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Acp70A regulates *Drosophila* pheromones through juvenile hormone induction

Gwénaëlle Bontonou, Haq Abdul Shaik, Béatrice Denis, Claude Wicker-Thomas*

CNRS UPR 9034, Université de Paris Sud, 91198 Gif sur Yvette, France

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ABSTRACT

Mated *Drosophila melanogaster* females show a decrease in mating receptivity, enhanced ovogenesis, egg-laying and activation of juvenile hormone (JH) production. Components in the male seminal fluid, especially the sex peptide ACP70A stimulate these responses in females. Here we demonstrate that ACP70A is involved in the down-regulation of female sex pheromones and hydrocarbon (CHC) production. *Drosophila* G10 females which express *Acp70A* under the control of the vitellogenin gene *yp1*, produced fewer pheromones and CHCs. There was a dose-dependent relationship between the number of *yp1-Acp70A* alleles and the reduction of these compounds. Similarly, a decrease in CHCs and diene pheromones was observed in *da* > *Acp70A* flies that ubiquitously overexpress *Acp70A*. Quantitative-PCR experiments showed that the expression of *Acp70A* in G10 females was the same as in control males and 5 times lower than in *da* > *Acp70A* females.

Three to four days after injection with 4.8 pmol ACP70A, females from two different strains, exhibited a significant decrease in CHC and pheromone levels. Similar phenotypes were observed in ACP70A injected flies whose ACP70A receptor expression was knocked-down by RNAi and in flies which overexpress ACP70A N-terminal domain. These results suggest that the action of ACP70A on CHCs could be a consequence of JH activation. Female flies exposed to a JH analog had reduced amounts of pheromones, whereas genetic ablation of the corpora allata or knock-down of the JH receptor Met, resulted in higher amounts of both CHCs and pheromonal dienes.

Mating had negligible effects on CHC levels, however pheromone amounts were slightly reduced 3 and 4 days post copulation. The physiological significance of ACP70A on female pheromone synthesis is discussed.

1. Introduction

In *Drosophila melanogaster*, mating is largely dependent on sex pheromones, although visual and auditory signals are also involved (Antony and Jallon, 1982). Sex pheromones are long-chain cuticular hydrocarbons (CHCs) that act by contact or at short distances. CHCs are sexually dimorphic: on males, the predominant hydrocarbons have 23 or 25 carbon chains and one double bond. The most abundant are 7-tricosene (7-T; C23:1) and 7-pentacosene (7-P; C25:1) (Antony and Jallon, 1982; Jallon, 1984; Antony et al., 1985). Virgin females have high levels of CHCs with 27- and 29-carbon chains and two double bonds. The 7,11-heptacosadiene (7,11-HD; C27:2) and the nonacosadiene (7,11-ND; C29:2), account for about forty percent of the total CHCs in most females (Antony et al., 1985; Jallon and David, 1987). 7-T has been found to inhibit male courtship and to stimulate mating in females (Jallon, 1984; Grillet et al., 2006), whereas the 7,11-dienes enhance male preference (Antony et al., 1985; Billeter et al., 2009). Some minor compounds, such as 7-P and 7-heptacosene (7-H, 27:1) were also shown to stimulate male courtship (Antony et al., 1985; Ferveur and Sureau, 1996).

In most insects, mating elicits a behavioral and physiological switch in females, triggered by components in the male ejaculate, which are transmitted, along with sperm, into females (Avila et al., 2011). These post-mating responses have been extensively studied in *Drosophila* and include increased ovogenesis (Soller et al., 1999), ovulation (Heifetz et al., 2000) and decreased sexual receptivity (Wolfner, 1997). These changes are triggered by a complex set of more than a hundred of proteins and peptides (Findlay et al., 2008). Among these, the accessory gland proteins (ACPs), especially ACP70A (also named sex peptide), play a major role (Chen et al., 1988; Chapman et al., 2003; Liu and Kubli, 2003). This 36-amino-acid peptide passes from the reproductive tract into the

* Corresponding author. Tel.: +33 1 69 823 708; fax: +33 1 69 823 736.
E-mail address: Claude.Wicker-thomas@legs.cnrs-gif.fr (C. Wicker-Thomas).

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 1  2  3  4  5  6  7  8  9  10 11 12 13 14 15 16 17 18 19 20 21 22
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CTTAATTTGGGGCCCGCCTGGGGTGGAAGATGTTAA
 L  N  L  G  P  A  W  G  G  R  C  *
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Fig. 1. Nucleotide and peptide sequences of Canton-S Acp70A. The N-terminal part of Acp70A used in transgenic flies is shown in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article)

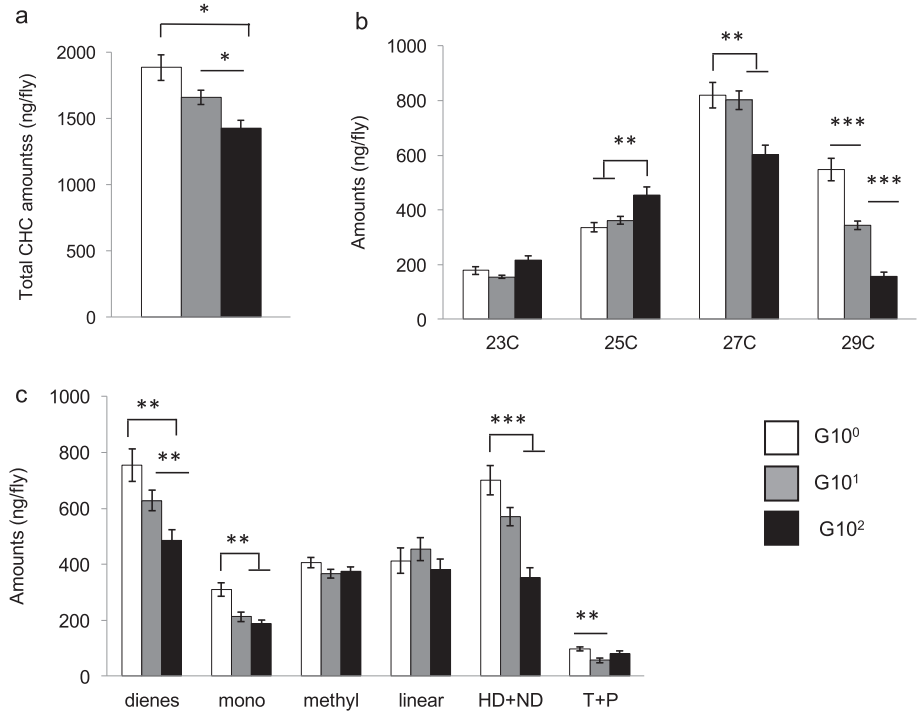

Fig. 2. Hydrocarbon amounts extracted from female flies derived from the G10 line and carrying 0 (white bars),1 (grey bars) or 2 (black bars) *Acp70A* alleles ($G10^0$, $G10^1$, $G10^2$, respectively). a: total hydrocarbon amounts (C23 to C29); b: amounts of hydrocarbons of different lengths; c: amounts of hydrocarbons of different classes. Each bar represents mean ± SEM ($n = 10$). *, ** and *** above bars indicate significant differences (one-way ANOVA, $P = 0.05$, 0.01 and 0.001, respectively) between means. HD: 7,11-heptacosadiene; ND: 7,11-nonacosadiene; T: 7-tricosene; P: 7-pentacosene; dienes: alkadienes; mono: alkenes; methyl: 2-methyl-alkanes; linear: linear alkanes.

Table 1
Analysis of differences between the CHC profiles of females issued from the G10 line: *P{Acp70A$^{g\ Yp1\ hs}$}/P{Acp70A$^{g\ Yp1\ hs}$}*, ($G10^2$); *P{Acp70A$^{g\ Yp1\ hs}$}/+* ($G10^1$) and +/+ ($G10^0$). CHC identities are given in the first column. Statistical analysis was performed using a one-way ANOVA followed by Tukey's multiple comparison post-hoc test. Values in bold indicate significant CHC variations with Acp70A dose. The last three columns give the mean quantities (±SEM) of CHCs (ng/fly) produced by ten 5-day old females at 25 °C.

CHC	*F*	P_{0-1}	P_{0-2}	P_{1-2}	$G10^0$	$G10^1$	$G10^2$
Total CHCs	10.45	**<.01**	0.55	0.07	1884.6 ± 97.2	1660.6 ± 54.8	1427.5 ± 58.1
9-T	1.24	0.52	0.91	0.30	6.9 ± 1.6	3.7 ± 1.9	12.7 ± 4.3
7-T	6.10	**0.02**	0.99	**0.01**	41.1 ± 3.5	19.0 ± 2.9	42.0 ± 8.0
23:0	4.61	0.96	**0.03**	**0.05**	121.1 ± 9.1	123.6 ± 3.3	148.0 ± 7.1
7,11-PD	14.15	0.99	**<.0001**	**<.0001**	32.9 ± 3.5	33.7 ± 3.8	104.3 ± 18.1
Me-24	28.00	**0.05**	**<.0001**	**<.0001**	33.0 ± 3.1	42.0 ± 2.9	86.1 ± 8.3
9-P	0.27	0.96	0.89	0.75	70.2 ± 5.9	68.3 ± 4.3	75.5 ± 5.1
7-P	4.53	**0.04**	**0.04**	1.00	55.7 ± 4.9	37.7 ± 6.1	37.3 ± 3.4
25:0	3.18	0.10	0.99	0.09	134.5 ± 9.4	172.2 ± 13.9	133.1 ± 13.6
7,11-HD	1.70	0.93	0.21	0.36	360.1 ± 28.7	347.2 ± 22.2	296.4 ± 26.0
Me-26	3.48	0.58	0.26	**0.04**	237.4 ± 14.3	255.7 ± 12.7	208.2 ± 11.4
9-H	17.20	1.00	**<.0001**	**<.0001**	49.4 ± 4.2	49.2 ± 5.3	16.1 ± 4.3
7-H	32.79	**0.002**	**<.0001**	**<.0001**	55.4 ± 5.3	32.5 ± 4.6	7.3 ± 2.1
27:0	2.59	0.99	0.16	0.13	105.2 ± 17.0	108.1 ± 14.6	64.1 ± 14.2
7,11-ND	56.46	**<.0001**	**<.0001**	**<.0001**	340.2 ± 28.6	223.7 ± 11.7	55.6 ± 11.5
Me-28	38.83	**<.0001**	**<.0001**	0.98	129.1 ± 9.3	61.2 ± 2.3	63.0 ± 4.8
9-N	3.28	0.10	0.009	0.01	0.9 ± 0.9	1.2 ± 1.0	8.5 ± 2.4
7-N	4.70	0.93	0.002	**<.0001**	1.8 ± 1.3	0.5 ± 0.3	10.3 ± 2.0
29:0	0.39	0.99	0.71	0.75	51.6 ± 18.5	50.0 ± 13.8	34.2 ± 13.2

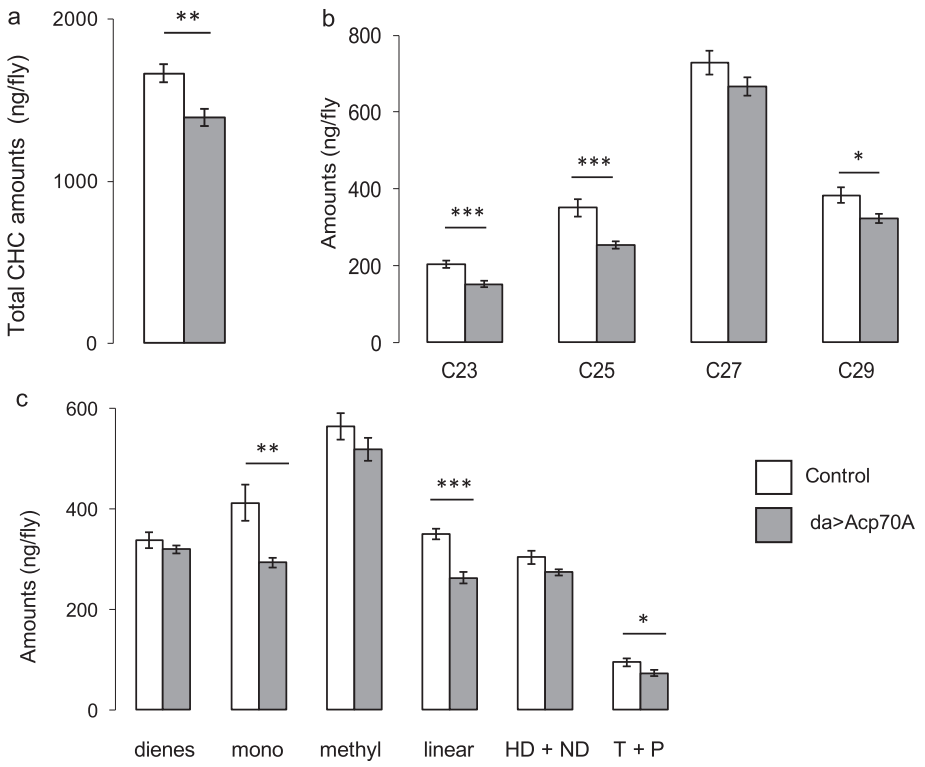

Fig. 3. Hydrocarbon amounts extracted from control flies (white bars) or those ubiquitously overexpressing *Acp70A* (grey bars). a: total hydrocarbon amounts (C23 to C29); b: amounts of hydrocarbons of different lengths; c: amounts of hydrocarbons of different classes. Each bar represents mean ± SEM ($n = 10$). *, ** and *** above bars indicate significant differences (one-way ANOVA, $P = 0.05$, 0.01 and 0.001, respectively) between means. HD: 7,11-heptacosadiene; ND: 7,11-nonacosadiene; T: 7-tricosene; P: 7-pentacosene; dienes: alkadienes; mono: alkenes; methyl: 2-methyl-alkanes; linear: linear alkanes.

haemolymph (Pilpel et al., 2008) and ultimately targets the nervous system of the female (Kubli, 2003; Häsemeyer et al., 2009). Its gradual release from stored sperm, facilitates long term post mating changes (Peng et al., 2005). The C-terminal part of ACP70A can bind to a neuronal sex peptide receptor (SPR), resulting in increased egg laying and reduced acceptance of male-mating attempts (Yapici et al., 2008; Häsemeyer et al., 2009). The N-terminal part can activate the corpora allata (CA), resulting in increased synthesis of juvenile hormone (JH) (Moshitzky et al., 1996), which triggers ovogenesis and vitellogenic oocyte progression (Soller et al., 1999).

In some species, mating may also induce a drastic reduction in female sex pheromone production. In the female corn earworm, three days following a single mating, a 93% reduction in pheromone titer, was observed (Raina et al., 1986). However mating had no significant effect on sex pheromone production in five other noctuid species (Shorey et al., 1968). In *Helicoverpa armigera*, injection of *D. melanogaster* ACP70A suppressed pheromone production but activated JH production (Fan et al., 1999). The C-terminus was largely responsible for pheromostasis (Fan et al., 2000). In *Drosophila*, we found that female flies that constitutively expressed *Acp70A* had lower CHC levels. We therefore investigated the effect of injection or overexpression of ACP70A on female CHCs. Our results suggest that the ACP70A induced changes to female CHCs and pheromones production is dependent on the N-terminal part, which stimulates an increase in JH synthesis.

2. Material and methods

2.1. Drosophila strains and rearing

Flies were maintained at 25 °C with 12:12 light–dark (LD) cycles, on standard yeast/cornmeal/agar medium. Flies were separated by sex at emergence and kept in sex-specific groups of 10 in fresh food vials until testing.

Two wild-type strains were used: CS (Canton-S) and Chav (Chavroches, France). CS has been kept in the laboratory for 20 years, Chav since 2008.

Lines from the Boomington *Drosophila* Stock Center included: *daughterless (da)-Gal4*, an ubiquitous driver; *elav-Gal4*, a driver

Table 2
Analysis of differences between the CHC profiles of females issued from the *da-gal4* × *UAS-Acp70A* cross. CHC identities are given in the first column. Statistical analysis was performed using a one-way ANOVA followed by Tukey's multiple comparison post-hoc test. Values in bold indicate significant CHC variations. The 4th and 5th columns give the mean quantities (±SEM) of CHCs (ng/fly) produced by ten 5-day old females at 25 °C.

CHC	*F*	*P*	Control	da > Acp70A
Total CHCs	9.92	**0.006**	1665.8 ± 74.9	1396.3 ± 41.3
9-C23:1	7.49	**0.01**	10.9 ± 1.4	6.5 ± 0.9
7-C23:1	0.36	0.56	43.4 ± 4.4	39.1 ± 5.7
C23:0	40.91	**<.001**	135.5 ± 3.8	90.5 ± 5.9
7,11-C25:2	40.09	**<.001**	20.2 ± 1.9	36.4 ± 1.7
2-Me-C24	43.13	**<.001**	23.0 ± 1.3	12.5 ± 1.0
9-C25:1	24.88	**<.001**	131.3 ± 15.1	55.3 ± 2.2
7-C25:1	13.91	**0.002**	51.4 ± 4.2	34.2 ± 1.9
C25:0	0.98	0.34	123.7 ± 6.5	131.4 ± 7.0
7,11-C27:2	11.23	**0.004**	178.2 ± 11.1	219.8 ± 5.6
2-Me-C26	10.36	**0.004**	300.0 ± 15.7	238.4 ± 10.9
9-C27:1	2.11	0.16	96.8 ± 11.8	78.6 ± 4.1
7-C27:1	0.26	0.62	62.2 ± 3.7	64.8 ± 3.4
C27:0	35.29	**<.001**	83.3 ± 4.4	54.8 ± 2.0
7,11-C29:2	81.34	**<.001**	125.9 ± 7.3	54.2 ± 3.1
2-Me-C28	2.15	0.16	231.4 ± 12.5	256.4 ± 11.6
9-C29:1	5.40	**0.03**	5.7 ± 1.6	1.9 ± 0.4
7-C29:1	5.46	**0.03**	4.1 ± 0.8	2.1 ± 0.2
C29:0	15.29	**0.001**	16.6 ± 1.7	8.9 ± 1.0

expressed in the nervous system; *Aug21-Gal4/Cyo*, carrying a driver specifically expressed in the corpora allata (Siegmund and Korge, 2001). The UAS-death activator line used was *UAS-hid, rpr* (*UAS-hid,UAS-reaper*) (Zhou et al., 1997). Crossing this line with *Aug21-Gal4/Cyo* resulted in *Aug21>UAS-hid,UAS-reaper* progeny. In these individuals, most of the cells of the corpora allata are lacking, resulting in a reduction in juvenile hormone synthesis (Gruntenko et al., 2010). *P{Acp70A$^{g\ Yp1\ hs}$}*, called G10, contain a transgene with the *Yp1* enhancer which drives the *Acp70A* gene (Aigaki et al., 1991). Virgin G10 females produce ACP70A constitutively in their fat body; G10 flies with 0, 1 or 2 copies of the *P{Acp70A$^{g\ Yp1\ hs}$}* transgenes were noted G10^0, G10^1 and G10^2, respectively.

Two UAS-RNAi lines were purchased from the VDRC Stock Center (Vienna): *UAS-SPR RNAi*, is directed against the receptor for ACP70A (Yapici et al., 2008). *UAS-Met RNAi*, is an RNAi line directed against *Methoprene-tolerant* (*Met*), a juvenile hormone receptor (Wilson et al., 2003).

2.2. Quantitative RT-PCR

Total RNA was extracted from 5 day-old virgin flies (n = 20/sample) using Nucleospin RNAII kit (Macherey–Nagel), and reverse transcription was performed by using Oligo-dT primers and random hexamers. Primers of equal amplification efficiency were designed for *Acp70A* and the three endogenous controls *RpII140*, *Rp132* and *TATA*. The PCR primers used were as follows: ATTCTTGGTT CTCGTTTGCG and TAACATCTTCCACCCCAGG for *Acp70A*, ATGGTGG CTTGCGTTTCGGTG and CCATCGCCAATCTGCGCAACAAT for *RpII140*,

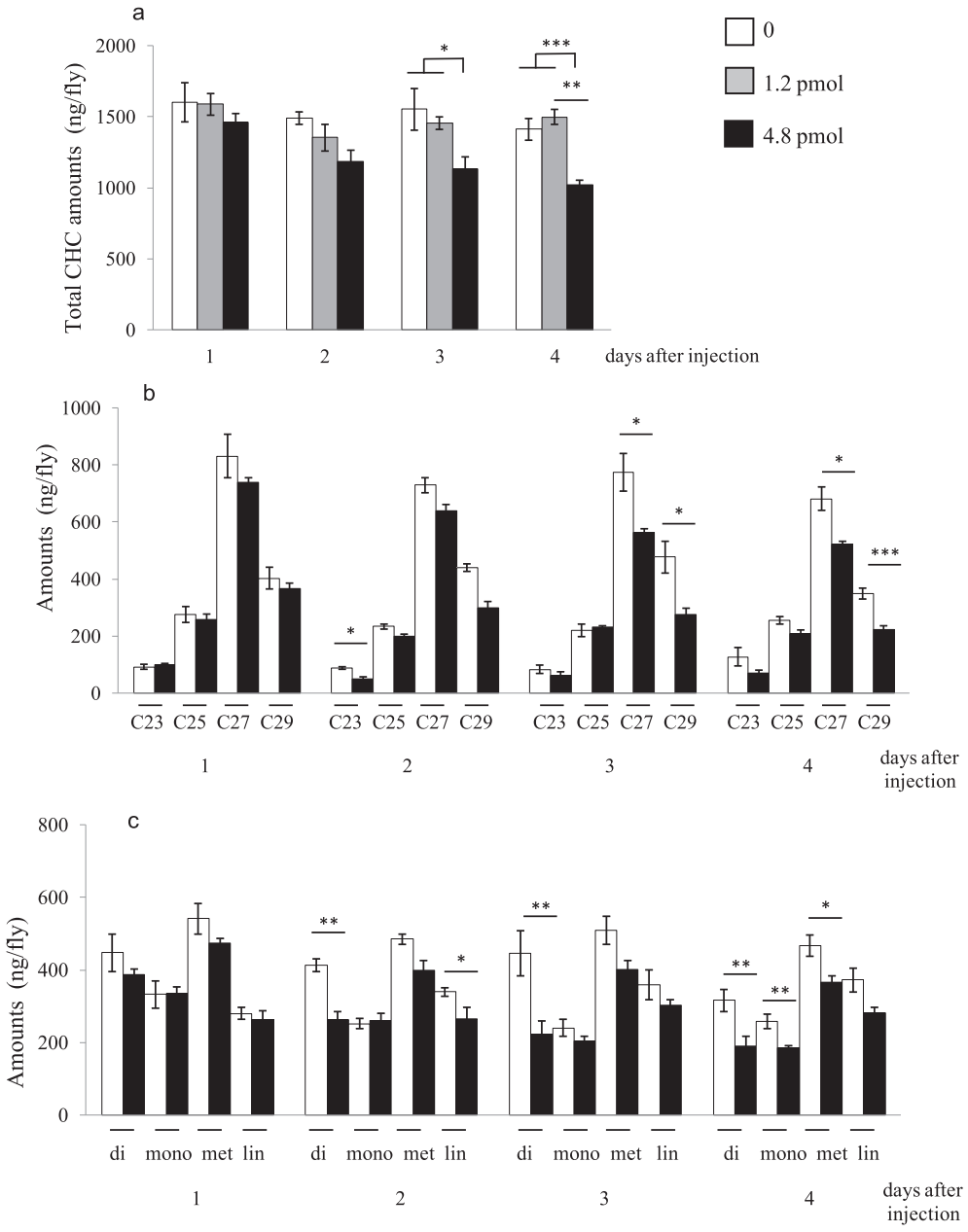

Fig. 4. Hydrocarbon amounts extracted from female Canton-S, flies injected with Ringer's solution (0, white bars), low (1.2 pmol, grey bars) or high (4.8 pmol, black bars) doses of synthetic ACP70A. a: total hydrocarbon amounts (C23 to C29); b: amounts of hydrocarbons of different lengths; c: amounts of hydrocarbons of different classes. Each bar represents mean ± SEM (n = 10). *, ** and *** above bars indicate significant differences (one-way ANOVA, P = 0.05, 0.01 and 0.001, respectively) between means. di: alkadienes; mono: alkenes; met: 2-methyl-alkanes; lin: linear alkanes.

AACATCGGTTACGGATCGAA and GACAATCTCCTTGCGCTTCT for *Rp132*, TAGCATTGCATGCGAGAAAC and AACCGAGCTTTTGGATGATG for *TATA*.

Analysis of total RNA was performed on a CFX96 real-time cycler (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad). Cycling conditions were 30 s at 95 °C and (210 s at 95 °C, 15 s at 60 °C, 20 s at 72 °C) × 35. PCR reactions were performed in biological duplicate and technical duplicate for each RNA sample.

Melting curves were established for all conditions to check for the absence of unspecific amplifications. The determination of reference genes was made with Bestkeeper (version 1, Pfaffl et al., 2004), geNorm (version 3.4, Vandesompele et al., 2002) and Normfinder (version 0.953, Andersen et al., 2004). Expressions of RpII140, Rpl32 and TATA were used to normalize the experimental samples. All relative quantification was calculated using REST-MCS software (version 2, Pfaffl, 2001) using the pair-wise fixed randomization test with 10,000 permutations.

2.3. Molecular biology

To generate the *UAS-Acp70A* transgenic lines, the *Acp70A* cDNA from Canton-S was cloned into a pUAST vector using standard molecular techniques (Brand and Perrimon, 1993). Plasmid constructs were injected by BestGene.

A UAS line was also generated with the sequence corresponding to the N-terminal part of the ACP70a protein (41 amino acids) (Fig. 1).

Protein deduced sequence of Canton-S ACP70A was synthesized by Proteogenix®. The synthetic peptide was dissolved in Ringer's solution at 2 different concentrations: 1.2 and 4.8 pmol. These concentrations are within the range of those used by different

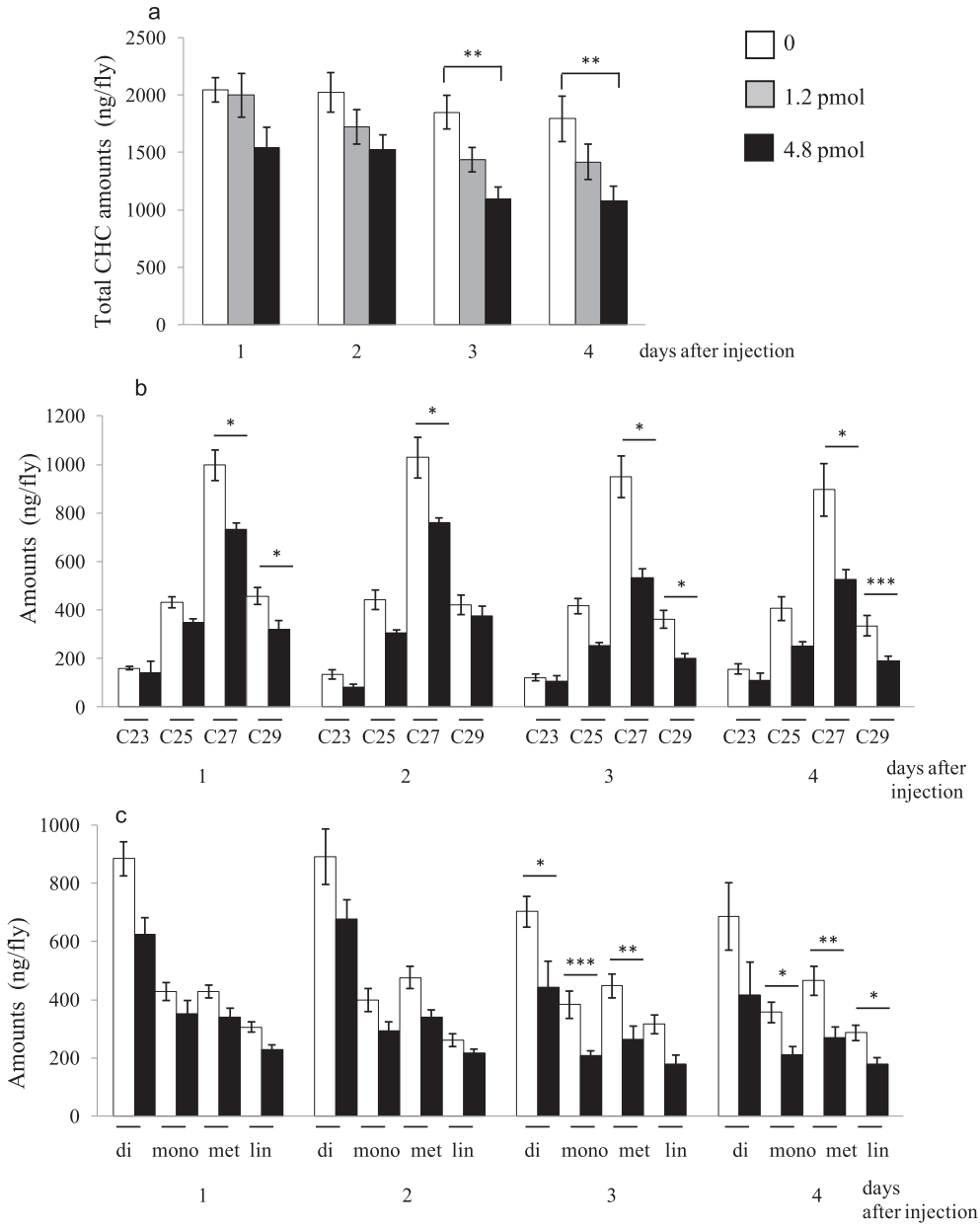

Fig. 5. Hydrocarbon amounts extracted from female Chavroches flies injected with Ringer's solution (0, white bars), low (1.2 pmol, grey bars) or high (4.8 pmol, black bars) doses of synthetic ACP70A. . a: total hydrocarbon amounts (C23 to C29); b: amounts of hydrocarbons of different lengths; c: amounts of hydrocarbons of different classes. Each bar represents mean ± SEM ($n = 10$). *, ** and *** above bars indicate significant differences (one-way ANOVA, $P = 0.05$, 0.01 and 0.001, respectively) between means. di: alkadienes; mono: alkenes; met: 2-methyl-alkanes; lin: linear alkanes.

authors: 3 pmol (Soller et al., 1999) and 1–10 pmol (Fan et al., 1999). Flies were chilled on ice and injected with 50 nL of Ringer's solution without (controls) or with synthetic ACP70A.

2.4. Mating experiments

5-day-old virgin females were individually introduced into a vial with a 5-day-old virgin male. After 2 h, the unmated females were discarded from the experiment. The mated females were kept in groups of 10 in fresh vials until hydrocarbon analysis.

2.5. Exposure to methoprene

Methoprene (M6662, Sigma) was dissolved in ethanol and applied to the surface of the food (25 μg per vial). Newly emerged females were transferred into these vials and the medium was changed four times once every day. This protocol is very effective because the flies are exposed to methoprene by feeding and contact/vapour (Wilson et al., 2003).

2.6. Hydrocarbon analyses

Flies were separated by sex just after emergence and kept in groups of ten flies for 5 days at 25 °C. For quantitative analysis, hydrocarbons were removed from single 4-day-old females by washing them for 5 min in 100 μl heptane containing 500 ng hexacosane (*n*-C26) as an internal standard. The fly was then removed from the vial, which was then sealed with a PTFE (polytetrafluoroethylene) cap to avoid heptane evaporation. All sample extracts were stored at 4 °C prior to gas chromatography. Five microliters of each sample were injected into a Perichrom Pr200 gas chromatograph, fitted with a flame-ionization detector, with a BP-1 capillary

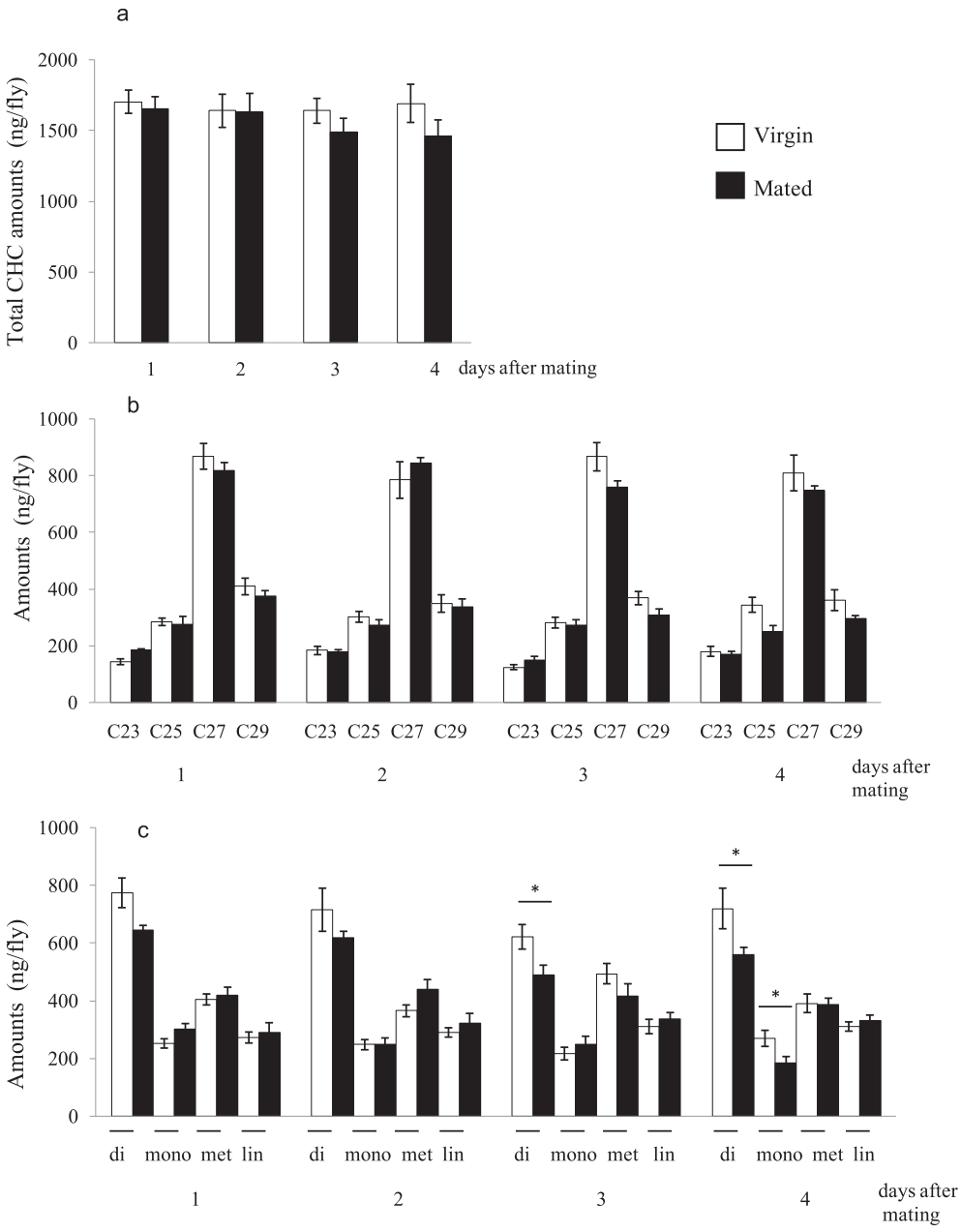

Fig. 6. Hydrocarbon amounts extracted from virgin (white bars) or mated (black bars) female Canton-S flies. a: total hydrocarbon amounts (C23 to C29); b: amounts of hydrocarbons of different lengths; c: amounts of hydrocarbons of different classes. Each bar represents mean ± SEM ($n = 10$). * above bars indicates significant differences (one-way ANOVA, $P = 0.05$) between means. di: alkadienes; mono: alkenes; met: 2-methyl-alkanes; lin: linear alkanes.

column (SGE, 25 m long, 220 μm in diameter and 0.1 μm ID) and hydrogen as the carrier gas (25 cm/s velocity). The injector and detector temperatures were 250 and 260 °C, respectively. The oven-temperature started at 180 °C, ramped at 3 °C/min to 300 °C, for a total run of 40 min. The data were automatically computed and recorded using Winilab III software (version 04.06, Perichrom) as previously described (Wicker-Thomas et al., 2009). We quantified 18 CHCs in female flies, all with a chain length between 23 and 29 carbons. Data are presented as means ± SEM ($n = 10$ for all tests).

2.7. Statistical analyses

CHC amounts were arcsine-square root transformed before normalization. We performed one-way analyses of variance (ANOVAs) separately for each CHC and then used Tukey's post-hoc test (including a correction for multiple comparisons) to identify significant differences among groups. All statistical analyses were performed using R version 2.13.1 (R Development Core Team 2011).

3. Results

3.1. Hydrocarbons in flies overexpressing Acp70A

3.1.1. Females expressing constitutively Acp70A

The original G10 line (*P{Acp70A$^{g\ Yp1\ hs}$}/P{Acp70A$^{g\ Yp1\ hs}$}*) was crossed with wild-type males to obtain females with 0, 1 or 2 *P{Acp70A$^{g\ Yp1\ hs}$}* copies (and noted G10^0, G10^1 and G10^2,

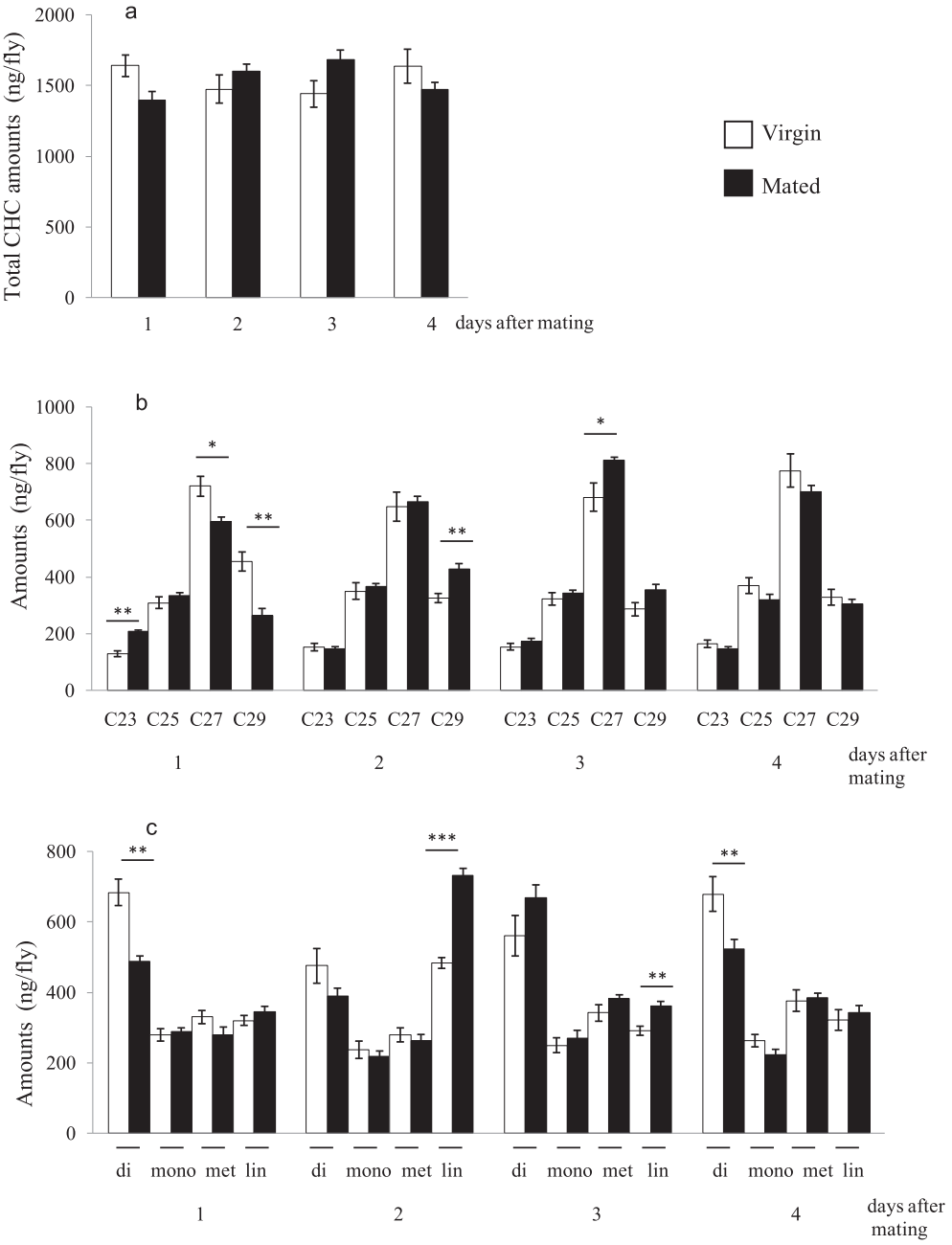

Fig. 7. Hydrocarbon amounts extracted from virgin (white bars) or mated (black bars) female Chavroches flies. a: total hydrocarbon amounts (C23 to C29); b: amounts of hydrocarbons of different lengths; c: amounts of hydrocarbons of different classes. Each bar represents mean ± SEM ($n = 10$). *, ** and *** above bars indicate significant differences (one-way ANOVA, $P = 0.05$, 0.01 and 0.001, respectively) between means. di: alkadienes; mono: alkenes; met: 2-methyl-alkanes; lin: linear alkanes.

respectively). $G10^2$ and $G10^1$ females synthesized less CHC amounts than $G10^0$ females (−24% and −12%, respectively, Fig. 2; Table 1).

There was a dose-effect relationship between the number of transgenes and the amount of total CHCs ($F_{2,24} = 10.45$, $P < 0.001$), leading to a 12 and a 24% decrease in $G10^1$ and $G10^2$ flies, compared with $G10^0$ (Fig. 2; Table 1). This effect was principally due to a drop in C29 CHCs (−37 and −71% in flies with 1 and 2 transgenes). An increase in C25 (+34%) and decrease in C23 CHCs (−26%) was also observed in $G10^2$ flies. Concerning the CHC classes, the levels of monoenes (−32 and −40%) and dienes (−17 and −35%) were markedly reduced in $G10^1$ and $G10^2$ flies, respectively. The pheromones (HD + ND) dramatically dropped: −18.5 and −49.7% in $G10^1$ and $G10^2$ females.

3.1.2. Females expressing ubiquitously Acp70A

As the G10 strain was generated in 1991, we wondered whether the CHC profile was due to the expression of *Acp70A* or to the drift of the line, which might have brought several mutations on the chromosome that carries *yp1-Acp70A* allele. Therefore, we generated flies containing a *UAS-Acp70A* transgene and overexpressed *Acp70A*, using the ubiquitous *da-gal4* driver.

There was a 16% decrease in CHC amount of da-gal4>UAS-Acp70A flies, compared to controls (Fig. 3; Table 2). The decrease concerned almost all the chain lengths (−25, −27 and −16% for C23, C25 and C29 CHCs, respectively). Monoene and saturated linear CHC quantities were also decreased (−29 and −25%, respectively). The pheromone amount (HD + ND) was not significantly affected.

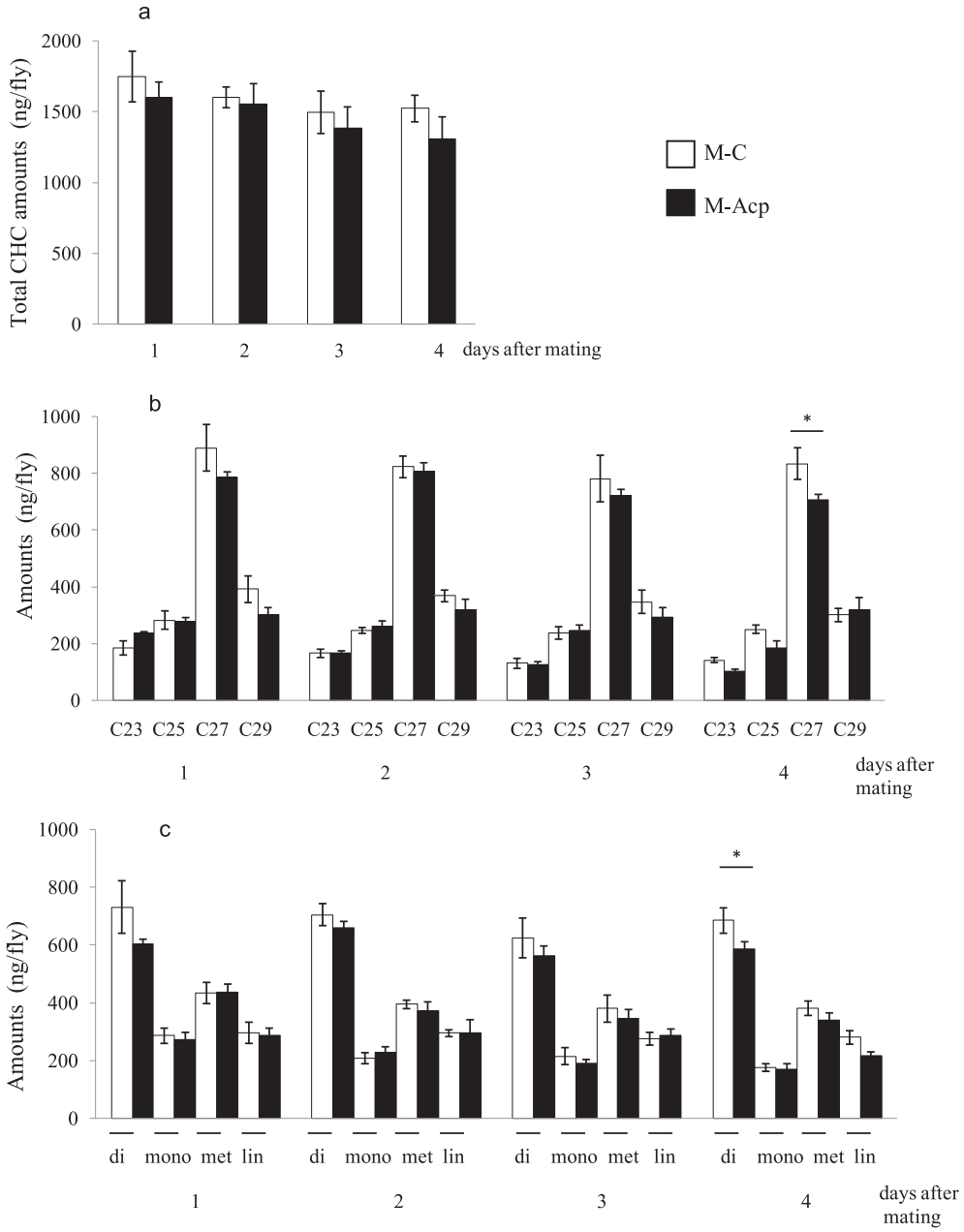

Fig. 8. Hydrocarbon amounts extracted from Canton-S females which were mated with control (M−C) (white bars) or *da > Acp70A* (M-Acp) males (black bars). a: total hydrocarbon amounts (C23 to C29); b: amounts of hydrocarbons of different lengths; c: amounts of hydrocarbons of different classes. Each bar represents mean ± SEM ($n = 10$). *above bars indicates significant differences (one-way ANOVA, $P = 0.05$) between means. . di: alkadienes; mono: alkenes; met: 2-methyl-alkanes; lin: linear alkanes.

3.2. Hydrocarbons after injection of ACP70A

CHC levels depended significantly on the dose of injected synthetic ACP70A ($F_{2,105} = 18.55$, $P < 0.001$ and $F_{2,105} = 17.49$, $P < 0.001$ in Canton-S and Chavroches respectively); (Figs. 4 and 5; Tables S1–S8). In both CS and Chavroches flies, the total CHC levels of those injected with 1.2 pmol synthetic ACP70A, were not significantly different from those injected with Ringer's solution. Conversely, both strains exhibited a marked decrease in CHC levels 3–4 days after injection with the higher dose (4.8 pmol) of synthetic ACP70A, compared to controls: CHC levels decreased by 28 and 40% in CS and Chavroches respectively. CHCs in C27 and C29 were particularly affected (−25 and −40% in Canton-S, −43 and −44% in Chavroches). Concerning the classes of CHCs, there was a diminution of the amount of dienes and monoenes (−45 and −20% in Canton-S, −38 and −43% in Chavroches).

Three to four days after injection with 4.8 pmol of synthetic ACP70A, levels of C27 and C29 CHCs were particularly affected (−25 and −40% in Canton-S, −43 and −44% in Chavroches) (Figs. 4b and 5b). All the classes of CHCs were affected in Chavroches 3 and 4 days after injection and in Canton-S, 4 days after injection (Figs. 4c and 5c).

3.3. Hydrocarbons after mating

3.3.1. Mating with wild-type males

5-day-old virgin females were mated and CHCs were extracted for analysis 1, 2, 3 and 4 days after mating (Tables S9–S12, Figs. 6 and 7). In both strains, mating did not modify total CHC amounts (Figs. 6a and 7a), nor did mating have an effect on individual CHCs in either strain (Figs. 6b and 7b). However, in Canton-S females, there was a 32 and 35% decrease in the quantity of total dienes 3 and 4 days after mating. Four days after mating a 33% decrease in the quantity of monoenes was also observed (Fig. 6c). Concerning the Chavroches strain, a 23% increase in C23 and an 18 and 42% decrease in C27 and C29 was observed the first day after mating. Total linear saturated CHC amounts were 24% higher 3 days after mating and the total amount of dienes was 23% lower 4 days after mating (Fig. 7c). Taken together, these results show that the effect of mating lies between the effects of 1.2 and 4.8 pmol ACP70A injection: 23–35% decrease in dienes (−15 to −19% at 1.2 pmol and −37 to −50% at 4.8 pmol); −20 and −26% in pheromones (7,11-HD + 7,11-ND) in mated Canton-S and Chavroches, respectively (−20 and −39% in both strains at 1.2 and 4.8 pmol, respectively).

3.3.2. Mating with males overexpressing Acp70A

The same experiment was performed with Canton-S females which were mated with control (M–C) or with those over expressing *Acp70A*; *da > Acp70A* (M-Acp) (Tables S13, S14, Fig. 8). Total CHCs levels were not significantly different in females that were mated with Canton-S or *da > Acp70A* males (Fig. 8a). The total C27 and total dienes amount was slightly decreased (−15% each) in females, 4 days after copulation with M-Acp males compared to females mated with CS (Fig. 8b and c). However, when compared to virgin Canton-S females, the total CHCs in M-Acp females were reduced by 16 and 23% 3 and 4 days after mating, respectively. The amount of monoenes and linear CHCs were particularly reduced at day 4 (−37 and −31%, respectively) (Figs. 6c and 8c).

3.4. Expression of Acp70A in flies overexpressing Acp70A

Next, we wanted to compare the relative change in Acp70A transcripts in flies overexpressing *Acp70A* (G10 and *da > Acp70A*) with male Canton-S (controls) and $G10^2$ females.

There were 5 times more *Acp70A* transcripts in *da > Acp70A* males, compared to control males (Fig. 9A). The expression of *Acp70A* was approximately 3.5 times higher in *da > Acp70A* females than in $G10^2$ (Fig. 9B).

3.5. ACP70A acts on CHCs through its or N- terminal part

It is known that the C-terminal part of ACP70A binds to the SPR receptor. To determine whether this interaction mediates changes in CHC levels, we injected SPR-knocked-down and control females with synthetic ACP70A or Ringer's solution and compared CHC levels. (Fig. 10; Tables S15–S18). ACP70A injection had similar effects in control and SPRi females: in particular there was a similar decrease in total CHCs (−20% in control and −17% in SPRi) (Fig. 10a) and total dienes (−24% in control and in SPRi) 4 days after ACP70A injection, compared to Ringer's solution injection (Fig. 10d and e).

To test the N-terminal part of the peptide, we could not follow the same protocol, because its receptor is unknown. We therefore overexpressed the N-terminal part of the peptide (Figs. 1 and 11; Table S19). Overexpression of the ACP70A N-terminal part resulted in a 12% decrease in total CHC amount compared to controls (Fig. 11a). Specifically, lower quantities of C27 (−14%) and C29 (−31%) CHCs were observed (Fig. 11b). The amounts of dienes and

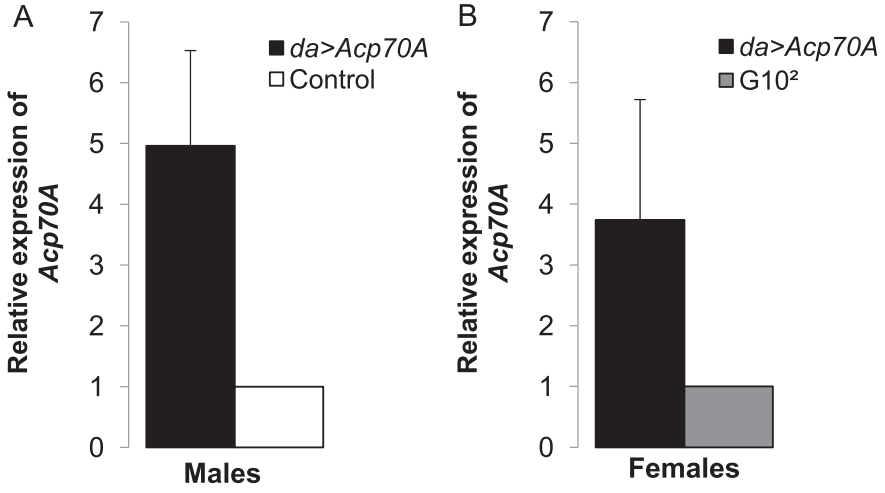

Fig. 9. Real-time PCR analysis: Values represent the fold change of *Acp70A* mRNA in *da > Acp70A* flies relative to control (males) or $G10^2$ (females). Results are expressed as mean ± SEM ($n = 4$).

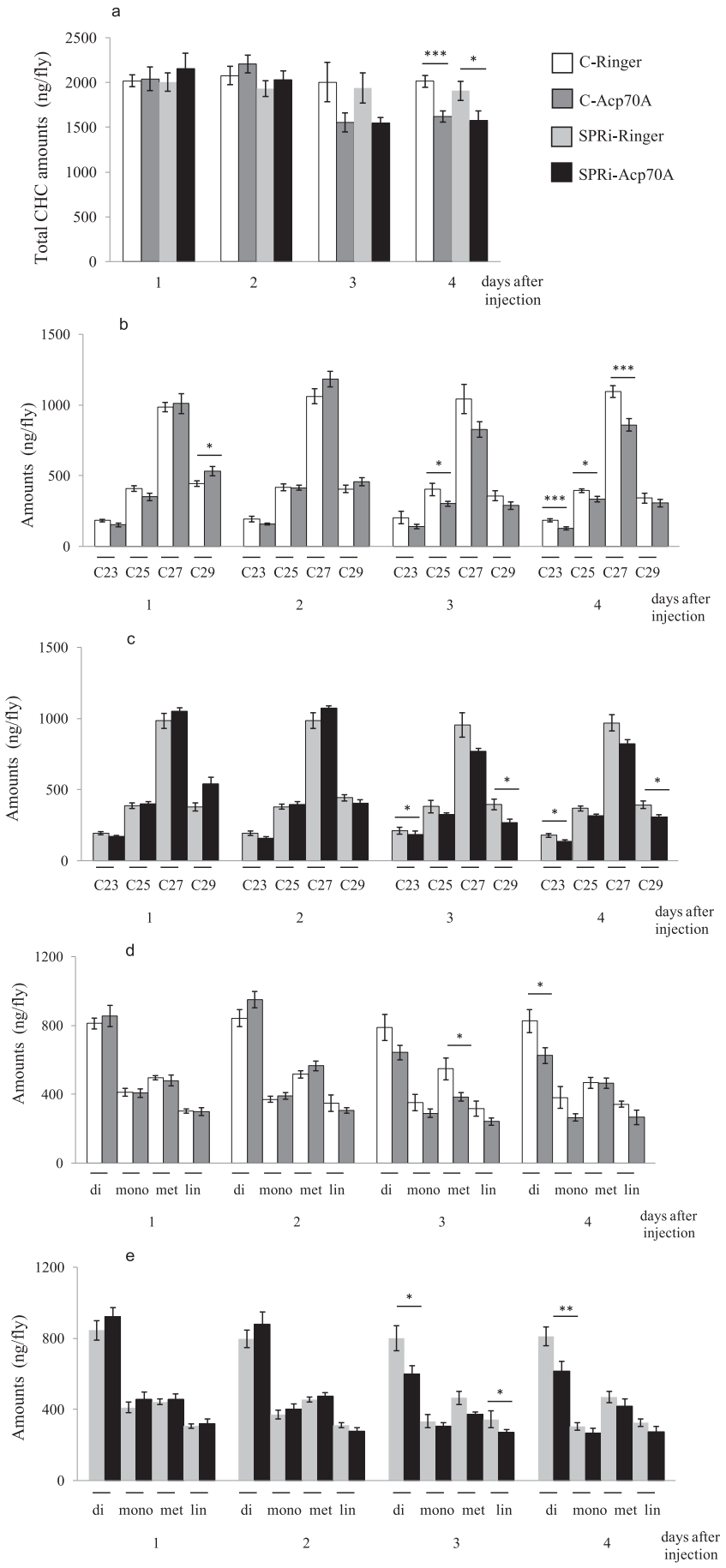

Fig. 10. Hydrocarbon amounts extracted from control (*SPR RNAi/Cy*) or SPRI (*elav > SPR RNAi*) females injected with Ringer or synthetic ACP70A (4.8 pmol). a: total hydrocarbon amounts (C23 to C29); b: amounts of hydrocarbons of different lengths in C females; c: amounts of hydrocarbons of different lengths in SPRi females; d: amounts of hydrocarbons of different classes in C females; e: amounts of hydrocarbons of different classes in SPRi females. Each bar represents mean ± SEM ($n = 10$). *above bars indicates significant differences (one-way ANOVA, $P = 0.05$) between means. di: alkadienes; mono: alkenes; met: 2-methyl-alkanes; lin: linear alkanes.

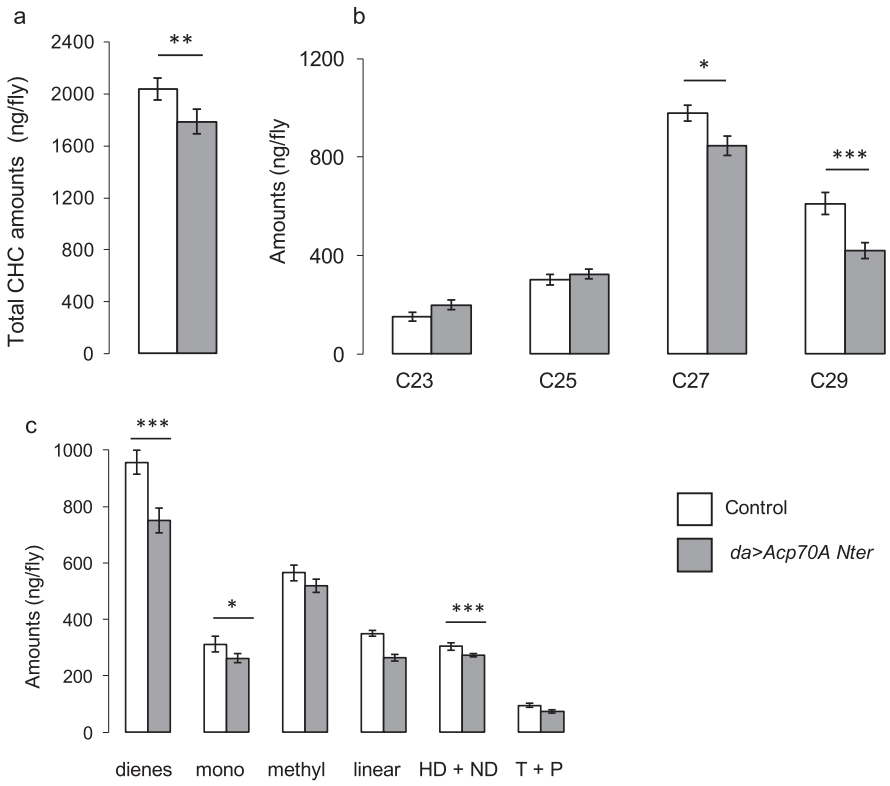

Fig. 11. Hydrocarbon amounts extracted from control females (white bars) or those overexpressing *Acp70A Nter* (grey bars). a: total hydrocarbon amounts (C23 to C29); b: amounts of hydrocarbons of different lengths; c: amounts of hydrocarbons of different classes. Each bar represents mean ± SEM ($n = 10$). *, ** and *** above bars indicate significant differences (one-way ANOVA, $P = 0.05$, 0.01 and 0.001, respectively) between means. HD: 7,11-heptacosadiene; ND: 7,11-nonacosadiene; T: 7-tricosene; P: 7-pentacosene; dienes: alkadienes; mono: alkenes; methyl: 2-methyl-alkanes; linear: linear alkanes.

monoenes were also reduced (−22% for both dienes and the pheromones HD + ND, −16% for monoenes) (Fig. 11c).

3.6. Does ACP70A act on CHCs through juvenile hormone?

As the N-terminal part of ACP70A links to the corpora allata and is responsible for higher JH synthesis, we tested the effects of methoprene, an analog of JH on CHC synthesis. The levels of CHCs in Canton-S females exposed to food supplemented with methoprene or ethanol, were not significantly different (Fig. 12a; Table S20). However, in females exposed to methoprene, the amounts of CHCs with 23 and 29C were decreased by 39% (Fig. 12b). Similarly, dienes and female pheromones were reduced by 25% compared to ethanol exposed flies (Fig. 12c).

In *Aug > hid, rpr* females, whose corpora allata cells had been partially ablated, total CHC (+31%), C23 (+158%) and C25 (+125%) levels were elevated (Fig. 13a). There was also a concurrent decrease in C29 (−49%) in *Aug > hid, rpr* compared to controls (Fig. 13b). The dienes and monoenes were 56 and 53% higher in *Aug > hid,rpr*, respectively, but the pheromone amounts (HD + ND) were not significantly different from the control. The quantities of 7-T + 7-P were 3 times higher (Fig. 13c; Table S21).

RNAi knock-down of the JH receptor *Met* in females was followed by an increase in total CHCs (+23%) (Fig. 14a) due to increased amounts of C25 (+46%) and C27 (+37%) (Fig. 14b). The dienes and monoenes were also increased (+37 and 29%, respectively) and 7-T + 7-P quantity was doubled (Fig. 14c; Table S22).

4. Discussion

Females constitutively expressing *Acp70A* in their fat body (G10) had reduced levels of total CHCs. This was largely due to a decline in C29 chain CHCs and pheromones. We are confident that this phenotype is due to expression of the transgene because there is a clear relationship between the number of transgenes and the strength of the phenotype. Similar results were obtained when *Acp70A* was ubiquitously expressed, using the *da-Gal4* driver. In G10 females, *Acp70A* was expressed at a level comparable to that of Canton-S males. When expressed ubiquitously, *Acp70A* transcript levels were five times higher. In this respect, the physiology of G10 females is probably closer to that of mated females (who receive ACP70A after mating).

To better understand the action of this peptide, we injected 1.2 or 4.8 pmol of synthetic ACP70A into virgin females. The lower dose did not alter the CHC amount but resulted in significant decrease in dienes and pheromones. The higher dose resulted in a decrease in CHC level, especially in long chain CHCs (C27, C29) and dienes. Two different strains were used in this experiment; Canton-S, adapted for a long time in the laboratory and Chavroches, which was more recently introduced. ACP70A injections drove more pronounced changes in Chavroches. A 40% drop in CHC levels was observed in Chavroches 4 days after injection compared to a 28% decline in Canton-S. This result shows that strains may react differently to the same ACP70A dose.

A decrease in CHCs quantity occurred as early as day 1 following ACP70A injection, however, this decline was not significant due to the large variations in CHC amounts among females. The decrease was more and more pronounced over the following days and became significant at three and four days after injection, in both Canton-S and Chavroches. In *Helicoverpa*, suppression of pheromone biosynthesis occurred immediately after *Drosophila* ACP70A injection (Fan et al., 1999). In this moth, pheromone biosynthesis is induced during the scotophase which restarts every night. In *Drosophila* hydrocarbons accumulate on the cuticle from emergence and the duration of their turn-over is not known. Even a

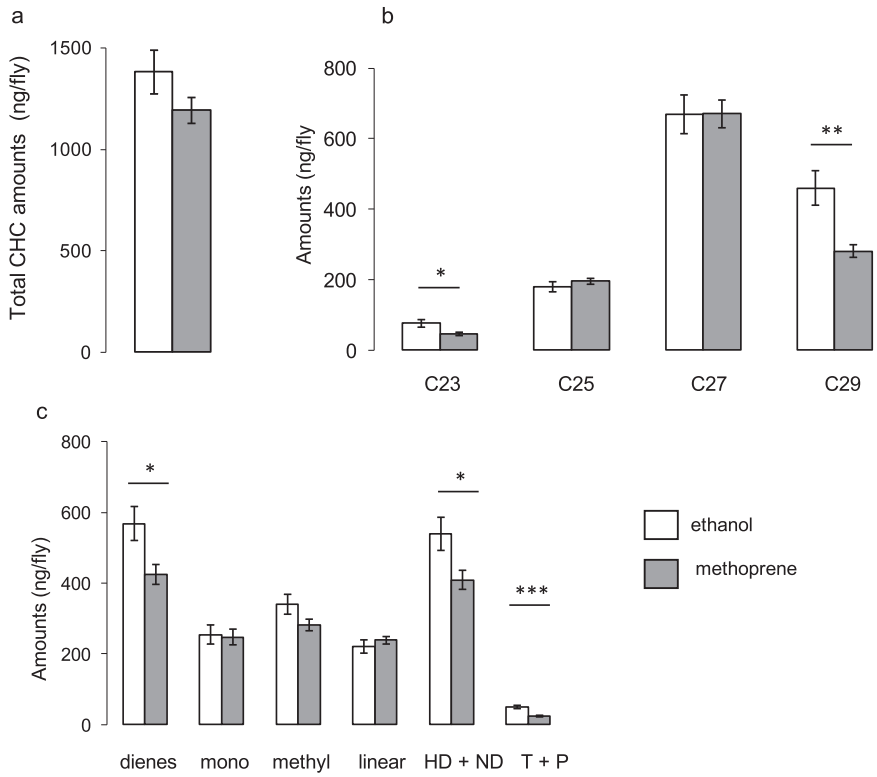

Fig. 12. Hydrocarbon amounts extracted from Canton-S female flies exposed to ethanol (white bars) or methoprene (grey bars). a: total hydrocarbon amounts (C23 to C29); b: amounts of hydrocarbons of different lengths; c: amounts of hydrocarbons of different classes. Each bar represents mean ± SEM ($n = 10$). *, ** and *** above bars indicate significant differences (one-way ANOVA, $P = 0.05$, 0.01 and 0.001, respectively) between means. HD: 7,11-heptacosadiene; ND: 7,11-nonacosadiene; T: 7-tricosene; P: 7-pentacosene; dienes: alkadienes; mono: alkenes; methyl: 2-methyl-alkanes; linear: linear alkanes.

small decrease in the overall CHC quantity may represent a large reduction of their synthesis.

Intriguingly, inhibition of *Drosophila* CHC synthesis persisted for at least four days after peptide injection. ACP70A induces both short-term (1 day) and long-term (1 week or more) responses. The long-term responses include increased egg-laying and reduced receptivity, which was described by Manning, (1962), (1967) and are due to the binding of the peptide to −and gradual release from-sperm in the female (Chapman et al., 2003; Liu and Kubli, 2003). The steady release of ACP70a from stored sperm requires the cleavage of the peptide at a trypsin cleavage site (Peng et al., 2005). Once transported into the haemolymph, ACPs are rapidly degraded by proteases (Monsma et al., 1990; Lung and Wolfner, 1999; Ravi Ram et al., 2005). Our results show that the effects of ACP70A on CHCs levels persist several days after its likely degradation, suggesting that the peptide acts indirectly.

The action of ACP70A on pheromones is independent on its C-terminal part. Indeed, overexpression of the peptide lacking the 14 C-terminal amino acid residues had the same effects as the entire peptide and injection of ACP70A to control and SPR knocked-down females caused similar effects. Action through the N-terminal part might explain why the effects of the peptide persist long after injection. Transient effects on receptivity and oviposition have been observed by injecting purified ACP70A into the abdomen (Chen et al., 1988). One explanation for these transient effects is the presence of haemolymph serine proteases that rapidly inactivate ACP70A (Pilpel et al., 2008). All the short-term effects described in the literature are mediated through the receptor to the C-part of the peptide. ACP70A stimulates the biosynthesis of JH in the corpora allata through its N-part (Moshitzky et al., 1996; Fan et al., 1999). We therefore wondered if ACP70A action on CHCs was due to higher JH levels. Application of methoprene, a JH analog to food resulted in a moderate decreased in dienes and pheromones. In contrast, partial genetic ablation of the corpora allata or RNAi knock-down for *Met* resulted in higher CHC and diene levels. We also saw higher levels of CHCs and less dienes in female flies whose JH esterase and JH epoxide hydrolases (four enzymes which are involved in JH degradation) were knocked-down using RNAi (CWT, unpublished results). Bilen et al. (2013) observed a delay in production of dienes after corpora allata genetic ablation and increased diene levels in *Met* null mutant females. All these data are in favor of an indirect action of ACP70A via the corpora allata.

Fan et al. (1999) showed that in the moth *Helicoverpa*, pheromone synthesis was suppressed by *Drosophila* ACP70A. This suppression persisted even after decapitation. In a later paper (Fan et al., 2000), these authors demonstrated that the C-terminal part of the peptide was involved in pheromone synthesis inhibition. However, this does not seem to be the case in *Drosophila*, since females knocked-down for the receptor SPR (which binds the C-terminal part) still exhibit CHC changes after ACP70A injection.

The physiological role of pheromone regulation by ACP70A in *Drosophila* females is still unclear. Mating has little but significant effect on pheromone production and the main effect is a decrease in dienes and pheromones 3 and 4 days after mating. This effect corresponds to a dose range of ACP70A between 1.2 and 4.8 pmol, although the quantity of ACP70A injected by the male to the female during mating has never been determined. These variations are certainly too low to inhibit male courtship. In support of this, Everaerts et al. (2010) did not detect differences in pheromone levels between virgin and mated wild-type *D. melanogaster* females. The inhibition of pheromone synthesis, exerted by ACP70A might be a side-effect of JH induction which plays a crucial role in physiological processes including development, reproduction and sexual maturation.

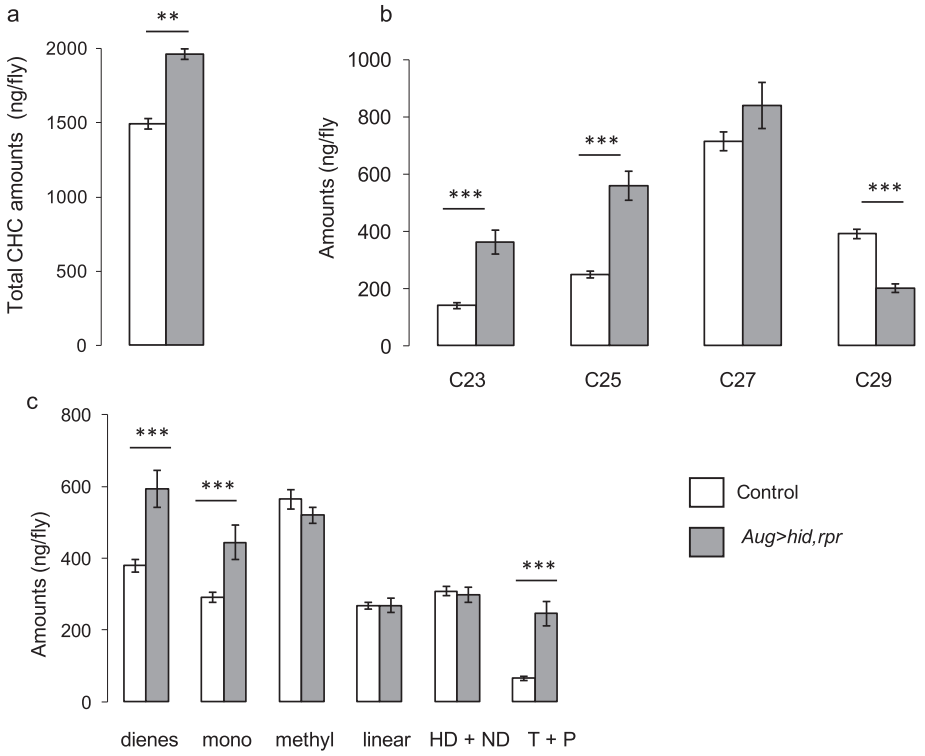

Fig. 13. Hydrocarbon amounts extracted from control females (white bars) and those whose corpora allata was genetically ablated (*Aug > hid,rpr*) (grey bars). a: total hydrocarbon amounts (C23 to C29); b: amounts of hydrocarbons of different lengths; c: amounts of hydrocarbons of different classes. Each bar represents mean ± SEM ($n = 10$). *, ** and *** above bars indicate significant differences (one-way ANOVA, $P = 0.05$, 0.01 and 0.001, respectively) between means. HD: 7,11-heptacosadiene; ND: 7,11-nonacosadiene; T: 7-tricosene; P: 7-pentacosene; dienes: alkadienes; mono: alkenes; methyl: 2-methyl-alkanes; linear: linear alkanes.

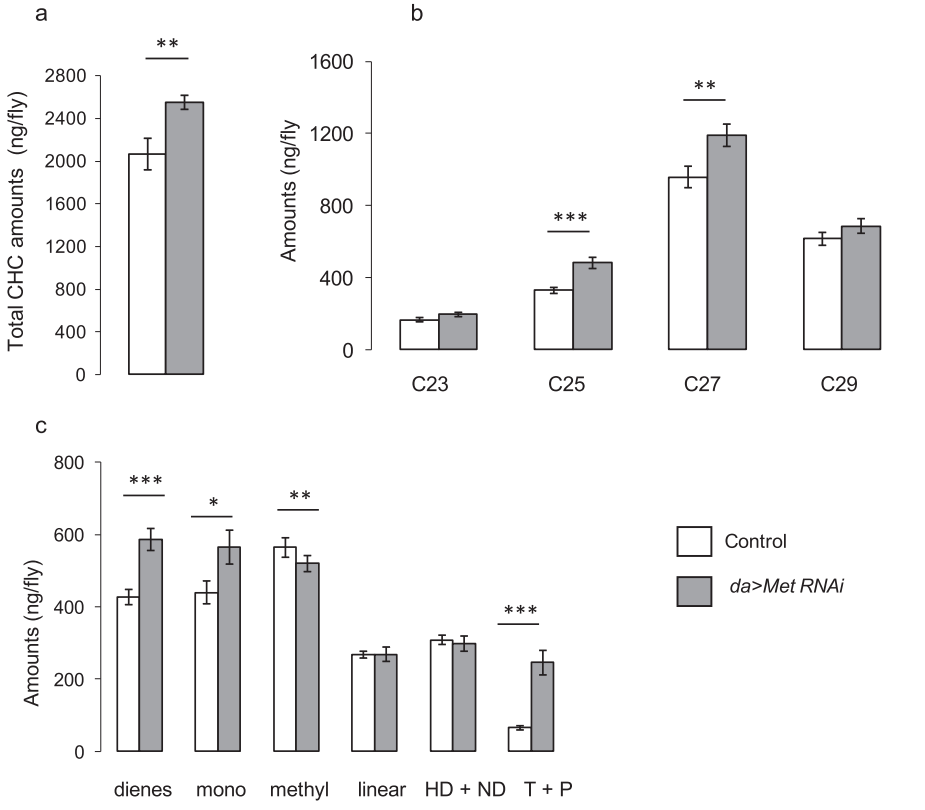

Fig. 14. Hydrocarbon amounts extracted from control females (white bars) or RNAi knocked-down for *Met* (grey bars). a: total hydrocarbon amounts (C23 to C29); b: amounts of hydrocarbons of different lengths; c: amounts of hydrocarbons of different classes. Each bar represents mean ± SEM ($n = 10$). *, ** and *** above bars indicate significant differences (one-way ANOVA, $P = 0.05$, 0.01 and 0.001, respectively) between means. HD: 7,11-heptacosadiene; ND: 7,11-nonacosadiene; T: 7-tricosene; P: 7-pentacosene; dienes: alkadienes; mono: alkenes; methyl: 2-methyl-alkanes; linear: linear alkanes.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2014.11.008.

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