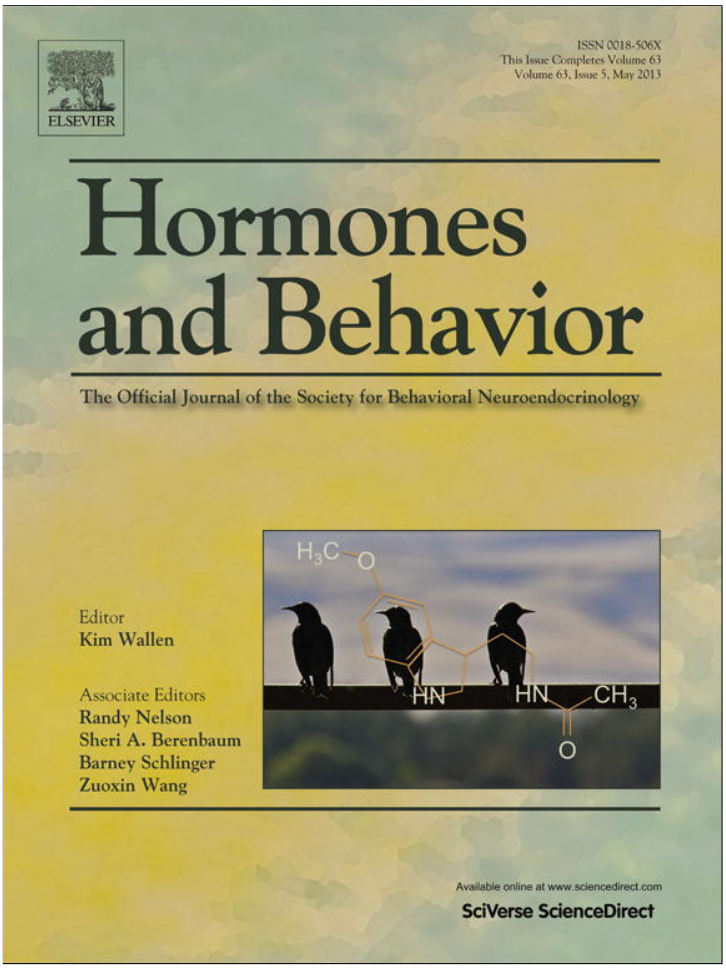

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# Is the rapid post-mating inhibition of pheromone response triggered by ecdysteroids or other factors from the sex accessory glands in the male moth *Agrotis ipsilon*?☆

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ABSTRACT

In many animals, male copulation is dependent on the detection and processing of female-produced sex pheromones, which is generally followed by a sexual refractory post-ejaculatory interval (PEI). In the male moth, *Agrotis ipsilon*, this PEI is characterized by a transient post-mating inhibition of behavioral and central nervous responses to sex pheromone, which prevents males from re-mating until they have refilled their reproductive tracts for a potential new ejaculate. However, the timing and possible factors inducing this rapid olfactory switch-off are still unknown. Here, we determined the initial time delay and duration of the PEI. Moreover, we tested the hypothesis that the brain, the testis and/or the sex accessory glands (SAGs) could produce a factor inducing the PEI. Lastly, we investigated the possible involvement of ecdysteroids, hormones essential for development and reproduction in insects, in this olfactory plasticity. Using brain and SAG cross-injections in virgin and newly-mated males, surgical treatments, wind tunnel behavioral experiments and EIA quantifications of ecdysteroids, we show that the PEI starts very shortly after the onset of copulation, and that SAGs contain a factor, which is produced/accumulated after copulation to induce the PEI. Moreover, SAGs were found to be the main source of ecdysteroids, whose concentration decreased after mating, whereas it increased in the haemolymph. 20-Hydroxyecdysone (20E) was identified as the major ecdysteroid in SAGs of *A. ipsilon* males. Finally, 20E injections did not reduce the behavioral pheromone response of virgin males. Altogether our data indicate that 20E is probably not involved in the PEI.



## Introduction

In many animal species, male reproduction is dependent on the detection and processing of female-produced sex pheromones. Responses to such pheromones depend not only on their chemical properties as signals, but also on environmental conditions and the physiological state of the receiver (Kolb and Whishaw, 1998; Meinertzhagen, 2001). Male reproductive success depends both on the ability to locate and copulate with a female, and to effectively transfer an ejaculate (Dewsbury, 1982). Contrarily to females, in which mating induces drastic long-lasting physiological and behavioral changes (Flanagan-Cato et al., 2006; Gillott, 2003; Huck et al., 1987), males can often remate after a variable time delay. However, remating in males is limited by the number of ejaculates they can deliver and the time required to replenish depleted reserves. Therefore, newly-mated males should delay the risk-taking and energy-consuming search for new sexual partners. By avoiding unsuccessful reproduction, an individual may increase both its probability of surviving to the next reproductive opportunity and the amount of energy available to undergo a next reproductive event. Although males of many vertebrate and few invertebrate species are also known to enter a post-ejaculatory refractory interval (PEI) (Aversa et al., 2000; Fischer and King, 2008; Phillips-Farfán and Fernández-Guasti, 2009; Reddy and Guerrero, 2000; Ureshi and Sakai, 2001), the mechanisms that lead to this sexual abstinence are far from being understood.

In the noctuid moth, *Agrotis ipsilon*, evidence is accumulating that the modulation of pheromone responses occurs through neuronal plasticity (Anton et al., 2007). In this species, we previously showed

☆ Sex accessory glands and post-mating olfactory inhibition in a moth.

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that newly-mated males are no longer attracted to sex pheromone, and that the response to pheromone is restored during the next night (Gadenne et al., 2001). This plasticity is not only seen at the behavioral level, but is accompanied by a decrease in the sensitivity of pheromone-specific neurons within the primary olfactory centre, the antennal lobe (AL): most neurons have much higher pheromone response thresholds after mating (Barrozo et al., 2010a; Gadenne et al., 2001). This olfactory switch-off is restricted to the responses to sex pheromone as newly-mated males still respond to plant odors (Barrozo et al., 2010a). This transient olfactory plasticity thus allows newly-mated males to enter a PEI in order to replenish their sex accessory glands (SAGs) for a potential new female encounter and copulation (Duportets et al., 1998).

The fast change in neuron sensitivity leading to this transient olfactory switch-off following mating could involve a down- or up-regulation of neuroregulatory peptides, biogenic amines or hormones such as juvenile hormone (JH) and ecdysteroids. Although biogenic amines have been shown to influence pheromone sensitivity in animals (including insects), we recently showed that octopamine and serotonin are probably not involved in the transient post-mating olfactory switch off in *A. ipsilon* males (Barrozo et al., 2010b). Although JH is necessary to elicit high sensitivity to sex pheromone in males during adult maturation, the positive effect of this hormone is rather slow (1–2 days) (Gadenne and Anton, 2000), and therefore it is rather unlikely that this hormone is involved in the fast post-mating switch off (hours or less). Moreover, no change in JH biosynthetic activity following mating in males was observed (Duportets et al., 1998).

In many vertebrates, steroids are known to control male sexual behavior by affecting the detection of sensory signals (Hull and Dominguez, 2007). In male mice, sex steroids control the processing of female odors, by modulating pheromone-induced immediate early genes in the accessory olfactory system (Yoshikage et al., 2007) and the expression of pheromone receptor genes in the vomeronasal organ (Alekseyenko et al., 2006). In male hamsters, gonadal steroids regulate behavioral responses to sex pheromones by acting on the medial preoptic nucleus (Swann, 1997). In a cichlid fish, reproductive status was found to modulate the expression of sex steroid receptors in the olfactory bulb (Maruska and Fernald, 2010).

In insects ecdysteroids have essential roles in coordinating developmental transitions such as larval moulting and metamorphosis, and also reproduction events, through their major active form: 20-hydroxyecdysone (20E) (Spindler et al., 2009). Indeed, 20E has been detected in the haemolymph and reproductive tissues of species of various taxa, including moths (reviewed in Brown et al., 2009). Although ecdysteroids are known to be present throughout life in both male and female adults, their functions in male physiology and behavior are not fully understood. In male *Drosophila melanogaster*, 20E mediates courtship behaviors (Dalton et al., 2009; Ganter et al., 2011), courtship memory and memory and sleep through the action of its receptors (Ishimoto and Kitamoto, 2011; Ishimoto et al., 2009). In the noctuid moth *Spodoptera littoralis*, 20E was recently shown to control the peripheral detection of pheromone (Bigot et al., 2012).

Here, we studied the time characteristics of the PEI in *A. ipsilon*. Moreover, we tested the hypothesis that factors present in the brain or reproductive tissues could induce the observed PEI. Lastly, we investigated the possible involvement of ecdysteroids in this olfactory plasticity.

## Materials and methods

### *Insects*

Adult males and females of *A. ipsilon* Hufnagel (Lepidoptera: Noctuidae) originate from a laboratory colony in Bordeaux. Wild insects are introduced into the colony each spring. The animals were reared on an artificial diet in individual cups until pupation. Pupae were sexed and males and females were kept separately in an inversed light/dark cycle (16 h light: 8 h dark photoperiod; scotophase starts at 10 am until 6 pm) at 22 °C, 50% relative humidity. Newly emerged adults were removed every day and were given access to a 20% sucrose solution *ad libitum*. The day of emergence was considered as day-0.

### *Mating experiments*

Mating experiments were performed as previously described (Barrozo et al., 2010a). Briefly, virgin 5-day-old sexually mature males and virgin 3-day-old sexually mature females were individually paired in cylindrical plastic containers before the onset of scotophase in a room under the same inverted light/dark cycle and temperature as cited above. Observations of the pairs were performed to detect the onset and the end of the copulation. In our photoperiod conditions, copulation occurs between 12:30 and 17:30 (mean onset and end of copulation at 14:10 ± 0:56 and 16:03 ± 0:55 respectively; $n = 270$) and lasts between 1 and 2 h (mean duration of copulation: 1 h 53 min ± 28 min; $n = 270$) (Fig. 1). Once copulation had ended, newly-mated males were removed from the observation room. For behavioral tests, they were transferred to the wind tunnel room, and females were dissected to check for the presence of the spermatophore, in order to confirm that mating was successful.

Time onset of PEI was analyzed by testing the behavioral pheromone response of males, which had been allowed to copulate for various time durations (Fig. 1). After the onset of copulation, males *in copula* were manually delicately separated from the females at different time intervals (5, 15 or 30 min) throughout copulation. For the 30-min copulation assays, visual observations of the onset of copulation were performed every 10 min with a red lamp. For the 5 and 15-min copulation assays, observations were performed every 5 min. As males were separated at different times after the onset of copulation, we chose to wait 2 h after the separation time in order to test the behavioral response of mated males at a time, which exceeded the naturally occurring mating duration of 113 min (Fig. 1).

Our previous results showed that mated males did not respond to the sex pheromone when they were tested within 1 h after the end of copulation (Gadenne et al., 2001). To analyze the duration of PEI within the scotophase, we therefore extended the time-window between the end of copulation and the test time by testing the behavioral pheromone response of newly-mated males at least 2 h after the end of copulation, and up to the end of the scotophase (Fig. 1). We thus mainly used males that had started and ended copulation early during the scotophase in order to test a long time-window before the return of the photophase. Control experiments were performed by testing the behavioral response of virgin males to sex pheromone up to the end of the scotophase.

### *Surgical treatments*

Castrated males were obtained by removing the testes of last instar larvae. Larvae were immobilized on a dissecting pad under Ringer's saline, and a dorso-lateral incision was performed in the 4th abdominal segment. Although noctuid larvae can theoretically be sexed according to external structures (Hinks and Byers, 1973), we found it difficult in *A. ipsilon* larvae, and therefore chose to dissect larvae at random. In the case of a male larva, the testes were quickly removed. The wound was dried with a tissue, allowed to heal naturally, and the larva was transferred to the rearing medium. Sham-operated larvae were obtained by performing an incision and gently touching the testes, without extracting them.

Adult males lacking SAGs were obtained by removing the Herold gland (Herold, 1815), i.e. the imaginal disc for the seminal vesicles, SAGs, common duct, and external genitalia of the adult male

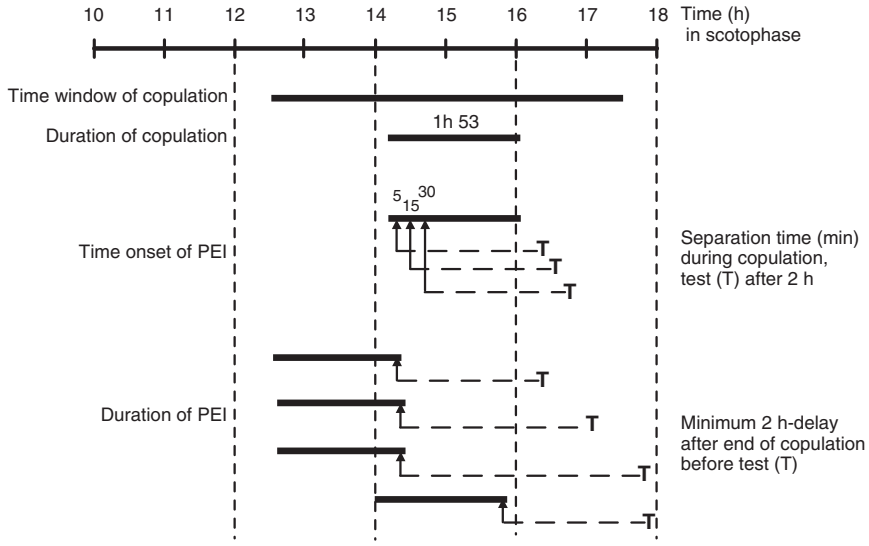


**Fig. 1.** Schematic representation of mating experiments performed for the study of the initiation and duration of the post-ejaculatory interval inducing the post-mating olfactory switch off in *A. ipsilon* males. The time window of copulation starts at 12:30 and ends at 17:30. The main duration of copulation is 1 h 53 min. Time onset of the PEI was studied by separating males and females in copula after 5, 15 and 30 min. Behavioral tests were performed 2 h after separation. Duration of the PEI was studied by testing mated males at least 2 h after the end of copulation and up to the end of the scotophase.

(Verson and Bisson, 1896) as described previously (Shirk et al., 1983). Briefly, larvae were immobilized on a dissecting pad under Ringer's saline, and a small opening was performed on the 9th abdominal sternite. The Herold gland was localized and quickly removed. The wound was dried with a tissue, and the larva was transferred to the rearing medium. Sham-operated larvae were obtained by performing an incision and gently touching the Herold gland, without extracting it.

Mortality of castrated and SAG-deprived animals did not differ from overall mortality in the rearing (ranging from 40 to 50% between larval and adult stage).

The behavioral response to sex pheromone of 5-day-old castrated and Herold gland-deprived adults was tested in wind tunnel experiments and compared to that of 5-day-old sham-operated adults. Directly after the behavioral tests, castrated and Herold gland-deprived males were checked for the effectiveness of surgery (absence of testes or SAG, respectively).

*Tissue collections, extraction, and injections*

Brains, testes, and SAGs were dissected from 5-day-old mated and virgin males in the second half of the scotophase, when this species shows the highest reproductive activity (Gadenne et al., 1993; Xiang et al., 2010). Tissues of newly-mated males were dissected out within 1 h following the end of mating. Dissections were conducted in Ringer's solution (8.76 g NaCl; 0.441 g $CaCl_2H_2O$; 0.224 g KCl; 2.29 g TES Buffer in 1 l ultrapure $H_2O$; +NaOH for pH 6.9; 8.55 g sucrose). In virgin males, the fused SAGs appear thick and strongly reddish colored, whereas SAGs of newly-mated males are thin and translucent.

For cross-injection experiments, tissues were then immediately placed in Eppendorf tubes in liquid nitrogen and stored at −80 °C. Ringer's solution was then added to the frozen tissues: 1 μL for each brain, and 2 μL for each SAG. Tissues were then allowed to thaw on ice and subsequently homogenized with a tissue homogenizer Polytron (Model P200, Fisher Scientific SAS, Illkirch, France) five times on ice for 1 s each. SAGs from virgin males were further centrifuged at 4 °C at 1500 rpm for 10 s, and the supernatant was collected for injections. For injections into the abdomen, each male received a dose of 2 brain-equivalents (2 μL of crude solution) or a dose of 1 SAG-equivalent (1 SAG represents the two fused SAGs from one male; 2 μL of supernatant/4 μL of crude solution). All injections were performed 1 h prior to the behavioral test. Both virgin and mated males were injected with brain and SAG extracts. Virgin males were injected with tissues from mated males, and with tissues from virgin males as controls. Mated males were injected with tissues of virgin males. Untreated (non injected) virgin and mated males served as controls.

For ecdysteroid quantification, fresh organs (brains, testes, SAG) were weighed on an analytical balance (Mettler H54AR, Viroflay, France) as batches of 5 to 10 organs, then stored at −18 °C in methanol (Merck, Semoy, France) (250 μL/10 organs). They were then homogenized with a Polytron (ProScientific Inc, Oxford CT, USA), centrifuged (10 min, 10,000 rpm) and the supernatant was collected. The residue was reextracted with methanol (250 μL) and the pooled supernatants were dried under vacuum in a SpeedVac Concentrator (Eppendorf, Hamburg, Germany). Haemolymphatic ecdysteroid quantifications were also performed in virgin, and in mated males as soon as copulation was terminated (0 h post-mating) and 1 h after the end of copulation (1 h post-mating). To collect haemolymph, the top of an insect's head was cut, and the whole animal was placed in a 0.2 mL tube with a hole in the bottom. This tube was then placed inside a 1.5 mL Eppendorf vial and centrifuged at 500 rpm for 10 min. Haemolymph pooled from 5 insects (10–50 μl/insect were collected) was suspended in ten volumes of methanol (Merck, France) and centrifuged at 10,000 rpm for 10 min. The supernatant was collected and dried as described above, samples were then stored at −80 °C until use.

For high-pressure liquid chromatography (HPLC) analysis, ecdysteroids were extracted from whole virgin males. First, wings were removed, and animals were cut into pieces with scissors, then ground in a glass-Teflon homogenizer (5 males in 5 mL methanol). All the extracts were stirred during 3 h then sonicated and centrifuged (15 min, 4000 rpm). The pellets were resuspended in methanol for a second extraction. All supernatants were pooled and dry-evaporated. After the methanol phase had evaporated, a partition with chloroform/water (1:2, v/v) was performed twice. The aqueous phase was purified on a Sep-Pak $C_{18}$ cartridge (according to Lafont et al., 1982): a polar fraction was first eluted with 5 mL of 30% methanol and free ecdysteroids were then eluted with 5 mL of absolute methanol. Only this fraction was further analyzed by HPLC. The same purification steps were used for a large batch of SAGs (64) from 5-day old virgin males, to analyze their ecdysteroid content by HPLC.

*HPLC analyses of ecdysteroids*

Ecdysteroids were analyzed on a Beckman apparatus (System Gold, Fullerton, CA, USA), with UV detection at 250 nm, at a flow rate of 1 mL $min^{-1}$ using two HPLC systems: a normal-phase system (NP-HPLC) using a silica column (250 mm × 4.6 mm i.d., Hypersil, Zorbax silica 5 μm, AIT Chromato, Le Mesnil Le Roi, France) and dichloromethane/propan-2-ol/water (125:30:1.5, v/v/v) as solvent; and a reverse-phase system, using a Spherisorb ODS2 column (250 mm × 4.6 mm, C18 5 μm, AIT) and methanol:water (50:50,v/v) as solvent. Fractions were collected every 42 s, evaporated until dry and resuspended in EIA buffer for ecdysteroid quantification (see below). The retention times of immunoreactive fractions were compared to the following reference ecdysteroids: 20E, ecdysone (E), and 2-deoxyecdysone (2dE). All ecdysteroids were generous gifts from Pr René Lafont (UPMC, Paris).

*Quantification of ecdysteroids*

Ecdysteroids were quantified with an enzyme immunoassay (EIA) adapted from the method described by Porcheron et al. (1989), by using goat anti-rabbit IgG (Jackson Immunoresearch Lab, West Grove, PA, USA) and 2-succinyl-20E coupled to peroxidase as enzymatic tracer. The enzymatic activity was measured using orthophenylenediamine (Sigma Aldrich, Saint-Quentin Fallavier, France) as substrate. For quantification in haemolymph, we used a polyclonal anti-20E antiserum AS4919 (Porcheron et al., 1976, 1989), which displays the same cross-reactivity towards E and 20E (Porcheron et al., 1989). For ecdysteroid quantification in other tissues, the polyclonal anti-ecdysone antiserum L2 (generous gift from Dr. M. De Reggi, Marseille) was used because of its great sensitivity (De Reggi et al., 1992). L2 displayed the highest affinity towards E (10.7 pg yielding 50% maximum binding), and recognized 20E fivefold less than E in our experimental conditions (antiserum L2 and enzymatic tracer used respectively at 1/50,000 and 1/100,000 initial dilution). Dried samples were resuspended in EIA buffer solution. In routine experiments, calibration curves were generated with 20E (ranging from 7.5 pg to 1920 pg) and results were given as 20E equivalents.

Statistical differences in ecdysteroid titers between physiological states were assessed by pairwise comparisons using either Student's t-test or a Mann–Whitney U-test, according to whether prerequisites for the use of parametrical testing were met (Sokal and Rohlf, 1995).

*20E injection treatments*

20-Hydroxyecdysone was a gift from Pr. R. Lafont (UPMC, Paris, France). Stock solutions of 20E were prepared in ethanol at a concentration of $10^{-2}$ M, then stored at −20 °C. For experiments, the stock solutions were diluted to $10^{-5}$ M in a NaCl (145 mM) solution.

Five-day-old virgin males (0.2 ± 0.02 g body weight) were anesthetized with carbon dioxide and received an injection of 2 μL $10^{-5}$ M 20E (approximately 10 ng) in the abdomen 6 h (at 9 am) or 2 h (at 1 pm) before the behavioral test. The behavioral response to sex pheromone of injected males was then tested during the same day in wind tunnel experiments in the second half of scotophase. Control experiments were performed by injection of 2 μL NaCl solution. At the concentration of $10^{-5}$ M, 20E has been previously reported to affect the responsiveness of males to sex pheromone in several species of moths (Bigot et al., 2012; Hoelscher and Barrett, 2003).

*Wind tunnel experiments*

Experiments were performed using a 2 m long wind tunnel under the same inverted photoperiod as for the mating experiments as previously described (Barrozo et al., 2010a,b; Gadenne et al., 2001). Experiments started 4 h after lights off (14:00) and lasted up to lights on (18:00). Environmental conditions during the bioassay were held constant: 22 °C, 50 ± 10% relative humidity, wind speed of 0.3 m $s^{-1}$. A cage containing a single experimental male was introduced in the wind tunnel. After 30 s during which the male adjusted to the airflow, a filter paper containing the stimulus was placed 160 cm upwind from the cage.

Pheromone stimulation was performed with an artificial pheromone blend containing (Z)-7-dodecen-1-yl acetate (Z7-12:OAc), (Z)-9-tetradecen-1-yl acetate (Z9-14:OAc), and (Z)-11-hexadecen-1-yl acetate (Z11-16:OAc) (Sigma Aldrich, Saint-Quentin Fallavier, France) at a ratio of 4:1:4 (Gemeno and Haynes, 1998; Picimbon et al., 1997). 10 ng of pheromone blend were used for all behavioral tests as this dose was shown to give the best behavioral results with sexually mature virgin males (Barrozo et al., 2010b).

The behavior of the moth was observed during 3 min, and oriented partial flight (at least 1/2 of the distance), complete flight (up to the source without landing), and landing on the pheromone source were considered as oriented response (Jarriault et al., 2009). Oriented as well as random flights were counted altogether in order to quantify the general flight activity of insects. Both untreated virgin males (which are expected to orient towards the pheromone) and treated experimental males were tested in the wind tunnel during each experimental day.

Statistical differences between groups of copulation-length manipulated and cross-injected experimental males were evaluated using a $R \times C$ test of independence by means of a G-test and applying the Williams's correction (Sokal and Rohlf, 1995). In addition, individual *post hoc* comparisons were carried out and the experimentalwise error rate was adjusted by using the Dunn-Šidák method (Sokal and Rohlf, 1995). Statistical differences between groups of NaCl- and 20E-injected males were evaluated by means of a G-test and applying the Williams's correction (Sokal and Rohlf, 1995). Statistical differences between groups of surgically treated experimental males were assessed by means of 3 × 2 $\chi^2$-tests (Sokal and Rohlf, 1995).

## Results

*Time onset of PEI*

Males were tested in the wind tunnel for their behavioral response to sex pheromone. In virgin males, the general flight activity was high (roughly 90%), and 55% of them performed an oriented response to pheromone (Fig. 2). None of the newly-mated males that were left to copulate normally (average duration 113 ± 28 min) showed an oriented response (Fig. 2, control).

Males manually separated from females during copulation were tested 2 h later. Males separated 30 min and 15 min after the onset of copulation did not show any oriented behavioral response (Fig. 2). Twenty-two percent of the males that were separated from their partners 5 min after the onset of copulation showed an oriented response, however still statistically different from the responses of virgin males ($G = 14.13$; $df = 1$; $P \leq 0.001$) (Fig. 2). The general flight activity of mated males tested after the different copulation durations was statistically different from that of virgin males ($G = 20.79$; 69.39; 56.75; 52.03; $df = 1$; $P \leq 0.001$ between virgin and durations of 5 min; 15 min; 30 min; and control copulation, respectively) (Fig. 2). However, general flight activity was significantly higher in males that were mated for 5 min as compared with males mated for 15 min ($G = 15.05$; $df = 1$; $P \leq 0.001$), 30 min ($G = 8.28$; $df = 1$; $P \leq 0.01$), and control males ($G = 7.2$; $df = 1$; $P \leq 0.01$), but still differed from that of virgin males ($G = 20.79$, $df = 1$; $P \leq 0.001$) (Fig. 2).

Altogether these results show that the initiation of olfactory switch-off occurs a few minutes after the onset of copulation.

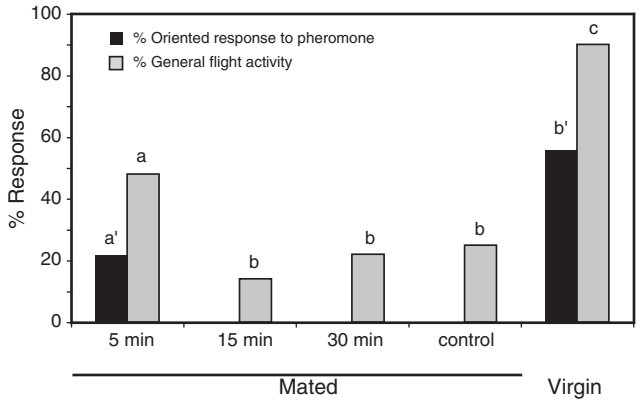


**Fig. 2.** Effects of mating duration on pheromone response in *A. ipsilon* males. Day-5 males were allowed to copulate with day-3 mature females, and then forced apart from the females at different times (5, 15, 30 min) after the onset of copulation (n = 50, 50, and 56 males respectively). They were then tested for their response to sex pheromone in a wind tunnel 2 h later. A control group of males (n = 50) was left to copulate without separation (mean copulation time: 113 min), and they were tested 1 h after the end of copulation (control). Virgin males (n = 66) were also tested in the wind tunnel. Bars with the same letters are not statistically different (G-test, $P \leq 0.05$).

*Duration of PEI*

Not a single mated male out of 31 tested at least 2 h after the end of copulation responded to the pheromone, independently of the delay between the end of copulation and the behavioral test.

This result shows that the PEI lasts at least the whole scotophase, i.e. the period of activity, after copulation.

*Effects of tissue cross-injection on pheromone response of virgin and mated males*

Tissues of virgin and mated males were injected into mated and virgin experimental males respectively, and their response to an artificial pheromone blend was quantified in a wind tunnel (Fig. 3). A general $R \times C$ log likelihood test of independence (G-test) revealed significant differences between groups (Gadj = 232.26, df = 7, $P \leq 0.001$). More precisely, control mated males did not show any oriented responses as compared with control virgin males (Gadj = 63.17, df = 1, $P \leq 0.001$) (Fig. 3). Also, compared with virgin control males, mated males did not respond to pheromone after the injection of brain (M + vBr) (Gadj = 72.62, df = 1, $P \leq 0.001$) or SAG (M + vSAG) (Gadj = 64.61, df = 1, $P \leq 0.001$) extracts from virgin males (Fig. 3).

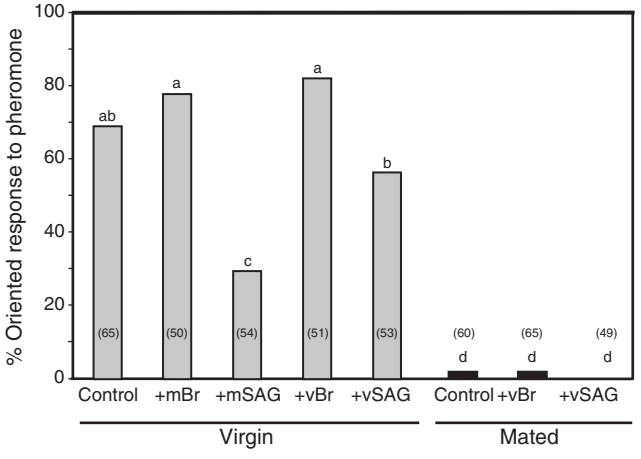


**Fig. 3.** Effects of brain/SAGs injections on the behavioral response of *A. ipsilon* male moths to sex pheromone. Brains (Br) and sex accessory glands (SAGs) from virgin (vBr/vSAG) and mated males (mBr/mSAG) were injected into mated and virgin males respectively. vBr and vSAG were also injected into virgin males as controls. See Materials and methods section for details. Number in brackets represent the number of tested males. Grey bars: virgin males, black bars: mated males. Bars with the same letters do not differ statistically (G-test, $P \leq 0.05$).

Responses of virgin males injected with brain extracts from virgin males (V + vBr) or mated males (V + mBr) did not differ from that of control virgin males (Gadj ≤ 2.41, df = 1, n.s.) (Fig. 3). The response of virgin males injected with SAG extracts from virgin males (V + vSAG) was not different from that of control virgin males (Gadj = 1.93, df = 1, n.s.) (Fig. 3). However, the response of virgin males injected with SAG extracts of mated males (V + mSAG) was significantly lower than that of virgin control males (Gadj = 18.14, df = 1, $P \leq 0.001$) (Fig. 3).

These results show that the behavioral pheromone response of mated males could not be restored by any of the performed tissue injections. On the contrary, the response of virgin males injected with SAG extracts from mated, but not virgin, males was significantly reduced.

*Effects of mating on ecdysteroid levels in brain and reproductive tract*

In brains of both virgin and mated males, ecdysteroids were below the EIA detection levels. In testes, ecdysteroid levels were very low in virgin 5-day old males (10.4 ± 0.8 ng 20E equivalents/organ) and below the EIA detection limit in newly-mated males (Fig. 4A). In SAGs of virgin males, ecdysteroid levels were significantly higher per animal (103 ± 37 ng 20E equivalents) than in SAGs of post-mated males (8.8 ± 0.9 ng/animal) (Mann–Whitney U-test; df = 20, P = 0.00021) (Fig. 4B).

*Effects of mating on haemolymph ecdysteroid levels*

Ecdysteroid levels were quantified in the haemolymph of virgin and mated males, 0 h and 1 h after mating (Fig. 5). Compared with virgin males, ecdysteroid titers were significantly higher in newly-mated males at 0 h (t-test; df = 21, P = 0.038) and 1 h (t-test; df = 27, P = 0.026) after the end of copulation (Fig. 5).

*Identification of ecdysteroids in A. ipsilon males*

Using HPLC separation of ecdysteroids followed by EIA, we analyzed the nature of ecdysteroids present in whole virgin 5-day old males and their SAGs. In NP-HPLC, the major immunoreactive peak in both samples co-migrated with reference 20E (Figs. 6A and B). Two other immunoreactive smaller peaks were detected in whole animals, co-migrating with 2dE and E, respectively (Fig. 6A). The fractions corresponding to each of the three ecdysteroid peaks from whole animals were pooled, purified and further separately analyzed

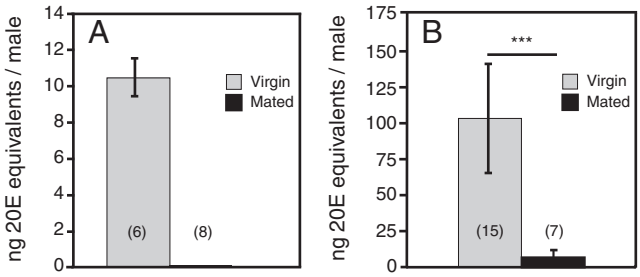


**Fig. 4.** Ecdysteroid titers in testes (A) and sex accessory glands (SAGs) (B) as a function of mating status in *A. ipsilon* males. Quantification by EIA, expressed as ng 20E equivalents per male (mean ± SD). Ecdysteroid titers were under detection limits in testes from mated males (A). Numbers in brackets represent the number of repetitions of 10 organs. Asterisks indicate a statistical difference between groups (Mann–Whitney U-test, $P \leq 0.05$).

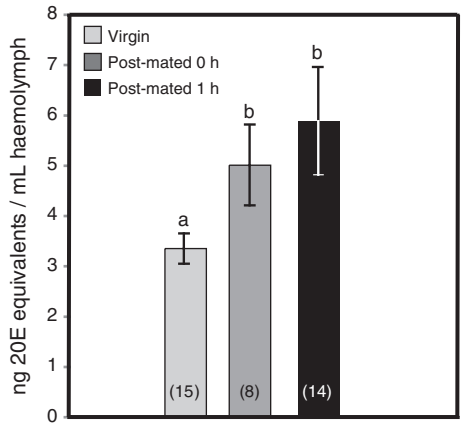


**Fig. 5.** Ecdysteroid titers in the haemolymph as a function of mating status in *A. ipsilon* males. Ecdysteroids were quantified by EIA, and titers expressed as ng 20E equivalents per mL haemolymph. Ecdysteroid levels are lowest in virgin males (grey bar), and increased in males straight after (0 h) or 1 hour (1 h) after the end of copulation. Bars (mean ± SD) with the same letters are not statistically different (t-test, $P \leq 0.05$). Numbers in brackets represent the number of repetitions of 5 pooled males.

with a RP-HPLC system followed by EIA. Each peak gave rise to an immunoreactive compound co-migrating with the same ecdysteroid as observed in NP-HPLC (data not shown). This confirms the presence of 20E, 2dE and E in *A. ipsilon* males. The same ecdysteroids were also detected in SAGs, with 20E being predominant (Fig. 6B).

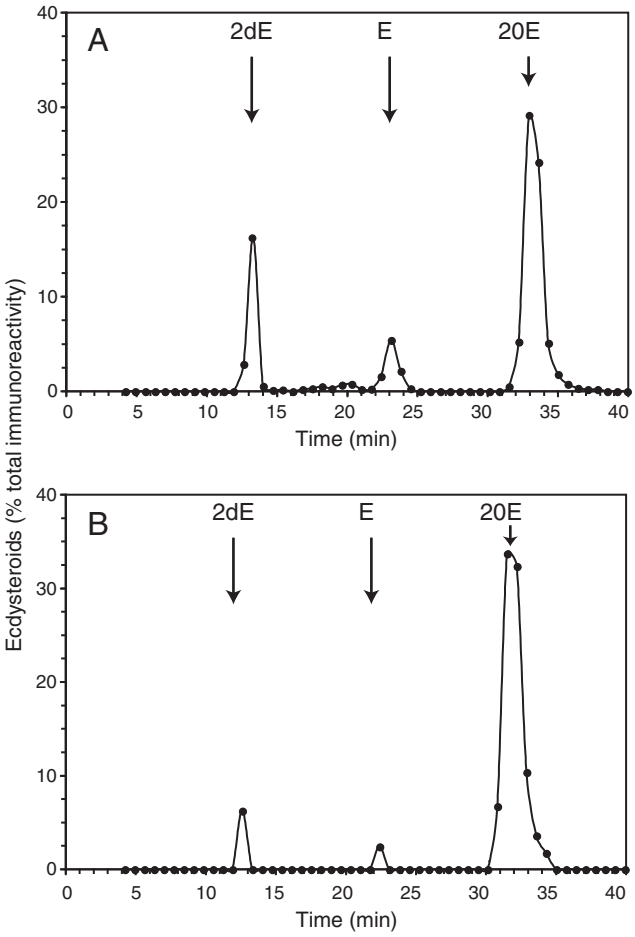


**Fig. 6.** Normal-phase HPLC-EIA analysis of free ecdysteroids in 5-day old virgin males of *A. ipsilon*. (A) Whole animals, (B) SAGs. HPLC conditions : Silica column Zorbax Sil; solvent, dichloromethane/propan-2-ol/water (125:30:1.5, v/v/v); flow rate of 1 mL $min^{-1}$. Arrows indicate retention times of ecdysteroid standards: 20E, 20-hydroxyecdysone; E, ecdysone; 2dE, 2-deoxyecdysone. Ecdysteroids were quantified by EIA in 0.7-min fractions as 20E equivalents, and are expressed as percentage of total immunoreactivity.

*Effects of 20E injection on pheromone response in sexually mature virgin males*

There was no statistical difference in the oriented responses between 20E- and NaCl-injected 5-day-old virgin males to sex pheromone when injections were performed 6 h ($G = 0.040$, $df = 1$, $P = 0.841$) or 2 h ($G = 0$; $df = 1$; $P = 1$) before the behavioral test (Fig. 7).

*Effects of testes or SAG deprivation on pheromone response*

We analyzed the behavioral response of virgin males (responding to the pheromone) lacking testes or SAGs. There was no statistical difference in the proportion of testes-deprived males ($n = 34$), sham-operated ($n = 36$), and control males ($n = 73$) responding to an artificial sex pheromone blend ($\chi^2 = 3.96$, $df = 2$, $P = 0.13$) (Fig. 8A).

Similarly, there was no statistical difference in the proportion of SAG-deprived males ($n = 47$), sham-operated ($n = 26$), and control males ($n = 77$) responding to an artificial sex pheromone blend ($\chi^2 = 1.01$, $df = 2$, $P = 0.602$) (Fig. 8B).

## Discussion

Although the post-mating effect on female receptivity has been studied in detail in many insect species including moths (review in Gillott, 2003), very little is known on the effects of mating on the sexual inhibition in male insects. Since a few years, we have focused our studies on the PEI, characterized by a fast and transient inhibition of pheromone responses, in *A. ipsilon* males. Results of the present study allow us to extend our knowledge on i) the timing of this PEI, ii) the tissues involved in the PEI, and iii) the possible role of ecdysteroids.

*The inhibition of the pheromone response starts very early after the onset of copulation and lasts up to the end of the scotophase*

When copulating males were forced apart from the females only 15 min after the onset of copulation, we found that their olfactory system was already switched off (no response to pheromone in the wind tunnel). Post-hoc dissection of females showed an absence of spermatophore in their reproductive tracts. Only a whitish milky

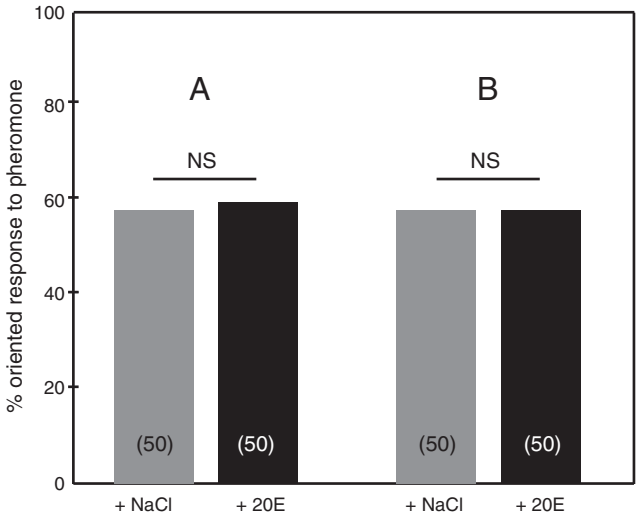


**Fig. 7.** Effects of 20E injections on pheromone responses in virgin *A. ipsilon* males. 20E or NaCl was injected in males 6 h (A) or 2 h (B) before the behavioral test. Numbers in brackets represent the number of tested males. G-test, $P \leq 0.05$. See Materials and methods section for details.

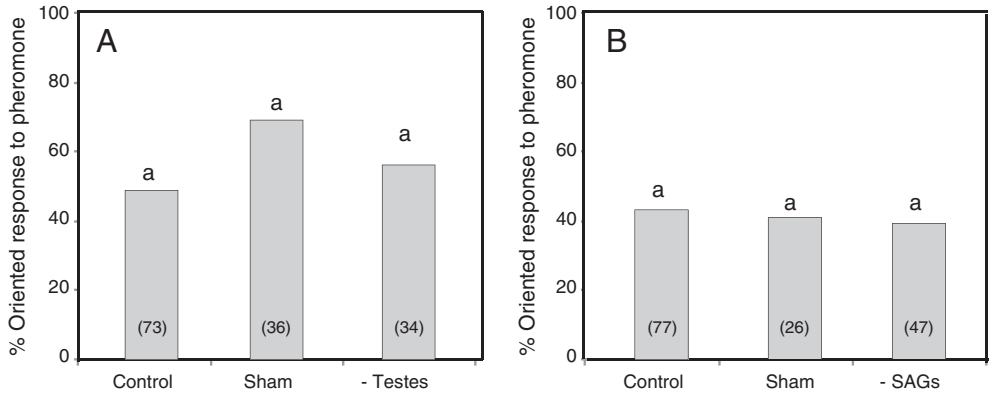


**Fig. 8.** Effects of testes (A) and sex accessory gland (SAGs) deprivation (B) on pheromone responses in *A. ipsilon* males. For each experiment, bars with the same letters are not statistically different ($\chi^2$ test, $P \leq 0.05$). Numbers in brackets represent the number of tested males. See Materials and methods section for details.

liquid was seen in the tracts. Similarly, the absence of a spermatophore was observed in females of *Heliothis zea* (Raina, 1989) and *Spodoptera litura* (Seth et al., 2002), when males were separated after a few minutes of copulation (5–15 min). In contrast to the lack of response after 15–30 min copulation, males that were forced apart from females after 5 min of copulation still showed a positive behavioral response to sex pheromone. When we performed detailed observations of copulation behavior, we sometimes noticed copulation attempts that were not successful: males and females went apart after few seconds or minutes of copulation. The very same males successfully mated afterwards during a second attempt. We hypothesize that the olfactory switch off in *A. ipsilon* males takes place only after the copulation has been secured (males and females are really in copula), i.e. when the formation of a spermatophore is engaged. This does not seem to be the case in *Nasonia* wasps, in which the post-mating behavioral switch in females has been shown to be independent of any genital contact (Ruther et al., 2010). However, the observed delay of the PEI in *A. ipsilon* would allow males which have failed to start a true copulation to be still responsive to another female for a possible new copulation attempt.

Although a few studies have shown a post-mating decrease of sexual responsiveness in males of insect species (Fischer and King, 2008; Lachmann, 2000; Norville et al., 2010; Reddy and Guerrero, 2000; Sakai et al., 1995; Ureshi and Sakai, 2001), information on the latency of this PEI is scarce.

In contrast, the delay of the post-mating decrease in sexual receptivity has been described in insect females. In the parasitic wasp *Nasonia vitripennis*, mated females cease to respond to the male pheromone as soon as 5 min after the end of copulation (Ruther et al., 2007). In *Ceratitis capitata*, mated females start to respond to fruit odor and stop to respond to the male pheromone, but no information on the latency is given (Jang, 1995).

In *A. ipsilon* the male PEI lasts up to the end of the scotophase: no behavioral response was observed between the end of copulation and the end of the scotophase. We previously showed that males were unable to remate within the same scotophase (Gadenne et al., 2001). Altogether these results show that as soon as the male has engaged in copulation (i.e., presumably started formation of a spermatophore) and up to the end of scotophase, its pheromone-specific olfactory system is switched off.

### *The inhibition of pheromone response is induced by a putative factor produced in the SAGs after or during copulation*

The behavioral response of virgin males to pheromone is decreased after injection of SAGs of mated donor males. Contrarily, the response of mated males could not be restored by injection of SAG extracts of virgin donor males. This could be due to a factor produced in the SAGs, inducing the inhibition of pheromone response observed in mated males. Surprisingly, although we assumed that this SAG factor would act at the central nervous level to induce the PEI, injection of brain extracts from mated males had no effect on virgin males. However we cannot exclude the possibility that the quantity of brain extract injected (2-brain equivalent) was not sufficient to elicit an effect in virgin males.

Our data show that surgical deprivation of virgin males from either their testes or SAGs did not reduce their responsiveness towards sex pheromone. Moreover, we show that soon after onset of copulation, males presumably engaged in spermatophore production ceased to respond to sex pheromone. This lead us to hypothesize that a factor is newly or over-produced in SAGs after mating (rather than decreased) to induce the PEI. Unfortunately we could not confirm the role of this SAG factor in the induction of the PEI by testing their behavioral response after mating, because SAG-deprived males cannot copulate, as they lack the external genitalia.

It was found in Drosophila and moths that SAGs contain a peptide, named sex peptide, which is transferred into the female during copulation, switching off its pheromone production (reviewed in Kubli, 2003). Recently this peptide has been shown to act in females at the central nervous level through its receptors in order to induce the cascade of events leading to the switch off of pheromone production (Rezával et al., 2012).

Although we have not yet searched for its existence and possible role in this moth, previous data showed that haemolymph titers of sex peptide were similar in virgin and mated males of *H. zea* (Raina, 1989), thus reducing the probability that this peptide could indeed act in the male itself and induce the PEI.

Other factors such as ecdysteroids (*D. melanogaster*: Harshman et al., 1999; *Anopheles gambiae*: Pondeville et al., 2008) and JH (*Heliothis virescens*: Park et al., 1998; *Apriona germari*: Tian et al., 2010) are also produced in male SAGs and transferred to females during copulation. JH is probably not involved in the PEI, as we previously showed that levels of JH biosynthesis did not change in males following mating (Duportets et al., 1998).

### *20E is present in SAGs but probably not involved in the induction of the pheromone-specific post-mating inhibition in A. ipsilon males*

Our analysis of ecdysteroids in *A. ipsilon* revealed 20E as the major form. No ecdysteroids were detected in brains of virgin males. This is not surprising, as brains of most insect species appear to be incapable of ecdysteroid synthesis (Warren et al., 1999). However, brains from honeybee workers cultured *in vitro* could release high amounts of 20E, but did not store it (Yamazaki et al., 2011). In vitro incubation

of brains from *A. ipsilon* males did not allow us to detect any ecdysteroid in the culture medium (data not shown). Presence of 20E has been described in adult testes of different insect species (reviewed in Brown et al., 2009). However, in *A. ipsilon* testes, ecdysteroid levels were low in virgin males and not detectable in post-mated males. They were 10 times higher in SAGs of virgin males. These organs have been shown as a site of ecdysteroid secretion in *Blattella germanica* (Gillott and Ismail, 1995) and *A. gambiae* (Pondeville et al., 2008). Levels of 20E in SAGs were significantly lower in post-mated males than in virgin males. In mosquito males, SAG levels of 20E also decrease after mating, and it was shown that 20E is transferred during mating to the female (Pondeville et al., 2008). This could also be the case in *A. ipsilon*. Haemolymph titers of 20E increased significantly after mating, suggesting that copulation could induce a shift of the hormone from the SAGs into the haemolymph. However, the post-mating 20E increase in the haemolymph could also originate from other tissues that we have not identified so far. More generally, there is a lack of knowledge in the endocrinology of reproduction in male moths (De Loof, 2006).

Given that injection of mated male SAGs containing low levels of 20E modifies the behavior of virgin males, it is unlikely that 20E alone induces the observed post-mating inhibition. Moreover, the injections of 20E in *A. ipsilon* virgin males performed at two different times before the test did not induce the expected decrease in their behavioral response, in opposition to recent results in *S. littoralis* (Bigot et al., 2012). Although the quantity of 20E injected in virgin males (10 ng) was higher than that in the haemolymph (3–6 ng/mL haemolymph: approximately 0.25 ng/male), we cannot exclude the possibility that low levels of another form of ecdysteroids are involved in the PEI of *A. ipsilon*.

Work is now in progress to identify the putative factor(s) produced/accumulated in the SAGs during mating, which could elicit the very fast olfactory switch-off at the central nervous level. Transcriptomic and peptidomic comparative profiles of both SAGs and brains from mated and virgin males might elucidate the modulators at the origin of the switch off.

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