males fed with sugars and sodium had a circulating JH-II level twice as high as those fed with water at D3 (Student t-test, $t = -9.68$, $df = 3.27$, $p = 0.002$) and at D5 (Student t-test, $t = -6.43$, $df = 2.52$, $p = 0.013$).

Furthermore, the *in vitro* quantification of JH-II acid biosynthesis by CAs was positively correlated with the circulating JH-II levels (Fig. 2B). Indeed, whatever the diet, the JH-II acid production increased significantly between D1 and D3 (Tukey's test, $p < 0.001$) and remained constant until D5 (Tukey's test, $p \geq 0.835$). However, the rate of the biosynthetic activity of CAs was dependent on the diet: CAs of males fed with water secreted at most 26 ± 2 pmol/h of JH-II acid, while CAs of males fed with sugars and sodium produced 51 ± 2 pmol/h (Student t-test, $t \geq -9.09$, $df \leq 14.96$, $p < 0.001$).

After quantifying the JH-II acid biosynthesis, we assessed the expression of *HMGR* within the CAs in 1-, 3- and 5-day-old males (Fig. 2C). Similarly to the *in vitro* JH-II acid production, whatever the diet, the *HMGR* expression level in CAs increased significantly between D1 and D3 (Tukey's test, $p < 0.001$) and remained identical until D5 (Tukey's test, $p \geq 0.850$). Nevertheless, the transcriptional activity of the *HMGR* in CAs was under the influence of diet. Indeed, 3-day-old males fed with sugars and sodium expressed *HMGR* twice as much as those fed with water (Student t-test, $t = -10.88$, $df = 15.62$, $p < 0.001$). This difference was also observed in 5-day-old males (Student t-test, $t = -11.59$, $df = 15.15$, $p < 0.001$).

3.3. Various sugars supplemented with sodium promote the expression of *InR* in CAs and *ILPs* in brain

In parallel with the CA *HMGR* expression, we examined the *InR* expression by the CAs in 1-, 3- and 5-day-old males fed with water or with sugars and sodium (Fig. 3A). Here again, whatever the diet, the *InR* expression increased with aging. While the expression of *InR* increased slightly from D1 to D5 in water-fed males (Tukey's test, $p \leq 0.013$), for those fed with various sugars and sodium, the *InR* expression increased drastically to reach a maximum from D3 (Tukey's test, $p < 0.001$). In addition, at D3 and D5, a diet rich in sugars and sodium led to an *InR* expression on average twice as high as a diet composed exclusively of water (Student t-test, $t \geq -15.12$, $df \leq 14.40$, $p < 0.001$).

After evaluating the diet effect on the CA *InR* expression, we quantified the expression levels of *ILP1*, *ILP2*, and *ILP4* in the brain of 1-, 3- and 5-day-old males fed with sugars and sodium or with water. The *ILP1*

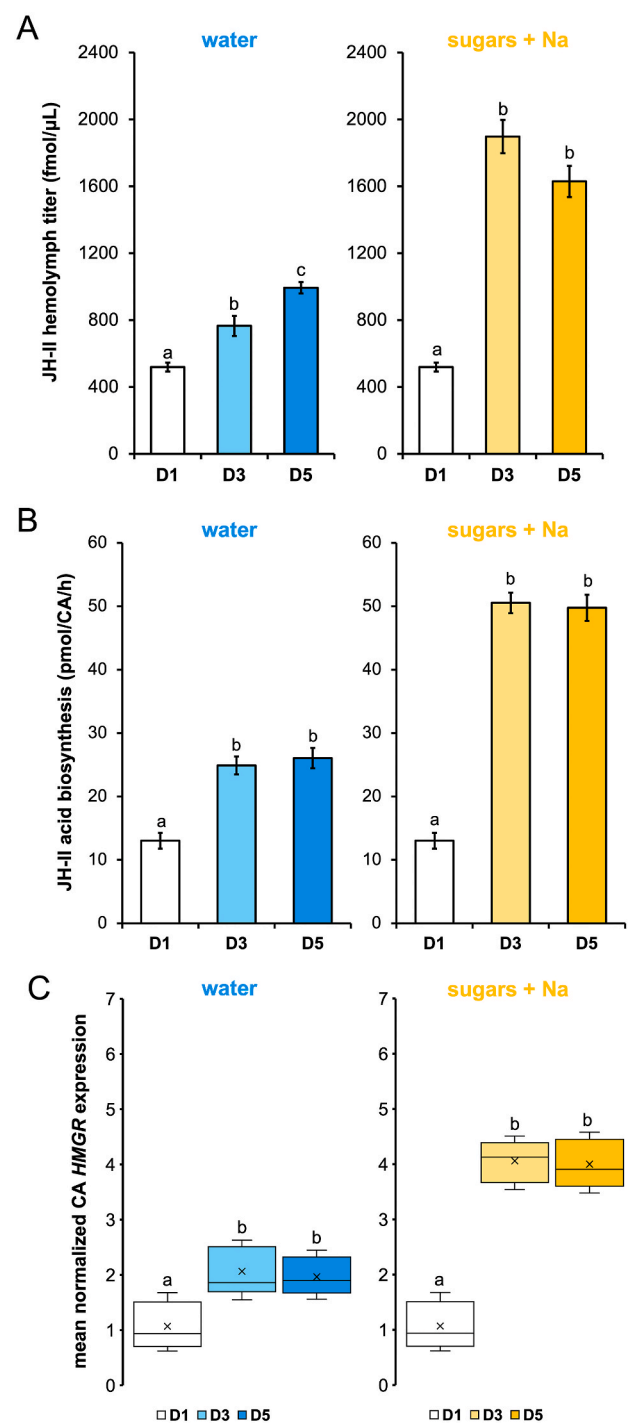


Fig. 2. Effect of diet on the rate of the circulating JH-II and the *in vitro* JH-II acid biosynthesis in *A. ipsilon* males. The circulating rate of JH-II (A), the JH-II acid biosynthesis by the *corpora allata* (CAs) incubated *in vitro* (B), and the expression level of *HMGR* in CAs (C) were evaluated for 1- (D1), 3- (D3), and 5-day-old (D5) males fed with water or with sugars and sodium. For the circulating rate of JH-II, sample size ranged from 21 to 38 males; for the JH-II acid biosynthesis by CAs, sample size was equal to 18 CAs (either 9 pairs) for each cohort. Values correspond to mean \pm SEM, and the letters indicate a statistically significant difference (Tukey's test, $p < 0.05$). The amounts of CA *HMGR* mRNA were evaluated by qPCR from 3 technical replicates and 9 independent biological replicates. The bars represent the value distribution, crosses denote the mean, and the letters indicate a statistically significant difference (Tukey's test, $p < 0.05$).

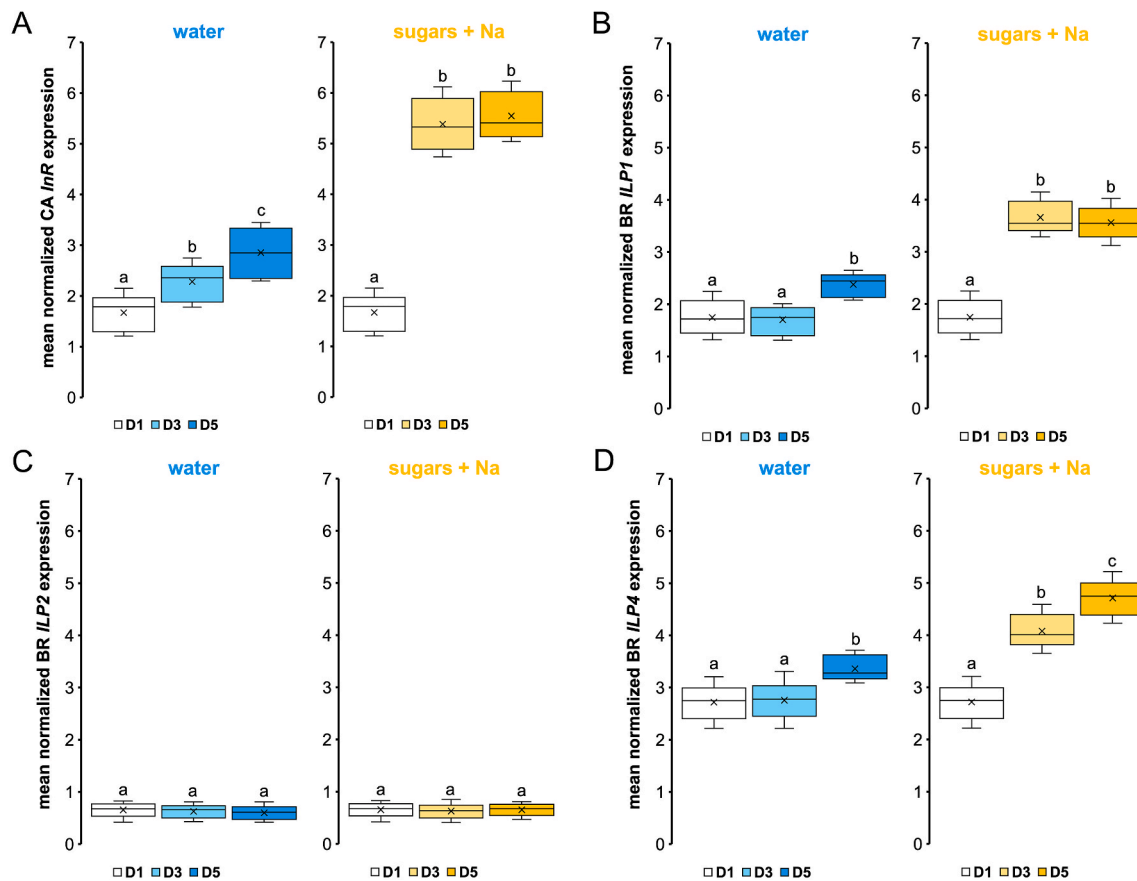


Fig. 3. Effect of diet on the expression level of *InR* in corpora allata (CAs) and *ILP1*, *ILP2*, and *ILP4* in brain (BR) of *A. ipsilon* males. The amounts of CA *InR* (A), and BR *ILP1* (B), *ILP2* (C), and *ILP4* (D) mRNA were evaluated for 1- (D1), 3- (D3), and 5-day-old (D5) males fed with water or with sugars plus sodium. Each qPCR was run in 3 technical replicates and 9 independent biological replicates. The bars represent the value distribution, crosses denote the mean, and the letters indicate a statistically significant difference (Tukey's test, $p < 0.05$).

expression level was significantly higher only for D5 water-fed males (Tukey's test, $p < 0.001$), whereas in males fed with sugars and sodium, the *ILP1* expression increased considerably from D3 and remained identical until D5 (Tukey's test, $p < 0.001$) (Fig. 3B). Furthermore, at both D3 and D5, males fed a sugary diet supplemented with sodium expressed *ILP1* approximately two times higher than males fed with water (Student t-test, $t \geq -11.59$, $df \leq 15.15$, $p < 0.001$). Then, regardless of diet, the *ILP2* expression was invariant according to the male age (ANOVA test, $F_{2,24} \leq 0.379$, $p \geq 0.688$) (Fig. 3C). As observed for *ILP1*, the expression level of *ILP4* increased only at D5 for males fed with water (Tukey's test, $p < 0.001$). By contrast, for males fed with sugars plus sodium, the *ILP4* expression increased with age without reaching a plateau at D3 (Tukey's test, $p < 0.001$) (Fig. 3D). At D3 and D5, the level of *ILP4* expression for males fed a sugary diet supplemented with sodium was significantly higher than that of water-fed males (Student t-test, $t \geq -9.74$, $df \leq 14.13$, $p < 0.001$).

3.4. Insulin signaling pathway upregulates the expression of *HMGR* in CAs and the *JH-II* acid biosynthesis by CAs

Finally, we tested whether the ISP modulates the biosynthetic activity of CAs by examining the effects of injection of exogenous insulin into 1-day-old males fed with water on both the expression of *HMGR* and the *in vitro* biosynthesis of *JH-II* acid by CAs two days after administration (Fig. 4A & B). The insulin injection significantly increased the CA *HMGR* expression compared to saline-injected control males (Student t-test, $t = 6.59$, $df = 15.96$, $p < 0.001$) (Fig. 4A); and nearly doubled the *JH-II* acid biosynthesis by CAs, which ranged from approximately 25 pmol/CA/h for control males to 46 pmol/CA/h for insulin-treated males

(Student t-test, $t = 8.51$, $df = 15.74$, $p < 0.001$) (Fig. 4B).

To support the regulatory action of ISP on the biosynthetic activity of CAs, we also evaluated the impact of injection of RNAi-mediated silencing of *InR* into 1-day-old males fed with sugars plus sodium on both the transcriptional activity of *HMGR* and the *in vitro* biosynthesis of *JH-II* acid by CAs two days after administration (Fig. 4C & D). Injection of *InR*-dsRNA caused a significant decrease in the CA *InR* (70% *InR*-dsRNA effectiveness, Supplementary Fig. S1) and *HMGR* expression (Fig. 4C) compared to LacZ-dsRNA-treated males (Tukey's test, $p < 0.001$). Moreover, in *InR*-dsRNA-treated males, the *JH-II* acid biosynthesis by CAs was decreased by approximately 20 pmol/CA/h compared to saline-injected males (Tukey's test, $p < 0.001$) (Fig. 4D).

4. Discussion

In the male moth *Agrotis ipsilon*, sexual maturation occurs after metamorphosis between the third- and fifth-day post-emergence under standard rearing conditions (Gemeno and Haynes, 2000) and is characterized by the maturation of ASGs concomitantly to the ontogeny of sexual behavior after the development of ALs (Aguilar et al., 2023; Bozzolan et al., 2015; Gadenne and Anton, 2000; Gassias et al., 2021). The orchestration of these events has been previously shown to be dependent on *JH* and diet. Here, we used *A. ipsilon* as a model to unravel, for the first time, functional relationships between nutrition, the ISP acting as a systemic nutritional sensor, and *JH* in male reproductive physiology.

While the natural diet of the adult *A. ipsilon* remains unknown, this insect has several generations per year and can migrate over long distances (Causse et al., 1989), supposing that its food comes from diverse

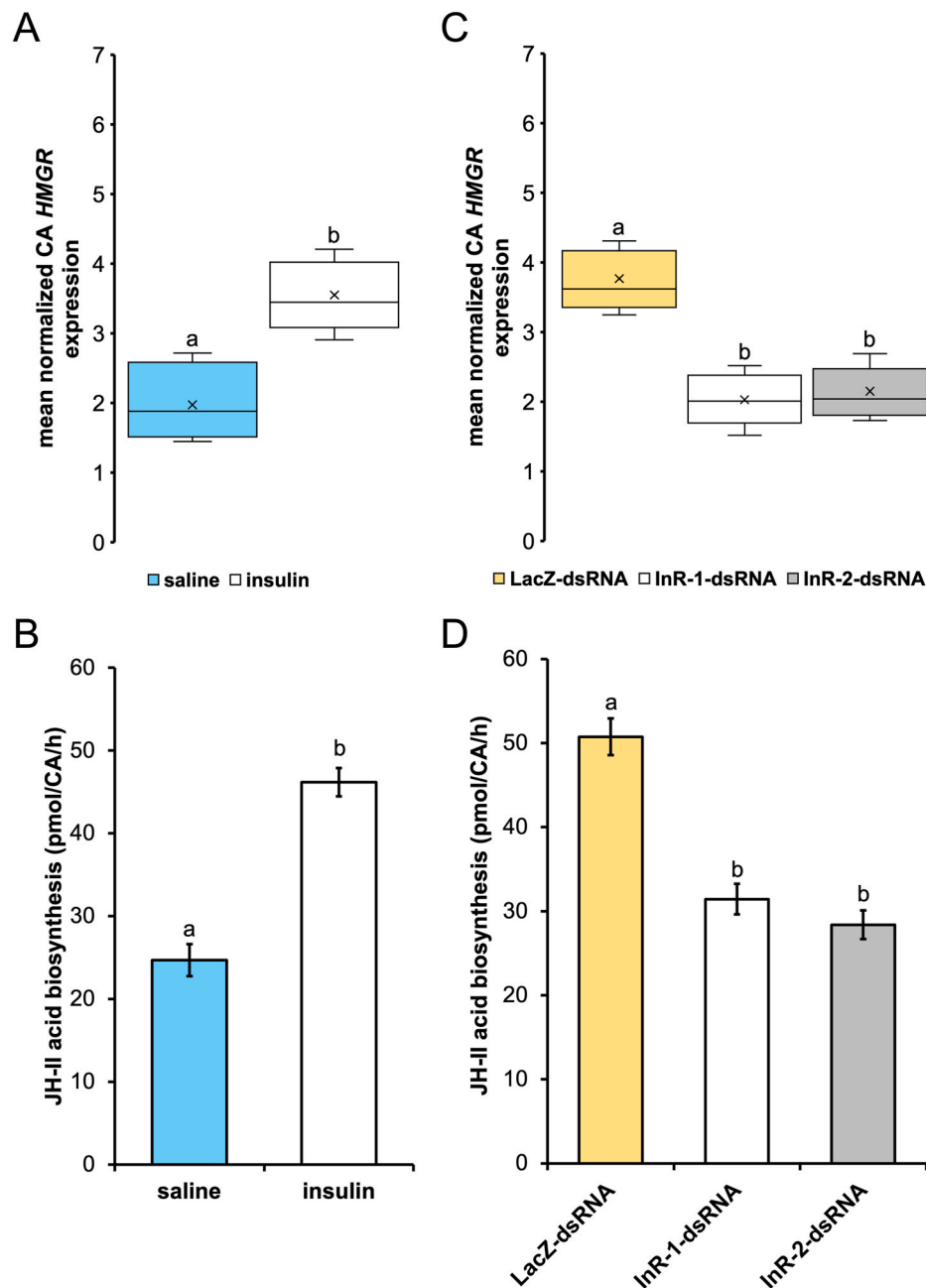


Fig. 4. Close link between insulin-like peptides and juvenile hormone-II acid biosynthesis by *corpora allata* (CA) in 3-day-old *A. ipsilon* males. (A) Effect of *in vivo* insulin injection on the level of *HMGR* expression in water-fed males. Each qPCR was run in 3 technical replicates and 9 independent biological replicates. The bars represent the value distribution, crosses denote the mean, and the letters indicate a statistically significant difference (Student t-test, $p < 0.05$). (B) JH-II acid biosynthesis by CA incubated *in vitro* in water-fed males after insulin injection. Sample size was equal to 18 CAs (either 9 pairs) for each cohort. Values correspond to mean \pm SEM, and the letters indicate a statistically significant difference (Student t-test, $p < 0.05$). (C) Effect of InR-dsRNA injection on the level of *HMGR* expression in males fed with sugars + Na. Each qPCR was run in 3 technical replicates and 9 independent biological replicates. The bars represent the value distribution, crosses denote the mean, and the letters indicate a statistically significant difference (Tukey's test, $p < 0.05$). (D) JH-II acid biosynthesis by CA incubated *in vitro* in males fed with sugars + Na after InR-dsRNA injection. Sample size was equal to 18 CAs (either 9 pairs) for each cohort. Values correspond to mean \pm SEM, and the letters indicate a statistically significant difference (Tukey's test, $p < 0.05$).

nutritional resources (Liu et al., 2016). More broadly, most adult moth species feed from flower nectars mainly composed of water with solubilized carbohydrates such as sucrose, glucose, and fructose (Broadhead and Raguso, 2021; Nepi et al., 2012; Roy et al., 2017). Among carbohydrates, sucrose is generally the primary component of many flower nectars (Nicolson and Thornburg, 2007), although glucose and fructose are present in many of them (Nicolson and Thornburg, 2007; Roy et al., 2017). Furthermore, another feeding behavior has been described in several lepidopteran males that consume liquid excrements or puddles

of brackish water (Ravenscraft and Boggs, 2016). This puddling behavior provides sodium essential for homeostasis (Ankola et al., 2021; Boggs and Dau, 2004; Molleman, 2010), but is mainly known to be necessary for locomotor activity by improving flight (Ankola et al., 2021; Xiao et al., 2010) and also for reproduction (Mitra et al., 2016; Molleman, 2010; Smedley and Eisner, 1996).

Recent studies showed the accelerating effects of a diet rich in sucrose, fructose, glucose, and sodium both on the development of sexual behavior (Force et al., 2024b) as well as on the maturation of ASGs in

male *A. ipsilon* (Force et al., 2023a). Thus, such a diet induces sexual maturity from the third day of adult life, instead of five days for water-fed males. In this species, it is well known that the JH signaling pathway plays a crucial role in male sexual maturation by promoting the growth and secretory activity of ASGs (Gassias et al., 2021) and the development of ALs (cerebral areas involved in the processing of pheromonal information) for the elicitation of male-oriented flight towards female sex pheromones (Aguilar et al., 2023; Gadenne and Anton, 2000). Taking into consideration these data, we hypothesized that a diet composed of various sugars and sodium may affect the JH signaling pathway within target tissues involved in the male sexual maturation of *A. ipsilon*. Our results demonstrated that such a diet significantly impacted the expression of JH signaling actors (*Met1*, *Tai*, and *Kr-h1*) in both ALs and ASGs. From the third day of adult life, males fed with sucrose, fructose, glucose, and sodium quickly and strongly expressed the actors of JH signaling compared to those fed water, for which the expression levels of *Met1*, *Tai*, and *Kr-h1* reached a peak only at the fifth-day post-emergence (Fig. 1). Thus, a diet rich in various sugars plus sodium potentializes of JH biological actions on ALs and ASGs.

Knowing that the activation of the JH signaling pathway is dependent on the local concentration of the hormone, it was reasonable to believe that a diet composed of various sugars with sodium may induce an increase in the circulating JH levels. As expected, we observed that this diet considerably increased the level of JH-II in the hemolymph to reach a maximum from the third day of adult life, while males fed with water exhibited a lower maximum of JH-II circulating level at the fifth post-emergence day (Fig. 2A). In addition, it is well known that JH is a lipophilic molecule commonly produced and released into the hemolymph by CAs, a pair of endocrine glands connected to the brain (Tobe and Stay, 1985). In adult male moths, CAs produce different types of JH acids; one of the most predominant forms is the JH-II acid, which is transformed into JH-II and released into hemolymph under the action of JHAMT in ASGs (Guo et al., 2021; Shirk et al., 1983). We then quantified the *in vitro* JH-II acid biosynthesis by CAs and we noticed that the diet of adult male *A. ipsilon* drastically affected the production of this hormone (Fig. 2B). Indeed, males fed with sucrose, fructose, glucose, and sodium synthesized twice as much JH-II acid as those fed with water. Such a diet effect on the JH biosynthesis by CAs has also been noted in female mosquitoes (Perez-Hedo et al., 2014).

In CAs, JH is synthesized by the MVAP pathway, an ancient metabolic pathway present in all three domains of life including bacteria, archaea, and eucaryotes (Lombard and Moreira, 2011). The MVAP consists of a main trunk followed by subbranches that generate a diverse range of biomolecules. Although insects do not have the branch that synthesizes cholesterol as in vertebrates, they have MVAP involved in the synthesis of JH within CAs (Bellés et al., 2005; Noriega, 2014). Initially, three units of acetyl-CoA are condensed to mevalonate through three sequential steps involving enzymes such as HMGR (Bellés et al., 2005; Noriega, 2014). Interestingly, by analogy with vertebrates, HMGR is likely a key enzyme in the MVAP regulation in insects (Feyereisen et al., 1981). For this reason, we also examined the expression level of HMGR in CAs according to the diet of *A. ipsilon* males (Fig. 2C). In concordance with the results obtained for the *in vitro* quantification of JH-II acid biosynthesis by CAs, we found that males fed with a diet composed of sugars and sodium expressed twice as much HMGR from the third-day post-emergence in comparison with water-fed males. It is well documented that changes in the biosynthetic activity of CAs are in part due to a coordinated transcriptional regulation of genes encoding JH biosynthetic enzymes (Kinjoh et al., 2007; Nouzova et al., 2011; Rivera-Perez et al., 2014). Thus, it is likely that in the male *A. ipsilon*, the increased transcriptional activity of HMGR in CAs is partly responsible for the increased biosynthetic activity of CAs in response to a diet rich in sugars with sodium. We cannot rule out the possibility of the transcriptional regulation of other JH biosynthetic enzymes as reported in the female *Rhodnius prolixus* after a blood meal (Leyria et al., 2022).

Several studies performed in different insect species brought in

evidence that the ISP acts on the biosynthetic activity of CAs by modulating the expression of the JH biosynthetic enzymes (Abrisqueta et al., 2014; Pan et al., 2022; Pérez-Hedo et al., 2013; Tu et al., 2005; Zhu et al., 2020). For instance, in *D. melanogaster*, the inactivation of *InR* inhibited the *HMGR* expression in CAs (Belgacem and Martin, 2007). In view of these data, we wondered whether a diet rich in various sugars and sodium may contribute to ISP activation, particularly by promoting the *InR* expression in CAs of adult male *A. ipsilon*. As expected, from the third day of adult life, we showed that such a diet induced an approximately twofold increase in the expression level of *InR* in CAs compared to water-fed moths (Fig. 3A). To support a functional link between the ISP and the biosynthetic activity of CAs, we demonstrated that the administration of insulin in water-fed males raised the *HMGR* expression in CAs, thus resulting in increased *in vitro* JH-II acid biosynthesis (Fig. 4A & B). By contrast, opposite effects were observed by the RNAi-mediated silencing of *InR* in males fed with various sugars and sodium (Fig. 4C & D). These results revealed the existence of a direct regulatory action of the ISP on the JH biosynthesis through the upregulation of *HMGR* expression in CAs as a response to positive nutritional status in the male *A. ipsilon*. Furthermore, there is evidence demonstrating that JH interacts with the ISP in female insects. For example, in the adult beetle *Tribolium castaneum*, RNAi-mediated knockdown of *Met* led to a decline in the expression level of ILPs (Sheng et al., 2011).

It is well documented that insect ILPs are mainly synthesized in the brain by the medial neurosecretory cells (MNSCs) (Defferrari et al., 2016; Leyria et al., 2021; Nässel and Vanden Broeck, 2016). Axonic extensions of MNSCs project into the CCs and the CAs, indicating that ILPs are directly delivered to the CAs (Vafopoulou and Steel, 2012). Moreover, it has been revealed in *A. aegypti* that the transcriptional activities of several ILPs were highly induced in the brain following sugar ingestion in female mosquitoes (Marquez et al., 2011). In the male *A. ipsilon*, a recent study showed the presence of three ILPs (ILP1, ILP2, and ILP4) near to CCs-CAs complex (Diesner et al., 2018) and here, we demonstrated that the brain expression of *ILP1* and *ILP4* are dependent of diet (Fig. 3B–D). Indeed, males fed with sucrose, fructose, glucose, and sodium strongly expressed *ILP1* and *ILP4* from the third day of adult life, while those fed with water weakly expressed these two ILPs only on the fifth day post-emergence. In concordance with this result, in *Bombyx mori*, the administration of glucose provoked the production and release of an ILP, named bombyxin, from the MNSCs (Masumura et al., 2000). Thus, this carbohydrate appears to be a common nutritional signal to stimulate the synthesis of insect and also mammalian insulins (Mizoguchi and Okamoto, 2013).

Taking into consideration the data reported in the literature and the results obtained in this study, we proposed a hypothetical scenario of the mechanisms underlying the functional connection between diet, ISP, and JH in the sexual maturation of male *A. ipsilon* (Fig. 5). After a supply of sugars (including glucose), ILPs produced and stored in the MNSCs are likely released towards the CC-CA complex. ILPs act directly on CAs by binding to their InRs, thus increasing the expression of *HMGR* and consequently the JH biosynthesis. Thereafter, the rise in the circulating JH levels simultaneously activates the JH signaling within ASGs (Gassias et al., 2021) and ALs (Aguilar et al., 2023) through the induction of *Met1*, *Tai*, and *Kr-h1* to promote concomitantly the maturation of ASGs and the development of ALs involved in the processing of pheromonal information. Thus, such a model illustrates the endocrine communications by which a sugary diet supplemented with sodium increases the reproductive fitness of male *A. ipsilon* (Force et al., 2023a, 2024a, 2024b), and in a broader perspective, also provides a framework for further investigations.

In conclusion, our study highlighted the complex regulatory network in which the ISP and JH interact to promote the insect male reproductive physiology in response to a positive nutritional state. In metazoans, due to the high degree of similarity of insulin and lipophilic hormone signaling pathways, including receptor proteins and their downstream participating elements, our study also provides the opportunity to

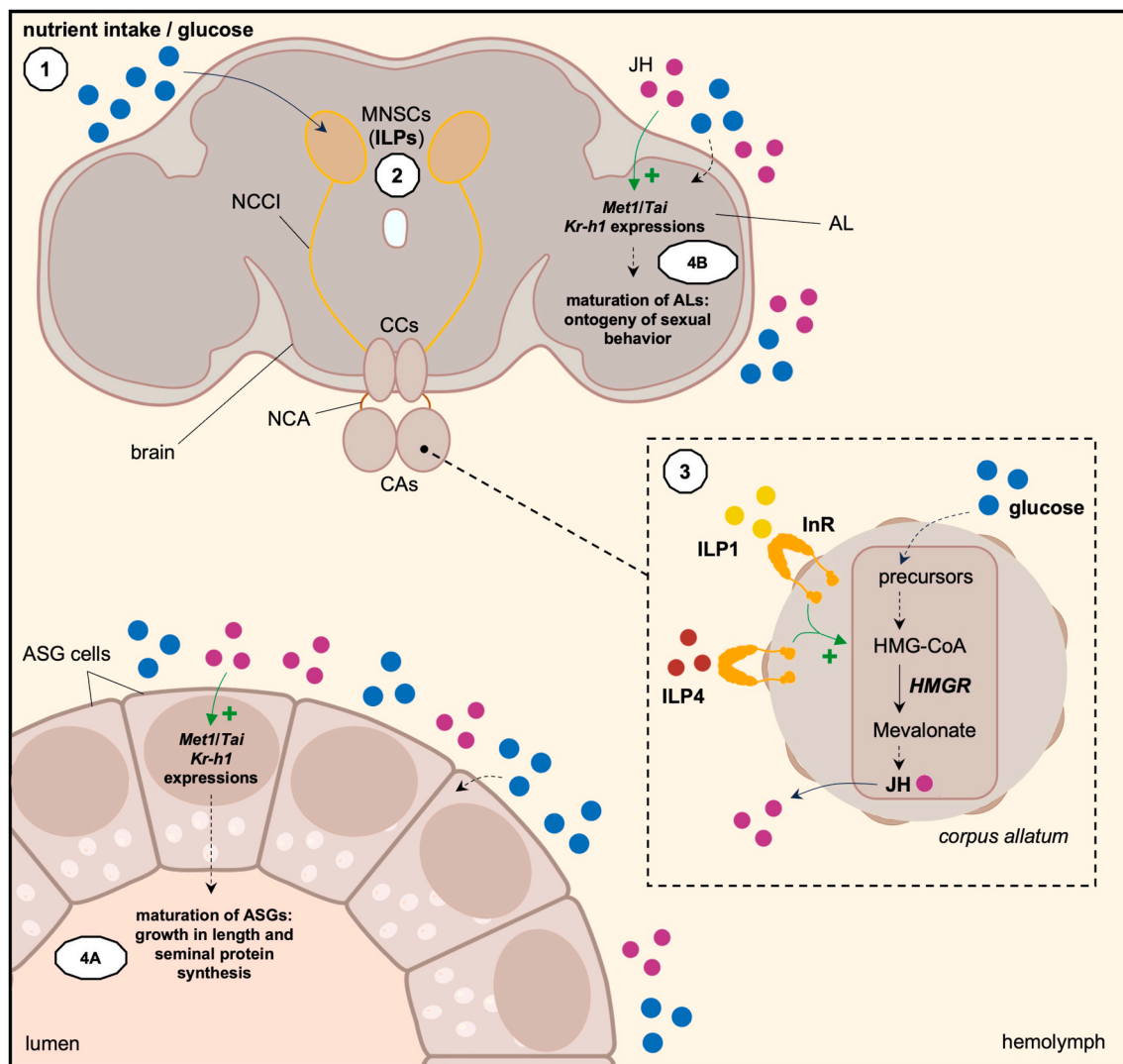


Fig. 5. Overview of the interactions between nutrition, insulin-like peptides (ILPs), juvenile hormone (JH) and its signaling pathway underlying male sexual maturation in the moth *Agrotis ipsilon*. After a supply of sugars (including glucose) (1), ILPs produced and stored in the median neurosecretory cells (MNSCs) might be released towards the *corpura cardiaca* (CCs) and the *corpura allata* (CAs) (2). In CAs, ILPs stimulate the expression of HMG-CoA reductase (HMGR) enzyme intervening into the JH biosynthetic pathway (3). Glucose might also modulate the JH production by stimulating the release of ILPs from the brain and/or acting as a source of precursors for JH biosynthesis. Then, circulating JH simultaneously stimulates the expression of *Met1*, *Tai*, and *Kr-h1* within accessory sex glands (ASGs) (Gassias et al., 2021) and antennal lobes (ALs) (Aguilar et al., 2023). Thus, these peripheral and central JH signaling pathway are known to promote concomitantly the maturation of ASGs crucial for the seminal protein biosynthesis (4A) (Gassias et al., 2021) and the development of ALs involved in the processing of sex pheromonal signals leading to the display of sexual behavior (4B) (Aguilar et al., 2023). NCCI: nervus *corpura cardiacum* I; NCA: nervus *corpura allatum*; Met1: Methoprene-tolerant 1; Tai: Taiman; Kr-h1: Krüppel homolog 1.

broaden the understanding of the functional relationships between these endocrine signals in the regulation of reproduction to other taxa. In addition, our findings will find potential applications in agricultural pest control and ecotoxicology. Many species of moths play a pivotal role in the ecosystem as pollinators, although the larvae (often pests of crops) may have negative impacts on agriculture (Donahue, 1995; MacGregor et al., 2015). Because they are taxonomically diverse, found in many different habitats, and are extremely sensitive to human-altered abiotic conditions, moths are particularly useful as indicators of ecosystem health (Maleque et al., 2009). Due to the crucial function of the ISP in the sexual maturation of male moths, our study projects the InRs as potential targets for the CRISPR-based gene drive technology, which is highly efficient in noctuid pests (Chen et al., 2018; Koutroumpa et al., 2016), and for the development of new control strategies (Zhang et al., 2022). Besides, the ILPs might be used as molecular biomarkers for monitoring and assessing the impact of environmental xenobiotic contaminants on the reproduction of pollinators and other economically

important insects.

CRediT authorship contribution statement

Evan Force: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Claudia Alvarez:** Validation, Methodology, Investigation. **Annabelle Fuentes:** Validation, Investigation. **Annick Maria:** Validation, Investigation. **Françoise Bozzolan:** Validation, Investigation. **Stéphane Debernard:** Writing – review & editing, Validation, Supervision, Conceptualization.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ibmb.2024.104252>.

Data availability

Data will be made available on request.

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