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PHYSIOLOGY

RNA interference reveals allatotropin functioning in larvae and adults of *Spodoptera frugiperda* (Lepidoptera, Noctuidae)

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Abstract

The allatotropin of *S. frugiperda* (Spofr-AT) and its cDNA sequence were characterized 10 years ago, but no functional analyses of the peptide are available. Here we used the RNA interference technique to study the effects of *Spofr-AT* gene suppression on juvenile hormone (JH) and ecdysteroid titers in the hemolymph of larvae, virgin and mated females, and of males. *Spofr-AT* gene silencing in last instar larvae resulted in an increase in the amount of JH III and 20-hydroxyecdysone in the hemolymph of the animals, corresponding to an acceleration of the prepupal commitment and transformation to the pupa. Mated females showed much higher JH titers in their hemolymph than virgins and laid almost twice the number of eggs. *Spofr-AT* gene silencing in freshly ecdysed females led to a further increase in egg production and oviposition, but had only a minor effect on the hemoylmph JH titer. Mated females contain considerable amounts of JH I and JH II in their hemoylmph, which are thought to be received from males during copulation. To confirm this hypothesis, we measured the amount of JH homologs in the male accessory reproductive glands (MARG) before mating and in the bursa copulatrix (BC) of the female after mating. MARG contained high amounts of JH I and JH II, which are transferred to the BC during copulation. One day after mating, JH disappeared from the BC and was then found in the hemolymph of the females. In conclusion, Spofr-AT acts as a true allatotropin in larvae and adults of both sexes of the armyworm.

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Contributions: IH, MMV and KH planned the study. IH performed the RNAi experiments and measured the JH and ecdysteroid titers. MMV supervised the PhD work of IH. IH and KH wrote the manuscript.

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Introduction

In insects, the sesquiterpenoid juvenile hormone (JH) regulates both larval development and adult reproduction (Riddiford, 2012). Its presence during larval molting prevents metamorphosis. In the last larval stage, the JH titer declines and adult development is initiated by ecdysteroids or molting hormones in the absence of JH (Hiruma, 2003). JH reappears in the adult to regulate female reproductive maturation (Jindra *et al.*, 2013). The concentration of JH in the hemolymph is regulated by its biosynthesis in the corpora allata (CA), but also by its degradation, sequestration, and excretion. Synthesis is thought to be the most important among these regulated processes (Gilbert *et al.*, 2000).

The biosynthesis of JH in the CA can be either stimulated or inhibited by neuropeptides termed allatotropin (AT) and allatostatin (AS) (Stay, 2000). Three families of structurally unrelated AS have been isolated and characterized from various insect orders: the type A AS (FGLamides), type B AS [W(X_6)Wamides], and type C AS (*Manduxa sexta* type). Allatostatins are pleiotropic in function. Besides their allatoregulating activity, AS modulate the contraction of visceral muscles (Hoffmann *et al.*, 1999; Weaver & Audsley, 2009), inhibit ovarian ecdysteroid biosynthesis (Lorenz *et al.*, 1997) and the synthesis of vitellogenins (Martin *et al.*, 1996), or stimulate midgut enzyme activity (McNeil & Tobe, 2001). An allatotropin was first structurally characterized from *M. sexta* (Kataoka *et al.*, 1989), and a structurally related AT in dipterans (Li *et al.*, 2003). In addition to their stimulating effect of JH biosynthesis, AT is a cardioacceleratory peptide (Veenstra *et al.*, 1994).

The fall armyworm, *S. frugiperda*, is an agricultural important pest species. In the polyandric moth, oogenesis is completed only after adult emergence and stimulation of egg production as well as oviposition are strictly dependent on mating associated with an elevated JH titer (Ramaswamy *et al.*, 1997). Since we hope that pest control strategies based on disruption of the insect endocrine system will aid in finding new, promising substances that can be used in agricultural biocontrol (Hoffmann & Lorenz, 1998; Cusson & Palli, 2000), we began to study the endocrine control of development and reproduction in *S. frugiperda* about 10 years ago. Investigations on the regulation of vitellogenesis in adult females demonstrated a crucial role of 20-hydroxy-

ecdysone in the induction of vitellogenin (Vg) synthesis, while JH seemed to be essential for a continued uptake of Vg by the developing oocytes (Sorge *et al.*, 2000). From methanolic brain extracts of adult females we purified a peptide, which caused an up to sevenfold increase in JH biosynthesis and was identified as *M. sexta* allatotropin (Oeh *et al.*, 2000). Repeated injections of Manse-AT (but not Manse-AS) into larvae and adults of *S. frugiperda* retarded their development and reduced fecundity (Oeh *et al.*, 2001). The structure of the formerly identified Manse-AT and the existence of a C-type allatostatin (Manse-AS) in this species was verified by cloning the cDNA, which encodes the precursors of Manse/Spofr-AT and Manse/Spofr-AS, respectively (Abdel-latief *et al.*, 2003). The *Spofr-AT* gene is expressed in three mRNA isoforms, differing from each other by alternative splicing. The three mRNA of the *Spofr-AT* gene and the *Spofr-AS* gene are expressed in the brains of last instar larvae, pupae, and adults of both sexes with variable intensity (Abdel-latief *et al.*, 2004b).

Functional analysis of the *Spofr-AS C-type* gene by RNA interference confirmed an allatostatic activity for this peptide (Griebler *et al.*, 2008). Besides Spofr-AS (C-type AS), a gene encoding Spofr AS type A was isolated from brain cDNA containing nine FGLamides (Abdel-latief *et al.*, 2004c). The gene is expressed in the brain and midgut of larvae and adults in a time- and tissue-specific manner, but also in the reproductive tissues of adult females and males. Functional analysis of the *Spofr-As type A* gene by RNAi revealed allatostatic activity also for this type of peptides (Meyering-Vos *et al.*, 2006). A cDNA encoding a novel *S. frugiperda* preprohormone was isolated and cloned by Abdel-latief *et al.* (2004a) and contained a decapeptide sequence RVRGNPISCF-OH with a C-terminus identical with the Spofr-AS (type C AS), but the peptide strongly stimulated the synthesis and release of JH *in vitro* by the CA. The peptide was termed Spofr-AT2. The preprohormone was expressed in the brain, midgut, and ovary in a tissue- and developmental-specific manner (Abdel-latief *et al.*, 2004a). Injection of Spofr-AT2 dsRNA into penultimate instar larvae resulted in an increased JH titer in last instar larvae and the last larval stage was prolonged. In adult females, *Spofr-AT2* gene silencing clearly reduced the JH titer in the hemolymph and the females produced significantly less eggs than Ringer injected controls (Griebler *et al.*, 2008).

Molecular functional analyses with the genuine lepidopteran AT, Spofr-AT/Manse-AT, in larvae and adults of *S. frugiperda* are at this time not available. To address this issue, we used the RNAi technique and designed a dsRNA that selectively knocked down the *Spofr-AT* gene. We conducted *Spofr-AT* gene expression studies using real-time PCR to evaluate the RNAi efficiency in specific tissues of *S. frugiperda*. Then we analyzed the effect of *Spofr-AT* gene suppression on the JH and ecdysteroid titers in the hemolymph of larvae, virgin and mated adult females, and of males at different age. Finally, we investigated how *Spofr-AT* gene suppression in females and males may interfere with the male to female JH transport during copulation and with the fertility of the females.

Materials and Methods

Insect rearing

Pupae and eggs of *S. frugiperda* were provided by Bayer CropScience (Bayer, Monheim, Germany) and reared at 27°C and 70% rh under a L16: D8 photoperiod as described (Oeh *et al.*, 2000). Under these conditions, the 5th (penultimate) larval stage lasted 3 days and the 6th (last) larval stage 3 to 4 days, followed by a period of 48 hours prepupation. Adults emerged after a pupation period of 9 to 10 days and females laid their first eggs 45 to 48 hours after emergence. Copulation occurred every 24 hours. Eggs were collected on each day of the adult life. During the last larval stage, physiological parameters (cession of feeding and wandering, loss of mobility, body size/weight change and color change) were used as developmental markers to determine the beginning of the prepupal phase.

dsRNA synthesis

A PCR method was used to generate the template for the dsRNA synthesis corresponding to 398-621 nucleotides (nt) of the Spofr-AT isoform A sequence (Abdel-latief *et al.*, 2003). T7 promotor sites were added to the specific primers T7ATF7 forward primer (5'-TAA TAC GAC TCA CTA TAG G↓GC TTC AAG GTC GAC ATG ATG ACC-3') and T7ATr9 reverse primer (5'-TAA TAC GAC TCA CTA TAG G↓GC GAC CAC AGA TCG CGC GAA TTC-3') for amplification of the AT fragment. The PCR program used to yield dsDNA was 95°C for 5 min, then 94°C for 30 s, 10 cycles of 68°C for 45 s, 68°C for 60 s, 94°C for 30 s, followed by 45 cycles of 60°C for 45 s and 68°C for 90 s; finally a 10 min extension step at 68°C was performed. Generation of the dsRNA was carried out with the T7 MEGAscript RNAi kit (Ambion, Huntington, UK), including a DNase digestion step and a LiCl precipitation. A final denaturation for 5 min at 95°C and an annealing step at room temperature followed. Quantification of the dsRNA was done spectrophotometrically. For control experiments with unspecific dsRNA in the moth, a transcript was produced derived from the sulfakinin sequence of a cricket, *Gryllus bimaculatus* (Meyering-Vos & Müller, 2007; Griebler *et al.*, 2008).

Injections

1.5 µg dsRNA in 2 µL noctuid Ringer (Davenport & Wright, 1985) were injected once with a 10 µL Hamilton syringe (Hamilton AG, Bonaduz, CH) into the third segment of the ventral abdomen of adult moths shortly after adult emergence. Penultimate and last instar larvae were injected directly after molting into the base of the last pseudopodium.

Hemolymph and tissue collection

Hemolymph was collected from adult moths through the intersegmental membranes with a 20 µL micropipette (Blaubrand® intra Mark Brand, Wertheim, Germany). Since adult moths produce only small amounts of hemolymph, hemolymph from 4 to 5 animals had to be combined to get a final volume of 20 µL. Larvae were cut at the pseudopods and 20 µL hemolymph could be collected from single animal. The JH was extracted as described (Westerlund & Hoffmann, 2004).

Moths were dissected under a binocular microscope, and covered with cricket Ringer saline (Lorenz *et al.*, 1997). Brains, ovaries, male accessory glands (AG; MARG), and the bursa copulatrix (BC) from females were isolated and freed from adhering tissue. Organs were transferred into a mixture of 200 µL methanol/isooctane (1:1; v/v) and carefully grinded with a glass homogenizer (0.5 mL; Motor cordless Kontes, Vineland, USA) for 90 s, vortexed and incubated at room temperature for 20 min. The extracts were stored at –70°C until use.

RNA extraction and real-time PCR

Pooled tissue material of 40 adult brains or 20 ovaries was homogenized with a tissue homogenizer (Kontes, Vineland, USA). Total RNA was extracted from 50 mg of each homogenized tissue utilizing PeqGold RNAPure® kit (peQlab, Erlangen, Germany) according to the manufacturer's instruction. To eliminate potential genomic DNA contamination, the DNase I (Fermentas, St. Leon-Rot, Germany) was used to digest the DNA. The RNA was quantified and the quality verified by spectrophotometrical detection.

500 ng of the extracted total RNA was reverse-transcribed primed by random hexamers in a final reaction mixture volume of 10 µL as described in the protocol of the high-capacity cDNA Reverse Transcription kit (Applied Biosystems, Warrington, USA) (Griebler *et al.*, 2008). Real-time PCR reactions were performed in triplicate each in a 20 µL reaction mixture following the manufacturer's instructions for the Power SYBR® green PCR MasterMix (Applied Biosystems) on

an ABI Prism 7300 sequence detection system (Applied Biosystems) as described (Griebler *et al.*, 2008). Rates of gene expression are shown both by absolute (ß-actin as reference gene) and relative quantification (Livak & Schmittgen, 2001).

Juvenile hormones and ecdysteroid titers

Concentrations of juvenile hormone homologs JH I, JH II, and JH III, and free ecdysteroids (ecdysone, E; 20-hydroxyecdysone, 20E) in hemolymph and tissues were quantified by the LC-MS method as described earlier (Westerlund & Hoffmann, 2004).

Statistics

Statistical analysis was performed using the Student's t-test or the Mann-Whitney U-test. A level of $P<0.05$ was considered as significant.

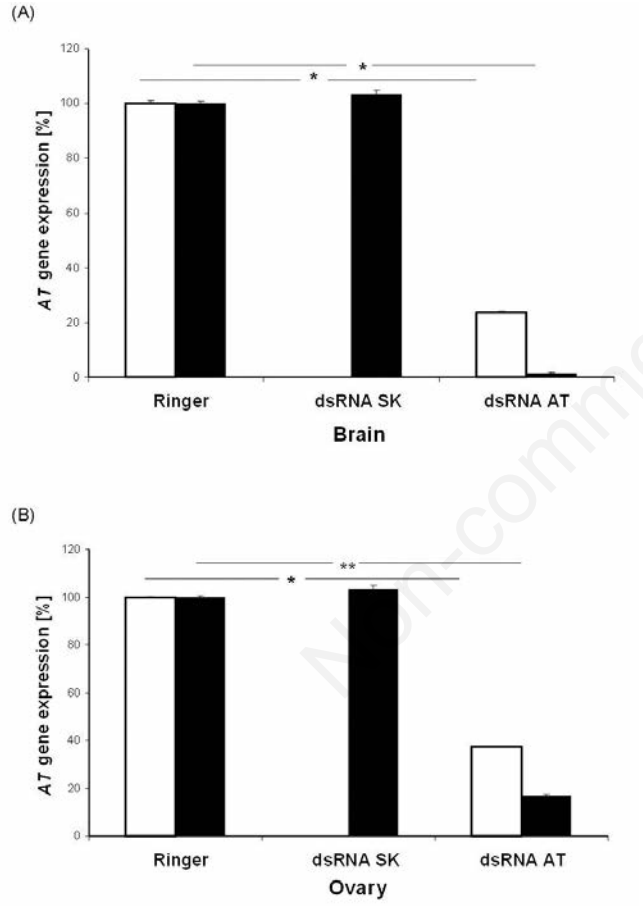

Figure 1. Expression of the *Spofr-AT* gene after RNAi silencing. Relative transcript levels of the *Spofr-AT* gene in the brain (A) and ovary (B) of 2 day old adult females were determined by reverse transcriptase real-time PCR. Newly emerged females were injected with 2 µL noctuid Ringer or 1.5 µg Spofr-AT dsRNA. For control of unspecific effects of the dsRNA injection, females were injected with dsRNA derived from the *sulfakinin (SK)* gene of *Gryllus bimaculatus*. Quantities of transcripts were determined either by absolute (black columns) or by relative quantification method (white columns) (see Materials and methods). Ringer injected controls were set to 100%. Means ±SEM; n=2; PCR was run in triplicate; *P<0.05; **P<0.01.

Results

Efficiency of RNA interference

The accumulated mortality (14 days) of *S. frugiperda* caterpillars and moths after a single injection of 1.5 µg Spofr-AT dsRNA was less than 20% and did not differ from that for untreated or Ringer injected animals (data not shown). A single injection of 1.5 µg Spofr-AT dsRNA into freshly ecdysed female moths resulted in a significant reduction of *AT* gene expression in the brain (Figure 1A) and ovaries (Figure 1B) 48 h later. The specifity of the knockdown was proven by injection of 1.5 µg sulfakinin dsRNA from the cricket, *G. bimaculatus*, which had no effect on the Spofr-AT transcript level. Efficiency and specificity of gene knockdown in larvae and male adults has been demonstrated earlier (Meyering-Vos *et al.*, 2006; Griebler *et al.*, 2008; Terenius *et al.*, 2011, unpublished data).

Effects of *Spofr-AT* gene knockdown on larval-adult development

Injection of 1.5 µg Spofr-AT dsRNA into penultimate instar larvae on the day of molting (L5/1) did not affect the body weight increase of the larvae nor the time of last larval molting and pupation (Figure 2). Caterpillars generally contained low amounts of JH I and JH II, but significantly higher concentrations of JH III in their hemolymph. Injection of Spofr-AT ds RNA on the first day of the penultimate larval stage did not affect the JH titer of the caterpillars compared to Ringer injected controls (Figure 3A). Titers of free ecdysteroids in the hemolymph were generally low in penultimate and last instar larvae, but with a small increase in 20E at the time of the last larval molt and a drastic increase during pupation (Figure 4B). Spofr-AT treatment of L5/1 larvae did not affect the titer of free ecdysteroids in the hemolymph of the caterpillars until L6/3 (Figure 3B).

A single injection of 1.5 µg Spofr-AT dsRNA into L6 larvae at the day of molting (L6/1) resulted in an acceleration of the prepupal commitment and the transformation into the pupa of about 24 hours (Figure 5A, B) when compared with Ringer injected controls. The duration of the pupal stage (average of 9 days in females and 10 days in males) and the percentage of emerging adults were not affected by the Spofr-AT

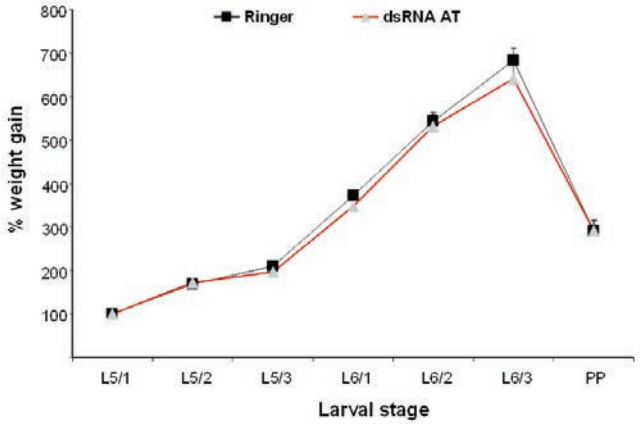

Figure 2. Effect of *Spofr-AT* gene silencing on development of penultimate and last instar larvae. Freshly molted penultimate instar larvae (L5/1) were injected with 2 µl noctuid Ringer or 1.5 µg Spofr-AT dsRNA. The body weight of the larvae was determined daily with a microbalance and the weight of the freshly ecdysed L5 larvae was set to 100% (97.2±6.0 mg). Means±SEM; n=12. No significant differences between treatments.

injection (not shown). Adults usually emerge during the early scotophase. dsRNA injection, but also Ringer injection, shifted the maximum of emerging females and males to the late photophase (not shown). Injection of Spofr-AT dsRNA into day 1 last instar larvae (L6/1) led to a significant increase in JH III at the end of the last larval stage (L6/4), but not in the prepupa (PP1) (Figure 4A), and to a still higher increase in the concentration of free ecdysteroids in the hemolymph of the wandering larvae and the prepupa (Figure 4B). The decrease of JH I at L6/4 occurred at a very low absolute level.

Effects of *Spofr-AT* gene knockdown in adult females on egg production and oviposition

In adult moths the first mating occurred 24 to 25 hours after emergence, and another 21 to 24 hours later females laid the first eggs. The time of first mating and oviposition were independent of a dsRNA treatment of freshly ecdysed females (not shown). Virgin females also started oviposition on day 2 after emergence, but the percentage of females ovipositing on that day was lower than in mated females. Moreover, virgin females laid a much lower total number of eggs than mated females

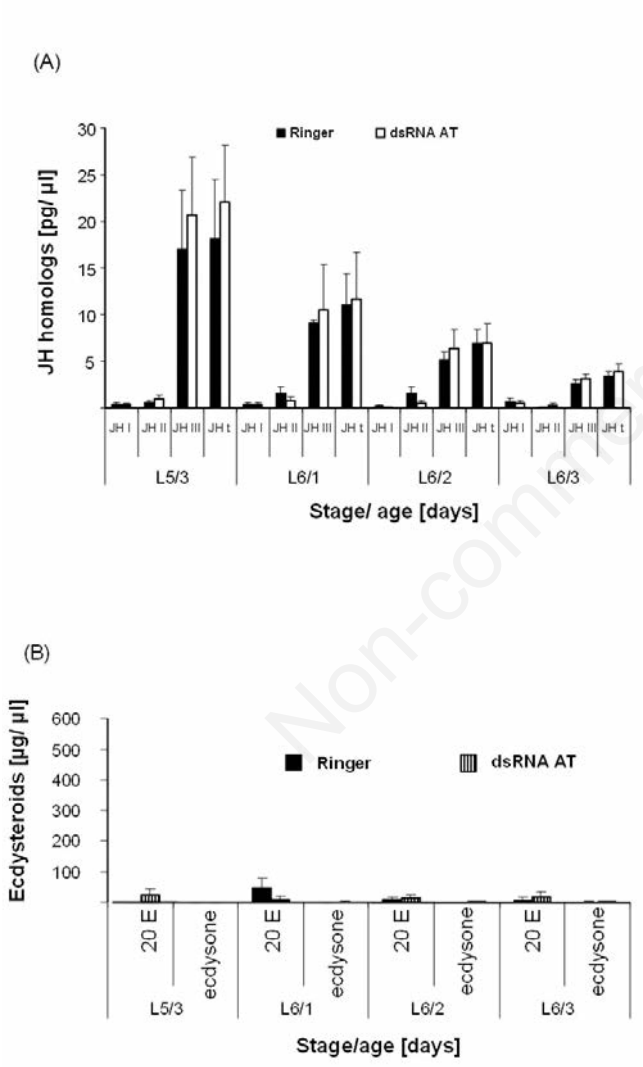

Figure 3. Effect of *Spofr-AT* gene silencing on juvenile hormone (JH I to JH III, total [t] JH) and ecdysteroid titers (ecdysone; 20-hydroxyecdysone, 20E) in the hemolymph of penultimate (L5) and last instar larvae (L6). Freshly molted L5 larvae (L5/1) were injected with either 2 µL noctuid Ringer or 1.5 µg Spofr-AT dsRNA. Hemolymph was taken from individual larvae at respective days and JH homologs and free ecdysteroids were determined by LC-MS. Scale on Y-axis for ecdysteroids as in Figure 4B. Means±SEM; n=8-10.

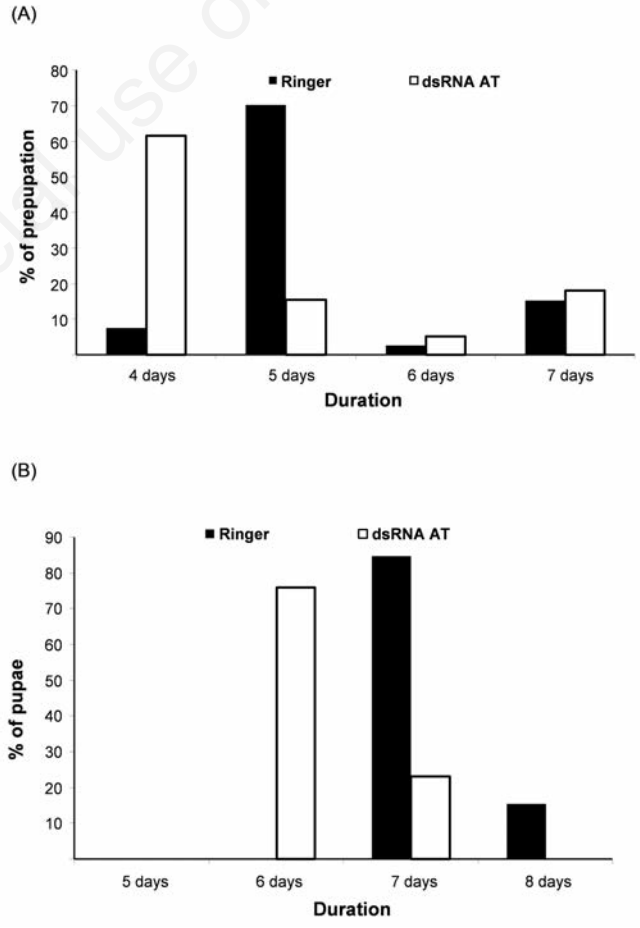

Figure 4. Effect of *Spofr-AT* gene silencing on juvenile hormone (JH I to JH III, total [t] JH) and ecdysteroid titers (ecdysone; 20-hydroxyecdysone, 20E) in the hemolymph of 4 d old last instar larvae (L6/4) and young prepupa (PP1). Freshly molted L6 larvae were injected with either 2 µL noctuid Ringer or 1.5 µg Spofr-AT dsRNA. Hemolymph was taken from individual larvae and prepupae at the respective days and JH homologs (A) and free ecdysteroids (B) were determined by LC-MS. Means±SEM; n=8-10; *P<0.05; ***P< 0.001.

(Figure 6). Injection of 2 µL Ringer into newly eclosed females did not affect egg laying, the total number of eggs nor the pattern of daily oviposition. Ringer injected mated females laid almost twice as many eggs as virgin females (Figure 6) with a maximum on days 3/4 after emergence (about 220 eggs per female and day), compared to day 6 for virgins with about 120 eggs per female and day. Injection of 1.5 µg Spofr-AT dsRNA into newly emerged females led to another increase in egg production and oviposition with more than 1600 eggs per female and a maximum of about 350 eggs per female and day on day 3 of adult life (Figure 6).

The JH titer in the hemolymph of adult females increased from the day of emergence to day 7 of adult life in virgin females and to day 4 in mated females, but decreased thereafter (Figure 7A, B). In contrast to larvae, the hemolymph of adult females contained considerable amounts of JH I and JH II, in addition to JH III. In virgin females, the hemolymph JH titer was lower (40 pg/µL on average and 75 pg/µL maximal) than in mated ones (90 pg/µL on average and a maximum of 130 pg/µL). Injection of 1.5 µg Spofr-AT dsRNA into freshly ecdysed females had only minor effects on the JH titer of the animals, except on day 7 of adult life, where JH III and total JH significantly decreased in virgin females, but increased in mated ones.

Eggs deposited by young females (days 2 to 5 after emergence) were significantly lighter in weight than eggs from older females (d 6/7) (Figure 8A). However, the hatching rate of the eggs was reversed. From eggs of younger females, 60 to 80% first instar larvae hatched successfully, but only about 20% from eggs laid by older females (Figure 8B). Injection of Spofr-AT dsRNA into newly ecdysed females hardly affected the weight of the deposited eggs or the hatching rate.

Effects of *Spofr-AT* gene knockdown in males on the transfer of juvenile hormone from males to females during copulation

The MARG contain significant amounts of JH I and JH II, but only traces of JH III. In the glands of unmated males, the amounts of JH I and JH II increased from the day of emergence to day 2 of adult life, whereas JH III remained low (Figure 9A). Mating on day 2 led to a drastic decrease of JH I and JH II in the MARG (Figure 9B). The female BC contains only traces of JH at the day of emergence, but concentrations of JH I and JH II increased significantly immediately after mating (Figure 9C). JH I and JH II disappeared again from the BC 1 day after mating (Figure 9C) and thereafter were found in the hemolymph of the females (Figure 10).

Injection of Spofr-AT dsRNA into newly ecdysed males only slightly affected the amounts of JH I and JH II transferred from the MARG to the female BC during mating (Figure 9C), but led to a highly significant increase in JH I and JH II in the hemolymph of the females. The number of eggs laid by females mated with such treated males was not different from the oviposition rate of females mated with Ringer injected males (Figure 11) (total of 1210±98 eggs with a maximum of 270 eggs per female and day at day 3 of adult life).

Discussion and Conclusions

In *S. frugiperda*, two types of allatostatins, AS type A or FGLamides and AS type C or Manse-AS, but no AS type B or [W(X_6)Wamides] were found. When we used double-stranded RNA (dsRNA) for RNA interference (RNAi) to knockdown the gene expression of Spofr-AS type A in penultimate instar larvae of *S. frugiperda*, a drastic increase in their hemolymph JH titer was observed, mainly due to an increase in JH I and JH III, indicating that at least one of the nine FGLamides acts as a true allatostatin (Meyering-Vos *et al.*, 2006) (Table 1). However, in

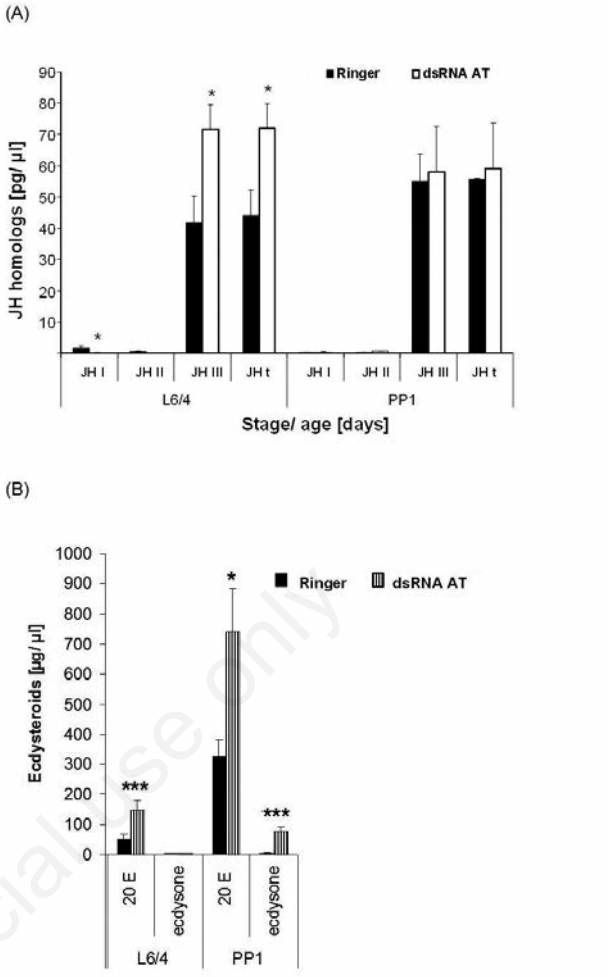

Figure 5. Effect of *Spofr-AT* gene silencing on larval/pupal development. L6/1 larvae were injected with 2 µL noctuid Ringer or 1.5 µg Spofr-AT dsRNA. Animals were kept individually in assortment boxes and duration until prepupal commitment (A) and transformation into the pupa (B) were observed. N=29 (Ringer); n=45 (dsRNA).

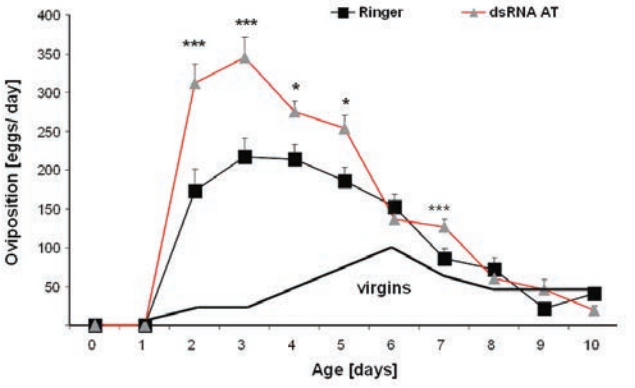

Figure 6. Effects of mating and *Spofr-AT* gene silencing on the oviposition of adult females. Virgin females were kept separated in boxes without males. For mating, males were added to the females in a ratio of 1:1 on day 1 of adult life and a new male was added to each female on day 4. Daily laid eggs per female were counted. Freshly emerged females were injected with 2 µl noctuid Ringer or 1.5 µg Spofr-AT dsRNA. Means±SEM; n=52-55 (Ringer); n=30-69 (dsRNA); *P<0.05; ***P<0.001.

spite of the high JH titer in the dsRNA treated larvae, no additional molting or *superlarva* were observed. When adult females were injected with various amounts of AS type A dsRNA, the oviposition rate was clearly reduced, but no JH titers had been measured in these animals. Functional analysis of the *Spofr-AS C-type* gene by RNA interference revealed allatostatic activity also for this peptide (Griebler *et al.*, 2008). Injection of Spofr-AS C-type dsRNA into penultimate larvae resulted in an elevated JH titer and the duration of the last larval stage was prolonged. In adult females the effect on the JH titer was inversely dependent on the age of the moths, indicating that the peptide acts either allatostatic or allatotropic. Moreover, gene silencing reduced the oviposition rate of adult females.

Besides the *classical* lepidopteran allatotropin (Manse/Spofr-AT), larvae and adults of the fall armyworm, *S. frugiperda*, contain the decapeptide RVRGNPISCF-OH, with a C-terminus as in the Spofr-AS (C-type allatostatin), but which acts as an allatotropin on JH release from the CA *in vitro* (Spofr-AT2; Abdel-latief *et al.*, 2004a). Knockdown of the *Spofr-AT2* gene revealed an allatostatic activity for the peptide in the larva, but an allatotropic activity in adult females (Griebler at el., 2008) (Table 1). Functional analysis with the genuine lepidopteran AT (Spofr-AT) in *S. frugiperda* has not yet been done, although Oeh *et al.* (2001) had shown that repeated injections of the peptide into larvae and adults retarded their development and reduced their fecundity.

Here, we used the technique of RNA interference (RNAi) for a specific knockdown of the *Spofr-AT preprohormone* gene. The amount of dsRNA required to produce RNAi effects is gene-dependent (Tomoyasu & Denell, 2004). In our experiments, a single injection of 1.5 µg dsRNA into caterpillars or moths was performed, which caused more than 80% silencing of the respective gene and the mortality of the animals remained low (<20 %). Injection of *G. bimaculatus* SK dsRNA as a control did not affect *Spofr-AT* gene expression.

The significant increase in the amount of JH III in the hemoylmph of the larvae following *Spofr-AT* gene knockdown in L6/1, together with a drastic increase in the concentration of free ecdysteroids, ecdysone

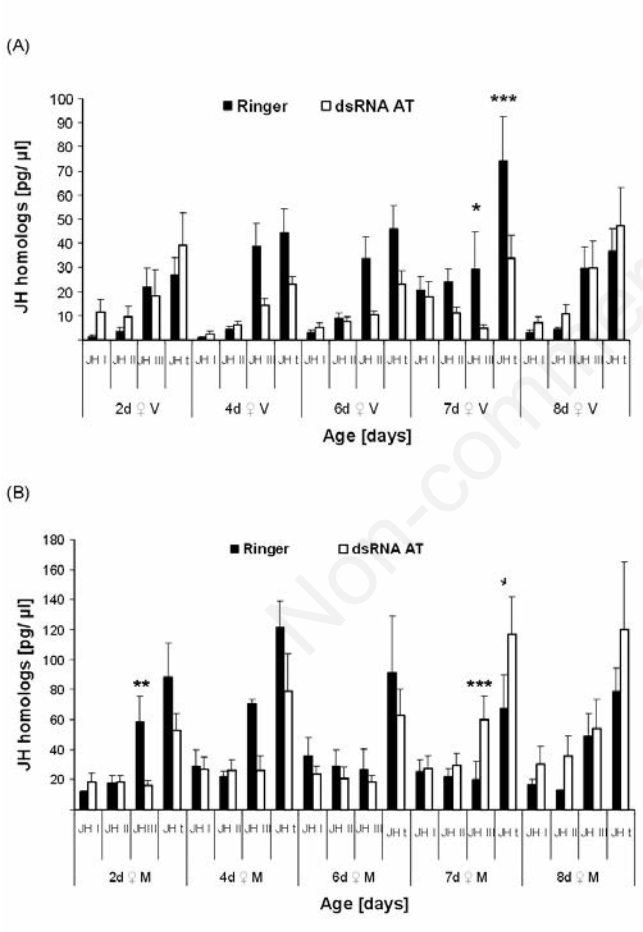

Figure 7. Effects of mating and *Spofr-AT* gene silencing on the juvenile hormone titer (JH I to JH III, total [t] JH) in the hemolymph of adult females. Newly emerged females were injected with 2 µL noctuid Ringer or 1.5 µg Spofr-AT dsRNA. Animals were kept either separated (A) (V, virgin) or (B) with males in a ratio of 1:1 (M, mated). Hemoylmph was collected from 2 d, 4 d, 6 d, 7 d, and 8 d old females during the early scotophase and JH titers were determined by LC-MS. Means±SEM; n=7-9 (Ringer); n=8-10 (dsRNA); *P<0.05; **P<0.01; ***P<0.001.

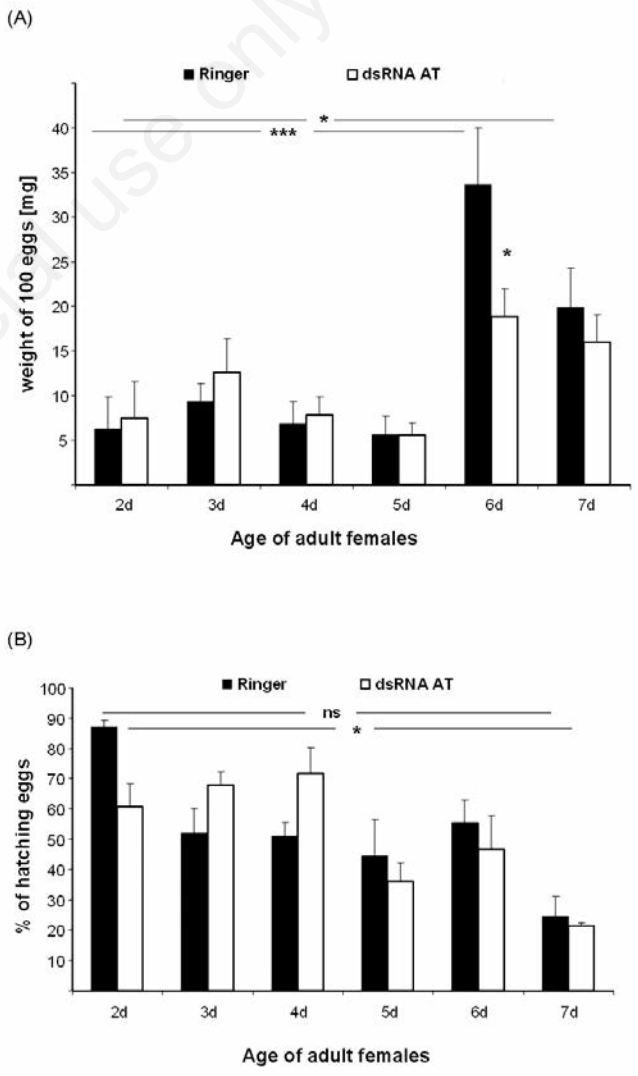

Figure 8. Effects of *Spofr-AT* gene silencing in young females on the egg weight (A) and viability of the eggs (B). Newly emerged females were injected with 2 µL noctuid Ringer or 1.5 µg Spofr-AT dsRNA. Males were added to the females in a ratio of 1:1. Filter paper was provided for egg laying. Eggs were collected daily. Means±SEM; n=8; *P<0.05; ***P<0.001.

and 20-hydroxyecdysone, means that the Spofr-AT peptide acts as an allatostatin (and not as an allatotropin) in armyworm larvae (Table 1), and that the *Spofr-AT* gene knockdown eliminated the block on JH release. Whether the ecdysteroid release following *AT* gene knockdown is directly or indirectly triggered, is not known. As expected, the increase of hemolymph ecdysteroids in L6/4 larvae following *Spofr-AT* gene knockdown led to a 24 hours earlier prepupal commitment and transformation to the pupa, compared to controls.

Spofr-AT gene knockdown in freshly ecdysed adult females followed by mating 24 hours later only slightly affected the concentration of JH III in the hemolymph, compared with Ringer injected controls (Table 1), but these females laid about 30% more eggs. The reason for the higher fecundity in *Spofr-AT* gene silenced females is unclear, but we speculate that *AT* gene knockdown may affect the food uptake (Müller, unpublished data). Geister *et al.* (2008) have shown that in another lepidopteran species, *Bicyclus anynana*, adult diet had pronounced effects on fecundity and egg hatching success. It is not surprising that an increased number of eggs in *S. frugiperda* gene silenced females results in a reduction of egg size. Such a trade-off between egg number and egg size has been observed in many invertebrates and vertebrates (Gibbs *et al.*, 2005; Olofsson *et al.*, 2009). It is also not surprising that the hatching rate from larger eggs of older females was lower than that of smaller eggs of young females. Such a phenomenon of higher infertility of older females has been observed in several insect species and may result from increasing ROS (reactive oxygen species) in older animals. ROS are known to have detrimental effects on eggs (Dowling & Simmons, 2009).

Virgin females laid about half as many eggs as mated once. Thus, mating seems to provide the correct stimuli for enhanced oogenesis and egg laying, and this is correlated with an elevation in the hemolymph JH titer. Similar results were obtained by Edwards *et al.* (1995), who demonstrated that virgin females of the tomato moth, *Lacanobia oleracea*, exhibit much lower JH titers than mated females. The switch from a virgin to a mated female is mediated by the sperm and the seminal fluid in the bursa copulatrix. During copulation, the male may trigger either a neural or humoral response in the female, thus stimulating the release of the endogenous gonadotropic signal JH (Ramaswamy *et al.*, 1997), or JH itself is transferred from the male to the female during mating. This was at first suggested by Shirk *et al.* (1980) in the *Cecropia* silkmoth, but the significance of such a JH transfer was not recognized. Park *et al.* (1998) clearly demonstrated a transfer of JH to females by males during mating in *Heliothis virescens*.

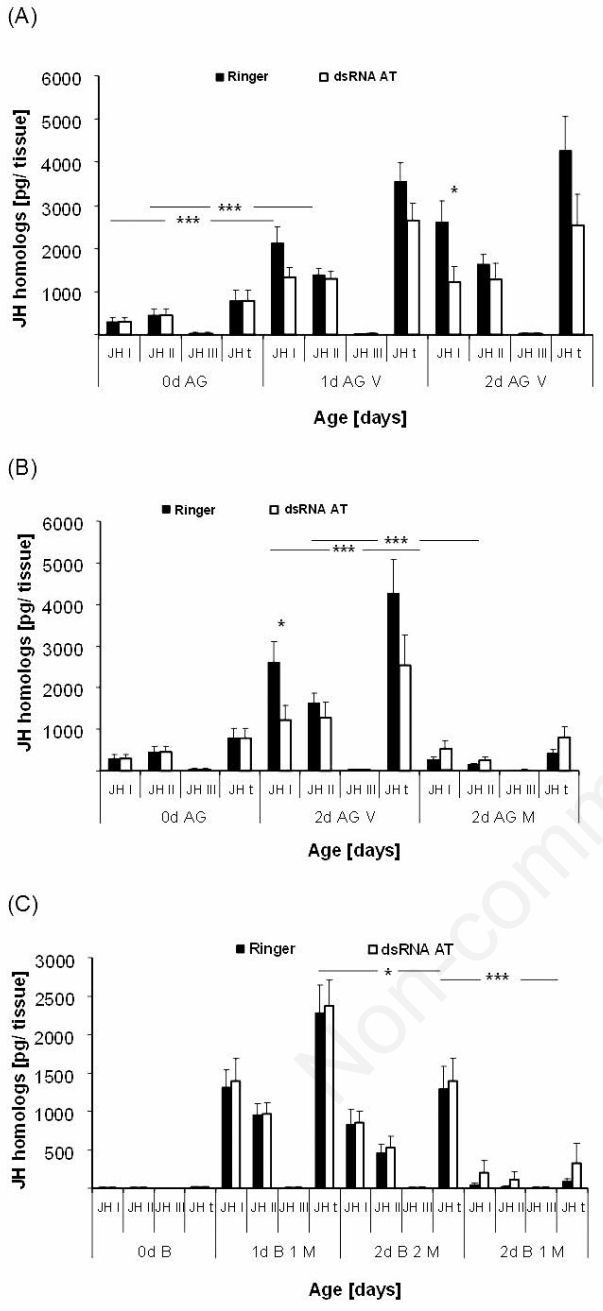

Figure 9. Effects of *Spofr-AT* gene silencing in males on the amount of JH homologs (JH I to JH III, total [t] JH) in the male accessory reproductive glands (AG) of unmated (virgin, V) (A, B) and mated (M) (B) males at days 0, 1, and 2 of adult life and on the amount of JH homologs in the bursa copulatrix (B) of females mated with such treated males (C). Newly emerged males were injected with 2 µL noctuid Ringer or 1.5 µg Spofr-AT dsRNA. 1dB1M=bursa copulatrix of 1 day old female mated on day one; 2dB2M=bursa copulatrix of 2 day old female mated twice on day 1 and 2; 2dB1M=bursa copulatrix of 2 day old female mated once on day 1 of adult life. JH titers were determined by LC-MS. N=12; *P<0.05; ***P<0.001.

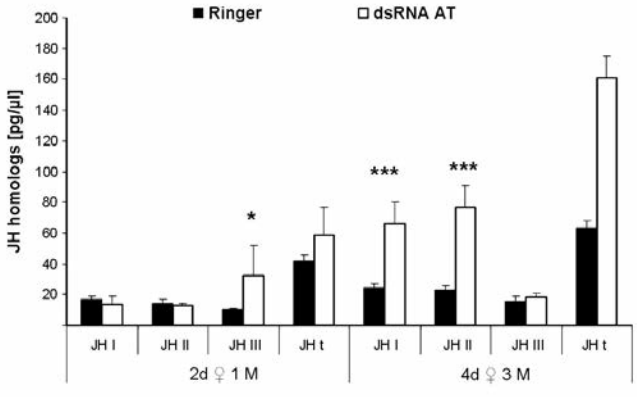

Figure 10. Effect of *Spofr-AT* gene silencing in males on the JH titer (JH I to JH III, total [t] JH) in the hemoylmph of females mated with treated males. Newly emerged males were injected with 2 µL noctuid Ringer or 1.5 µg Spofr-AT dsRNA. Untreated females were reared in a ratio of 1:1 with treated males. 2d♀1M=2 day old females one day after mating; 4d♀3M=4 day old female after 3 successive matings. JH titers were determined by LC-MS. Means±SEM; n=10; *P<0.05; ***P<0.001.

Table 1. Effects of allatoregulating neuropeptides on the amounts of juvenile hormone homologs (JH I, JH II, JH III) in the hemolymph of larvae and adults of *S. frugiperda* revealed by RNA interference.

Peptide	Larvae	Adult females	Adult males
Spofr-AS type A	Allatostatic (Meyering-Vos *et al.*, 2006)	Not determined	Slightly allatostatic (unpublished data)
Spofr-AS type C	Allatostatic (Griebler *et al.*, 2008)	Allatostatic or allatotropic (Griebler *et al.*, 2008)	Allatostatic (unpublished data)
Spofr-AT	Allatostatic (this paper)	No clear effect (this paper)	No clear effect (this paper)
Spofr-AT2	Allatostatic (Griebler *et al.*, 2008)	Allatotropic (Griebler *et al.*, 2008)	Allatotropic (unpublished data)

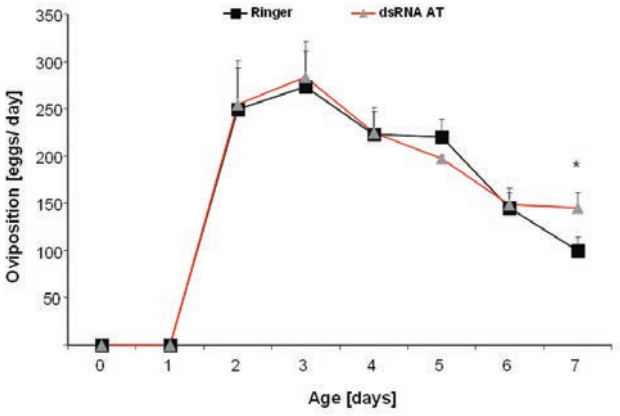

Figure 11. Effect of *Spofr-AT* gene silencing in males on the oviposition of females mated with treated males. Newly emerged males were injected with 2 µL noctuid Ringer or 1.5 µg Spofr-AT dsRNA. Untreated females were reared in a ratio of 1:1 with treated males and the number of deposited eggs was counted daily. Means±SEM; n=25 (Ringer); n=26 (dsRNA); *P<0.05.

Pszczolkowski *et al.* (2006) then showed that the JH transferred from the male to the promiscuous female promotes JH synthesis and egg development in the female. The present data confirm a significant transport of mainly JH I and JH II from the MARG to the female BC during copulation, followed by an increase of JH in the hemolymph of the mated female. *Spofr-AT* gene knockdown in newly ecdysed males, however, only slightly affected the JH production/transfer and had no effect on the oviposition rate of the females. We assume that other allatoregulatory neuropeptides than Spofr-AT control the synthesis of JH in the MARG and their transport into the female during copula (Table 1). Functional studies on Spofr-AS type C, Spofr-AS type A, and Spofr-AT2 in male/female JH transfer are in progress.

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