Chapter 5

Role of Sex Peptide in *Drosophila* Males

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Abstract

Drosophila male sex peptide ACP70A is a small peptide mainly produced in the accessory glands. It elicits a high number of post-mating responses in mated females; yet its function in male physiology is not well known. Here, we explore its role in male sex behavior and pheromone biosynthesis, using males either mutant or RNAi knocked-down for *Acp70A*. Courtship was severely affected in both *Acp70A* mutants and *Acp70A* knocked-down males, with only 2% of the males succeeding copulation. Cuticular hydrocarbon amounts were moderately affected with 25% decrease in *sp0* mutant (without *Acp70A* expression) and 10–22% increase in flies overexpressing *Acp70A*. *Acp70A* knock-down either ubiquitously or in the testes surprisingly resulted in an overproduction of hydrocarbons, whose amounts were double of the controls. We tested eight putative "off-target" genes but none of these led to an increase in hydrocarbon amounts. These results show that male courtship behavior is largely dependent on the presence of Acp70A and independent of cuticular hydrocarbons. The presence of potential "off-target" genes explaining the hydrocarbon phenotype is discussed.

Keywords: cuticular hydrocarbons, pheromones, sex peptide, Acp70A, courtship behavior, *Drosophila*

1. Introduction

In most reproducing animals, including *Drosophila*, seminal fluid is transferred along with sperm to females during mating. These seminal fluid components have important effects on female behavior and physiology and have been extensively studied in *Drosophila melanogaster* [1, 2]. Most of these seminal proteins are synthesized in the accessory glands (AGs) and therefore, named ACcessory gland Proteins (ACPs). One well-characterized ACP, ACP70A

(also called SP, sex peptide), plays a major role in eliciting postmating response: it modifies the female behavior, resulting in the rejection of courting males [3, 4]. It has a crucial role on female reproduction: it increases oogenesis [5], egg production [6] and egg-laying [3, 4]. It also induces dramatic effects on female nutrition: it increases food uptake [7], modifies food preference by altering nutrient balancing [8] and alters gut water absorption and intestinal transit [9]. The other physiological modifications are the inhibition of sleep [10] and the regulation of sperm release from the storage organs [11]. All these effects are caused by the binding of the C-terminal part of the sex peptide to a neuronal sex peptide receptor (SPR) in the female [12–14]. The central part of sex peptide elicits the expression of immune response genes [15], and the N-terminal part activates the corpora allata (CA), inducing increased synthesis of juvenile hormone (JH) [16], which triggers oogenesis and vitellogenic oocyte progression [5] and also leads to decreased pheromone biosynthesis [17].

Whereas, there are numerous studies on the role of male sex peptide on female physiology, there are no such studies concerning male physiology. As we observed that there was a defect in courtship behavior of sex peptide mutant males, we wanted to elucidate the possible roles of ACP70A in male behavior and physiology. In this study, we report clear defects in male sex behavior and moderate defects in hydrocarbon and pheromone synthesis concerning mutant males. Using sex peptide knocked-down males, we confirmed the control of sex peptide on male sex behavior. Conversely, ubiquitous expression of *Acp70A*-RNAi resulted in a two-fold increase in cuticular hydrocarbon (CHC) amounts. We could exclude the role of eight off-targets in this CHC augmentation and localize this RNAi effect in the accessory glands (responsible for a 35% increase) and in the testes (responsible for the rest of the effect). The presence of sperm in the testes does not affect CHC biosynthesis.

2. Materials and methods

2.1. *Drosophila* strains and rearing

Three strains mutant for sex peptide were used:

- the deficiency *Δ130*/*TM3* (covering the *Acp70A* gene);
- the point mutant *sp0*, produced by targeted mutagenesis by homologous recombination [4]. *sp0* males were used balanced by *TM3* (*sp0*/*TM3*: one copy of *Acp70A* is active) or homozygous (*sp0*/ *sp0*: no production of ACP70A), or crossed by *Δ130*/*TM3* (*sp0*/*Δ130*: no production of ACP70A).
- DTA-E [18], which are sperm-less and lack ACPs produced from the main cells (96% of the accessory glands).

The laboratory wild-type Canton-S strain was also used as a control.

The following Gal-lines from the Bloomington *Drosophila* Stock Centre were used: *daughterless (da)-Gal4*, a ubiquitous driver; *elav-Gal4*, a driver expressed in the nervous system [19], *dopa decarboxylase (ddc)-Gal4*, expressed in epidermis and nervous system [20], *1407-Gal4* and

PromE-Gal4, both expressed in pupal and adult oenocytes [21, 22], *c564-Gal4*, expressed in fat body [23], *Acp26A-Gal4*, expressed in accessory glands [3], *svp-Gal80*, which specifically blocks Gal4 activity in the oenocytes [24]. Using a *UAS-GFP* line, we could show that *1407-Gal4* was also expressed in testes and built a line with the following genotype: *1407-Gal4; svp-Gal80* that drives the expression only in the testes. Images were visualized and photographed on a Nikon eclipse E800 microscope with a Cool Snap camera.

A *UAS-Acp70A* line was generated in our laboratory and noted *UAS-Acp70A+* [17]. The following *UAS-RNAi*-lines were obtained from the VDRC Stock Center and directed against: *Acp70A*, SP (109,175 KK); *lamp1*, CG3305, (7309 GD); *dco*, CG4379 (101,524 KK); *rgk1*, CG44011 (108,710 KK); *CG5961* (100,023 KK); *CG15128* (100238KK); *CG9413* (108,867 KK); *CG8315* (105,654 KK); *tinc*, CG31247 (101,175 KK).

Drivers were maintained as heterozygous over a Balancer (Cyo or TM3). In all RNAi knock-down (or overexpression) experiments, balanced gal4-driver females were crossed to UAS males. Balanced progeny was taken as the control of RNAi knocked-down (or overexpression) progeny.

Flies were grown at 25°C with 12/12 light–dark (LD) cycles, on standard cornmeal medium. They were separated by sex at emergence and kept sex-separated in groups of 10 in fresh food vials until testing (4 days after emergence).

2.2. Hydrocarbon analyses

CHCs were removed from single 4-day-old flies 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 and 5 μL of each sample was injected into a Perichrom Pr200 gas chromatograph, with hydrogen as the carrier, using a split injector (split ratio 40:1). 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 [17]. As we did not observe significant variation in the CHC profiles, we only represented the total amount of CHCs as means ± SEM (n = 10 for all tests).

2.3. Analysis of *Acp70A* expression

Quantitative PCR was performed as described [25] using RNA TRIzol™ (Invitrogen) to extract RNAs from 10 adults for each sample. cDNAs were synthesized with SuperScript II, and PCR was perfumed with a LightCycler® 480 SYBR Green I Master (Roche Applied Science). Primers for Acp70A (5′-ATTCTTGGTTCTCGTTTGCG-3′ and 5′-TAACATCTTCCACCCCAGG-3′) were used. To normalize mRNA amounts, we tested six different genes and used one gene, which was shown to be very stable in all samples: CG7598 (5′-AACGGATGTGGTGTTCGATT-3′ and 5′-TAATGCCATCCTTGGTGTGA-3′). Samples were performed in independent triplicates (each consisting of two technical replicates).

2.4. Mating experiments

A 4-day-old Canton-S female was introduced into the observation chamber, consisting of a watch glass (28-mm diameter and 5-mm internal height) placed on a glass plate and left for

2 min before the introduction of the male. The following parameters were recorded: lengths of courtship, first copulation attempt and copulation latency (time from introduction of the male into the observation chamber to courtship, first copulation attempt or copulation), percentages of courtship, first copulation attempt and copulation (percentages of males performing courtship, copulation attempt or copulation). The effects of genotypes were evaluated by Kruskal-Wallis tests (latencies) and χ^2-tests (percentages of flies). N ≥ 50 for all tests.

3. Results

3.1. Expression of *Acp70A* in adult males

Acp70A expression was not significantly different in controls (Canton-S and *Acp26A*) and in *sp0* mutants that possess a point mutation in the signal sequence. In contrast, *Acp70A* expression was dramatically inhibited in *Acp26A > Acp70A-RNAi* males (–99%) and higher expression was observed in *Acp26A > Acp70A* males (+63%) (**Figure 1**).

3.2. Effect of *Acp70A* on male sex behavior

Males mutant for *Acp70A* (*sp0/+* and *sp0/sp0*), overexpressing *Acp70A* (*Acp26A > Acp70A*) or RNAi knocked-down (*Acp26A > Acp70A-RNAi* and *da > Acp70A-RNAi*) were tested in face of wild-type females (**Figure 2**).

All the steps of courtship were affected in *sp0* mutants: the number of heterozygous males that attempted or succeeded copulation decreased by 54 and 73%, respectively. The effect of the homozygous mutation was dramatic: *sp0/sp0* males performing courtship (wing vibration) were 5 times fewer than heterozygous or control males, and out of the 50 homozygous

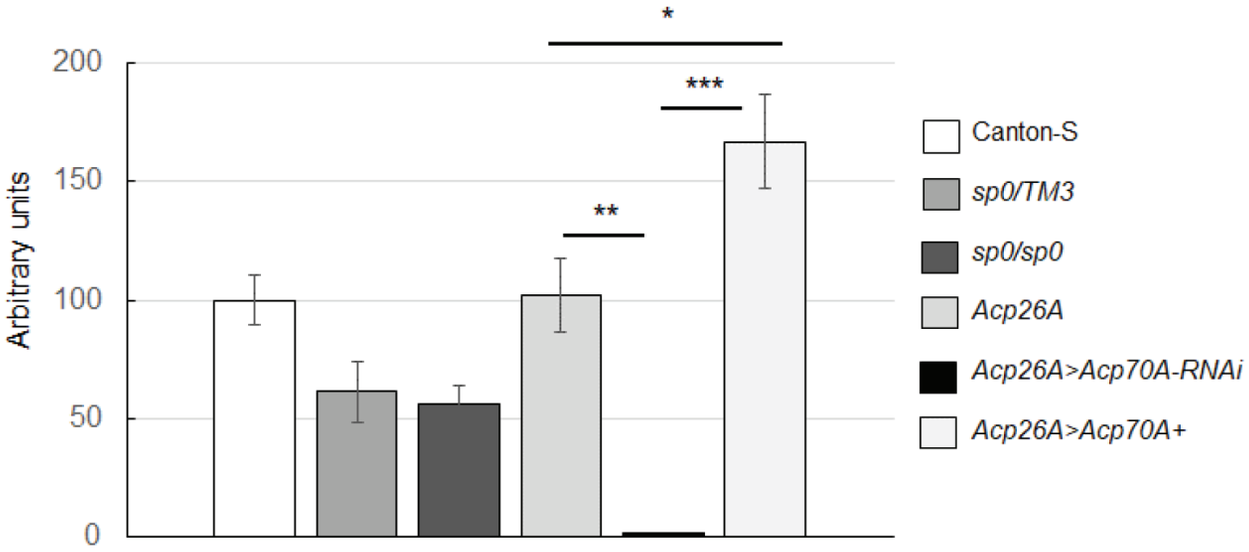

Figure 1. Transcriptional expression of *Acp70A* in control, mutant, knocked-down or overexpressing male flies. Each bar represents mean ± SEM of three independent trials. *, ** and *** indicate significant differences (P = 0.05, 0.01 and 0.001, respectively).

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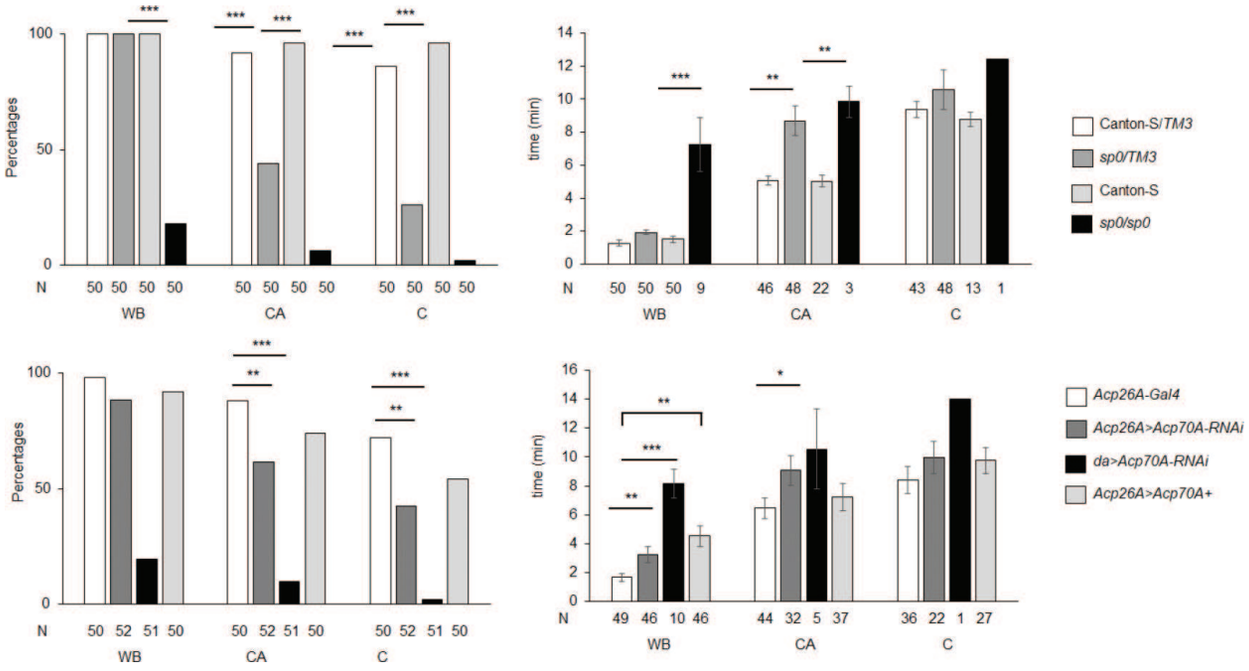

Figure 2. Courtship and mating experiments in fly pairs composed of a wild-type (Canton-S) female and a male of a different genotype: percentages of males performing courtship (WB), copulation attempts (CA) and copulation (C) and time needed to initiate these tasks. Effect of the *sp0* mutation and overexpression or RNAi knock-down of *Acp70A* in males (drivers *Acp26A-Gal4* and *da-Gal4*). Each bar represents mean ± SEM of 50 trials. *, ** and *** indicate significant differences (P = 0.05, 0.01 and 0.001, respectively). N is indicated below each bar.

males tested, only 3 attempted to copulate and 1 succeeded copulation. Time needed to perform these tasks was higher as well: homozygous *sp0* males needed 5 times more than *sp0*/+ or wild-type males to initiate courtship and the time to attempt copulation was 1.7 and 2 times longer in heterozygous and homozygous mutants, compared to control males.

Overexpression of *Acp70A* in the accessory glands did not modify the proportion of males performing the different steps of courtship behavior. On the other hand, the time necessary to perform wing vibration was double.

Courtship of males knocked-down for *Acp70A* in accessory glands was also affected: the percentage of these males performing copulation attempts and copulation was, respectively, 30 and 41% lower when compared to control males. It took them 2 and 1.5 times longer to perform wing vibration and copulation attempts when compared to control males. When knock-down was induced ubiquitously (*da* > *Acp70A-RNAi*), the inhibition of courtship was more severe and similar to that observed in homozygous *sp0* males.

These results show that there is a significant inhibition of courtship behavior in absence of sex peptide expression.

3.3. Effect of *Acp70A* on CHCs

Heterozygous *sp0* male CHCs were not significantly different from wild-type ones (**Figure 3**). Conversely, homozygous *sp0* males as well as males bearing one *sp0* over a deficiency covering the entire *Acp70A* gene showed a 25% decrease in the total CHC amount. This result

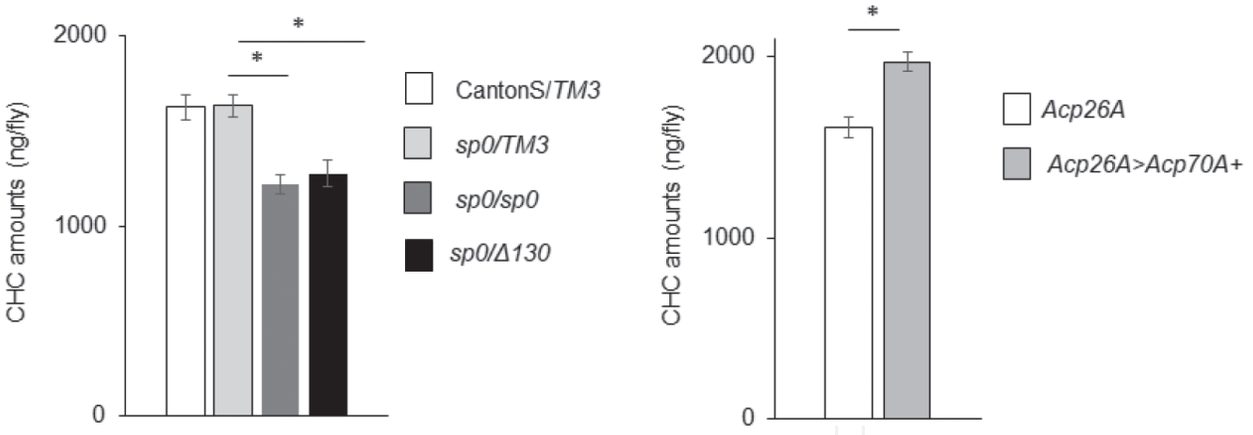

Figure 3. Cuticular hydrocarbon amounts in adult males either mutant for *sp0* (left) or overexpressing *Acp70A* (right) under the *Acp26A-gal4* driver. Each bar represents mean ± SEM (n = 10). * indicates significant differences ($P = 0.05$).

confirms that *sp0* is a null mutant. Inversely, a ubiquitous overexpression of *Acp70A* led to a small but significant increase in CHCs (+22%).

Acp70A ubiquitous knock-down (*da > Acp70A-RNAi*) was followed by a twofold increase in CHC amount (**Figure 4**). We thus wondered whether this increase could be due to off-target effect. The RNAi line was described as having no off-target sequence (no gene covering a 19-mers sequence of the RNAi sequence). We performed a Blast analysis with different 16-mers from the RNAi sequence and obtained eight putative off-target genes containing a stretch of coding sequence identical to at least 15-mers of *Acp70A* sequence (**Figure 5**). The RNAi of these genes was expressed ubiquitously to measure their effect on male CHCs. For five RNAi tested, we obtained no effect on CHCs and for three RNAi (directed against *lamp1*, *rgk1* and *tinc*), there was a lower amount of CHCs (from −14 to 22%, depending on the RNAi) (**Figure 6**). In

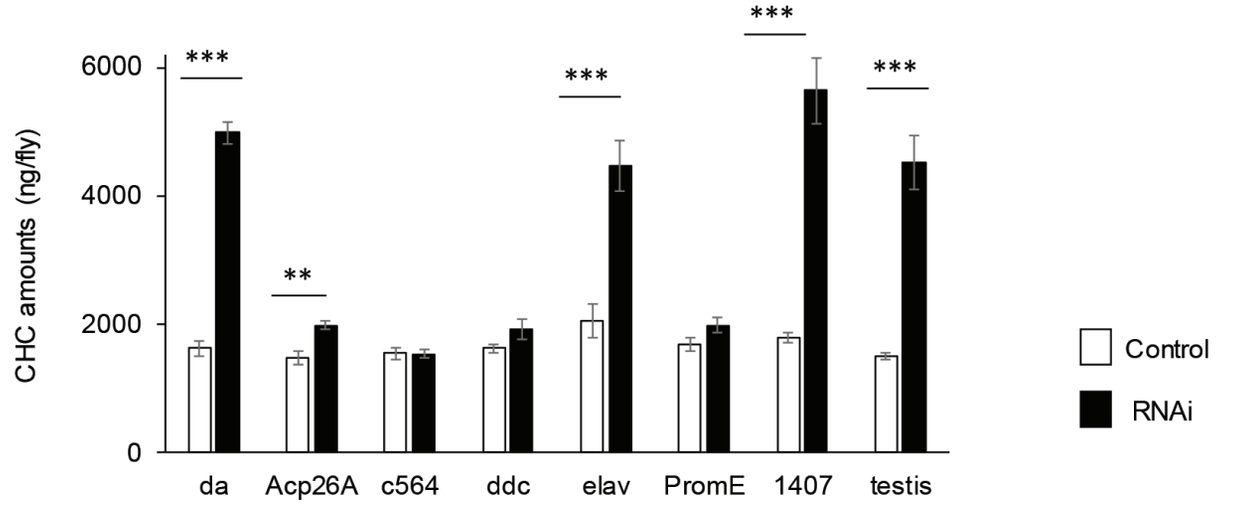

Figure 4. Cuticular hydrocarbon amounts in adult males that were RNAi knocked-down for *Acp70A* in different tissues: ubiquitously (da), in the accessory glands (Acp26A), in fat body (c564), in epidermis (ddc), in nervous system (elav), in oenocytes (Prome), in oenocytes and testes (1407) and in testis (1407; svpgal80). Each bar represents mean ± SEM (n = 10). ** and *** indicate significant differences ($P = 0.01$ and 0.001, respectively).

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ATGAAAACTCTAGCTCTATTCTTGGTTCTCGTTTGCGTACTCGGCTTGGT    CG5961 CG9413
CCAGGCCTGGGAATGGCCGTGGAATAGGAAGCCTACAAAGTTTCCAATTC    CG8315 CG44011
CAAGCCCCAATCCTCGTGATAAGTGGTGCCGTCTTAATTTGGGGCCCGCC    Lamp1  dco  tinc
TGGGGTGGAAGATGTTAA                                    CG15128
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Figure 5. Putative off-target genes containing a stretch of coding sequence identical to at least 15-mers of *Acp70A* sequence.

conclusion, the dramatic increase in CHC amount following ubiquitous *Acp70A* knock-down cannot be explained by an off-target effect due to these genes.

3.4. Characterization of the tissue involved in CHC control

In males, *Acp70A* is expressed at a very high level in accessory glands and at a moderate level in testis and carcass (8631, 100 and 95 arbitrary units, respectively; FlyAtlas).

We then wanted to determine the tissue responsible for this effect by targeting *Acp70A-RNAi* to various tissues. We confirmed the locations of expression of the different *Gal4* lines used in this study and showed that *1407-Gal4* was additionally expressed in the testes (**Figure 7**).

No significant effect on CHCs was obtained when *Acp70A RNAi* was expressed in fat body (c564 > *Acp70A RNAi*), in epidermis (*ddc* > *Acp70A RNAi*) and in oenocytes (*PromE* > *Acp70A RNAi*). On the other hand, *Acp70A* knock-down in accessory glands led to a moderate (+35%) increase in CHC amount. CHC amount was multiplied by a factor of 2 *in elav* > *Acp70A RNAi* (nervous system) and a factor of 3 in *1407* > *Acp70A RNAi* (oenocytes + testes) and *1407; svp-Gal80* > *Acp70A -RNAi* (testes). This last result shows an essential role of the testes on CHC production (**Figure 4**).

3.5. CHC profile of the DTAE-line

The DTA-E line is characterized by the absence of accessory glands and some defects in testes, among them, a lack of sperm. DTA-E males were found to produce 1.4-fold more CHCs. We

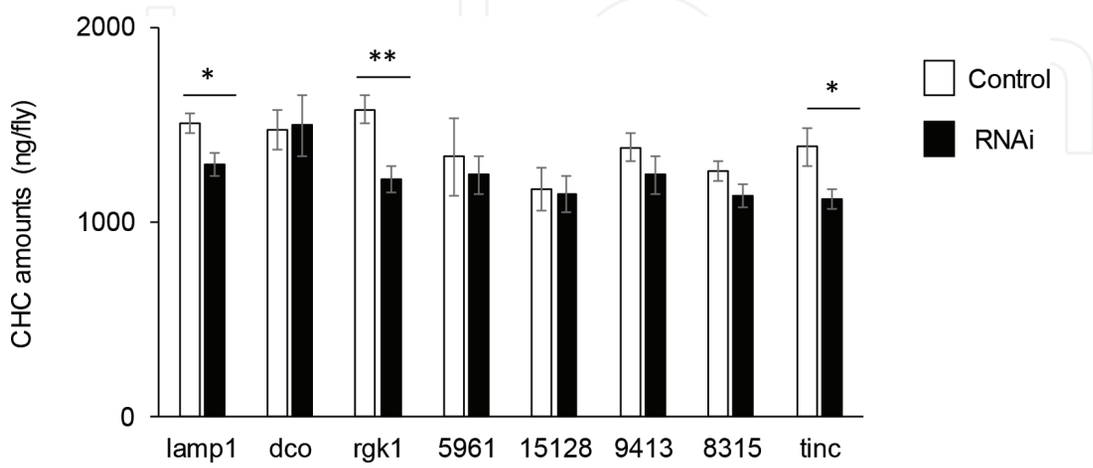

Figure 6. Cuticular hydrocarbon amounts in adult males knocked-down for putative off-target genes. Each bar represents mean ± SEM (n = 10). * and ** indicate significant differences (P = 0.05 and 0.01, respectively).

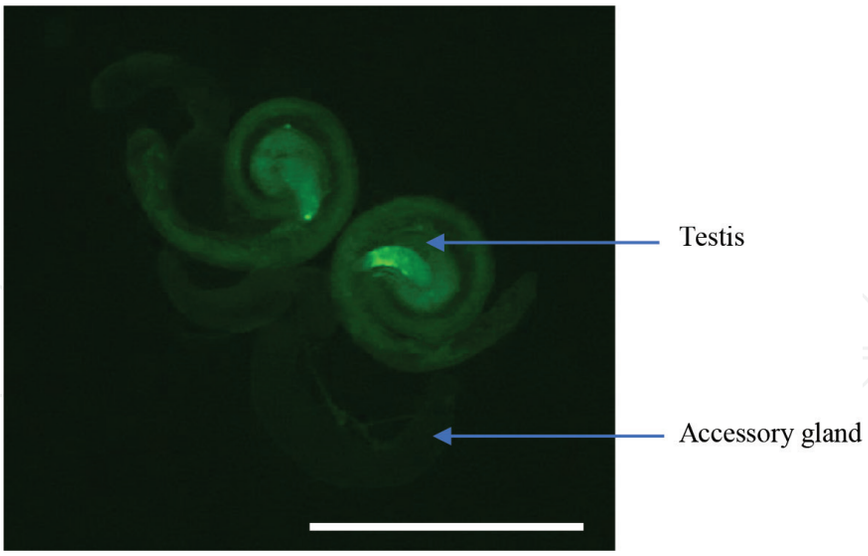

Figure 7. Photomicrographs showing GFP expression in male reproductive apparatus from *1407-Gal4; svp-Gal80*. Fluorescence could be detected only in the testes. Scale bar: 0.5 mm.

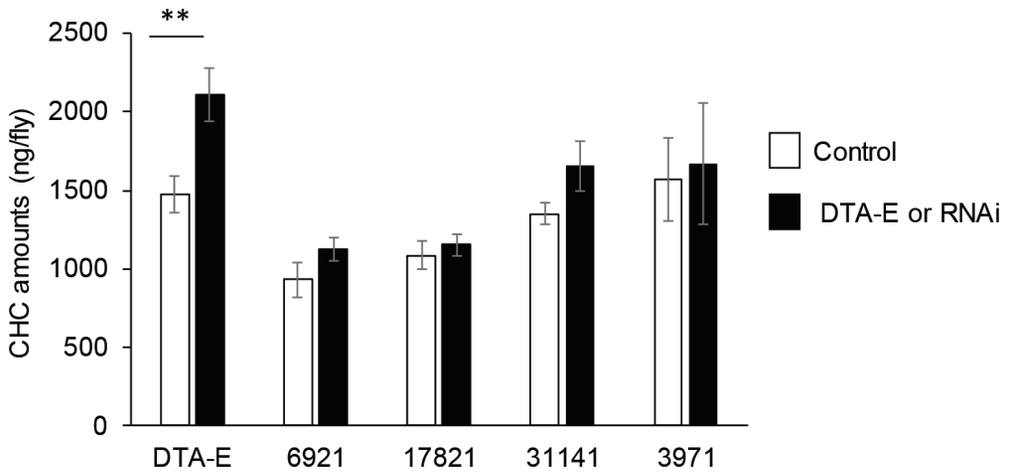

Figure 8. Cuticular hydrocarbon amounts in adult males that do not produce sperm: either DTA-E or knocked-down for CG6821, CG17821, CG31141 and CG3971. Each bar represents mean ± SEM (n = 10). ** indicates significant difference ($P = 0.01$).

wanted to evaluate the effect of the absence of sperm on CHCs. Four elongase genes are essential to spermatozoid development and the lack of expression in testes leads to sterile males without sperm [26–28]. We knocked-down these genes in the testes, using the 1407-Gal4 line. We verified the absence of sperm in the RNAi males. None of these genes had any effect on male CHC production (**Figure 8**).

4. Discussion

4.1. Sex behavior

Ubiquitous overexpression of sex peptide had no significant effect on male sex behavior: the percentage of males performing the different steps of courtship (wing vibration, copulation

attempts and copulation) was unchanged and only the time to begin courtship was lengthened. Conversely, *sp0* males showed difficulties to court and the effect was dependent on the dose of the mutant allele: heterozygous *sp0* males courted wild-type females the same way as wild-type males did but only a half of them attempted copulation and one-eighth succeeded to mate. The inhibition was more drastic in homozygous *sp0* males, as less than one-fifth courted the females and only 2% succeeded to mate.

We tested the males that were RNAi knocked-down for sex peptide in the accessory glands. To target the expression in the accessory glands, we used the driver *Acp26A-Gal4*. *Acp26A* gene is almost exclusively expressed in the accessory glands (3589 and 97 units in the accessory glands and the testes, respectively; FlyAtlas). Courtship behavior of *Acp26A > Acp70A RNAi* males was affected, but less than that of *sp0* males: they courted wild-type females the same way as wild-type males did, two-third knocked-down males attempted copulation and less than a half copulated. This result raised the question: does *sp0* affect tissues other than accessory glands? When we ubiquitously expressed sex peptide RNAi, we obtained courtship results similar to those with *sp0*. Taken together, the results suggest a positive control of sex peptide on male courtship behavior. They also pose the problem of the reason of the absence of mating in *sp0* and *da > Acp70A RNAi* males since Q-PCR results clearly show that the expression of *Acp70A RNAi* in accessory glands via *Acp26A-Gal4* reduces *Acp70A* expression to only 1%.

4.2. Cuticular hydrocarbons

In the female, the transfer of ACP70A during mating induces a decrease in cuticular hydrocarbon amount. This decrease occurs 3 and 4 days after mating and might be due to the overproduction of juvenile hormone following mating, caused by the action of *Acp70A* on the corpora allata [17]. We therefore wondered whether *Acp70A* could regulate the production of hydrocarbons in the male. The *sp0* mutation as well as *Acp70A* ubiquitous overexpression led to moderate effects on male CHC production: whereas, wild-type and *sp0* heterozygous males had similar CHC amounts, there was a 25% decrease and a 10–22% increase in homozygous *sp0* that do not produce ACP70A and da > *Acp70A* (overproduction of ACP70A) males, respectively. This result seems to be in favor of a positive regulation of sex peptide on CHC production.

The results concerning the effect of *Acp70A RNAi* on cuticular hydrocarbons were unexpected: a 35% increase occurred when *Acp70A* expression was inhibited in the accessory glands, using *Acp26A-Gal4*. *Acp26A* gene is mainly, but not exclusively, expressed in the accessory glands (3589 and 97 units in the accessory glands and the testes, respectively; FlyAtlas). *Acp26A* expression in the testes represents 2.7% of the expression in the accessory glands, similar to *Acp70A* (1.1%). Moreover, a ubiquitous *Acp70A* knock-down led to a twofold increase in CHC amount; we firstly ascribed this dramatic effect to the presence of possible off-targets of the RNAi.

ACP70A is a small peptide (55 amino acids, including the signal sequence). The nucleic sequence of *Acp70A RNAi* covers almost the totality of the coding sequence, and also includes the small intron. We found eight putative off-target genes, containing a stretch of coding sequence identical to at least 15-mers of *Acp70A RNAi* sequence. However, none of these putative off-target genes could be accountable for the dramatic CHC increase resulting in *Acp70A RNAi* expression.

4.3. Search of the tissue involved in the control on hydrocarbon production

We knocked-down sex peptide in different tissues and could demonstrate that neither the fat body, nor the oenocytes or the epidermis could be responsible for the large rising level of CHCs. On the other hand, sex peptide expression in the testes or in the nervous system led to a CHC increase similar to ubiquitous overexpression.

Sex peptide Acp70A is mainly expressed in the accessory glands, but some expression is also observed in the testes and the carcass (FlyAtlas). Inside the accessory glands, it is exclusively produced by the main cells (96% of the accessory glands) [29]. When we used the DTA-E line in which accessory gland main cell function was genetically disrupted [18], we obtained as well a large-fold increase in CHCs. DTA-E line was obtained after the introduction of diphtheria toxin subunit A (DTA) into the accessory glands via the promoter of *Acp95EF* [18]. ACP95EF is also a sex peptide produced in the accessory glands and transmitted to the female after mating. It has the same place of production as ACP70A; in the accessory glands, it is exclusively produced in the main cells [29]. Within the fly, it is mainly expressed in the accessory glands and marginally in the testes (787 and 62 arbitrary units, respectively; FlyAtlas). DTA-E males lack ACPs produced from the main cells but have normal secondary cells as well as ejaculatory bulb and duct [30]. DTA-E males are sterile and the block of spermatogenesis occurs at the primary spermatocyte stage [18]. The occurrence of a faint expression of this gene in the testes (FlyAtlas) could explain the lack of sperm. However, the lack of sperm is not directly responsible for the large increase in CHC amounts since flies that did not produce sperm after RNAi knock-down for different elongases involved in sperm production did not increase their CHC production.

The question is: why does DTA-E line show a similar male CHC phenotype to *da > Acp70A-RNAi*? In the former line, no off-target can be involved. An explanation could be that a "leakage" of the Acp95EF promoter has resulted in a lack of sperm and probably other defects [18]. In males that have been RNAi knocked-down ubiquitously, one may suppose the effect of unknown "off-target" genes that are essential to testis function. This might suggest a role (yet unknown) of the testes in the control of male hydrocarbons.

5. Conclusion

This study demonstrates a role of sex peptide on male courtship behavior. Moreover, the data with DTA-E and RNAi knocked-down flies show the importance of the integrity of the testes (not the sperm) in the control of CHCs.

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