RNA interference with allatoregulating neuropeptide genes affecting circadian rhythm, development, mating and reproduction of *Spodoptera frugiperda* (Lepidoptera: Noctuidae)

Dissertation

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Dedication

To my beloved father

&

wonderful children

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1 Introduction

Butterflies and moths form the most diverse species that enrich the biodiversity on earth; there are 137 thousand named species of Lepidoptera (Allen et al., 2011). The fall armyworm, *Spodoptera frugiperda*, is a polyandrous moth which mates several times during its lifespan. Larvae of *S. frugiperda* are known as pest on pastures and corns in Canada and USA, which have received some attention because they represent a continuous and serious threat to the production of high quality agricultural products and have a destructive impact on the positive commercial importance of corns because of their rapid reproduction.

1.1 Neuropeptides

Neuropeptides are peptide hormones, which are secreted by nerve cells into the blood or hemolymph of animals. In insects, they regulate almost all physiological, developmental and behavioural processes. The largest group of neuropeptides in insects are those with myoregulatory function.

Myoactive peptides play various roles in pathways controlling tissue movement. They stimulate or inhibit visceral muscles of the gut and control heart beat rate in a dose/time-dependent manner (Merte and Nichols, 2001). The first identified neuropeptide with myoactive function was proctolin (Starrat et al., 1976). In Lepidopterans, Leu-callatostatin and helicostatins are FGLamide brain-gut peptides, which belong to a ubiquitous FGLamide family of insect neuropeptides (type A allatostatins) controlling gut motility. They were at first isolated from *Cydia pomonella* and *Helicoverpa armigera* larvae (Duve et al., 1997a; Davey et al., 1999). FLRFamides I, II and III, crustacean cardioactive peptide (CCAP), and cardioactive peptide 2b (CAP 2b) were at first identified from the tomato moth, *Lacanobia oleracea* (Audsley and Weaver, 2003a).

In other insects, leucomyosuppressin (LMS) stimulates digestive enzyme activity in gut lumen contents, whereas Dippu-allatostatins inhibit proctolin-induced contractions of midgut muscles of the cockroach *Diploptera punctata* (Fusé et al., 1999). LMS and Peram-MIP (myoinhibiting peptides of *Periplaneta americana*) inhibit the activity of the foregut, but not of other visceral muscles in the American cockroach (Predel et al., 2001). Four myoinhibitory neuropeptides (MIP I-IV; type-B allatostatins) were isolated and identified from the ventral nerve cord of adults of *Manduca sexta* and *L.*

oleracea and were found to inhibit *in vitro* spontaneous peristaltic movement of the ileum (Blackburn et al., 2001; Audsley and Weaver, 2003b). Moreover, MIP I inhibits ecdysteroid biosnthesis *in vitro* by prothoracic glands, indicating that it has a role in ecdysis behaviour of *Bombyx mori* (Davis et al., 2003).

1.2 Allatotropins

Allatoregulating neuropeptides have either stimulatory (allatotropin, AT) or inhibitory (allatostatin, AS) action on juvenile hormone (JH) biosynthesis by the corpora allata (CA). Allatotropins are members of a family of myoactive neuropeptides found in several invertebrate phyla. In Lepidoptera, they have stimulatory effect on JH biosynthesis, but also play myoactive (myotropic) roles (Elekonich and Horodyski, 2003).

Manse-AT (AT 1) was at first identified in *M. sexta* (Kataoka et al., 1989) and then in the tomato moth, *L. oleracea* (Audsley et al., 2000a), but occurs also in other moths. Another structural unrelated allatotropin (Spofr-AT 2) was identified and characterized only in *S. frugiperda* (Abdel-latief et al., 2004c). The AT 1 peptide consists of 13 amino acid residues and stimulates *in vitro* JH biosynthesis by the CA of various lepidopterans in a dose-dependent manner (Bogus and Cymborowski, 1984; Kataoka et al., 1989; Veenstra and Costes, 1999; Oeh et al., 2000; Audsley et al., 2000a; Edwards et al., 2001; Koladich et al., 2002; Li et al., 2005). The C-terminal amidated pentapeptide sequence of AT 1 is also a potent *in vitro* stimulator of JH biosynthesis in the CA of dipterans. Rachinsky et al. (2000) showed that the terminal steps of JH biosynthesis were affected in larval workers of the honey bee, *Apis mellifera,* by this peptide. Additionally, AT 1 accelerates heart beat rate (Koladich et al., 2002), stimulates heart muscle contractions and gut peristalsis, and inhibits ion transport across the midgut (Lee et al., 1998; Lee and Horodyski, 2002). Manse-AS (type C allatostatin of *M. sexta*) had no influence on the Manse-AT stimulatory effect in *L. oleracea,* but in *S. frugiperda* (Audsely et al., 2000a; Oeh et al., 2000). Injection of Manse-AT peptide into sixth instar larvae of *L. oleracea* had no effect on larval development and food consumption (Audsley et al., 2001), but induced high mortality, reduced body weight, and delayed prepupal development. Injection of AT 1 peptide into larvae of *S. frugiperda* did not affect JH biosynthesis by their CA *in vitro* (Oeh et al., 2000).

AT 1 cDNA that encodes 134 amino acids, has been cloned from several dipterans and lepidopterans (Veenstra and Costes, 1999; Truesdell et al., 2000; Abdel-latief et al., 2003a). The *AT 1* gene is expressed as three mRNA isoforms that differ from each other by alternative splicing (Abdel-latief et al., 2003a), thus providing a mechanism for the production of peptides specific to each isoform. The three mRNAs are expressed in brain, digestive tract, and reproductive organs of larvae, pupae, and adults of *S. frugiperda* in a time- and tissue-specific manner (Abdel-latief et al., 2003b). Its expression in the brain, gut and reproductive tract proves the dual role of the peptides as brain/gut neuropeptides and implies that they have functions in reproductive processes (Abdel-latief et al., 2004a). The expression of this gene increased in the nerve cord of last instar larvae of *M. sexta* that were starved, parasitized, or fed the edysteroid agonist RH 5992 (Lee and Horodyski, 2002). Each of these treatments resulted in reduction of feeding and an increased level of hemolymph JH.

AT 1-like peptides were identified in *L. oleracea* (Audsley et al., 2000a). Moreover, three additional Manse-AT-like sequences that are flanked by basic amino acid residues have been identified (Horodyski et al., 2001). These peptides are predicted to include three allatotropin-like peptides that exhibit limited structural identity to Manse-AT and overlapping biological activities (Lee et al., 2002). Manse-AT–like sequences revealed that they may be derived from a duplication of ancestral Manse-AT sequences followed by divergence (Horodyski et al., 2001).

1.3 Allatostatins

Besides the lepidopteran typical allatostatin C-type (Manse-AS), members of the Y/FXFGL-NH_2 allatostatins (A-type allatostatins) were identified immunologically from the nervi corporis cardiaci (NCC) of *M. sexta* (Stay and Tobe, 2007). However, Manse-AS C-type peptide represents the “true” lepidopteran allatostatin and has been shown to be present at first in the tobacco hornworm *M. sexta* (Kramer et al., 1991), sequenced by Audsley et al. (1998) and shown to inhibit *in vitro* JH biosynthesis by the CA of larval (*M. sexta*, Kramer et al., 1991; *L. oleracea,* Audsley et al., 2000b) and adult (*M. sexta*; Audsley and Weaver, 2003b) moths. The *Manse-AS* gene has been cloned from *Pseudaletia unipuncta* (Jansons et al., 1996) and *S. frugiperda* (Abdel-latief et al., 2003). Besides its allatoregulatory activity, the peptides showed inhibitory effects on gut peristalsis *in vivo*, suppressed feeding, retarded

growth and increased gut motility in *L. oleracea*, but they did not inhibit *in vitro* JH biosynthesis by the CA in *L. oleracea* (Audsley et al., 2001) and in *P. unipuncta* (Jansons et al., 1996). However, after suppression of *Manse-AS* gene expression in the 5th larvae stage and in adults of *S. frugiperda*, a positive effect on JH titers in the hemolymph was determined. Moreover, in *AS type C* gene silenced animals, the weight of the larvae was reduced causing a prolongation of the larval stage (Griebler et al., 2008).

The ubiquitous A-type F/YXFGL (I, V) amide cockroach allatostatins are released by exocytosis from brains in many invertebrates, and they inhibit JH biosynthesis by the CA of cockroaches, crickets, and termites, probably by targeting the JH biosynthesis pathway prior to the conversion of farnesol to JH (Woodhead et al., 1989; *M. sexta*, Kramer et al., 1991; Stay et al., 1991; *M. sexta*, Kramer et al., 1991; Lloyd et al., 2000). Their widespread localization in central and stomatogastric nervous system implies a more general role as neurotransmitters and neuromodulators (Duve et al., 1997a; Stay and Tobe, 2007). They were first discovered in larvae and adults of the viviparous cockroach *Diploptera punctata* (Woodhead et al., 1989) and later in other cockroaches such as *Blattella germanica* (Bellés et al., 1994), but also in crustaceans like *Carcinus maenas* (Duve et al., 1997b) and the tiger prawn, *Penaeus monodon* (Duve et al., 2002). Several peptides were isolated from the termite *Reticulitermes flavipes* (Yagi et al., 2008), the cricket *Gryllus bimaculatus* (Lorenz et al., 1999), and the stick insect *Carausius morosus* (Lorenz et al., 2000). Type A allatostatins inhibit JH biosynthesis in the CA of several insect orders (Lorenz et al., 1999; Li et al., 2005; Yagi et al., 2005; Clark et al., 2008; Abdel-latief and Hoffmann, 2010). Their effect on the CA of cockroaches depends on the dose and sensitivity of the CA as well (Stay et al., 1996).

The allatostatin A-type peptide receptor has been cloned; it was localized within the brain lateral neurosecretory cells, but also, for example, in the fat body, indicating the multifunctional role of the peptides (Stay et al., 1993; Auerswald et al., 2001). A type allatostatins are pleiotropic peptides, similar to somatostatins, which reflects parallel evolution of these peptides (Bendena et al., 1997). They affect many physiological processes including stimulation of digestive enzymes like invertase, α-amylase and carbohydrate metabolizing enzymes, as well as inhibition of JH (III) biosynthesis, muscle contraction, ovarian ecdysteroid biosynthesis, vitellogenin production, and cockroach reproduction (Bellés et al., 1987; Fusé et al., 1999; Hult et al., 2008).

AS A-type genes were cloned from several cockroach species, dipterans, lepidopterans, termites, and the cricket, *Gryllus bimaculatus* (Meyering-Vos et al., 2001; Abdel-latief et al., 2004b; Wang et al., 2004; Elliott et al., 2009). In *G. bimaculatus* the gene was shown to be expressed in brain, caecum, digestive tract (ileum, midgut, colon), and in various tissues such as fat body, ovaries and female reproductive glands (Meyering-Vos and Hoffmann, 2003).

The first lepidopteran type A allatostatin was isolated from *M. sexta* and has been named lepidostatin-1. Colocalization of the type A allatostatin with a diuretic hormone in the brain of *M. sexta* and its synchronous co-release implies that this peptide has a role in liquid transportation and myogenic contraction in the lepidopteran larval hindgut (Davis et al., 1997; Stay, 2000). However, the peptides do not affect JH biosynthesis in lepidopterans. The cDNA of the lepidopteran brain/gut *AS A-type* preprohormone encodes 9 to 10 members of the Y/FXFGL-a peptide family and was sequenced from *S. frugiperda* (Abdel-latief et al., 2004b).

1.4 Juvenile hormones (JH)

Juvenile hormones (JH) are sesquiterpenoids, which are synthesized by the corpora allatata (CA), released into the hemolymph, and transported by carrier proteins to their target cells. So far, seven JH homologs are known from various insect orders, whereas JH III is mostly widespread. In lepidopterans, JH I, II, and III were found. Because of their non-polar nature juvenile hormones easily penetrate the cell membrane by diffusion as well as do the ecdysteroids. Within the cell, juvenile hormones may bind to a receptor and interact somehow with the genome, thus activating the DNA machinery (Davey, 2000). Right now, the USP-subunit of ecdysteroid receptors and the transcription factors *Met-tolerant* and E 75A are discussed as putative JH receptors (Klowden, 2008). Juvenile hormones are crucial in all insect developmental and reproductive events (Riddiford, 2008) including embryogenesis, larval moulting, metamorphosis, vitellogenin synthesis, vitellogenin uptake by the ovaries and ovarian development, polymorphism, diapause regulation, and various aspects of metabolism associated with these functions. Besides their general juvenoid function in larvae and gonadotropic function in adult insects (Postlethwait and Jones, 1978; Yamamoto et al., 1988), they control caste differentiation e.g. in the damp-wood termite *Hodotermopsis sjostedti* (Isoptera: Termopsidae) (Cornette et al., 2008) and in honey bees *Apis mellifera* L. (Rachinsky

and Hartfelder, 1990), and are correlated with aggression in colony defence and establishment of dominance in burying beetles (Scott, 2006). Moreover, PBAN (pheromone biosynthesis activating neuropeptide) activation and the release of pheromones are controlled by JH II that up-regulates the putative pheromone receptor protein in female pupae, for example in *Helicoverpa armigera* (Rafaeli et al., 2003). JH also triggers the flight muscle hydrolysis in a wing dimorphic cricket, *Modiogryllus conformatus* (Tanaka, 1994).

JH is supposed to be involved in diapause regulation of overwintering insects (Taub-Montemayora et al., 2005), but Okuda et al. (1996) reported that other factors than JH evoke diapause of *Nomada succincta* and *N. japonica.* JH is involved in the maternal regulation of phase-dependent progeny characteristics in *Schistocerca gregaria* (Maeno and Tanaka, 2009), and Cisper et al. (2000) reported that JH is responsible for wing polymorphism in the cricket *Gryllus firmus.* Taub-Montemayora et al. (2005) stated that the sexual dimorphism in the boll weevil, *Anthonomus grandis*, is not due to JH titer differences between females and males.

Crustacean juvenile hormone (methyl farnesoate), an intermediary metabolite of JH biosynthesis in the CA of insects, is released by the mandibular organs from all crustacean species and affects larval development and reproduction (Borst et al., 1987).

The JH hormone analog methoprene stimulated vitellogenesis and ovarian development in sexually mature females of *Nomada succincta* and *N. japonica* (Okuda et al., 1996). Methoprene also stimulated vitellogenin production in intact males of the boll weevil, *A. grandis* (Taub-Montemayor et al., 2005). While JH III had no effect on the vitellogenin production in the fat body of adult females of *Locusta migratoria* (Taub-Montemayor et al., 2005), methoprene acted on the locust fat body to bring about the expression of the *Vg* gene (Wyatt et al., 1987). In conclusion, methoprene seems to act as a true JH analog in many adult insects.

1.5 Regulation of JH titer

The hemolymph juvenile hormone titer is regulated by JH synthesis and release from the corpora allata, activity of JH esterase in the hemolymph and tissues, availability of binding proteins in the hemolymph, hormone uptake by the tissues, and by hormone catabolism and hormone excretion (De Kort and Granger, 1981; Klowden, 2008). Brain stimulatory and inhibitory neurosecretion and ovary somatic signals

modulate the CA activity in different ways according to developmental stages. Control factors regulating JH biosynthesis in the CA may act via nerves or via the hemolymph (Tobe, 1980; Tobe et al., 1982; Rankin and Stay, 1983; Kataoka et al., 1989; Woodhead et al., 1989; Pratt et al., 1990; Kramer et al., 1991; Stay et al., 1991; Gu et al., 1995; Veenstra and Costes, 1999; Rachinsky et al., 2000; Audsley et al., 2000a; Audsley et al., 2000b; Lloyd et al., 2000; Elekonich and Horodyski, 2003).
The cDNAs of mevalonate pathway enzymes involved in JH biosynthesis were cloned (*Bombyx mori,* Kinjoh et al., 2007). JH binding proteins (JHBP) have a high affinity to the hormone and belong to the lipophorin fraction of hemolymph proteins (*Leptinotarsa decemlineata*; De Kort et al., 1987). JH binding proteins JHBPs and JHEHs (JH epoxide hydrolase) of two lepidopteran species were characterized (Prestwick et al., 1996). The concentration of binding proteins and the activities of degradative enzymes change during the insects' life cycle and may correlate with changes in hormone titers.
In many insects JH analogs inhibit JH synthesis in the CA, e.g. fenoxycarb in *D. punctata* (Lenkic et al., 2009). A similar effect was observed for endogenous JH III in intact females of *P. americana* (Edwards et al., 1987). It appears that the JH analog fenoxycarb as well as endogenous JH will control CA activity by a negative feedback mechanism (Edwards et al., 1987).
As shown above, many neuropeptides are regulating JH biosynthesis in a stimulatory or inhibitory way, and they may act at different steps of JH biosynthesis. *Aedes aegypti* allatotropin (Aedae-AT) and farnesoic acid probably act on the terminal steps of JH biosynthesis (Li et al., 2003a). However, Manse-AT had stimulatory effects on JH I to JH III release through increasing the supply of acetyl- and propionyl-CoA precursors (*M. sexta* Teal, 2002). Manse-AS, on the other side, acts prior to formation of the sesquiterpene alcohol precursors of JH (*Heliothis virscens;* Teal, 2002). Allatostatins act on JH biosynthesis through inhibition of the activity of final enzymes, converting farnesoic acid to methyl farnesoate and then to JH (Wang et al., 1995). Sutherland and Feyereisen (1996), however, proposed that the inhibition of JH III biosynthesis by an A-type allatostatin occurs at the first step of JH III synthesis i.e. the transfer of 2C units from mitochondria to the cytoplasm by the tricarboxylate carrier and/or the ATP-citrate lyase. Gruntenko et al. (2005) suggested that ecdysone controls JH synthesis through dopamine in *Drosophila*. Tu et al. (2005) reported that

the insulin signaling pathway may mediate JH biosynthesis through the JH regulatory neuropeptides.

1.6 Ecdysteroids

The prothoracotropic hormone PTTH stimulates ecdysteroid secretion by insect prothoracic glands (Gilbert et al., 2002). PTTH does not only activate glandular tyrosine kinase, but tyrosine phosphorylation is required for ecdysone secretion and occurs at a very early step in the PTTH signalling pathway (Smith et al., 2003). The neuropeptides generally stimulate the conversion of cholesterol to ecdysone and 20-hydroxyecdysone (20E). Levels of free ecdysteroids in the hemolymph are controlled by feedback mechanisms (Klowden, 2008). The nuclear ecdysteroid receptor controls the cell transcription machinery and represents a heterodimer of ecdysone receptor (EcR) and ultraspiracle (USP) (Klowden, 2008). JH III potentiates the transcriptional inducibility of edcysteroids acting via EcR also in mammals showing a strong functional relationship between the two hormones (Henrich et al., 2003). The JH analog ZR-515 has the ability to activate PTTH in last instar larvae and pupae of *Mamestra brassicae* (Hiruma et al., 1978).

In several lepidopterans, the *PTTH* gene was isolated and sequenced (Shionoya et al., 2003). A 226 amino acid preprohormone closely related to PTTH had been cloned from *Bombyx mori*, *Samia cynthia ricini*, *Antheraea perani*, and *Hyalophora cecropia* (Kataoka et al., 1991; Ishizaki and Suzuki, 1994). The recombinant PTTH produced by *E. coli* was shown to be biologically active to initiate both the larval and the adult moults in brainless *M. sexta*. Ecdysiotropins such as PTTH stimulate ecdysteroid biosynthesis (Koolman, 1989) and ecdysiostatins (PTSP) inhibit their biosynthesis. Hua et al. (1999) isolated PTSP (prothoracostatic) peptides from the brain of *B. mori*, which were similar to MIP I (see B-type allatostatins above) and showed high homology with vertebrate galanins. The inhibition of ecdysteroid biosynthesis by PTSP works through activation of cAMP (Hua and Koolman, 1995).

In adult insects, prothoracic glands may degenerate and ecdysteroids are released from other tissues such as follicle cells of the ovary, the abdominal fat body/epidermis or the testes (reviewed by Brown et al., 2009). Prothoracic glands usually secrete ecdysone which is converted to the physiologically active 20E in various peripheral tissues like the Malpighian tubules (Rees and Isaac, 1985). Females may transfer ecdysteroids into the eggs where they induce embryonic

moultings (Hoffmann et al., 1985). In general, ecdysteroids were found to induce embryonic, larval, pupal and adult moultings. Ecdysteroids and not JH III initiate *vitellogenin* (*Vg*) gene expression, synthesis and release of Vg into the hemolymph, and vitellogenin uptake into the oocytes in some adult insects, like in dipterans, and also in ticks (Thompson et al., 2005).

1.7 Larval development

Insect development and reproduction are regulated by ecdysteroids, juvenile hormones, and neuropeptides (Gäde and Hoffmann, 2005). Larval development is guided by the simultaneous presence of ecdysteroids and juvenile hormones for larval - larval moulting. In insects with holometabolic development, it has been shown quite clearly that the JH titer is high at the time of last-larval moulting, and then declines to a very low or undetectable level prior to larval commitment (Riddiford, 1972). In *S. fruigperda* JH I to JH III are present in larvae, but JH III is the predominant homolog. The JH titers are fluctuating during larval development, as well as the presence and distribution of Manse/Spofr-AT and Manse/Spofr-AS peptides (Abdel-latief et al., 2004a). Injection of Manse-AS peptide into larvae of *S. frugiperda* had no effect on the titer of JH homologs, body weight and mortality (Oeh et al., 2000), whereas, silencing of the *Manse-AS* gene of *S. fruigperda* larvae caused elevation of JH titers, reduction of body weight, increasing mortality and prolongation of larval stages (Griebler et al., 2008).

1.8 Metamorphosis

A small peak of ecdysteroids initiates pupal commitment in the Lepidoptera (Watson et al., 1987) and the ecdysteroids are released by the prothoracic glands (Sehnal et al., 1988). The timing of pupal commitment is gated by PTTH and dictated by the endogenous JH titer (Rountree and Bollenbacher, 1986). Moreover, enhancement of ecdysteroid receptors was shown by Riddiford and Truman (1993) during that time, indicating that they trigger the transcription machinery during metamorphosis. Ecdysteroids in the absence of JH activate two classes of genes: one responsible for the phenotype typical of the next (pupal) developmental stage and the other permitting a modified response to ecdysteroids (Wang et al., 1995). More ecdysone is then secreted and initiates metamorphosis. Various larval tissues transform into pupal tissues and become insensitive to JH (Riddiford, 1972).

Elimination of the CA from penultimate instar larvae of *M. sexta* accelerated pupation, adult eye differentiation and differentiation of the base of the forewings (Kiguchi and Riddiford, 1978). Allatectomy thus caused changes leading to metamorphosis including a transformation of the prothoracic glands from larval to pupal type.

On the other hand, inhibition of pupal commitment was reported by keeping JH titers high, that means by treating with JH or JH analogs before onset to metamorphosis. Retardation of metamorphosis in favour of supernumerary larval moults was observed following treatment with JH analogs (Kremen and Nijhout, 1998).

1.9 Reproduction

Vitellogenin (Vg) is synthesized by the fat body, secreted into the hemolymph and taken up into oocytes by endocytosis via specific Vg-receptors (Raikhel and Dhadialla, 1992). In most adult females JH induces vitellogenesis and controls oocyte growth and maturation (Bendena et al., 1997). In some species Vg production is accomplished by proper feeding (Fei et al., 2005). In dipterans, JH, or their mimics, and 20E act in combination to stimulate ovarian maturation and vitellogenin synthesis (Kelly et al., 1987). For example, production of vitellogenin requires both JH and 20E in *Musca domestica* (Adams and Filipi, 1988), whereas 20E did not affect vitellogenesis in the Culicidae (Redfern, 1982). JH III is important in chorion formation during later ovarian maturation (*Diatraea grandiosella;* Shu et al., 1997), whereas 20E is the responsible hormone in *Galleria mellonella* (Memmel et al., 1988).

In general, juvenile hormones and ecdysteroids have gonadotropic functions in adult insects (see above). The ecdysteroids are synthesized in the follicle cells of ovaries and testes, for instance, in adult females of *Blattella germanica* (Romana et al., 1995).

The cockroach allatostatin Dippu-AST 1 had an inhibitory effect on ovary development in a 4 day old mealworm female (Wasielwski et al., 2009). Moreover, type A and type B allatostatin epitopes were found in the ovary of last instar larvae and adult crickets, *Gryllus bimaculatus,* especially in the cortical cytoplasm of the oocyte anterior pole (Witek and Hoffmann, 2001). From these results it can be concluded that JH biosynthesis in adult insects is also controlled by allatoregulating neuropepides.

1.10 RNA interference (RNAi)

Biological events concerning survival, growth and differentiation occur as a respond to altering patterns in gene expression. Gene transcription level quantification has become crucial in gene functioning research (Zamorano et al., 1996). Moreover, many of the discovered genes are temporary with unknown functions (Bellés, 2010). The RNA interference (RNAi) technique, or post transcriptional gene silencing (PTGS), mediated by 21 to 22 nucleotide small interfering RNAs (Elbashir et al., 2000), which induce depletion of a chosen transcript, can help resolving this challenge (Bellés, 2010). So far, the possibility of studying the functions of homologous genes in different species can facilitate an evolutionary insight into developmental processes of insects (Bellés, 2010). Moreover, the RNAi can help studying mechanisms of action of known insecticides, and to develop new targets for new insecticides. RNAi itself could be envisaged as an insect control tool through targeting vital genes, although efficient systems of dsRNA formulation and delivery must be developed (Price et al., 2008; Whyard et al., 2009; Tao et al., 2010).

RNAi is a natural process to regulate gene expression (Lieber, 2010) by down-modulation of a specific mRNA (Bellés, 2010), triggered by short helical RNA molecules named small interfering RNA (siRNA) that are generated in the cells from larger double-stranded RNA precursors by enzymatic degradation through the RNase enzyme Dicer. These siRNAs are then incorporated into a silencing complex called RISC (RNA-induced silencing complex), which identifies and silences complementary messenger RNA (Meister and Tuschl, 2004). RNA interference already proved its usefulness in functional genomic research on insects. Huvenne and Smagghe (2010) described at least two pathways for dsRNA uptake in insects: the transmembrane channel-mediated uptake mechanism and an ‘alternative’ endocytosis-mediated uptake mechanism.

Pest insects should be able to take up the dsRNA through feeding and digestion into their midgut. Recently, we could show in our laboratory that *S. frugiperda* larvae take up dsRNA dissolved in a semi-synthetic diet and that this dsRNA suppresses gene expression (Griebler et al., 2008). The authors demonstrated that dsRNA taken up with the food was successful in suppression of *Manse-AS* and *Spofr-AT 2* genes in several tissues of larvae and adult moths, resulting in massive mortality in this pest species. Huvenne and Smagghe (2010) highlighted the achievement of implementing

RNAi in insect pest control with successful experiments using transgenic plants and a diversity of insect orders/species and target genes, respectively.

1.11 Problem statement and justification of the research project

Allatoregulating neuropeptides are numerous and have been localized in various insect tissues. There are some reports on *in vitro* studies, which investigated the bioactivity of these peptides, but *in vivo* studies are rare. The RNA interference is an up-to-date method used *in vivo* to suppress gene expression selectively and serves as a tool to analyze gene functions.

Allatostatins (AS) and allatotropins (AT) are neuropeptides that inhibit or stimulate the biosynthesis of juvenile hormones (JH). In the moth, *S. frugiperda*, at least two types of AS (type A and type C) and two AT (AT 1 and AT 2) are expressed, which are grouped by structural features. Both A- and C-type allatostatins and AT 1 peptides are localised in neurosecretory cells of the brain and are present in the CC, CA and ventral nerve cord, although variations exist in different sexes and at different stages of development (Abdel-latief et al., 2003; 2004a; 2004b). However, the widespread expression of the genes in various tissues corroborates their multifunctional roles. So far, expression and localization sites of AT 1 peptide suggest that the peptide may have functions distinct from regulation of JH biosynthesis.

Juvenile hormones and ecdysteroids are classical hormones controlling the development, metamorphosis and reproduction of insects. Several homologs of the juvenile hormones (JH I, II, and III) exist in the Lepidoptera (see above). There are indications that the hemolymph titers of defined isoforms are controlled in relation to the developmental stage and age of *S. frugiperda* by the allatoregulating neuropeptides.

The fall armyworm, *S. frugiperda,* is an agriculturally important pest species. Much effort has been performed to improve pest control strategies based on disruption of the insect endocrine system. One of the major targets is the control of JH biosynthesis in the CA through allatoregulatory neuropeptides (De Kort et al., 1987).

To address these issues, we used the RNA interference (RNAi) and designed double stranded (ds)RNA that selectively knocks down genes for the preprohormones of allatoregulating neuropeptides, here the type A allatostatin (AS) and the allatotropin (AT) 1 of *S. frugiperda*.

I aimed with this work at first to study the circadian rhythm of adult emergence, mating behaviour, and egg laying following *AT 1* gene suppression.

Secondly, I analysed the effect of RNAi *AT 1* gene suppression on the JH and ecdysteroid titers in the hemolymph of larvae, virgins and mated adult females and males at different age. This should shed light on the *in vivo* role of these neuropeptides on development, metamorphosis, and reproduction.

Thirdly, there are some factors synthesized in the reproductive tract of the male that are transferred from the male to the female of *S. frugiperda* during copulation and trigger the fertility of the female. In this work, I investigated how *AT 1 and AS A-type* gene suppression in females and males may interfere with the male to female JH transport and the fertility of the females including the maturation of reproductive tissues and egg deposition.

At fourth I checked the amplified AT 1 templates used to transcribe the dsRNA AT 1 by cloning and sequencing.

Finally, I conducted *AT 1* gene expression studies using real-time RT-PCR technique to evaluate the RNAi process efficiency in specific tissues.

2 Materials and Methods

2.1 RNA interference technique

In order to investigate the physiological functions of AT 1 peptide in development and reproduction of *S. frugiperda in vivo,* the *AT 1* gene was silenced by RNA interference and its effects on the development, the circadian rhythm of egg laying as well as on the JH and ecdysteroid hemolymph titers were measured. Effects of *AT 1* and *AS type A* gene silencing on the JH content in the male accessory glands (AG) and on the transfer of material into the female bursa copulatrix (BC) during copulation were also examined. To evaluate the efficiency of *AT 1* gene silencing on brains and ovaries of *S. frugiperda*, reverse transcriptase (RT)-PCR method was conducted. Silencing of *AT 1* and *AS type A* gene expression required multiple steps including amplification of their cDNA, gel electrophoresis, elution and purification, photometrically determination of DNA concentration, transcription of the DNA into ssRNA, digestion of template DNA, annealing of RNA, and injection of the purified dsRNA into insects, as described below in details.

2.1.1 Synthesis of DNA fragment

2.1.1.1 AT (allatotropin) 1 (*Manduca sexta* AT)

Polymerase chain reactions (PCR) were conducted to amplify a segment of DNA (from 397- 621 nt) that lies between two regions of a known sequence of AT 1 isoform A cDNA (Abdel-latief et al., 2003). Two oligonucleotides were used as primers, T7 ATF7 forward primer **5'-TAA TAC GAC TCA CTA TAG G↓GC TTC AAG GTC GAG ATG ATG ACC-3'** and T7 ATr9 reverse primer **5'-TAA TAC GAC TCA CTA TAG G↓GGC GAC CAC AGA TCG CGC GAA TTC-3'**, for a series of synthetic reactions that are catalyzed by a Taq-DNA polymerase. Both primers are prolonged at the 5`terminal ends of the final DNA fragment with a promotor sequence, which can be recognized by a T7 RNA polymerase. The forward and reverse oligonucleotides typically have different sequences and are complementary to sequences that lie on opposite strands of template dsDNA, and flank the segment of DNA that has to be amplified. The template DNA - in this case the T7AT fragment (provided by M. Meyering-Vos, Animal Ecology I, University of Bayreuth) - was first denatured by heating in the presence of a large molar access of each of the two

oligonucleotides and the four dNTPs (dNTP-Mix with dATP, dCTP, dGTP, dTTP; 2.5 mM Fermentas, St. Leon-Rot, Germany). The reaction mixture was then cooled to a temperature that allows the oligonucleotide primers to anneal to their target sequences, after which the annealed primers were extended catalysed by DNA polymerase. The cycle of denaturation, annealing, and DNA synthesis were repeated many times. Because the products of one round of amplification serve as template for the next, each successive cycle essentially doubles the amount of the desired DNA products. The major product of this exponential reaction is a segment of double stranded DNA whose termini are defined by 5' termini of oligonucleotide primers and whose length is defined by the distance between the primers. Primers used were synthesized by MWG Biotech Company, Ebersberg (Germany). For sensitive and appropriate detection of T7-Promotor sequences during *in vitro* DNA transcription the T7-Promoter sequences were labelled.

Taq-DNA-polymerase (1 U/µl) (Fermentas, St. Leon-Rot, Germany) was used, purified from the thermophilic bacterium *Thermus aquaticus*, which can survive extended incubation at 95°C, because it is not inactivated by heat denaturation step and does not need to be replaced at every round of the amplification as in case of in former times used *E. coli* DNA polymerase.

Under normal reaction conditions, the amount of Taq DNA polymerase becomes limiting after 25-30 cycles of amplification. The reaction was supplemented by $MgCl_2$ (25 mM) as a cofactor of the polymerase and buffered by 10 x Taq puffer with 750 mM Tris-HCl; 200 mM $(NH_4)_2SO_4$; 0.1 % Tween 20; pH 8.8 (Fermentas, St. Leon-Rot, Germany).

To amplify T7 AT 1 fragment the reaction was prepared in 50 µl by using sterile pipettes (Pipetman® P10, P20, P100, Gilson, Middleton, USA) with sterile pipette tips [10 µl pipette tips P2–10, Gilson®, Middleton, USA, 100 µl pipette tips Daslab®, 100 µl pipette tips Greiner bio-one, Frickenhausen, Germany]. Sterilisation was performed by autoclaving for 20 min in CertoClav CV-EL10 small field Labortechnik, Traun (Austria).

For all buffers and solutions sterile Milli-Q-H_2O was used (demineralization filter: Millipore 0.2 µm). The reaction was prepared in small PCR-tubes (0.2 ml) Brand, Wertheim (Germany), as described below (Table 1):

Sample	Volume (µl)
H_2O	34.5
Buffer 10x	5
dNTPs (2.5 mM)	4
$Mgcl_2$	3
T7 ATF7 primer (25 mM)	1
T7 ATr9 primer (25 mM)	1
T7AT template (~100 ng /µl)	0.5
Taq polymerase (Fermentas) 1 U/µl	1
Total volume	50

Table (1): Pipette scheme for the PCR reaction to amplify the T7 AT1 DNA fragment.

The mixture was shortly vortexed using vortexer REAX 2000 (Heidolph, Schwabach, Germany), centrifuged at 4000 x g and 25°C using centrifuge 5415C (Eppendorf, Hamburg, Germany), and run into serial of thermocycles during which the DNA template was denaturated, annealed by the pair of primers and elongated, using PCR Thermal Cycler T-Personal (Biometra, Göttingen, Germany), with following thermocycle profile (Table 2):

Step	Temperature (°C)	Time	No. of cycles
1	95	3 min	1
2 3 4	94 68 68	30 sec 45 sec 60 sec	10 x
5 6 7	94 60 68	30 sec 45 sec 90 sec	45 x
8 9	68 10	10 min ∞	1

Table (2): Temperature profile for the PCR reaction to amplify the T7 AT 1 DNA fragment.

2.1.1.2 AS (allatostatin) type A

A PCR method was used to generate the dsDNA templates for dsRNA synthesis corresponding to nucleotides 289 - 758 nt of the *S. frugiperda AS A-type* gene sequence (Abdel-latief et al., 2004). T7 promotor binding sites were added to the specific primers to get T7-sA1f **5' – TAA TAC GAC TCA CTA TAG GCC TCA CTA CGA CTT TGG - 3'** and T7-spoASTr1 **5' – TAA TAC GAC TCA CTA TAG GAG GCC GAAACT GAA ACC GTG CAT GTC - 3'**.

Mal 12 was used as a template (provided by M. Meyering-Vos, Animal Ecology I, University of Bayreuth), which encoded partly the Spofr-*AS-type A* gene sequence. It was ligated into a pGEM-Teasy vector. The reaction was prepared in 50 µl as described below (Table 3):

Sample	**Volume (µl)**
H_2O	34.5
Buffer 10x	5
dNTPs (2.5 mM)	4
$MgCl_2$	3
T7-sA1f primer (20 µM)	1
T7-spoASTr1primer (20 µM)	1
Mal 12 (~100 ng/µl)	0.5
Taq polymerase (Fermentas) 1 U/µl	1
Total volume	50

Table (3): Pipette scheme for the PCR reaction for amplification of DNA fragment from the template Mal 12.

The following thermocycle profile was used (Table 4):

Step	Temperature (°C)	Time	No. of cycles
1	95	3 min	1
2	94	30 sec	
3	68	45 sec	10x
4	68	60 sec	
5	94	30 sec	
6	62	45 sec	40x
7	68	90 sec	
8	68	10 min	1
9	10	∞	

Table (4): Temperature profile for the PCR reaction to amplify the AS A-type DNA fragment with Mal 12 plasmid as a template.

2.1.1.3 T7-SK fragment derived from the *sulfakinin* gene of *G. bimaculatus*

In RNAi suppression studies a nontarget control, which has not any counterpart at the nucleotide level in the observed organism, should be run in parallel. This accounts for effects evoked by unspecific reactions. In this study, a fragment of the *sulfakinin* gene of the cricket *Gryllus bimaculatus* was used.

Sulfakinin (SK) DNA fragment from *G. bimaculatus* elongated with T7 primers was amplified, primed by T7-SKf10 forward **5'- TAA TAC GAC TCA CTA TAG GG AAG CGC CCC TGC ACT CGC AC - 3'** and T7-SKr10 reverse primer **5'- TAA TAC GAC TCA CTA TAG GG ACT GCC TCT TGC TCA TCT CG – 3'**. The reaction was pipetted in a 50 µl reaction volume as described below (Table 5):

Sample	Volume (µl)
H_2O	34.5
Puffer 10x	5
$MgCl_2$	3
dNTPs (2.5 mM)	4
T7-SKf10 primer	1
T7-SKr10 primer	1
Template (~100 ng/µl)	0.5
Taq polymerase (Fermentas) 1 U/µl	1
Total volume	50

Table (5): Pipette scheme for the PCR reaction to amplify the T7 sulfakinin fragment.

The following thermocycle profile was used (Table 6):

Step	Temperature (°C)	Time	No. of cycles
1	95	4 min	1
2	94	30 sec	
3	67	1 min	1
4	72	2 min	
5	95	45 sec	
6	64	1 min	45
7	72	2 min	
8	72	10 min	1
9	4	∞	

Table (6): PCR temperature profile for amplification of the T7 sulfakinin fragment.

2.1.2 Gel electrophoresis

1.8% gels were casted by weighing 540 mg agarose (PeqGOLD Universal Agarose PeqLab, Erlangen, Germany) with analysis scale MC210P Sartorius, Göttingen (Germany), melting in the presence of 30 ml 0.5 x TBE puffer (Tris base, boric acid Sigma, Germany), 0.5 M EDTA (AppliChem, Germany), H_2O, pH 0.8, boiled in the

microwave (Bosch, Stuttgart, Germany) at 600 W for 1 min until the agarose was totally dissolved to get a clear, transparent solution. The most convenient method to visualize DNA in agarose gels is staining with the fluorescent dye ethidium bromide (Sharp et al., 1973). Two µl ethidium bromide (10 mg/ml water) (Promega, Madison, USA) was added to the gel for staining the bands shortly before the melted gel solution was poured into gel chambers and allowed to harden. Upon hardening, the agarose forms a matrix with defined density, which is determined by the concentration of the agarose in the solution. To make viscosity of DNA samples high and prevent degradation of the DNA samples, loading buffer (AppliChem, Darmstadt, Germany) containing bromo-phenol-blue as a dye, sucrose and EDTA was used. DNA solution was mixed with loading buffer added in a ratio of 5:1 (v/v) and transferred into the agarose gel slots. 12 µl 1000 bp DNA marker (fragment size: 100, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000 bp, Diagonal, Münster, Germany) was used. The gel was covered with 0.5 x TBE-buffer (445 mM Tris; 445 mM boric acid; 12.5 mM EDTA), scheduled with Milli-Q-water and adjusted to pH 8.0 (pH-Meter 766 Calimatic Knick, Berlin, Germany). Electrophoresis was run with electrophoresis power supply BioRAD power/PAC 3000 (Bio-Rad Laboratories GmbH, Germany) for 35 min and 5 V/cm. DNA, which is negatively charged at neutral pH, migrates towards the anode.

2.1.3 Visualisation of the DNA band and imaging

The location of DNA within the gel was determined directly by staining with low concentrations of the fluorescent dye ethidium bromide detected directly by puting the gel under UV light. “UV radiation at 254 nm is absorbed by the DNA and transmitted to the dye; radiation at 302 nm and 366 nm is absorbed by the bound dye itself which emits light around 600 nm presenting an orange colour” (Sambrook et al., 1989).

The DNA fragments were imaged by Image Master® VDS System (Version 3.0) Pharmacia Biotech, Freiburg (Germany), and analyzed using image master program. Photographs of gels were made using transmitted or incident ultraviolet light. The most sensitive film is Polaroid type 57 or 667 (ASA 3000) Roche Diagnostics, Mannheim (Germany). Photos were taken by “Image master system” (Amersham), saved and the intensity of bands was densimetrically analysed and the integrated density calculated.

Afterwards, the gel was observed under the ultra violet radiation, an UV-batwing radiator from Konrad Benda, Wiesloch (Germany) was used as protector from UV radiation. The target DNA band was cut out precisely, put into a 2 ml tube with known weight and the weight of the isolated gel part was determined by difference. DNA bands were recovered from the gel either utilized for dsRNA transcription used a long our RNAi and expression experiments or for Manse-AT fragment sequencing.

2.1.4 DNA purification

GFX® PCR DNA and Gel Band purification kit (GE health care, Freiburg, Germany) was used to extract the DNA bands from the gel according to manufacturer protocols. 10 µl capture buffer per 10 mg agarose was added to the gel and heated at 60°C for 5 min. The gel was completely dissolved. Using P1000 pipette (Gilson, Middleton, USA) and 1000 µl pipette tips (long tips) (Kisker Biotech, Steinfurt, Germany), 400 - 500 µl gel were transferred to a column and the column was centrifuged shortly at 10000 x g. Column was twice washed with 500 µl washing buffer, incubated for one min and centrifuged again for 1 min at 10000 x g. Then, 30 µl of elution buffer were added, incubated for one min and column was centrifuged for another 1 min to recover the purified DNA.

2.1.5 Determination of DNA concentration

Purified DNA diluted to a proper amount in 100 µl of highly purified milliQ water was measured using a spectrophotometer [UV/VIS Spectrometer Lambda 25 Perkin Elmer Instruments, Shelton, USA, UV Winlab Standard L 610-0025 Perkin Elmer (Version 4.0) Shelton, USA] at 260 nm wave length and a micro quarz cuvette from Hellma Analytics, Müllheim, Germany. One unit of optical density corresponds to 50 µg dsDNA/ml. In some experiments, DNA concentration was measured utilizing the Nanodrop Photometer®, Implen (USA).

2.1.6 *In vitro* transcription

Transcription is the circumscribing of a specific DNA into RNA. DNA template works as a matrix for the synthesis of the RNA cord. Pure DNA adjacent to promoter sites for DNA-dependent RNA polymerases can be used for *in vitro* transcription.

In a first step PCR was conducted to yield dsDNA and to generate the DNA fragments derived from the target lepidopteran sequence elongated with the proper promotor sequences. The DNA products were separated through gel electrophoresis and purified with GFX® PCR DNA and Gel Band purification kit. PCR was followed by generation of ssRNAs from both complimentary DNA-strands in one tube with the T7 MEGAscript® RNAi kit (Ambion, Huntingdon, UK). 0.8 -1 µg DNA (in a maximum of 6 µl) was used and completed to a final 20 µl of master mix, then the reaction tubes were shortly vortexed, centrifuged and afterwards incubated in an incubator (Haereus, Hanau, Germany) at 37°C overnight to transcribe the RNA; initiating synthesizing of big amount of RNA as possible. The reaction solutions were pipetted as described below (Table 7):

Solution	**Volume (µl)**
H_2O	Complete to total volume
ATP, CTP, GTP, UTP	Each 2
Reaction buffer 10x	2
Template band A	X, max. in 6 µl
Enzyme mix	2
Total volume	20

Table (7): Pipette scheme for the transcription reaction to generate AT 1 and AS A -type dsRNA. X = 0.8 -1 µg.

The incubation step was followed by a DNase digestion to remove the template DNA. 1 µl Turbo-DNase was carefully added to the reaction, mixed with tips, and incubated at 37°C for 15 min. Afterwards the DNase was denatured at 65°C for 15 min. For convenient precipitation of dsRNA and removing unincorporated nucleotides and most proteins, 15 µl nucleic acid free water and 30 µl of LiCl (Ambion, Huntingdon, UK) were added to the solution and mixed thoroughly.

The dsRNA was precipitated at -20ºC overnight followed by longer storage at -70ºC until use. For getting a good pellet the tubes were centrifuged at 4°C for 15 min at 12000 x g with the Multifuge 1 $_{LR}$ Heraeus Instruments, Hanau (Germany). Supernatant solution was discharged, the pellet washed with ice-cold 75**%** ETOH (p.a), mixed and centrifuged at 4°C for 5 min at 12000 x g. The pellet was dried at air

and resuspended in 50 µl RNase free water by pipetting up and down. A final denaturation for 5 min at 95ºC and an annealing step at room temperature overnight was followed. Quantification of dsRNA concentration was done by spectrophotometric determination using Nanodrop photometer. dsRNA was analyzed with gel electrophoresis to check the size and the success of annealing. Gel was prepared with the standard method but all solutions were prepared with RNase free water. 0.2 µg in 10 µl final sample volume was used for analysis.

2.1.7 Rearing of *S. frugiperda*

Pupae of *S. frugiperda* were kindly supplied by Bayer CropScience AG, Leverkusen (Germany). The animals were reared at long day conditions (16 h photophase: 8 h scotophase) at 27°C and a relative humidity of about 70%. Each pupa was individually kept in a separate compartment of assortment boxes (9 x 32 x 36 mm per compartment, Licefa GmbH & Co KG, Bad Salzuflen, Germany) until emergence, which was observed every ½ h during dark and light periods. Those insects that emerged were considered to be 0 day moths. Freshly emerged adult females and males were kept in 20 × 20 × 10 cm plastic boxes. Either two animals of same sex were put together or they were mixed in a 1:1 female to male ratio. They were fed with 10% sugar solution and given water. Filter paper was provided for egg laying. Larvae of *S. frugiperda* were fed with a food mixture for Noctuidae provided as dried food by Bayer CropScience AG, Leverkusen (Germany), which has been cooked in our lab.

2.1.8 Injection of *S. frugiperda*

1.5 µg dsRNA in 2 µl noctuid Ringer solution (7.1 mM $CaCl_2$, 22 mM Na-ß-glycero-phosphate, 13.5 mM $MgSO_4$, 29.6 mM $MgCl_2$ and 29.5 mM KCl, pH adjusted to 6.8 with KH_2PO_4 and filtered sterile with 0.2 µm meshes) or 2 µl noctuid Ringer solution as a control was injected abdominally into the third segment of adult moth directly after emergence or into the root of the last pseudopod of freshly moulted penultimate larvae, using Injections Syringes Microliter™ (10 µl) Hamilton, Bonaduz (CH).

2.1.9 Weight of ovaries and eggs and percentage of hatching

After injection of the moths (female or male) an untreated male or female was added, to each in a ratio of 1:1 for both sexes in separated Bellaplast boxes, according to the experimental purpose. The females were dissected at certain days from day 2 until day 7 after mating under binocular microscope (Binocular M7S, Wild, Heerbrugg, Switzerland). Decapitated animals were covered with modified cricket Ringer saline (86 mM NaCl, 5.4 mM KCl, 3 mM $CaCl_2$) (Lorenz et al., 1997), fixed with needles at the thorax and the abdomen was laterally opened by a sissor. The target tissues were withdrawn and adhering fat body or other tissues removed carefully. Clean ovaries were weighed using precision scale 1219 MP Sartorius, Göttingen (Germany).

For weighing 100 deposited eggs were collected by carefully removing egg packs from the box wall and cover and confirming their number under binocular microscope. The weighed eggs were kept in well closed plastic container until hatching (for rearing conditions see above) and the emerged larvae were counted.

2.2 Detection and analysis of hormones in the hemolymph

Many methods have been developed for measuring hormone production or hormone titers, such as rates of hormone biosynthesis by radiochemical assay or hormone titers by immunochemical methods or gas chromatography-mass spectrometry (GC-MS). In this work we used the liquid chromatograph-mass spectrometry method (LC-MS) developed by Westerlund and Hoffmann (2004), which is less time consuming and requires less solvents than other techniques, and makes it possible to measure JH homologs and their degradation products as well as ecdysteroids simultaneously.

2.2.1 Hemolymph and tissue collection for LC-MS analysis

Hemolymph was collected from adult moths by tapping the intersegmental membranes and collecting the leaking drop utilizing a micropipette (20 µl) (Blaubrand® intra Mark Brand, Wertheim, Germany). Since the adult moths bleed only marginally, the hemolymph had to be combined from several animals to yield the final 20 µl of hemolymph. Larvae were cut at the pseudopods and from one larva 20 µl could be collected. The hemolymph was transferred into a clean, tempered glass

tube (50 x 6-6.5 mm, Assistant, Sondheim/Rhön, Germany), heated at 240°C for 16 h in an oven T 5042K Heraeus, Hanau (Germany), which contained 100 µl methanol and 100 µl isooctane (for liquid chromatography, Merck KGaA, Darmstadt, Germany). The mixture was immediately vortexed for 20 sec and then incubated at room temperature for 20 min. For long time storage the vial was sealed with parafilm and aluminium foil and kept at -70°C.

Moths were dissected under binocular microscope, covered with modified cricket Ringer saline (Lorenz et al., 1997). Accessory glands or bursa copulatrix were isolated and adhering tissues carefully removed. Afterwards, the target tissue was transferred into a tempered glass tube which contained 100 µl methanol and 100 µl isooctane. The tissue was carefully ground by a small glass homogenizer (0.5 ml, Motor cordless Knots, Vineland, USA) for 90 sec, vortexed and incubated at room temperature for 20 min. The sealed extracted solution could be stored at -70°C until use.

2.2.2 Sample clean-up and hormone extraction

Hormones were extracted in multiple steps including removing of protein layer. Sample clean-up involves the precipitation of proteins by methanol/isooctane (1:1 v/v), centrifugation and partial evaporation of the organic solvents. Samples from -70°C were centrifuged at 1000 x g for 20 min, as a result solution separated into 3 phases. The upper phase of isooctane was transferred into a new glass tube and the resulting protein phase and methanol phase were re-centrifuged at 1000 x g for 5 min. The protein forms a pellet at the bottom of the glass and the overlaying methanol phase was taken and combined with the isooctane phase. This step was followed by evaporation of isooctane and concentrating the sample volume from approximately 200 µl to 20 µl using freeze-drying Christ alpha RVC 2-4 (Christ, Osterode, Germany). Afterwards the samples were again centrifuged at 1000 x g for 4 min. The final volume was determined by a Hamilton syringe and transferred into small autosampler vials (Carl Roth GmbH & Co KG, Wertheim, Germany).

2.2.3 Analysis and quantification of hormones

The simple, fast and sensitive liquid chromatography-mass spectrometry (LC-MS) developed for determination of juvenile hormones (JH), JH diols and JH acids in

insect hemolymph, as described by Westerlund and Hoffmann (2004), was used to quantify the JH titer of the various JH isoforms and of the free ecdysteroids. LC-MS apparatus composed of an autoinjector (SIL-10AD VP) and an Eldex MicroPro HPLC system with two HPLC pumps (LC-20AD) for liquid chromatography, coupled with an LCMS 2010A from Shimadzu, Duisburg (Germany). After extraction and clean-up of the hormones, the JH compounds and the ecdysteroids were separated on a C18 column (ReproSil-Pur ODS-3, 5 µm, Dr. Maisch GmbH, Ammerbuch, Germany) supplemented with a precolumn (C18, Phenomenex, Aschaffenburg, Germany) at 37°C by gradient elution of water (Merck KGaA, Darmstadt, Germany) - methanol (hypergrade, Merck KGaA, Darmstadt, Germany) at a flow rate of 200 µl/min. The elution gradient increased within 0-10 min from 30 to 100%, stayed at 100% from 10-15 min and then decreased within 15-16 min from 100 to 30% and stayed at 30% until minute 30. Samples were analysed by electrospray mass spectrometry (maximum of sample voltage 4.5 kV, detector voltage 1.5 kV and a CDL-voltage of 25 V, nitrogen flow 4 l/min, CDL-temperature 250°C and heating block 200°C). Due to the high abundance of Na^+ in insect hemolymph, $[M+Na]^+$ is primarily formed, but also the $[M+K]^+$ was detected. The limit of detection and quantification was 6 to 20 pg for JH, and 20 to 100 pg for ecdysteroids. Due to matrix effects, the calibration curve for each of the JH was compiled by spiking cricket (*G. bimaculatus*) hemolymph with a low JH-titer (0 d adult crickets) with standards JH I, JH II, and JH III (Fluka, Neu-Ulm, Germany). LC-MS software® (LabSolutions LCMSsolution, Version 3.0, Shimadzu Corporation, Duisburg, Germany) was used to analyse ecdysteriod and juvenile hormone titers as described by Westerlund and Hoffmann (2004).

2.3 Gene cloning

2.3.1 Introduction to T7 AT and T7 AS–type A DNA fragments cloning

Converting of poly $(A)^+$ mRNA into double stranded DNA and the convenient modern technique of cloning DNA into prokaryotic vectors have become fundamental tools of molecular biology. Different strategies were used to insert the double-stranded cDNA into a site that is closely flanked by two hexanucleotide restriction sites.

E. coli bacteria carrying recombinant plasmid DNA were grown in culture media to amplify T7 AT and T7 AS–type A DNA fragments. The process of gene cloning includes several steps. First the DNA fragment becomes amplified from cDNA (or DNA fragments) of the gene by means of a PCR, then the isolated DNA is ligated into a suitable vector and the recombinant plasmid transformed into competent microorganisms. Increasing (fermentation) of microorganisms is an important step of the gene cloning followed by extraction and purification of the plasmids out of the microorganisms (Sambrook et al., 1989).

2.3.2 Amplification of the DNA fragments

T7 AT 1 or T7 AS-type A DNA fragments, which were used along our research work, were checked and proven by cloning and sequencing. First DNA fragments were amplified, purified by gel electrophoresis, extracted by GFX® PCR DNA and Gel Band purification kit (GE health care, Freiburg, Germany) and photometrically quantified as described before (see 2.1.1 and 2.1.2).

2.3.3 Ligation

Gene pJET™ cloning Kit K 1231, K 1232 (Thermo Scientific, Germany) was used for cloning of T7 AT or AS-type A DNA fragments generated by PCR. Prior to ligation the DNA fragment was gel-purified and treated with a proof reading DNA polymerase to get blunt ends used in a 3:1 molar ratio with the plasmid PJET 12/blunt. The DNA blunting enzyme is a proprietary thermostable Taq DNA polymerase with proof reading activity. It removes 3´- overhangs and fills in 5´- overhangs. Nucleotides for the blunting reaction were supplied in the reaction buffer included in the kit.

1. Set up blunting reaction (Table 8):

Component	Volume recommended	Volume used
2x reaction buffer	10 µl	5 µl
PCR products	1- 2 µl	1 µl
Water, nuclease free	Up to 6 µl	2.5 µl
DNA blunting enzyme	1 µl	0.5 µl
Total volume	18 µl	9 µl

Table (8): Components of blunting reaction.

The blunting reaction was set up, vortexed briefly and centrifuged for short interval.

2. The mixture was incubated at 70°C for 5 min and chilled briefly on ice.

3. Ligation reaction was set up. The following solutions were added (Table 9).

Component	Volume recommended	Volume used
PJET 1.2/ blunt cloning vector (50 ng/µl)	1 µl	0.5 µl
T4 DNA ligase (5 U/µl)	1 µl	0.5 µl
Total volume	20 µl	10 µl

Table (9): Components of ligation reaction.

4. The ligation reaction was incubated at room temperature (22°C) for 5 min.

5. The ligation solution was used directly for bacterial transformation.

2.3.4 Competent cells

E. coli bacteria cells introduce DNA under normal conditions only in limited amount. However, to transform such types effectively, those cells were subjected to physical and/ or chemical treatments in order to become competent cells (Sambrook et al., 1989).

Bacteria were manipulated in fluid cultures of LB medium (Luria-Bertani-Medium) [1 l desalted water, 5 g yeast extract (AppliChem, Germany), 5 g trypton (ApplieChem, Germany),10 g NaCl**,** stirred and autoclaved]. 250 µl of a start suspension of E. *coli* JM109, Genotype: *rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17 (rK-, mK+), *rel*A1, *sup*E44, _

(*lacpro*AB), [F', *tra*D36, *pro*AB, *lac*lqZ_M15] Promega, Madison (USA), which was stored at -70℃, was used for inoculation of 50 ml LB-medium in a 500 ml flask and incubated at 160 x g and 37℃ for about 3 h. The amount of grown bacteria was checked at 550 nm after one hour and then every 15 min using 1 ml plastic cuvettes, until the growth curve reached 0.5 OD/550 nm. The tube was incubated on ice for 10 min and then centrifuged by 4000 x g for 10 min at 4℃. The liquid was carefully discarded and the pellet was resuspended in 10 ml cold freshly prepared 0.1 M $CaCl_2$ (20 ml steril milliQ water and 0.294 g $CaCl_2$) using sterile pipette with plastic sucker. Then the suspension was incubated on ice for 10 min and centrifuged at 4000 x g and 4℃ for 10 min. The liquid was carefully discarded and the white pellet was resuspended with 2 ml 0.1 M $CaCl_2$, 15% (v/v) glycerol.

Under a clean bench the resuspended solution (competent cells) was divided into 0.2 ml portions in reaction tubes (1.5 ml), shock frozen under liquid nitrogen and stored at -70° C until use.

2.3.5 Transformation

E. coli under normal condition is sensitive to the antibiotic ampicillin; they are carrying a gene that codifies ß-lactamase enzyme (Sambrook et al., 1989). However, each of the plasmid cDNA that encodes *lend* gene could inactivate ß-lactamase enzyme gene. Vector good receptivity of recombinant plasmid DNA converts *E. coli* to ampicillin resistant cells (Sambrook et al., 1989).

All the steps for transformation are performed under sterile conditions and were conducted in a clean bench. Pipette tips Pipetman® P P10, P20, P100, P200, P1000 (Gilson, Middleton, USA) were autoclaved for 20 min.

Agar LB - ampicillin plates were prepared one day before transformation in multiple steps:

SOB – medium was prepared from 20 g tryptone, 5 g yeast extract (AppliChem, Darmstadt, Germany), 0.5 g NaCl, 0.19 g KCl, 900 ml deionised water, pH adjusted to 7 and solution complemented to 990 ml with water, autoclaved and cooled to room temperature. Finally 10 ml steril 1 M $MgCl_2$ was added. SOC media was prepared from 450 µl SOB + 9 µl Glucose.

For the transformation, 5~100 ng (5 µl) of ligated plasmid were added to 2 ml competent cells [prepared according to the Hanahan method (Sambrock et al., 1989) and provided by the group of M. Meyering-Vos, University of Bayreuth], incubated on

ice for 30 min, heat shocked at 42°C for 2 min and followed by cooling for 2 min on ice. 409 µl SOC-medium was added to the tube, suspension was incubated at 37°C and 160 rpm in a shaker placed in a Thermo Heraeus Heracell 150 Incubator (Trading Company, USA) for 1 h. 200 µl and 400 µl from each sample was transferred onto LB-medium agar ampicillin plates (LB-plates with autoclaved LB-Agar, prepared by F. Wende, Bayreuth). Media were cooled down to nearly 60°C, 1 µl ampicillin solution (100 mg/ml in water) per 1 ml medium were added and the media were poured into petri dishes with 10 cm in diameter. The agar had been coated with 20 µl of 20 mg/ml X-Gal (5-bromo-4-chloro-indolyl-ß-D-galactopyranoside) from AppliChem, Darmstadt (Germany), dissolved in dimethyl formamide, and 100 µl IPTG-solution (isopropyl-β-D-thiogalactoside), IPTG AppliChem, Darmstadt (Germany) (100 mM in H_2O) and spread all over the LB-Agar Luria-Bertani-Medium with 1.5 % (w/v) plate by Drigalski spatula. IPTG is constituted of 100 mM IPTG in sterile H_2O/K-acetate-solution (Puffer N3) [3 M K^+, 5 M Ac^-, with acid tendency (pH 4.8) = 60 ml of 5 M KAc + 11.5 ml of HAc + 28.5 ml of H_2O)].
The Petri dishes were well closed and incubated at 37°C overnight. Plates could be stored for 2 weeks at 4°C.
Blue/white screening of the grown colonies has been conducted (Sambrook et al., 1989).

2.3.6 Fermentation

Plasmids were purified from liquid LB-media cultures containing the appropriate antibiotic. Three ml solutions of LB medium containing 3 µl ampicillin (100 mg/ml H_2O_{steril}) in each sterile tube were inoculated with a single white bacterial colony picked from an agar plate, incubated at 37°C and stirred at 240 x g on a shaker placed in a Thermo Heraeus Heracell 150 Incubator (Trading Company, USA) overnight. 1.5 ml transformed *E. coli* JM109 culture, in a 2 ml reaction tube, was centrifuged for 5 min by 4000 x g at room temperature. Liquid was discarded and the pellet collected.

2.3.7 Purification of plasmid DNA

In this work, Gene pJET™ Plasmid Miniprep Kit (Fermentas, St. Leon-Rot, Germany) was used for isolation of the plasmids out of the bacterium cells according to the

protocol of the manufacturer. All the steps were conducted at room temperature. The dried pellet was resuspended in 250 µl of the lysis solution, vortexed by pipetting up and down. 350 µl of the neutralization solution were added and mixed thoroughly by inverting the tube 4-6 times and centrifuged at 8000 x g and 25°C for 5 min to get pellet with cells debris and chromosomal DNA. The supernatant was transferred to the supplied GeneJET™ spin column by decanting and centrifuged for 1 min; the flow through was discarded and the column was placed back into the same collection tube. 500 µl of wash solution were added to the column and centrifuged for 30-60 sec and the flow through was discarded. The washing step was repeated two times, the flow through was thrown away and the column was centrifuged to remove residual wash solution. The GeneJET™ spin column was transferred into a new 1.5 ml tube, 50 µl of elution buffer was added to the spin column, incubated 2 min and centrifuged for 2 min at 12000 x g. The concentration of the eluted DNA was photometrically determined.

A PCR test was conducted in order to control whether the target DNA fragment was inserted. The DNA was used for sequencing at GATC (Biotech, Konstanz, Germany).

2.4 *AT 1* gene expression studies

2.4.1 RT-PCR

The reverse transcription polymerase chain reaction (RT-PCR) is the most precise method detecting even low abundance of mRNA obtained from very small tissues. “Many problems are associated with the complicity of the technique, beside those problems that are inherited in PCR” (Bustin, 2000).

In order to ascribe the effectiveness of the RNA interference on the suppression of the transcript level, the *AT 1* gene expression was examined by RT-PCR. The RNA was isolated from brains and ovary tissues, and the polyA$^+$ RNA circumscribed first of all into cDNA using oligo-dT primer. For precise quantification, the cDNA has been amplified then with gene specific primers in a computerized PCR.

2.4.2 Tissue dissection

For gene silencing the moths were injected with AT 1 or AS-type A dsRNA as described before (see 2.8.1). An additional group of animals was injected with the nonhomologous SK dsRNA derived from the gene *sulfakinin* of *G. bimaculatus*, which served as a negative control in the expression studies. At defined age, virgin female moths of *S. frugiperda* were decapitated and covered with modified cricket Ringer saline (Lorenz et al., 1997) and dissected under binocular microscope. Tissues, as brain and ovaries, were isolated and adhering tissues were carefully removed. Tissues were kept in safe-lock Eppendorf tubes (Eppendorf AG, Hamburg, Germany) and were flash frozen using liquid nitrogen and stored at -70º C until use.

2.4.3 Total RNA extraction from the tissues

Total RNA was extracted from 50 mg of each homogenized tissue utilizing PrepGold PureRNA® kit (peQlab, Erlangen, Germany) according to manufacturer’s instructions. Pooled tissues of 40 brains or 20 ovaries were homogenized with a tissue homogenizer (Kontes, Vineland, USA) fitted into 1.5 ml Eppendorf tube. At first 250 µl PrepGold PureRNA® kit (peQlab, Erlangen, Germany) solution was added to the tissues, homogenized for 1.5 min using stop watch (Rotilabo-Signal-Timer, Roth GmbH, Germany), and then 750 µl extraction solution was added and the tube vortexed.

The mixture was incubated at room temperature for 5 min. 0.2 ml chloroform (p.a.) (Sigma, Fisher Scientific, UK) was added, well shaken for 15 sec and incubated 3-10 min on ice (4°C). Afterwards the solution was centrifuged for 5 min at 4°C and 12000 x g. Thereby the solution separated into three phases.

The upper watery phase was transferred to a new tube; the same volume of isopropanol (0.5 ml) was added and centrifuged at 4°C with 12000 x g for 15 min.

Suspension was carefully discarded using 200 µl pipette tips and the pellet washed with 75% (v/v) ethanol (Sigma, St. Louis, USA). The RNA pellet was dried shortly under air.

The RNA was extracted in combination with an RNase-free DNase treatment (Qiagen, Hilden, Germany). The totally dried pellet was resuspended through multiple steps of sucking and discharging with pipette tips in 50 µl RNase free water and the solution was improved by heating at 55-60°C.

2.4.4 DNA digestion

The quantity of the extracted RNA was verified by spectrophotometry. To eliminate potential genomic DNA contamination, the DNase I (Fermentas, Life Sciences, Germany) was used to digest the DNA (Table 10). The reaction was activated by 10 x DNase buffer 100 mM Tris; 25 mM $MgCl_2$; 5 mM $CaCl_2$; pH 7.5; Fermentas, St. Leon-Rot (Germany).

Solution	**Volume [µl]**
RNase free water	X
RNA	1 µg, max. 8 µl
DNase buffer	1
DNase I (Fermentas)	1
Total volume	10

Table (10): Pipette scheme for genomic DNA digestion.

The solution was mixed by pipetting without vortexing, and incubated at 37°C for 30 min. Then 1 µl of EDTA (25 mM in RNase free water, pH = 8) was added and mixed by pipetting. DNase was inactivated by incubating the solution for 10 min at 65°C and stored at -70°C until use.

2.4.5 Reverse transcription

500 ng of the extracted total RNA were used for reverse transcription, primed by oligo (dT) in a final reaction mixture volume of 10 µl as described in the provided protocol of high capacity cDNA Reverse Transcription® kit (Applied Biosystem, Warrington, UK). The first strand cDNA was synthesized after hybridization of the oligo (dT) primer to the 3`poly (A) tails of the poly A^+ RNA.

500 ng of total extracted RNA from brains and ovaries were reverse transcribed under the assumption that the polyA^+ RNA is transcribed only one time. During constant reaction conditions the yielded cDNAs reflect that amount of poly A^+ RNA, which is contained in a defined amount of RNA. The master mix (Table 11) was produced by pipetting dNTP-Mix (100 mM), RNase inhibitor, Multiscribe reverse transcriptase and 10 x RT buffer from Fermentas, St. Leon-Rot (Germany), while oligo(dT) primer (0.5 µg / µl) was used from Promega (Madison, USA) and RT-PCR Grade Water introduced by Ambion (Austin, USA).

Solution	**Volume [µl]**
10x RT buffer	1.0
25 x dNTP mix (100 mM)	0.4
Oligo dT	0.5
MultiScribe™ reverse transcriptase	0.5
RNase inhibitor	0.5
Nuclease free H_2O	1.6
RNA (DNA digested)	5.5
Total volume	10

Table (11): Pippette scheme for reverse transcriptase reaction.

The reaction was mixed carefully with tip and placed into the thermal cycler with following program (Table 12):

Temperature (℃)	Time (min)
25	10:00
37	120:00
85	0:05
4	∞

Table (12): Temperature profile for reverse transcription PCR reaction.

2.4.6 Synthesis of standard curves

2.4.6.1 Amplification of the AT 1 fragment

For an evaluation of the expression data, manufacturing of external standard curves is necessary, which should be generated with a DNA fragment as a template composed of a part of the target gene sequence (Table 13). The plot of log of initial copy number of the DNA product versus ct (threshold value) of the PCR reaction is a straight line that represents the standard curve. In this work we used AT 1 and ß-actin RNAs as standard curves.

The *AT 1* gene has three mRNAs which differ from each other by alternative splicing, the AT isoforms A, B and C (Abdel-latief et al., 2003). For the amplification of the AT fragment, the primers AT5prf **5'- CAT TTC AAT GCA TTT GGC GGT AGC- 3'** and AT5prR **5'- CCC TGG CGG TCA TCA TCT C- 3'** were used. The primers address a gene sequence area, which covers all three mRNA isoforms.

Sample	Volume (µl)
H_2O	34.5
Puffer 10 x	5
dNTPs (2.5 mM)	4
$Cacl_2$	3
AT5prf primer	1
ATprR primer	1
Mal 7 fragments	0.5
Taq polymerase (Fermentas) 1 U/µl	1
Total volume	50

Table (13): Pipette scheme for amplification of standard templates.

2.4.6.2 Amplification of the ß-actin fragment

The internal standard should be expressed at a constant level in different tissues of an organism. RNA from housekeeping *ß-actin* gene was used to normalize pattern of *AT 1* gene expression. For analysis of *ß-actin* gene expression a standard curve was run in parallel. The standard template for such a PCR was generated by using Actf **5'- CCT CAA CCC TAA GGC CAA CAG G - 3'** and Actr **5'- CCA TCA CCG GAG TCC AAG ACG - 3'** primers and cDNA as a template (Table 14). For the optimization of this PCR reaction composed of 50 µl volume 1 µl of the cDNA was pipetted as the template. The concentration of the used primers was 5 µM. The PCR was run in a $T_{personal}$ Thermocycler (Biometra, Analytical Jena Company) with a defined thermo profile (Table 15).

Sample	**Volume (µl)**
H_2O	4
Puffer 10 x	1
dNTPs (2.5 mM)	1
$Cacl_2$	1
Actf primer	0.5
Actr primer	0.5
cDNA 1/10 (5 ng)	1
Taq DNA polymerase 1 U/ µl (Fermentas)	1
Total volume	10

Table (14): Pipette scheme for standard ß-actin amplification.

Step	Temperature (°C)	time	No. of cycles
1	95	3 min	1 x
2	94	30 sec	
3	68	41 sec	-1°C 10 x
4	68	60 sec	
5	94	30 sec	
6	60	45 sec	45 x
7	68	90 sec	
8	68	10	1 x
9	10	∞	

Table (15): Thermocycler programm SP3 was used to amplify ß-actin standard template.

ß-Actin and AT 1 PCR products were yielded in the range of 143 and 150 bp, respectively. The synthesized AT 1 and ß-actin fragments were analysed by gel electrophoresis, their bands were cut out precisely, photographed, purified from gel and their concentrations were measured using nanodrop photometer as described before (2.1.2- 2.1.5).

2.4.7 Optimization of standard curves for real-time PCR

The choice of suitable DNA polymerase and buffers were considered, and contamination tests were carried out for all samples and standards to check and avoid any contamination during the expression analysis. The amplified DNA fragments of the real-time PCR were analysed (10 µl aliquot out of PCR) by means of gel electrophoresis on an agarose gel.

At first, various concentrations from the AT 1 and ß-actin templates were generated as described (2.4.6.1 and 2.4.6.2) and were used in the real-time PCR to analyse the effectiveness and purity, as well. 10 µl of the pipetted reaction solutions (Table 16) were transferred to Optical 8-Tube strips (0.2 ml) from Applied Biosystems (Foster City, USA) that were covered by Optical Cups (Applied Biosystems, Foster City, USA). After set up the samples were centrifuged shortly by Micro20 centrifuge Hettich (Noblesville, USA). The optimal number of PCR cycles for each sample was

determined by analyzing the amount of PCR products after a series of PCR amplifications with accelerating cycles from 35 to 40 by gel electrophoresis.

Solution	Volume (µl)
PowerSYBR® Green PCR Master Mix	10
H_2O	7
Forward primer 5 µM	0.5
Reward primer 5 µM	0.5
Template (AT 1 or ß-actin DNA fragments) 100 nM	2
Total volume	20

Table (16): Pipette scheme for the reaction used for real-time PCR standard curve optimization.

Then, standard curves using SYBR Green I as a fluorescence dye, were generated, regarding their linear response over a large dynamic range by performing 10 samples with DNA concentrations between 2 pg and 0.002 pg running 40 PCR cycles (Table 17). All the results that gave positive products peaked in the melting curves between 87.4℃ and 88.2℃. The cDNA concentrations of 2 pg to 0.002 pg showed a linear range of amplification with an efficiency value (E) of 99.9 % and were chosen as AT1 and ß-actin standards for further experiments. Amplification of four independent serial dilutions of the cDNA constitutes the standard curve. Additionally, a negative control reaction by leaving out the template was run in parallel.

In general, the highest and lowest ct (cycle threshold) values were discarded to correct for pipetting errors and the remaining four values were averaged to give the final ct value for that defined dilution. The ct value is inversely proportional to the log of the initial copy number. Therefore, a standard curve is generated by plotting the ct values, with 99% confidence intervals, against the decade logarithm of the initial copy number.

Step	Temperature (°C)	Time	
1	95	3 min	
2	94	30 sec	40 x
3	62 (for AT and ß-actin)	45 sec	
4	68	90 sec	
5	68	10 min	
6	4	∞	

Table (17): PCR temperature profile program for amplification of standard AT 1 and ß-actin DNA fragments.

2.4.8 Real-time PCR

Reverse transcription polymerase chain reaction (RT-PCR) is a reliable technique measuring precise differences between mRNA levels among samples (Shiao, 2003). Two common methods were used to analyze the data for absolute and relative quantification. Absolute quantification determines the input copy number, usually by relating the PCR signal to a standard curve. Relative quantification relates the PCR signals of the target transcript in a treatment group to that of another sample such as untreated control. The $2^{-\Delta\Delta ct}$ method is a convenient way of analysing changes in gene expression relatively (Livak and Schmittgen, 2001).

Real-time PCR reactions were performed in triplicates each in a 20 µl reaction mixture volume (Table 18) following the manufacturer's instructions for the PowerSYBR® green PCR MasterMix (Applied Biosystems, Warrington, UK). They were run on an ABI Prism 7300 sequence detection system (Applied Biosystems).

Solution	Volume
PowerSYBR® Green PCR Master Mix	10 µl
H_2O	7 µl
Forward primer (5 nM)	0.5 µl
Reverse primer (5 nM)	0.5 µl
Template: cDNA	2.0 µl
Total volume	20 µl

Table (18): Pipette scheme for real-time PCR reactions to amplify fragments of the AT 1 or ß-actin cDNA. Forward/reverse primer: AT5prf/AT5prR or sfActf/ sfActr, respectively.

Two µl of the reverse transcription reaction mixture were used as template according to cDNA generated from 80 ng total RNA. To reduce differences during cDNA synthesis step, all RNA samples have been reverse-transcribed simultaneously. In parallel, reactions were run to detect genomic DNA contaminations by using 2 µl of the prepared template solution described before (see 2.4.5.). Additionally, negative controls by leaving out the templates were performed. Standard curves for the AT 1 and endogenous controls were generated by four serial dilutions from the cDNA of ß-actin and AT 1 and included in each real-time PCR run. All these reactions were set up in Optical 96 well reaction plates from Applied Biosystems (Foster City, USA). 96 well plates were covered with Optical Adhesive Film Kit from Applied Biosystems (Foster City, USA) and shortly centrifuged at 22°C and 4000 x g with a 96 well plate rotor adapted to Centrifuge 5415C (Eppendorf, Hamburg, Germany).

The thermal cycling profile (Table 19) was run on the 7300 Real-time PCR System connected to a Dell Laptop (Applied Biosystems, Foster City, USA).

Step	**Temperature (°C)**	**Time (min)**	**No. of cycles**
1	95	1:00	1
2	95	0:15	41
	62	1:00	
3	68	10:00	1
4	95	0:15	1
Dissociation	60	1:00	
stage	95	0:15	

Table (19): Real-time PCR thermocycler program.

The 7300 Real-time PCR System is able to detect fluorescence emission between 500 nm and 660 nm. The resulting fluorescence is induced during RT-PCR by interaction of SYBR Green with the DNA products of the PCR reaction. The quantification of gene expression can be achieved by plotting fluorescence against cycles and compare the sample signal with that of standards at cycle threshold.

2.4.9 Analysis of the real-time RT-PCR

In this study, the expression of synthesized AT 1 was measured as transcript levels in tissues normalized against that of the *ß-actin* housekeeping gene. The analysis of *AT 1* gene expression was carried out by both, absolute and relative quantification methods. The absolute values of the target transcript levels of AT 1 were normalized to that of the reference gene *ß-actin*. The SBYR Green I (Applied Biosystem) was used for quantification of differentially expressed genes. C_t (threshold cycle) values, defined as the fractional cycle number at which the fluorescence passes a fixed value, were used to estimate relative concentrations of target sequences (Livak and Schmittgen, 2001; Pfaffl, 2001). By measuring the threshold values for samples of known concentration, standard curves were produced. The linear range of amplification represents the range over which the logarithm of the target concentration versus the threshold value forms a linear relationship. The slope of the

standard curve over the linear range was used to determine the amplification efficiency, using the following equation:

$$\text{Efficiency} = 10^{(-1/\text{Slope})} - 1$$

To ensure that the expression in different samples differed very strongly, long PCR thermocycler programs had been used.

The analysis took place, therefore, for all samples via the standard curve and the basis line, which is grasped in the real-time PCR by the device automatically during the background phase of the PCR, was set for all 96 well plates discs automatically by means of the SDS 7300 Systems Software Applied Biosystems, Foster City (USA) related to the analysis. Afterwards, the threshold was set manually for each disc within the logarithmically linear phase. In addition, the logarithmic view in the amplification plot was selected, in which exclusively this phase of the PCR is represented. Out of the standing curve generated by the program automatically on the basis of the start copy number for each dilution, the quantity in all samples was calculated.

Additionally a relative quantification method was used. For the calculation to be valid, the efficiencies of the target and reference must be approximately equal. In that case the amount of target DNA products normalized to an endogenous reference control and relative to a calibrator, is given by $2^{-\Delta\Delta C}{}_T$ (Livak and Schmittgen, 2001). Briefly, ΔC_T (C_T differ value) is calculated for each sample by subtracting the average C_T value obtained from several PCR replicates of an endogenous control (in this study ß-actin) from the average ct value obtained from target sequences calibrator (in this study the AT 1). The ΔC_T value obtained for the calibrator (in this study Ringer controls) is then used to calculate concentrations in each sample relative to the calibrator.

Steps of calculation:

(1) Calibrator = C_T goal gene – C_T reference gene (ΔC_T calibrator)

(2) Sample = C_T goal gene – C_T reference gene (ΔC_T reference)

(3) C_T Sample – C_T Calibrator = ($\Delta\Delta C_T$)

(4) Relative Expression = $2^{-\Delta\Delta C}{}_T$

With goal gene = *AT 1*; reference gene = *ß-actin*; sample: dsRNA injected; calibrator: Ringer injected control.

2.5 Survey on data collection

The following data were recorded after gene silencing:

1. Relative *AT 1* transcript levels using RT-PCR in combination with real-time PCR were measured.
2. Mortality among injected *S. frugiperda* larvae and adults was followed.
3. Parameters of larval last instar development were followed (larval weight gain, prepupation, pupation, metamorphosis).
4. Hemolymph samples were collected from L5/3, L6/1, L6/2, L6/3, L6/4 larvae and prepupae and hormone titers were measured.
5. Circadian rhythms of adult emergence, mating behaviour and copulation duration were recorded.
6. Oviposition of mated females during the first 10 days of adult live after injection of dsRNA was determined. Either females or males were treated directly after the imaginal moult.
7. Oviposition of virgin females during the first 14 days of adult live after injection of dsRNA at imaginal moult was recorded.
8. Hemolymph samples were collected on days 2, 4, 6, and 8 after ecdysis from virgin and mated females for JH measurements. JH was also quantified in the bursa copulatrix of females and in the reproductive accessory glands of the males before and after mating.
9. Egg weight and larval hatching after treatment of the females or males with dsRNA was observed.

2.6 Statistical analysis

For the statistical analysis, the Sigma Plot ®version 11 (Systat Software Inc. (USA) was used. Normally distributed data were statistically analysed using Microsoft® Office Excel 2003 (Microsoft Corporation 2003), then analysed with the student's t-test in order to compare two test groups. In the case of not normally distributed data or when using percentage values a statistical analysis with the Mann-Whitney U-test was used. Statistical significant values are marked by asterisks and are shown as:
** = P <0.05; ** = P < 0.01; *** = P < 0.005; **** = P < 0.001.*

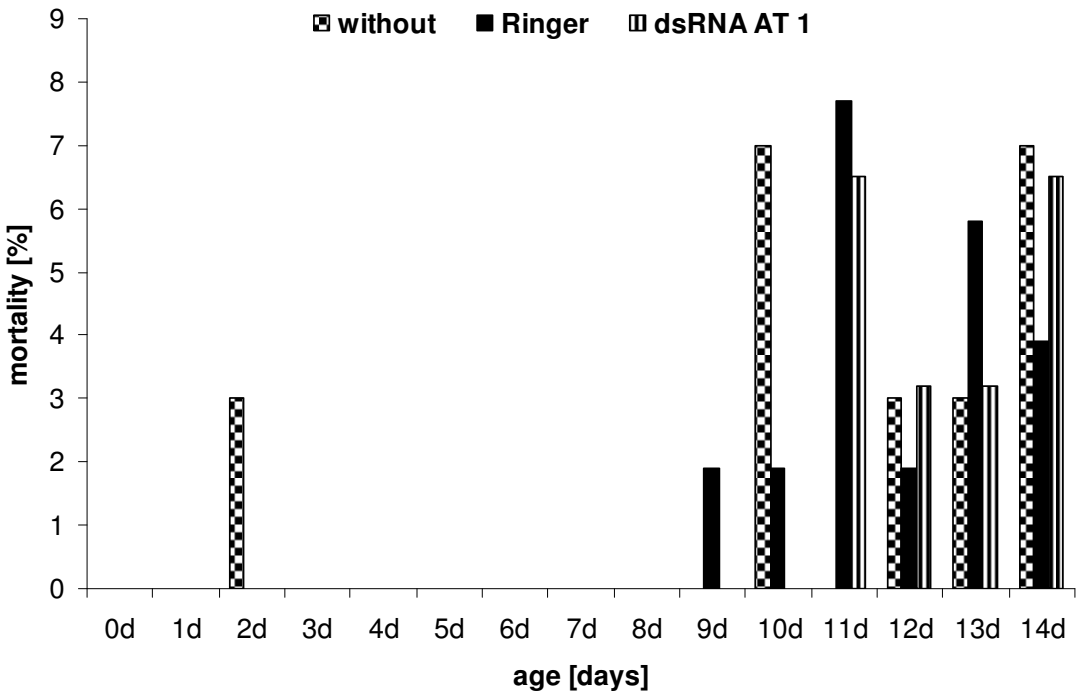
3 Results

3.1 Effects of *in vivo* gene silencing of *AT 1* in females of *S. frugiperda* on transcript level in tissues, metamorphosis, circadian rhythm of adult emergence, reproduction and hormone levels in the hemolymph

3.1.1 Mortality

The virgin females of *S. fruigperda* were reared individually either without treatment, or injected with 2 µl noctuid Ringer or 1.5 µg dsRNA AT 1 in 2 µl noctuid Ringer on day 1 after ecdysis. The percentage of mortality was calculated over 14 days. Few virgin untreated females died on day 2. Injection of dsRNA AT 1 into the adults did not cause any differences in the range and peak of mortality in comparison with controls. The profile of mortality in virgin females is shown in **Figure 1A**. The accumulated mortality of virgin females after 14 days was 22.4% for untreated females, 23.1% for Ringer injected controls and 20.1% for *AT 1* gene silenced animals **(Figure 1B)**.

(A)



(B)

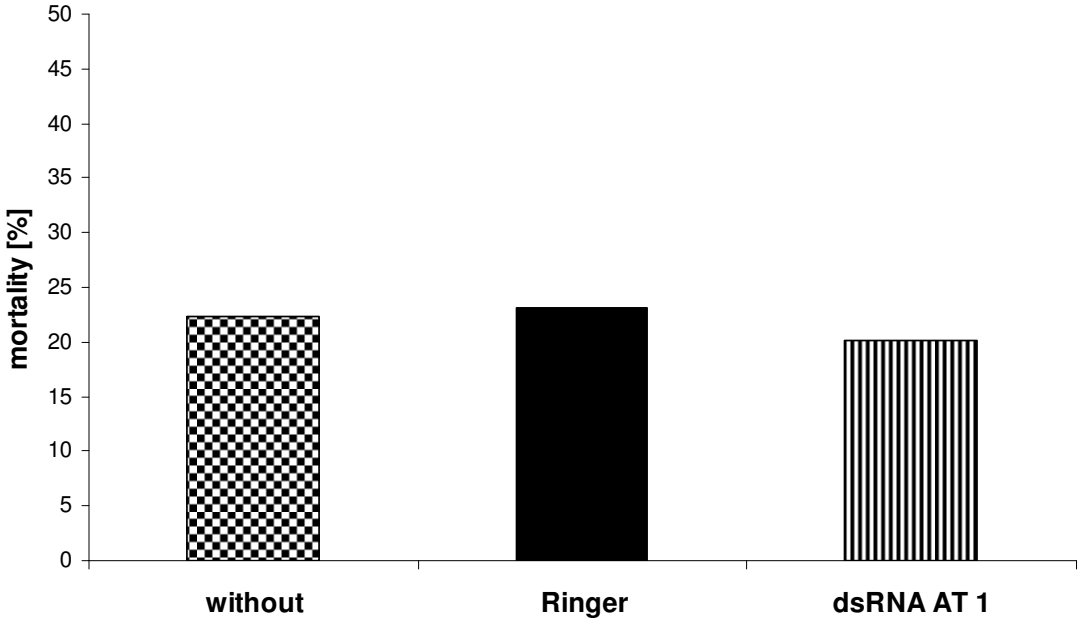


Figure (1): Daily percent mortality of virgin *S. frugiperda* females (A) and (B) accumulative mortality over 14 days of adult life. Virgin females were either without treatment or injected with 2 µl noctuid Ringer or 1.5 µg dsRNA AT 1 in 2 µl noctuid Ringer. 10% sucrose, water and filter paper for egg deposition were supplied. n without treatment = 31, Ringer = 55, dsRNA AT = 69.

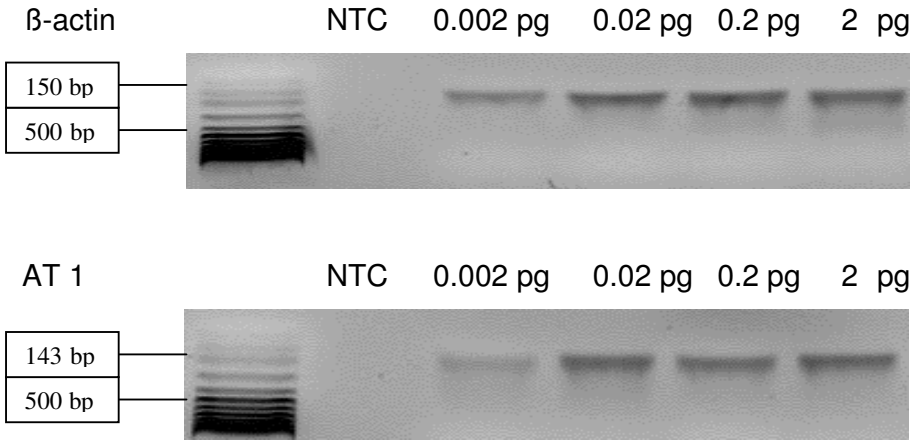
3.1.2 Transcript levels in brain and ovary

Highly precise two step, real-time polymerase chain reactions (RT-PCR) were conducted in triplicates for target gene (*AT 1*), negative gene (*SK*) and Ringer controls. The absolute quantities of mRNA were normalized to the transcript level of the housekeeping gene *ß-actin*. The relative quantification took place by determination of the ct values, which were standardized using AT 1 and ß-actin standard curves. We used both absolute and relative quantification in our experiments.

Absolute and relative method of calculations of results, after the injection of dsRNA AT 1 into the virgin adult females, yielded a reduction of the target mRNA in brain (76.2% and 98.5%*, P < 0.05.)* and ovary (66.7%, *P < 0.01* and 83.3%*, P < 0.05),* respectively **(Figures 2 and 3)**. The negative controls performed by injection of sulfakinin dsRNA from *Gryllus bimaculatus* showed similar transcript levels in brain and ovary to that injected with noctuid Ringer. This indicates that the effect of the RNAi is not locally limited but systemic, and that the specificity of the method is high.

Further controls were performed for larvae and adults of various age and sex in our laboratory, but are not shown here.

(A)



(B)

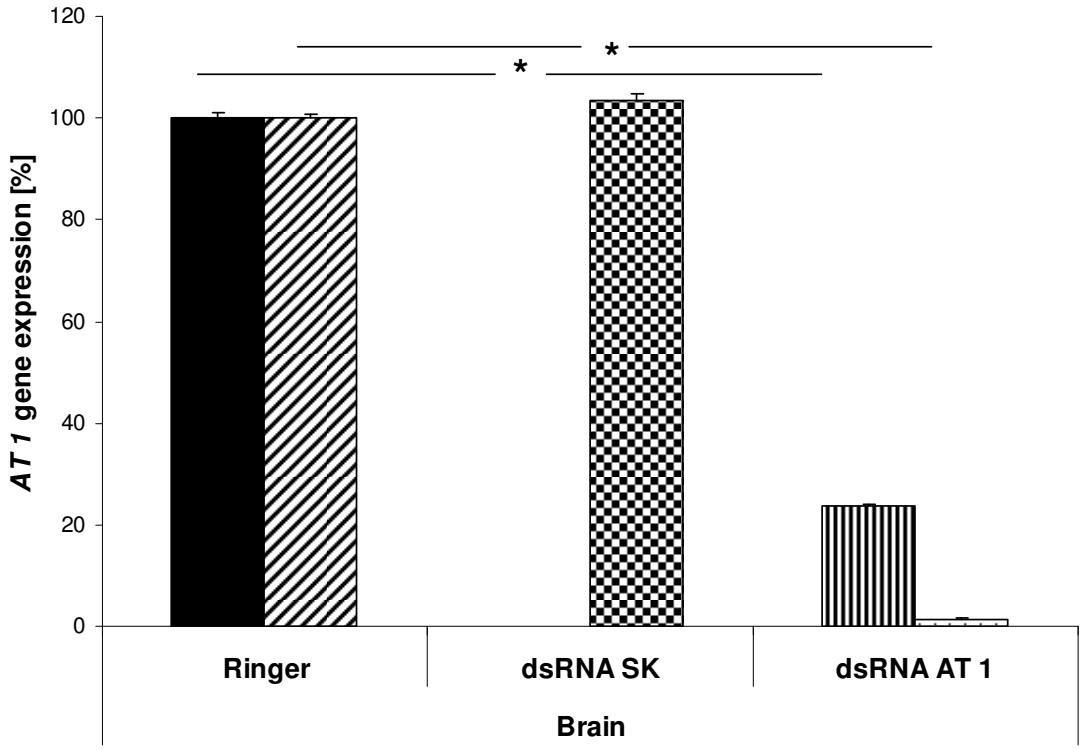


Figure (2): Gene expression of *Manse-AT* (AT 1) after silencing the gene transcript level in the brain of 2 day old virgin females of *S. frugiperda* (B). Newly eclosed females were injected with either 1.5 µg dsRNA AT 1 or dsRNA SK in 2 µl noctuid Ringer or 2 µl noctuid Ringer. 40 brains from 2 day old virgin females were used for the experiment. Results were analyzed by both absolute (right columns; except for dsRNA SK) and relative quantification (left columns) method. Means ± SEM, n = 2; PCR was run in triplicates. Mann-Whitney *U*-test, asterisks indicate significant differences, ** P < 0.05.* (A): The gel electrophoresis results of the negative controls (NTC) and for the DNA fragments of ß-actin and AT 1 at four different concentrations (0.002 to 2 pg).

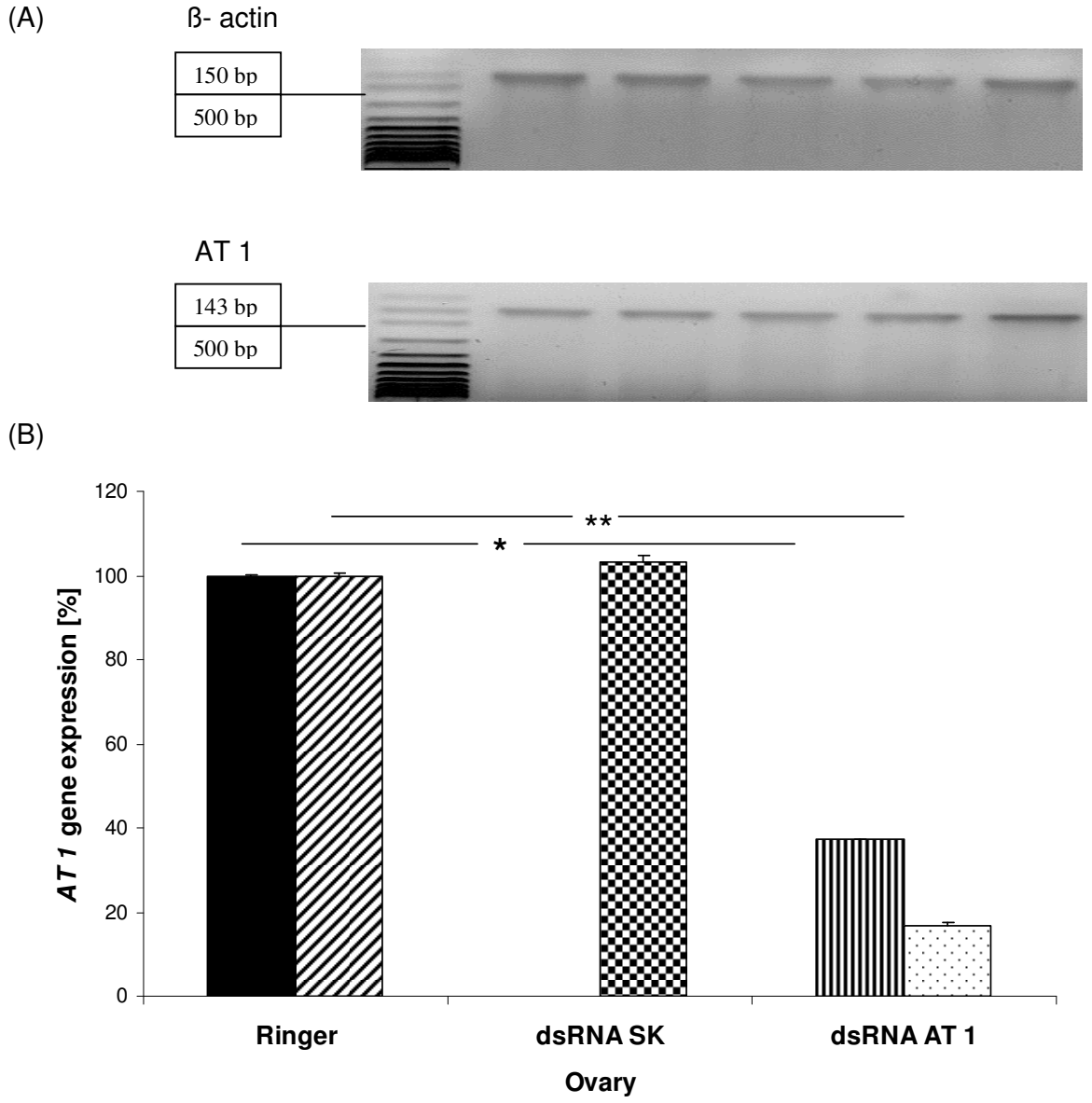


Figure (3): Gene expression of *Manse-AT* (AT1) after silencing the gene transcript level in the ovary of 2 day old virgin females of *S. frugiperda* (B). Newly eclosed females were injected with either 1.5 µg dsRNA AT 1 or dsRNA SK in 2 µl noctuid Ringer or 2 µl noctuid Ringer. 20 ovaries from 2 day old virgin females were used for experiment. Results were analyzed by both absolute (right columns; except for dsRNA SK) and relative quantification (left columns) method. Means ± SEM, n = 2; PCR was run in triplicates. Mann-Whitney *U*-test, asterisks indicate significant differences, ** P < 0.05, ** P < 0.01.* (A): The gel electrophoresis results of the negative controls (NTC) and for the DNA fragments of ß-actin and AT 1 at four different concentrations (0.002 to 2 pg).

3.1.3 Parameters of adult development

Animals were reared at 25ºC, 70% humidity and an L 16: D 8 photoperiod. Under these conditions the penultimate (L5) larval stage lasted 3 days and the last larval stage (L6) lasted 3 to 4 days followed by 2 days of prepupation. The pupal stage lasted 10 to 12 days and females ecdysed 1 to 2 days earlier than males in a ratio of 1: 2 (females to males). Each pupa was individually kept in a separate compartment of assortment boxes (9 x 32 x 36 mm) until emergence, and the animals were observed every 30 minutes during dark and light periods.

After injection of L6/1 (day 1 of 6th larval stage) with either 2 µl noctuid Ringer or 1.5 µg dsRNA AT 1 in 2 µl noctuid Ringer, animals in L6/4 showed different pattern of commitment into the prepupal stage. Those who were *AT 1* gene silenced prepupated 24 hours earlier (61.5 ± 12.5 %), namely on the 4th day after last larval moulting, than the controls, which pupated on the 5th day (74.6 ± 16.7 %) **(Figure 4)**.

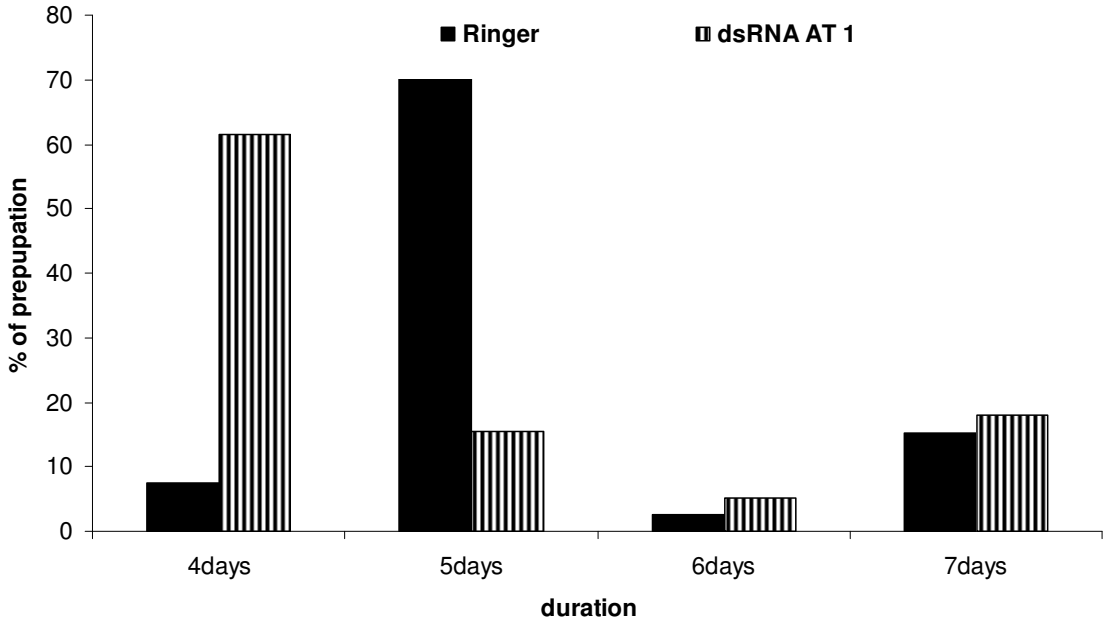


Figure (4): Duration until prepupal commitment after moulting into the last larval stage (L6) in the fall armyworm, *S. frugiperda.* L6/1 larvae were injected with either 2 µl noctuid Ringer or 1.5 µg dsRNA AT 1 in 2 µl Ringer. Animals were kept in assortment boxes, supplied with artificial food and continuously observed. Noctuid Ringer n = 29, dsRNA AT 1 n = 45.

Gene silencing of *AT 1* at L6/1 also led to earlier pupation of the animals. Those who were *AT 1* gene silenced at L6/1 pupated about 24 h earlier (76.6% pupated on day 6) than the Ringer controls. 84.6% of Ringer injected larvae pupated on day 7 **(Figure 5)**. Minor numbers of dsRNA injected animals pupated on day 7 (23.1 % of AT 1 animals) and some Ringer injected animals on day 8 (15.4 % of Ringer animals).

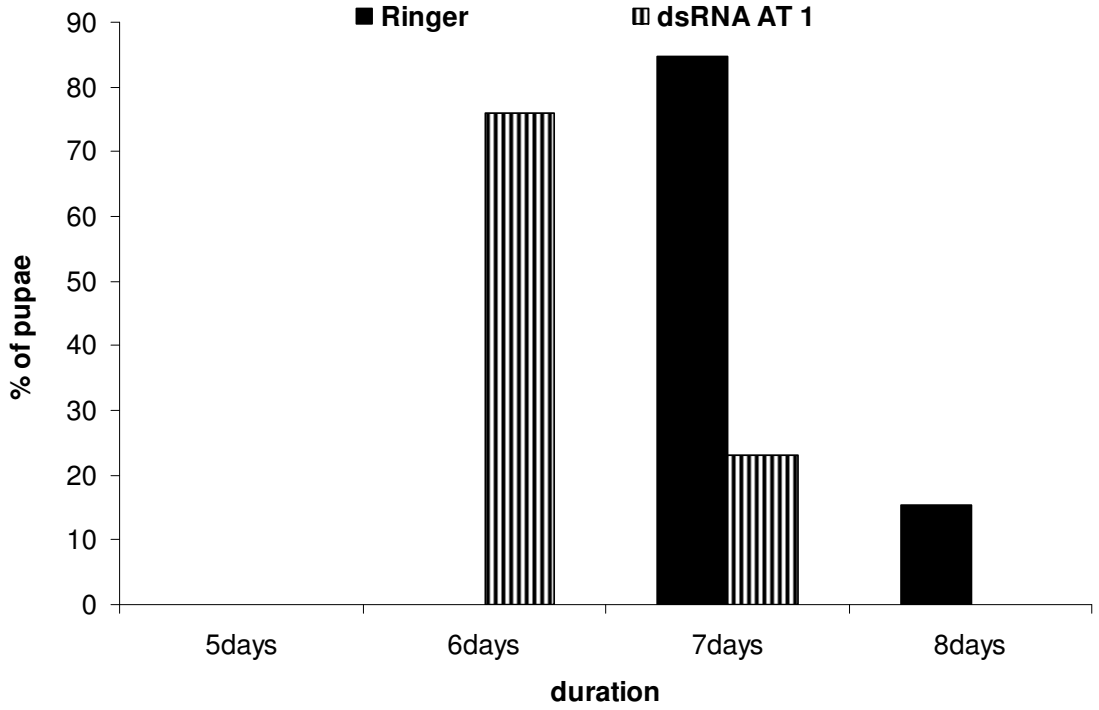
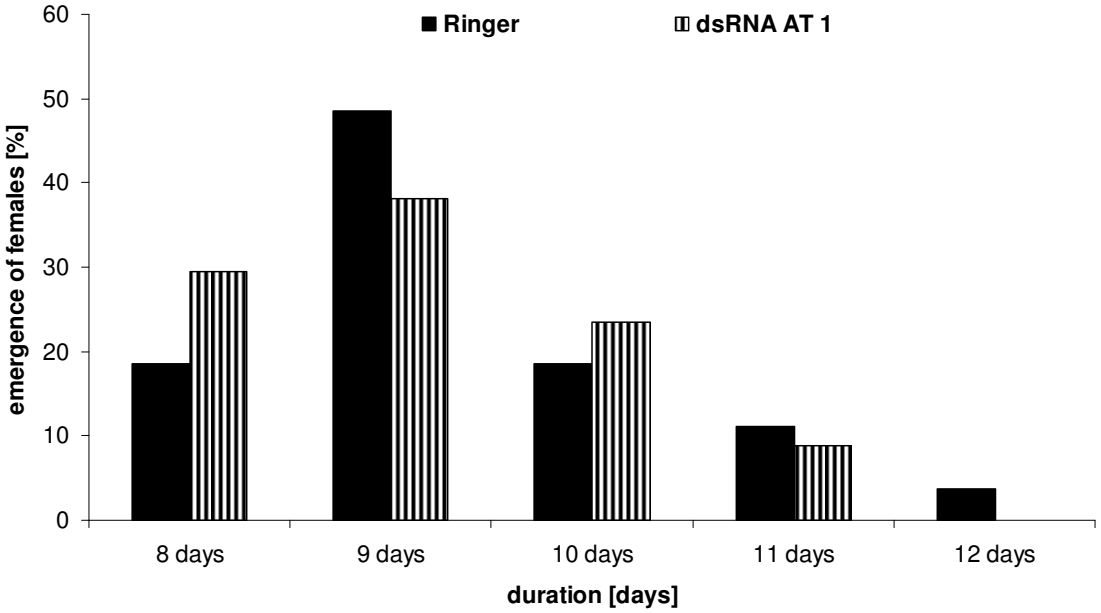


Figure (5): Duration of transformation into pupa from moulting into the last larval stage (L6*).* L6/1 larvae injected with either 2 µl noctuid Ringer or 1.5 µg dsRNA AT 1 in 2 µl Ringer at the time of moulting into the last larval stage. L6/1 were kept in assortment boxes, supplied with artificial food, and observed continuously. Noctuid Ringer n = 29, dsRNA AT 1 n = 45.

The pupal phase of untreated larvae took 10 to 12 days for females and 12 to 14 days for males (results not shown). Females from dsRNA AT 1 larvae injected at L6/1 emerged 8 to 11 days after pupation. 29.4 % of the animals started moulting on day 8 after pupation, while the mass (38.2 %) emerged on day 9. Females from Ringer injected L6/1 also started to emerge on day 8 (18.5 %), while the mass (48.2 %) ecdysed on day 9 **(Figure 6 A)**.

Ringer injected adults males emerged from pupal developed 8 to 12 days after pupation whereas some dsRNA AT 1 treated animals emerged on day 13 only. In both groups, maximum of emergence was on day 10 with about 40% for AT 1 treated animals and 70 % for the Ringer controls **(Figure 6 B)**.

(A)



(B)

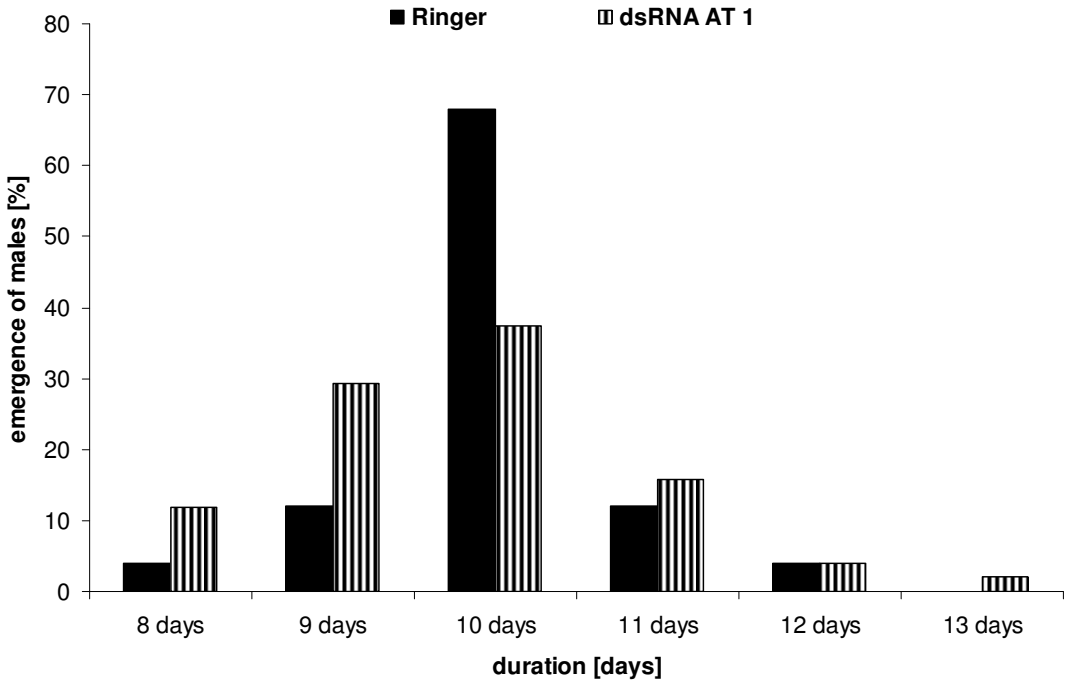


Figure (6): Percentage of adult females (A) and males (B) emergence. Newly eclosed L6/1 were injected with either 2 µl noctuid Ringer (n = 28) or 1.5 µg dsRNA AT 1 in 2 µl noctuid Ringer (n = 23). Pupae were kept in assortment boxes and observed continuously.

S. frugiperda larvae and pupae were reared under 16 L: 8 D photoperiod until emergence and time of adult moulting was observed. 48.3% of the females from untreated pupae emerged in the range of 3 hours at the end of the photophase, whereas most of them emerged during the early scotophase with a peak after two hours darkness **(Figure 7)**. Ringer injection caused some differences in the time of adult female emergence compared to untreated ones. Emergence was mainly shifted to the end of the light-phase, and only one third of the animals emerged at the beginning of the scotophase. A similar pattern was observed for dsRNA AT 1 injected larvae.

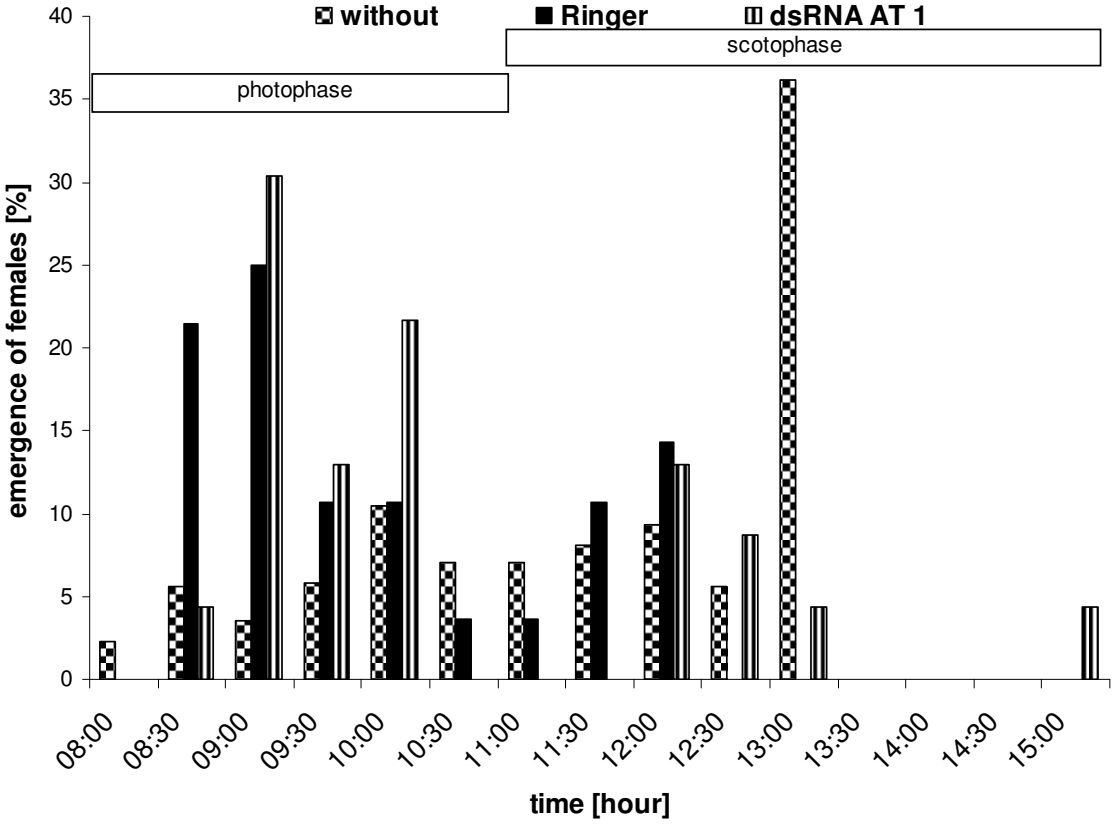


Figure (7): Percentage of emerging adult females of *S. frugiperda* in relation to the light-dark cycle (L: D 16: 8 h). 11:00 – 15:00 (MET) represents first hours of scotophase, 08:00 – 11:00 represents last hours of the photophase. Pupae were kept in assortment boxes and emergence was observed every 30 minutes. Untreated females n = 29, noctuid Ringer n = 86, and females *AT1* gene silenced n = 23.

16.1% of males emerged from untreated larvae/pupae in the range of 2.5 hours at the end of the photophase, whereas most of them emerged during the early scotophase, with peak emergence one hour after light off. Ringer injection caused some differences

in the time of adult emergence compared to untreated animals. Most of the males emerged 2 hours before the scotophase, and another peak occurred 30 min after lights off. The pattern of emergence after *AT 1* gene silencing was similar to that for the Ringer controls, but with a slight shift to the light phase (maximum 2 ½ hours before light off) **(Figure 8)**.

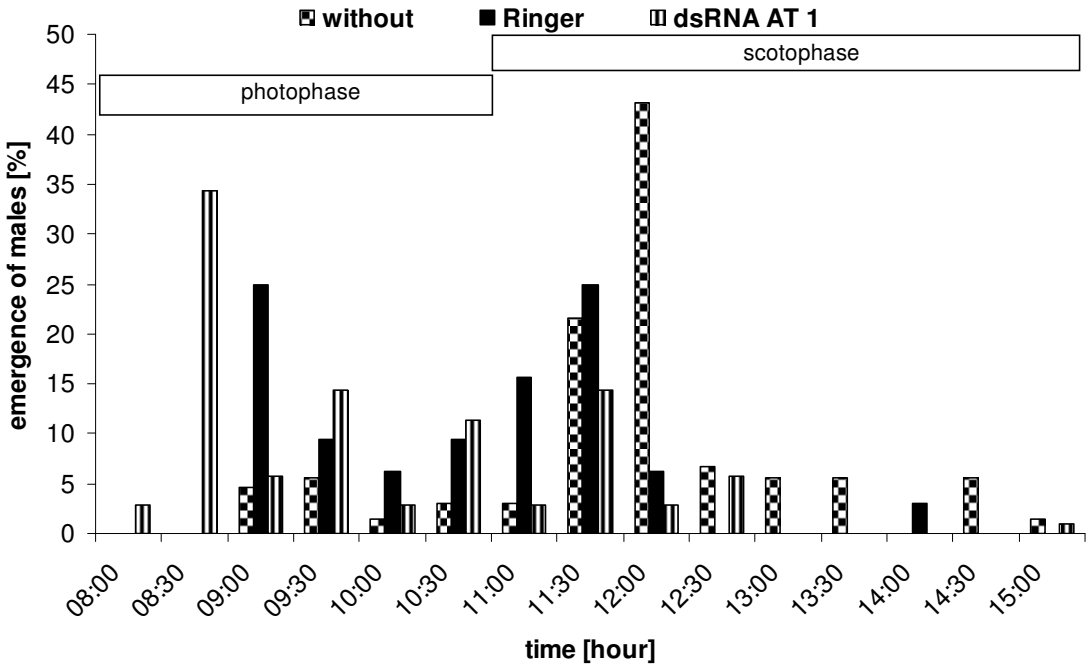


Figure (8): Percentage of emerging adult males of *S. frugiperda* in relation to the light-dark cycle (L: D 16: 8 h). 11:00 – 15:00 (MET) represents first hours of scotophase, 08:00 – 11:00 represents last hours of the photophase. Pupae were kept in assortment boxes and emergence was observed every ½ hour. Untreated males n = 65, noctuid Ringer n = 32, and males *AT 1* gene silenced n = 65.

24 $\pm$ 3 hours after adult moult, moths started to become active and feeding. The virgin females began to mate within 1 to 2 hours in the early scotophase and coupling lasted for 1.5 to 2 hours. Oviposition occurred in the succeeding scotophases. Ringer injected females mated between 21 to 30 hours after emergence with a peak at 24 hours. *AT 1* gene silenced females started mating 23 hours after emergence until 27 hours **(Figure 9)**. The peak of mating was shifted to 25 hours after emergence. Repeated mating occurred almost every 24 hours, shortly before or in the early dark phase.

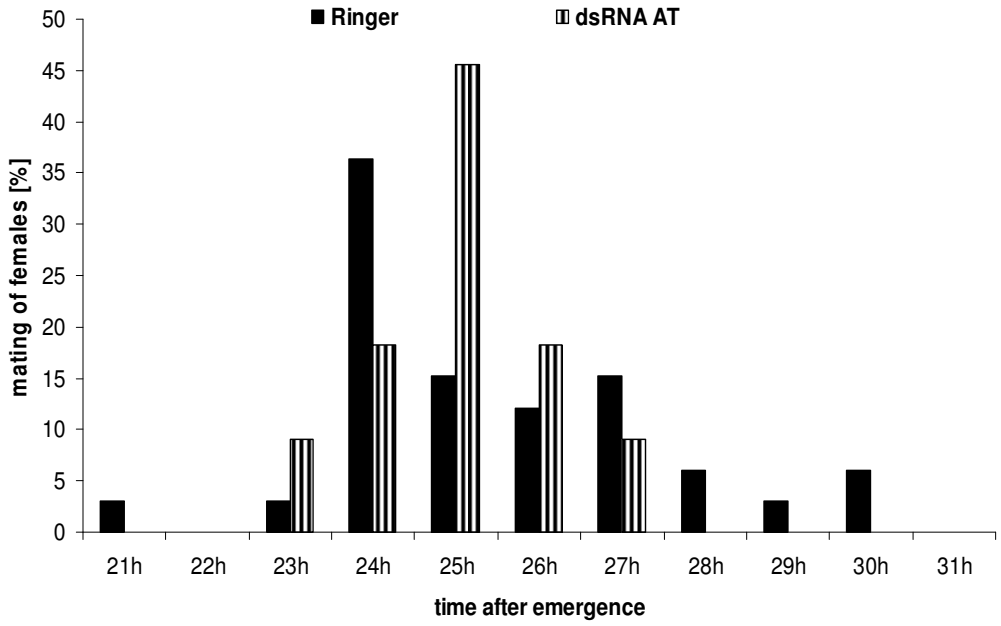


Figure (9): Effect of *AT 1* gene silencing on time from emergence to first mating in females of *S. frugiperda*. Freshly moulted females were injected with either 2 µl of noctuid Ringer (n = 55) or 1.5 µg dsRNA AT 1 in 2 µl noctuid Ringer (n = 67). Each female was kept individually in a box with an untreated male. 10% sucrose, water and filter paper for egg deposition were supplied and animals were observed each hour.

Mated females of *S. frugiperda* started laying eggs 45 to 54 hours after emergence, and daily oviposition was reported in this polyandrous moth **(Figure 10)**. From the deposited eggs first instar larvae hatched within 60 to 72 hours with no significant differences, neither in time nor in hatching rates, between *AT 1* gene silenced and control animals (results not shown).

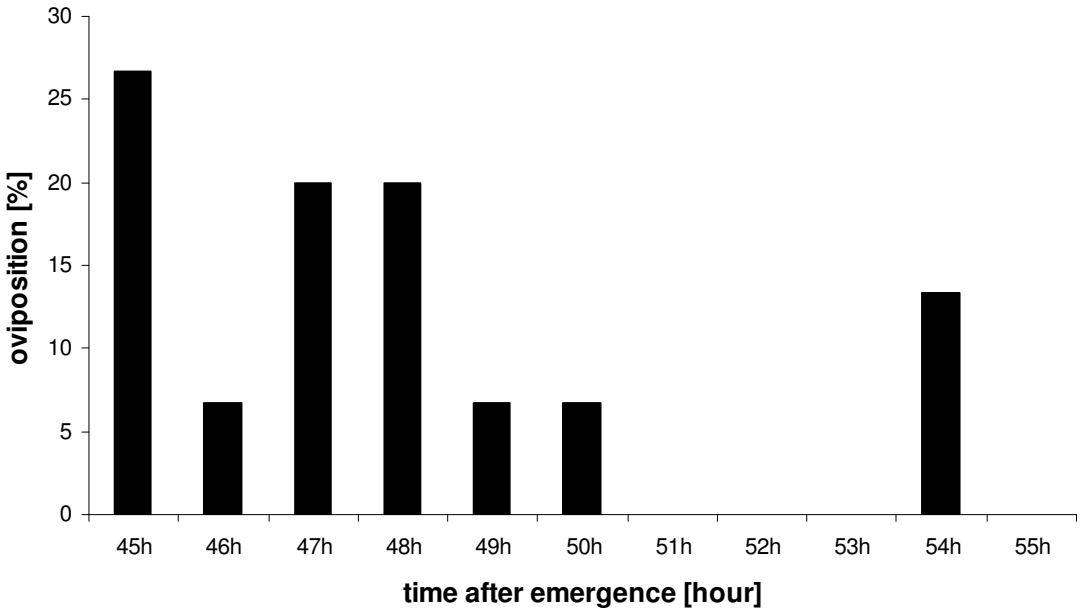


Figure (10): Time from emergence to first egg laying in untreated females. Freshly moulted females and males were kept in boxes in 1: 1 ratio and 10% sucrose, water and filter paper for egg deposition were supplied. Egg laying was observed each half hour during 2 hours at the end of photophase and during the first 5 hours of scotophase; n = 15.

The time of first egg deposition in virgin untreated females, virgin Ringer injected females and virgin AT 1 dsRNA treated females was measured. 16.1% of virgin untreated females, 23.1% of virgins treated with noctuid Ringer, and 46.7% of virgins *AT 1* gene silenced started to deposit eggs on 2 day after emergence **(Figure 11)**. In mated females, egg laying on day 2 after emergence was generally higher than in virgins. However, there was no difference in first oviposition time between Ringer injected controls and AT 1 dsRNA treated animals, neither when males (♂) nor when females (♀) had been treated with the AT 1 dsRNA. Thus *AT 1* gene silencing of females induces virgins to deposit more eggs earlier, but has no effect in mated females**.**

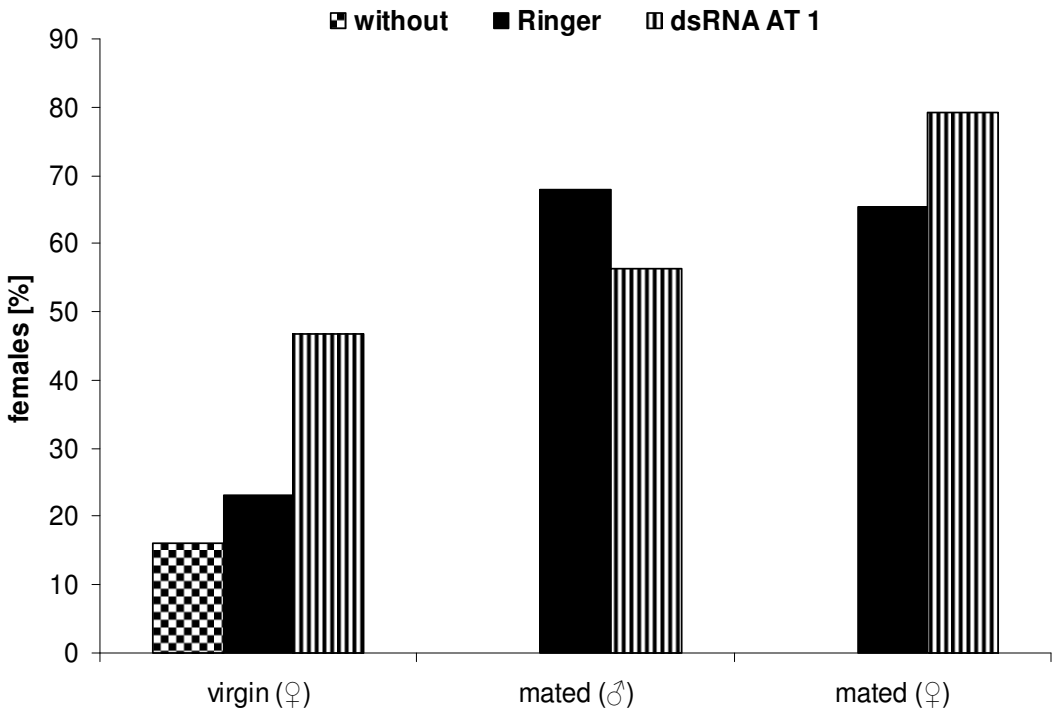


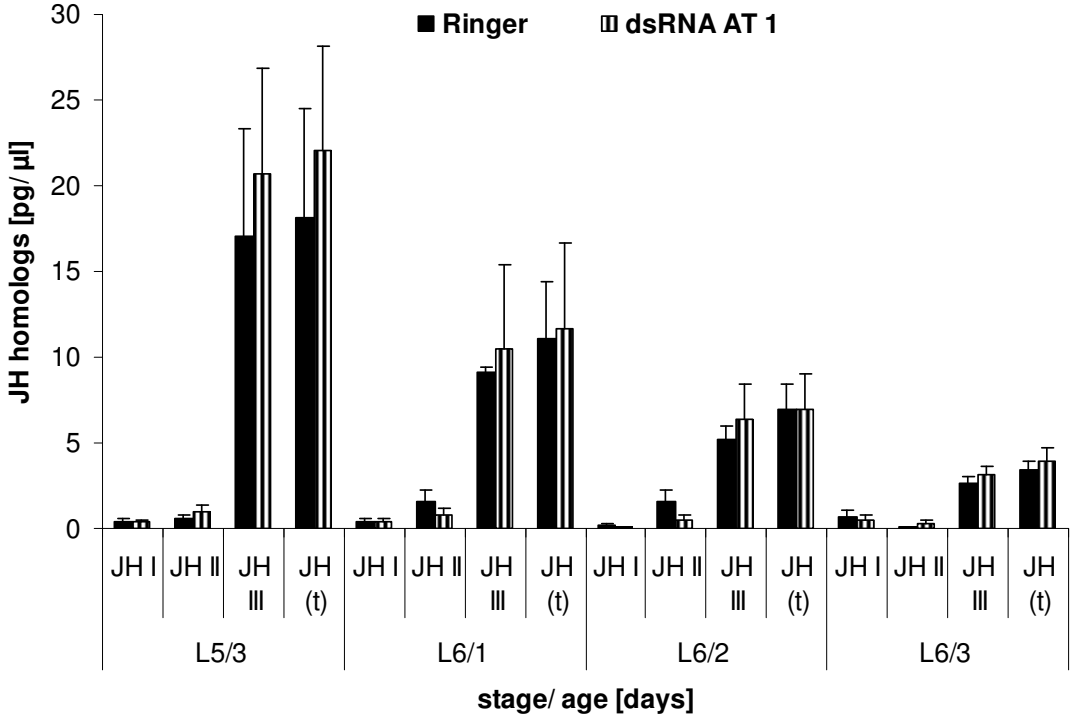
Figure (11): Percentage of virgin and mated females of *S. frugiperda* that started egg deposition on day 2 after emergence. Virgin females were without treatment or injected with 2 µl noctuid Ringer or 1.5 µg dsRNA AT 1 in 2 µl noctuid Ringer. Mated (♂): An AT 1 dsRNA treated male was kept together with an untreated female. Mated (♀): An AT 1 dsRNA treated female was kept together with an untreated male. 10% sucrose, water and filter paper for egg deposition were supplied. n without treatment = 31, Ringer = 52, dsRNA AT 1 = 30.

3.1.4 JH in the hemolymph of larvae

Juvenile hormone (JH) allows larval moulting in response to ecdysteroids but prevents the switching of gene expression necessary for metamorphosis. The JH titer is high at the time of last larval moulting, and then declines to a very low or undetectable level. In this work, larval pharate (L5) of *S. frugiperda* were injected with either 1.5 µg dsRNA AT 1 in 2 µl noctuid Ringer or 2 µl noctuid Ringer into anterior pseudopods immediately after their moulting. **Figure 12 A** shows that *AT 1 gene* silencing in L5/1 larvae had no effect on the JH titer of the hemolymph three days later (L5/3: 22.03 ± 6.1 pg/ µl) in comparison with controls (18.5 ± 6.3 pg/ µl). JH titers dropped during the following days of the 6th larval stage but, again, no differences between *AT 1* gene silenced animals and Ringer controls were observed (L6/1 11.7 ± 5 pg/ µl vs.11 ± 0.3 pg/ µl, L6/2 7 ± 2 pg/ µl vs. 7 ± 1.4 pg/ µl, L6/3 3.9 ± 0.8 pg/ µl vs. 3.4 ± 0.5 pg/ µl). In all cases, JH III was the main JH homolog and only low amounts of JH I and JH II were detected. *AT 1* gene

silencing of L6/1 larvae led to a significant reduction of JH I at the end of the larval stage (L6/4) (0.1 ± 0.2 pg/ µl compared to 1.6 ± 0.9 pg/ µl in the controls; *P < 0.05*), whereas the JH III titer (71.7 ± 7.9 pg/ µl) was significantly elevated in comparison to controls (42.0 ± 0 pg/ µl; *P < 0.05*). This resulted in a significantly increased total JH titer in *AT 1* gene silenced animals shortly before pupation. Prepupa (PP1), on the other hand, did not show any differences between the JH titers of *AT 1* gene silenced and control animals **(Figure 12 B)**. JH titers were generally higher in wandering phase (L6/4) and prepupal stage than in younger 6th instar larvae.

(A)



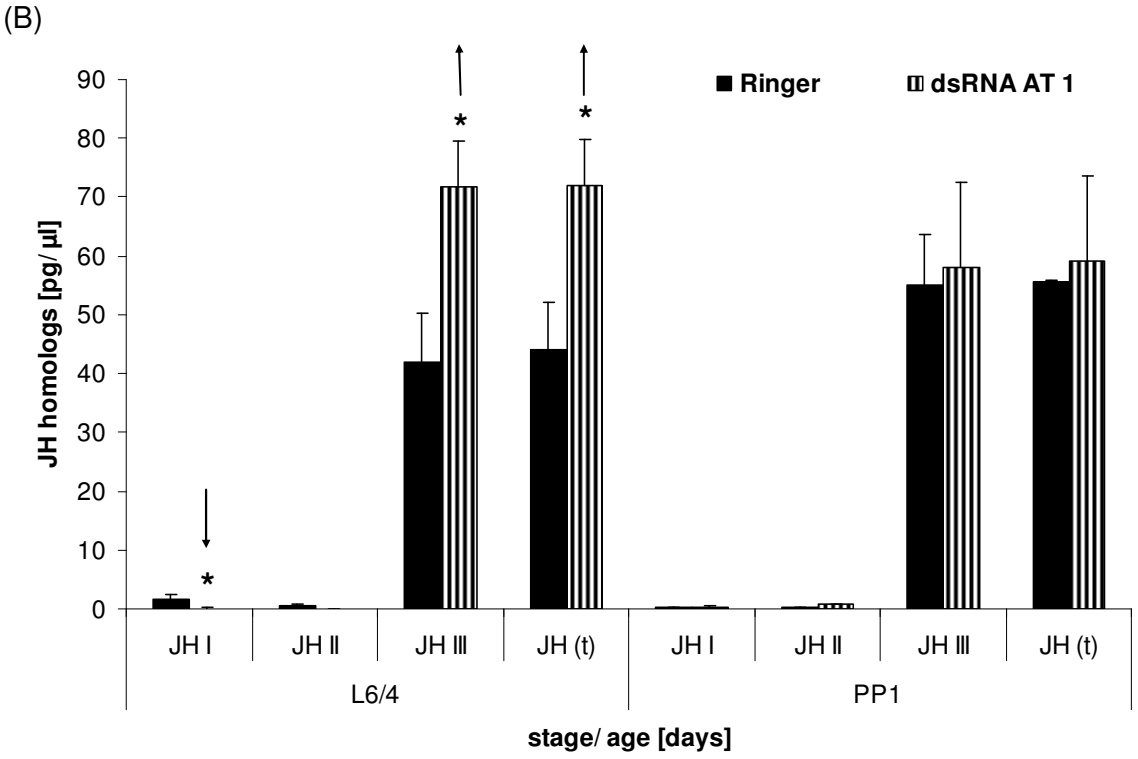


Figure (12): (A) Effect of *AT 1* gene silencing at the first day of the penultimate larval stage (L5/1) on the titer of the JH homologs in 3 day old penultimate larvae (L5/3) and in 1 to 3 day old last instar larvae (L6) of *S. frugiperda.* (B) Effect of *AT 1* gene silencing at the first day of the last larval stage (L6/1) on the titer of the JH homologs in 4 day old last instar larvae and in the young prepupa (PP1). Newly eclosed L5/1 and L6/1, respectively, were injected with either 2 µl noctuid Ringer or 1.5 µg dsRNA AT 1 in 2 µl noctuid Ringer. Hemolymph was taken from individual larva and JH homologs were measured by LC-MS. Means ± SEM; n (Ringer, dsRNA AT 1) = 8 - 10. Mann-Whitney *U*-test, asterisks indicate significant differences between treatments, * $P < 0.05$.

3.1.5 Ecdysteroids in the hemolymph of larvae

Pharate L5 larvae (L5/1) and young last instar larvae (L6/1) of *S. frugiperda* were injected with either 1.5 µl dsRNA AT 1 in 2 µl noctuid Ringer or 2 µl noctuid Ringer into anterior pseudopods immediately after moulting. Hemolymph samples were taken from individual larvae every 24 hours (L5/3 to PP1) and concentrations of free ecdysteroids (ecdysone, 20-hydroxyecdysone) were measured by LC-MS. Ecdysteroid titers of penultimate and last instar larvae were not affected by *AT 1* gene silencing until days 3 of the last larval stage, whereas a strong increase of free ecdysteroids in wandering larvae L6/4 and prepupae was observed. L6/4 exhibited much higher 20-

hydroxyecdysone concentrations (146.6 ± 34.6 pg/ µl) in comparison to the control (51.6 ± 15.3 pg/ µl, *P < 0.005*). Knockdown of *AT 1* gene expression also induced a significant increase of 20-hydroxyecdysone (741.6 ± 143.8 pg/ µl vs. 326.2 ± 57.3 pg/ µl, *P < 0.05*) and ecdysone (77.2 ± 14.4 pg/ µl vs. 5.03 ± 2.8 pg/ µl, *P < 0.*001) in the prepupa **(Figure 13)**. Free ecdysteroid titers were generally higher in wandering phase (L6/4) and prepupal stage than in younger 6th instar larvae.

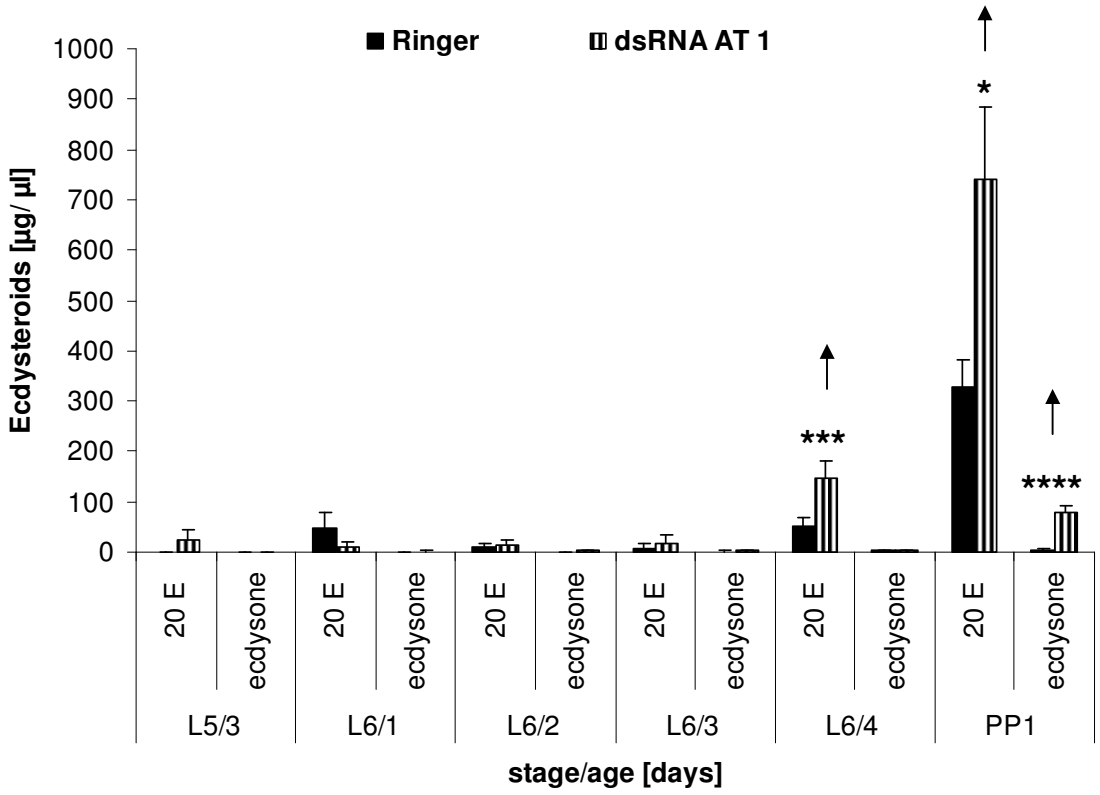


Figure (13): Effect of *AT 1* gene silencing on the titer of free ecdysteroids (ecdysone; 20E = 20-hydroxyecdysone) in the hemolymph of 3 day old penultimate larvae (L5/3), 1 to 4 day old last instar larvae (L6), and the early prepupal (PP1) stage of *S. frugiperda.* Newly eclosed L5/1 and L6/1 were injected with either 2 µl noctuid Ringer or 1.5 µl dsRNA AT 1 in 2 µl noctuid Ringer. Hemolymph was taken from individual animals and ecdysteroid concentrations were measured by LC-MS. Means ± SEM; n (Ringer, dsRNA AT 1) = 8 - 10. Mann-Whitney *U*-test, asterisks indicate significant differences between treatments, ** P < 0.05; ***P < 0.005, ****P < 0.001.*

3.1.6 Body weight of larvae

The timely onset of metamorphosis in holometabolous insects depends on reaching the appropriate size known as critical weight in the last larval stage. Once critical weight is reached, juvenile hormone (JH) titers decline, resulting in the release of prothoracicotropic hormone (PTTH) at the next photoperiodic gate and thereby inducing metamorphosis. The body weight of larvae L5/1 (97.2 ± 6 mg) increased continuously and reached a peak in 3 day old last instar larvae L6/3 (613.8 ± 16.7 mg). The body weight then dropped to lower value in the prepupal stage PP (262.9 ± 5.9 mg), and even less in pupal stage (not shown). RNA interference *in vivo* by abdominal injection of dsRNA AT 1 into young penultimate larvae (L5/1) did not affect body weight changes **(Figure 14)**.

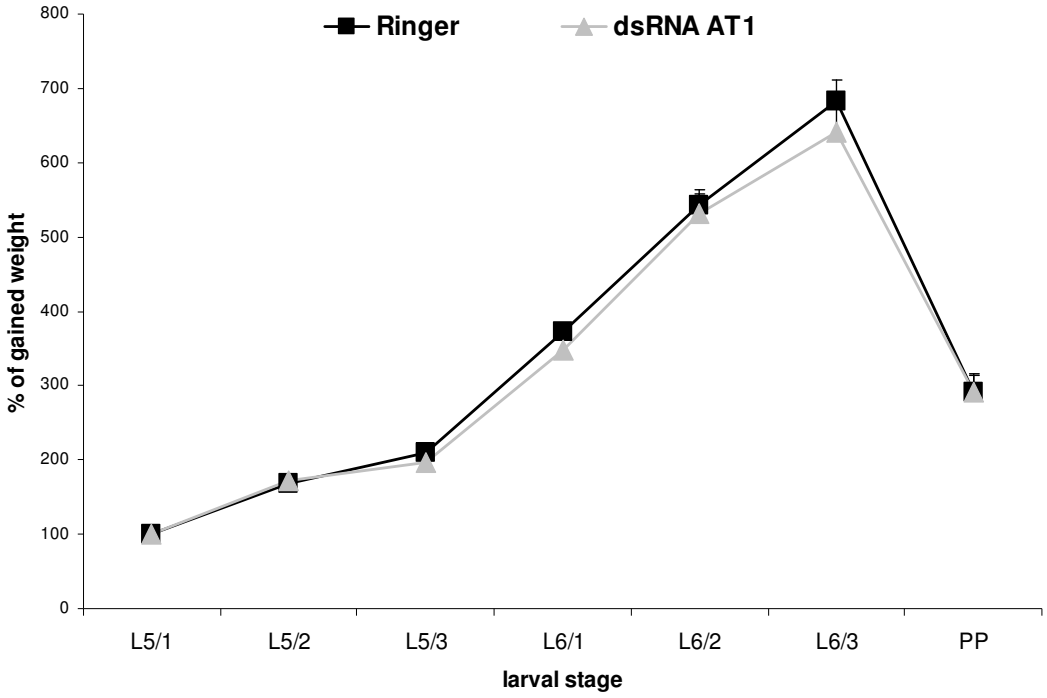


Figure (14): Effect of *AT 1* gene silencing on the body weight of 1 to 3 day old penultimate larvae (L5), 1 to 3 day old last larvae (L6), and the prepupa of *S. frugiperda*. Newly eclosed larvae L5/1 were injected either with 2 µl noctuid Ringer or 1.5 µg dsRNA AT 1 in 2 µl noctuid Ringer into the last pseudopodium. The body weight immediately after the moult into the penultimate larval stage was set to 100% (97.2 ± 6 mg). n (Ringer, dsRNA AT 1) = 12. Mann-Whitney *U*-test. No significant differences between treatments.

3.1.7 JH in the hemolymph of virgin females

"Juvenile hormone (JH) titer in virgin female lepidopterans is generally lower than in mated females of the same age. The JH titer in virgin females follows a diel pattern in which it begins to increase towards the end of photophase, remains high around the onset of scotophase, and declines during scotophase. The titer reaches its lowest level at the onset of photophase, and remains low during the first half of the photophase" (Ramaswamy et al., 2000). Here we followed the hemolymph JH titer of virgin females of *S. frugiperda* during the first 8 days of adult life, measured once a day during the early scotophase. In control females JH III titer was nearly constant with a small peak at day 4 (2 day old 22 ± 7.9 pg / µl, 4 day old 38.9 ± 9.7 pg / µl, 6 day old 33.9 ± 8.8 pg / µl, 7 day old 29.2 ±15.7 pg / µl, and 8 day old 29.6 ± 8.8 pg/ µl). However, in contrast to the larvae, the hemolymph of adult females contained considerable amounts of JH I and JH II at all ages. Total JH titer, therefore, peaked with about 75 pg / µl at day 8 of adult life. Following *AT 1 gene* silencing the JH III was not affected in younger females (day 2), whereas in older females (day 4 to 7) JH III was strongly reduced (4.7 ±1.7 pg JH III / µl vs. 29.2 ±15.7 pg JH III / µl at day 7, $P < 0.05$). This led to a significant reduction of total JH titer at that day ($P < 0.001$). Gene silencing also induced a significant reduction of JH II in 6 day old virgin females (7.7 ± 1.9 pg / µl in comparison to control with 9.2 ± 1.9 pg / µl, $P < 0.05$), and an elevation of JH II in 8 day old females (10.7 ± 3.8 pg / µl compared to control with 4.3 ± 0.9 pg / µl, $P < 0.005$) **(Figure 15)**.

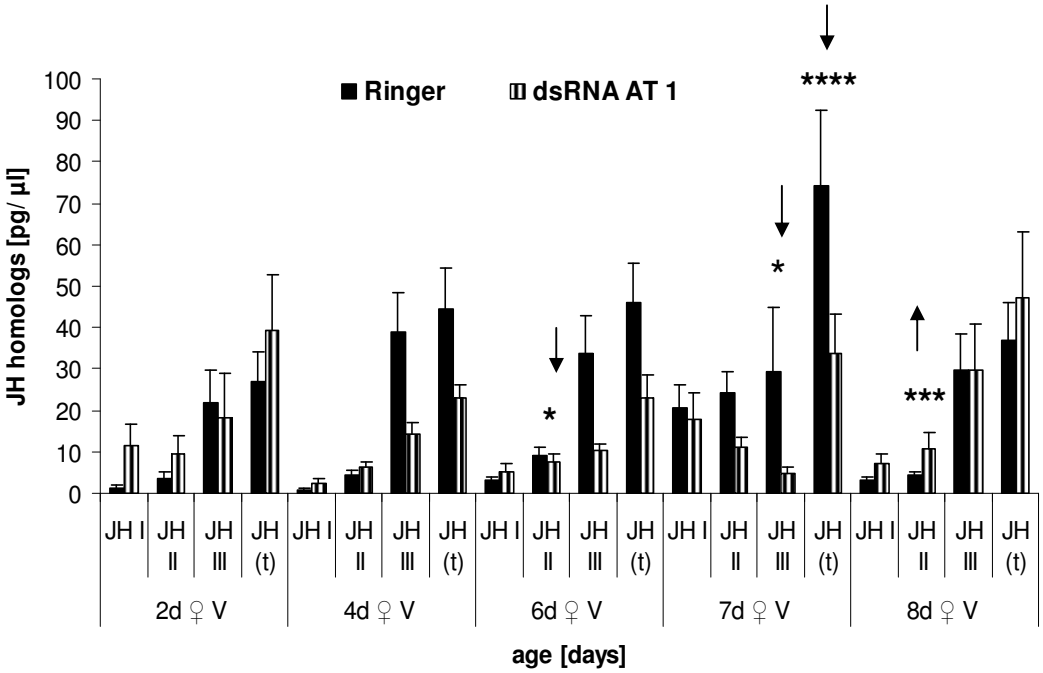


Figure (15): Effect of *AT 1* gene silencing on the titer of the JH homologs in the hemolymph of 2 to 8 day (d) old virgin (V) females of *S. frugiperda*. Newly eclosed females were injected with either 2 µl noctuid Ringer or 1.5 µg dsRNA AT 1 in 2 µl noctuid Ringer into the third abdominal segment. Females were reared without males, supplied with 10% sucrose, water and filter paper for egg laying. 20 µl of hemolymph was collected from 2 d, 4 d, 6 d, 7 d, and 8 d old virgin females during the first 2 to 3 hours of scotophase. Means ± SEM; n = 7 - 9 (Ringer), 8 - 10 (dsRNA AT 1). Mann-Whitney *U*-test, asterisks indicate significant differences between treatments, ** P < 0.05; *** P < 0.005, **** P < 0.001.*

3.1.8 Ecdysteroids in the hemolymph of virgin females

In some dipterans, the synthesis of yolk proteins (YP) depends on the level of circulating ecdysteroids (Siegenthale et al., 2009). The concentration of 20-hydroxyecdysone in the hemolymph oscillates and, at high levels, is followed by expression of YP (Siegenthale et al., 2009). In the present work, hemolymph samples were taken from virgin females *S. frugiperda* and the free ecdysteroid concentrations were measured during the first 8 days of adult life by LC-MS. The amount of 20-hydroxyecdysone (20E) was 6.5 ± 3.7 pg / µl in 2 day old virgin females, 22.1 ± 6.4 pg / µl in 4 day old females, 20.9 ± 6.8 pg / µl in 6 day old females, 15.2 ± 7.9 pg / µl in 7 day old females and 8.7 ± 2.7 pg / µl in 8 day old females, and thus peaked between day 4 and day 6. Concentrations of ecdysone were generally lower but followed the same time-pattern. Knockdown by AT 1 dsRNA injection at the day of emergence enhanced

the amount of free ecdysteroids in virgin females, especially in older animals (day 8). In 4 day old females concentration of ecdysone was significantly higher (23.9 ± 7.3 µg / µl in comparison to control with 6.6 ± 2.02 pg / µl, $P < 0.05$), whilst 8 day old females exhibited much higher 20E (44.1 ± 16.5 pg / µl vs. 8.7 ± 2.7 pg / µl, $P < 0.005$) and ecdysone (36.8 ± 19.2 pg / µl vs. 9.9 ± 3.5 pg / µl, $P < 0.05$) concentrations **(Figure 16)**.

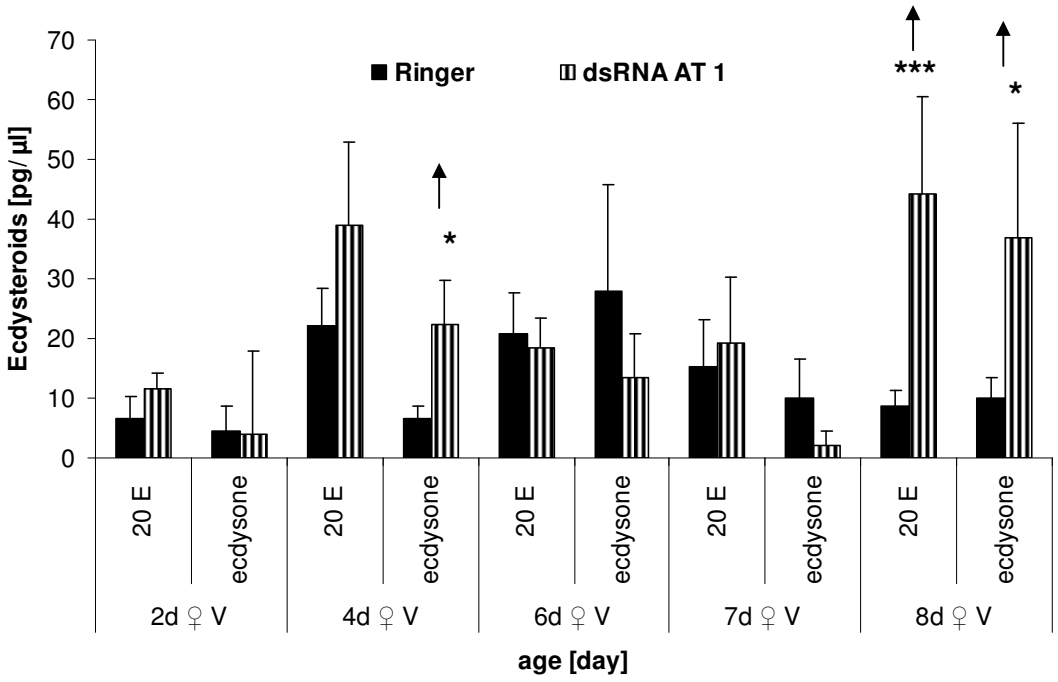


Figure (16): Effect of *AT 1* gene silencing on the titer of free ecdysteroids (ecdysone and 20-hydroxyecdysone, 20E) in the hemolymph of 2 to 8 day (d) old virgin (V) females of *S. frugiperda*. Newly eclosed females were injected with either 2 µl noctuid Ringer or 1.5 µl dsRNA AT 1 in 2 µl noctuid Ringer in the third abdominal segment. Females were reared without males, supplied with 10% sucrose, water and filter paper for egg laying. 20 µl of hemolymph was collected from 2 d, 4 d, 6 d, 7 d, and 8 d old virgin females during the first 2 to 3 h of scotophase. Means ± SEM; n = 7 - 9 (Ringer), 8 - 10 (dsRNA AT 1). Mann-Whitney *U*-test, asterisks indicate significant differences between treatments, ** $P < 0.05$, *** $P < 0.005$.*

3.1.9 Reproduction of virgin females

Freshly moulted untreated virgin females of *S. frugiperda*, or females treated by injection of 2 µl noctuid Ringer or 1.5 µg dsRNA AT 1 in 2 µl noctuid Ringer into the third abdominal segment were kept separately in boxes. The daily laid eggs by each female were counted. Virgin females started egg laying on day 2 after emergence with no

significant differences between the various groups (25.1 ± 11.9 eggs / day without treatment, 24.7 ± 8.6 eggs / day for Ringer injected animals, and 28.4 ± 11.2 eggs / day for *AT 1* gene silenced females) **(Figures 17 and 18)**. In all three groups, maxima in egg deposition were between days 5 and 8, whereas egg deposition rates dropped to day 14 of adult life. Injection of 2 µl noctuid Ringer induced a general increase in number of deposited eggs between day 5 and 9 compared to untreated control, which was significant at day 7 (102 ± 15 eggs vs. 67.7 ± 21.3 eggs, $P < 0.05$). Another significant difference was observed at day 14 of adult life with 26.1 ± 12 deposited eggs for Ringer injected females compared to 2.1 ± 1.2 eggs for untreated controls ($P < 0.05$) **(Figure 17)**.

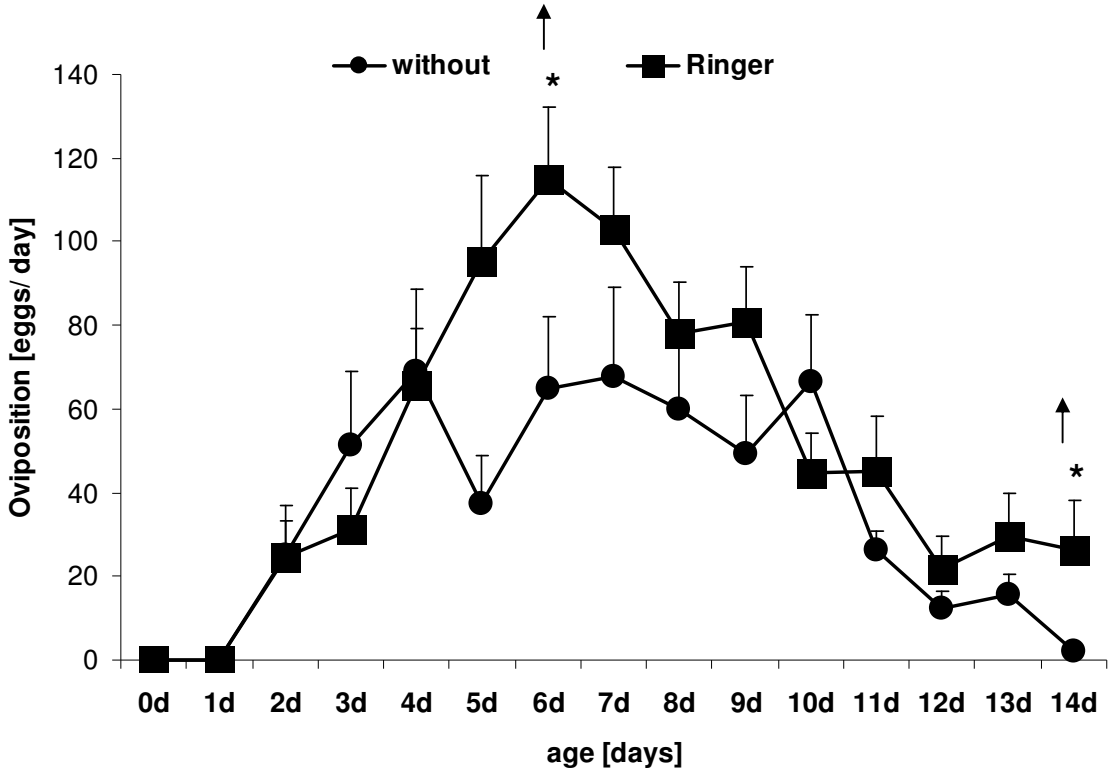


Figure (17): Effect of Ringer injection on the profile of egg laying for virgin females of *S. frugiperda.* Virgin females were either untreated (without) or injected with 2 µl noctuid Ringer into the third abdominal segment at the day of emergence. Females were reared without males. 10% sucrose, water, and filter paper for egg deposition were supplied. Daily laid eggs by each female were counted. Means ± SEM; n without treatment = 31, Ringer injection = 55). Mann-Whitney *U*-test, asterisks indicate significant differences between treatments, ** P < 0.05.*

AT 1 gene silenced females deposited significantly more eggs on day 3 (53.4 ± 12.2, *P< 0.005*), day 12 (41.2 ± 8.7, *P< 0.05*), and day 13 (26.5 ± 7, *P< 0.05*) than the Ringer controls (31.1 ± 10.1, 8.8 ± 1.1, and 10.8 ± 5, respectively), whereas egg deposition of *AT 1* knockdown animals was significantly lower on day 7 (*P< 0.05*) **(Figure 18)**.

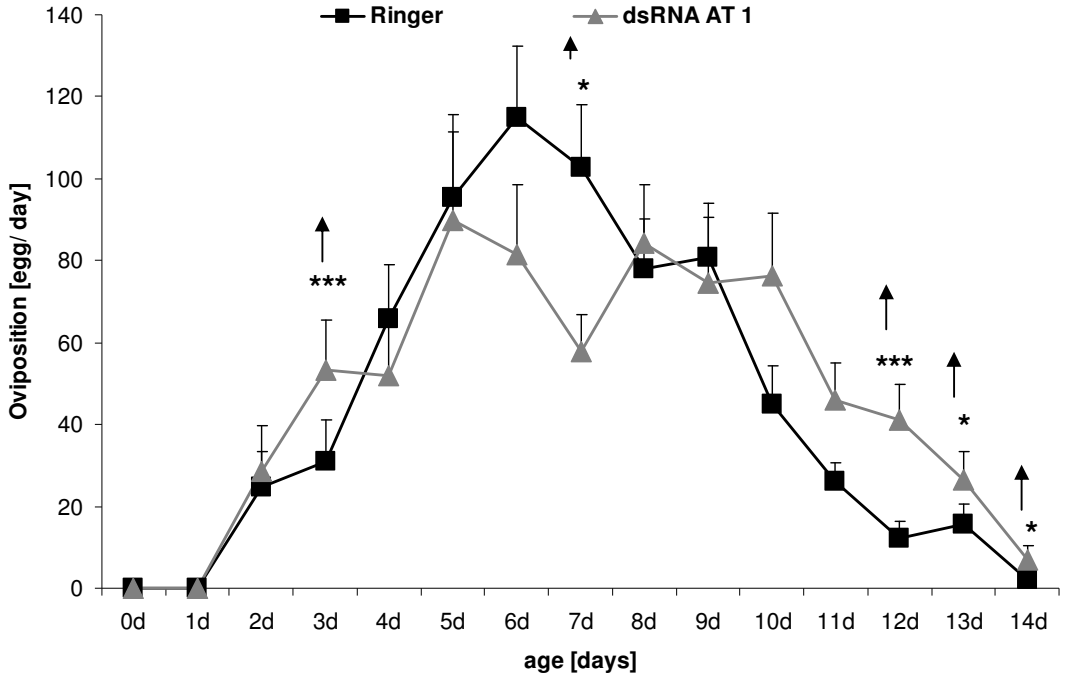


Figure (18): Effect of *AT 1* gene silencing on the profile of egg laying for virgin females of *S. frugiperda.* Virgin females were injected either with 2 µl noctuid Ringer or 1.5 µg dsRNA AT 1 in 2 µl noctuid Ringer into the third abdominal segment at the day of emergence. Females were reared without males. 10% sucrose, water, and filter paper for egg deposition were supplied. Daily laid eggs by each female were counted. Means ± SEM; n Ringer = 52, dsRNA AT 1 = 30. Mann-Whitney *U*-test, asterisks indicate significant differences between treatments, ** P < 0.05, *** P < 0.005.*

Figure (19) shows the accumulated deposited eggs during the life span of virgin females. Virgin untreated females laid 605.7 ± 116.7 eggs, *AT 1* gene silenced females deposited a total of 710.9 ± 116.3 eggs, and Ringer injected controls laid 684.9 ± 92.5 eggs; the differences were not significant.

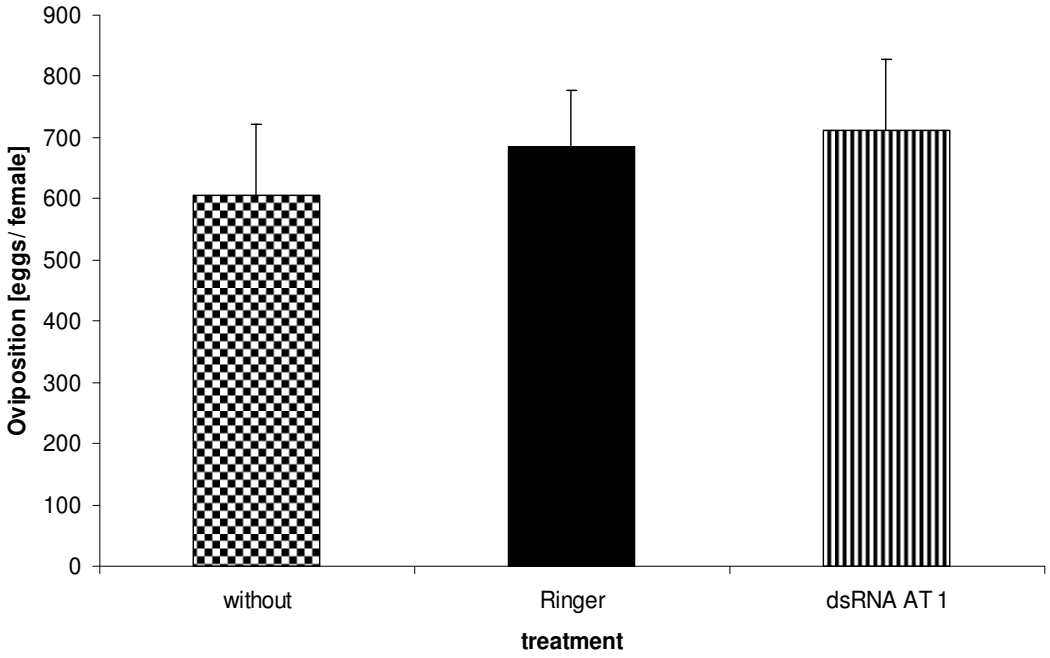


Figure (19): Effect of *AT 1* gene silencing on accumulated oviposited eggs by 2 to 14 day old virgin females of *S. frugiperda.* Virgin females were without treatment or injected with 2 µl noctuid Ringer or with 1.5 µg dsRNA AT 1 in 2 µl noctuid Ringer into the third abdominal segment at the day of emergence. Females were kept separated in boxes. 10% sucrose, water and filter paper for egg deposition were supplied. Means ± SEM; n without treatment = 31, Ringer = 52, dsRNA AT1 = 30). Mann-Whitney *U*-test. No significant differences between treatments.

3.1.10 JH in the hemolymph of mated females

In virgin females, hemolymph JH titers were low at emergence, but increased thereafter and reached a maximum at day 7 of adult life. In mated females, JH titers increased significantly after coupling and became nearly twice as high as those in virgin females. In Ringer injected controls the JH III titer was already high at the beginning of adulthood (2 day old 59 ± 16.3 pg / µl, 4 day old 70.8 ± 2.9 pg / µl), but then declined to 26.8 ± 13.9 pg / µl in 6 day old females and 20.3 ± 11.7 pg / µl in 7 day old animals. Considerable, but quite stable amounts of JH I and JH II (20 - 25 pg / µl) were measured in females of all ages, so that total JH titer followed that of JH III. In young adult mated females of *S. frugiperda AT 1* gene silencing induced a significant reduction of JH III at day 2 (16 ± 3.8 pg / µl compared to 58.96 ± 16.3 pg / µl, $P < 0.01$ in the control), whereas in 7 day old females *AT 1* gene silencing increased the concentration

of JH III (59.75 ± 15.7 pg/ µl compared to 20.3 ± 11.7 pg / µl, *P < 0.005* in the control) **(Figure 20)**.

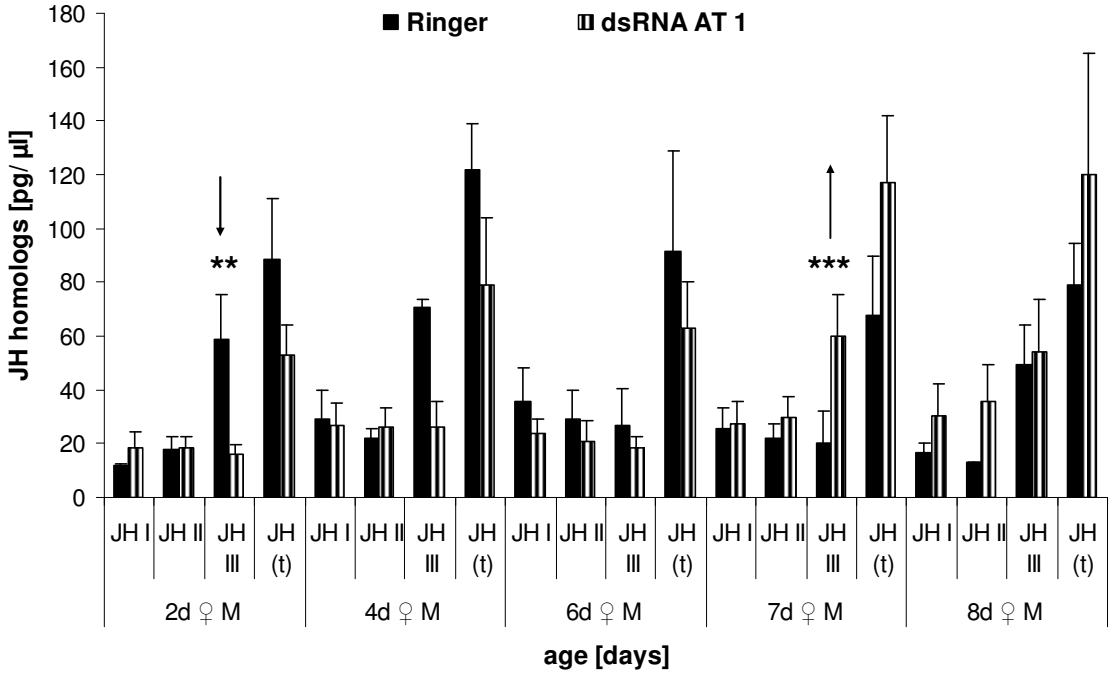


Figure (20): Effect of *AT 1* gene silencing on the titer of the JH homologs in the hemolymph of 2 to 8 day (d) old mated (M) females of *S. frugiperda*. Newly emerged females were injected with either 2 µl noctuid Ringer or 1.5 µg dsRNA AT 1 in 2 µl noctuid Ringer into the third abdominal segment. Animals were reared in a 1: 1 ration with untreated males and a new male was added at the 4th day. Each box was supplied with 10% sucrose, water and filter paper for egg laying. Hemolymph was collected from 2 d, 4 d, 6 d, 7d, and 8 d old mated females during the first 3 to 4 hours of scotophase. JH titers were measured by LC-MS. Means ± SEM; n = 7 - 9 (R), 8 - 10 (dsRNA AT 1). Mann-Whitney *U*-test, asterisks indicate significant differences between treatments, *** P < 0.01, *** P < 0.005.*

3.1.11 Edysteroids in the hemolymph of mated females

Mating accelerates the final stages of egg maturation and egg laying which may accelerate the production of ecdysteroids in the follicle cells of the ovary. 20-Hydroxyecdysone concentration in the hemolymph of mated females was already high at day 2 after emergence (15.2 ± 4.8 pg / µl) and fluctuated thereafter in an age-dependent manner (4 day old 9.9 ± 5.3 pg / µl, 6 day old 22.8 ± 7.9 pg / µl, 7 day old 5.8 ± 3.5 pg / µl, and 8 day old 12.6 ± 3.5 pg / µl). *AT 1* gene silencing of mated females

had only slight effects on the concentrations of 20-hydroxyecdysone in the hemolymph of the animals, whereas the concentration of ecdysone was significantly stimulated in 7 day old females (4.6 ± 2 pg / µl compared to a value close to detection limit in the control, *P < 0.001*) **(Figure 21)**.

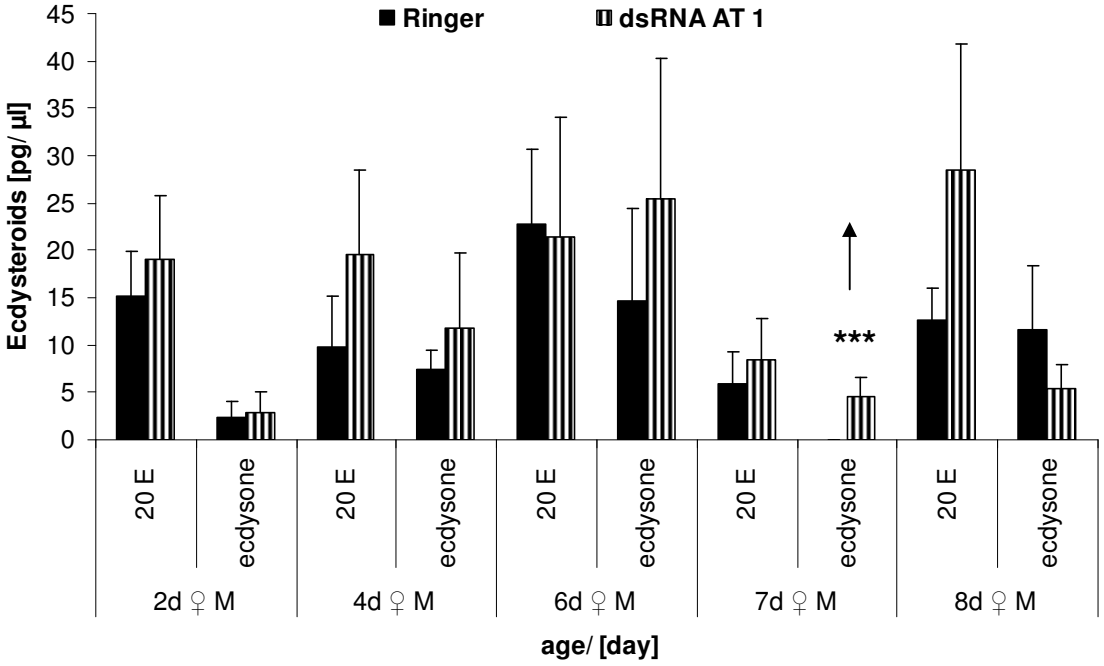


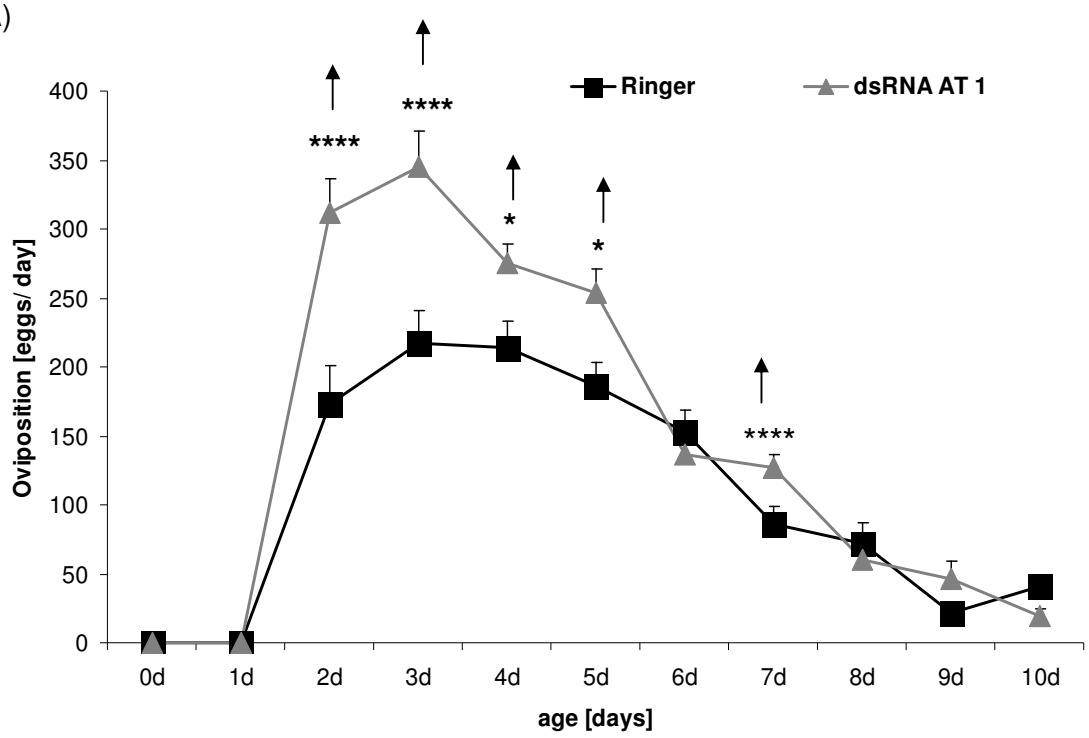
Figure (21): Effect of *AT 1* gene silencing on the titer of free ecdysteroids (ecdysone and 20-hydroxyecdysone, 20E) in the hemolymph of 2 to 8 day (d) old mated (M) females of *S. frugiperda*. Freshly emerged adult females were abdominally injected with either 1.5 µg dsRNA AT 1 in 2 µl noctuid Ringer or 2 µl noctuid Ringer as a control. Animals were reared in a 1: 1 ration with untreated males and a new male was added at the 4th day. Each box was supplied with 10% sucrose, water, and filter paper for egg laying. Hemolymph was collected from 2 d, 4 d, 6 d, 7 d, and 8 d old mated females during the first 3 to 4 hours of scotophase. Ecdysteroids were measured by LC-MS. Means ± SEM; n = 7 - 9 (R), 8 - 10 (dsRNA AT1). Mann-Whitney *U*-test, asterisks indicate significant differences between treatments, **** P < 0.005.*

3.1.12 Reproduction of mated females

Mated females laid much more eggs than virgin females (see Figures 18 and 19). Eggs were deposited continuously after mating from day 2 onwards with a maximum on day 3 (217.2 ± 25.3 eggs), but egg laying then declined slowly until day 9/10. The *AT 1* gene silenced mated females showed a similar oviposition profile as the Ringer controls, but

the peak on day 3 was significantly higher (345 ± 24 eggs) and a second smaller peak occurred on day 7 **(Figure 23 A)**. *AT 1* gene silenced mated females overall laid 1515 ± 131 eggs compared to the Ringer control with 1113 ± 146 eggs (*P < 0.001)* **(Figure 23 B)**. In conclusion, mating as well as *AT 1* gene silencing drastically increased oviposition.

(A)



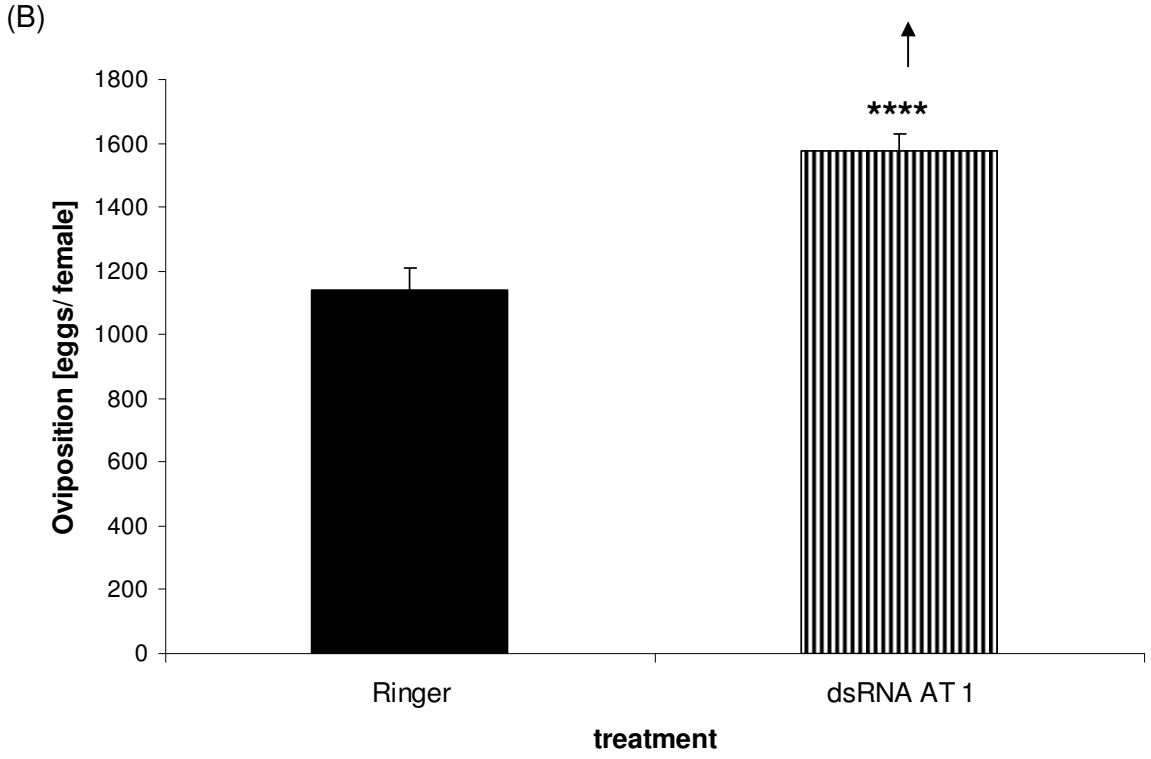


Figure (22): Effect of *AT 1* gene silencing on the profile of egg laying (A) and on accumulated egg deposition (B) for mated females of *S. frugiperda.* Freshly emerged females were injected with either 2 µl noctuid Ringer or 1.5 µg dsRNA AT 1 in 2 µl noctuid Ringer. Males were added to the females in 1: 1 ratio and a new male was added to each female at day 4. 10% sucrose, water, and filter paper were supplied for egg deposition. Daily laid eggs were counted. Means ± SEM; n (R) = 55, (dsRNA AT1) = 69. Mann-Whitney *U*-test, asterisks indicate significant differences between treatments, ** P < 0.05, **** P < 0.001.*

3.1.13 Ovary weight of mated females, weight of oviposited eggs and hatching rates

The ovary weight of mated females of *S. fruiperda* increased with age to a maximum at day 7 after emergence (60 to 90 mg) **(Figure 23)**, independent of treatment of the animals (Ringer injection or *AT 1* gene silencing). The ovary weight of control females increased significantly from 23 ± 17.9 mg in 3 day old females to 90.6 ± 7.6, *P < 0.05*, but not significantly in case of dsRNA AT 1 injected females (41.3 ± 12 mg and 61.8 ± 7.6 mg, respectively). Also it increased from 34.2 ± 6.1 mg in control 5 day old females to 90.6 ± 7.6 mg (*P < 0.05*) in 7 day old ones. dsRNA AT 1 injected females exhibited

an ovary weight of 20 ± 6 mg in 5 day old animals compared to 61.8 ± 7.6 mg (*P < 0.05*) in 7 day old ones **(Figure 23).**

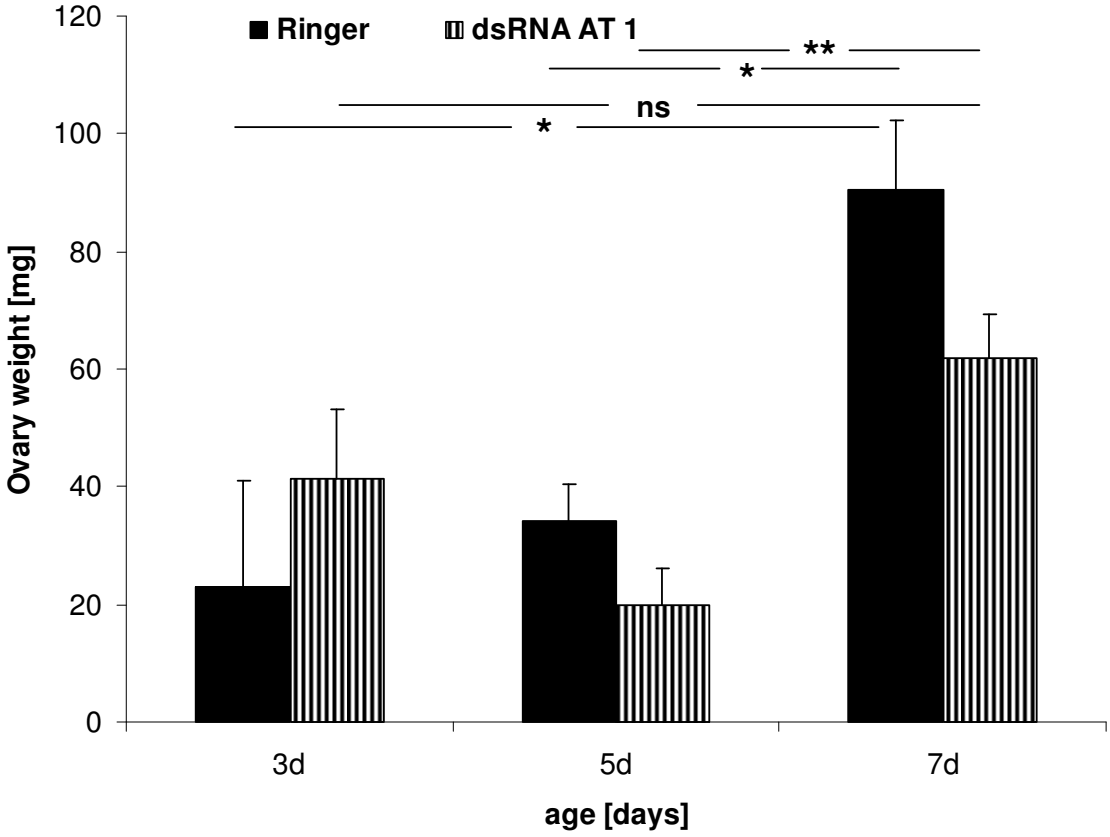


Figure (23): Effect of *AT 1* gene silencing on the ovary weight for mated females of *S. frugiperda*. Freshly emerged females were injected with either 2 µl noctuid Ringer or 1.5 µg dsRNA AT 1 in 2 µl noctuid Ringer. Males were added to the females in a 1: 1 ratio and animals were supplied with 10% sucrose, water, and filter paper for oviposition. Noctuid Ringer n = 8**,** dsRNA AT 1 n= 8. Means ± SEM. Mann-Whitney *U*-test, asterisks indicate significant differences between treatments, ** P < 0.05, ** P < 0.01,* ns = not significant.

The weight of laid eggs was low during the first days of the oviposition period (5 to 10 mg per 100 eggs, that means an average of 75 µg / egg), whereas older females (day 6 to 7 of adult life) laid heavier eggs (up to 340 µg / egg). *AT 1* gene silencing did not influence the weight of eggs laid by younger females, whereas the egg weight for older females (day 6) was significantly reduced **(Figure 24)**.

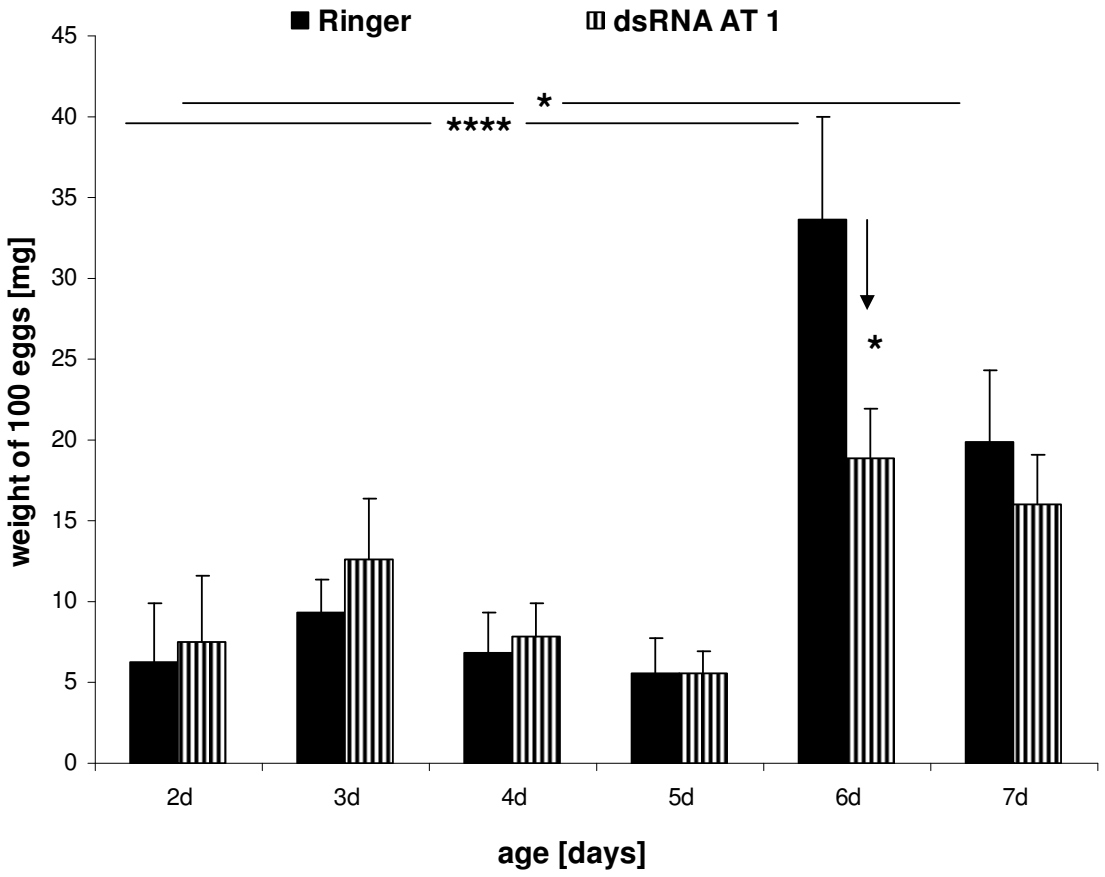


Figure (24): Effect of *AT 1* gene silencing on weight of laid eggs by mated females of *S. frugiperda*. Freshly emerged females were injected with either 2 µl noctuid Ringer or 1.5 µg dsRNA AT 1 in 2 µl noctuid Ringer. Males were added in 1: 1 ratio to females and a new male was added at day 4. Water, 10% sucrose, and filter paper were supplied for oviposition. Daily laid eggs were counted and weighed in portion of hundred using precise balance. Noctuid Ringer n = 8**,** dsRNA AT 1 n = 8. Means ± SEM. Mann-Whitney *U*-test, asterisks indicate significant differences between treatments, ** P < 0.05, **** P < 0.001.*

Oviposition was checked daily and fertility (% egg hatching) of eggs laid on days 2 to 7 was determined. Percentage of hatching from eggs of mated, Ringer injected females was highest for young females (87.1 ± 2.2 % for eggs from 2 day old females), but dropped towards eggs from older females (24.6 ± 8.2 % for eggs from 7 day old females). Percentage of hatched eggs deposited by mated *AT 1* gene silenced females did not significantly differ from the controls **(Figure 25)**, but the decrease of % hatching from day 2 to day 7 was significantly different (*P<0.05*).

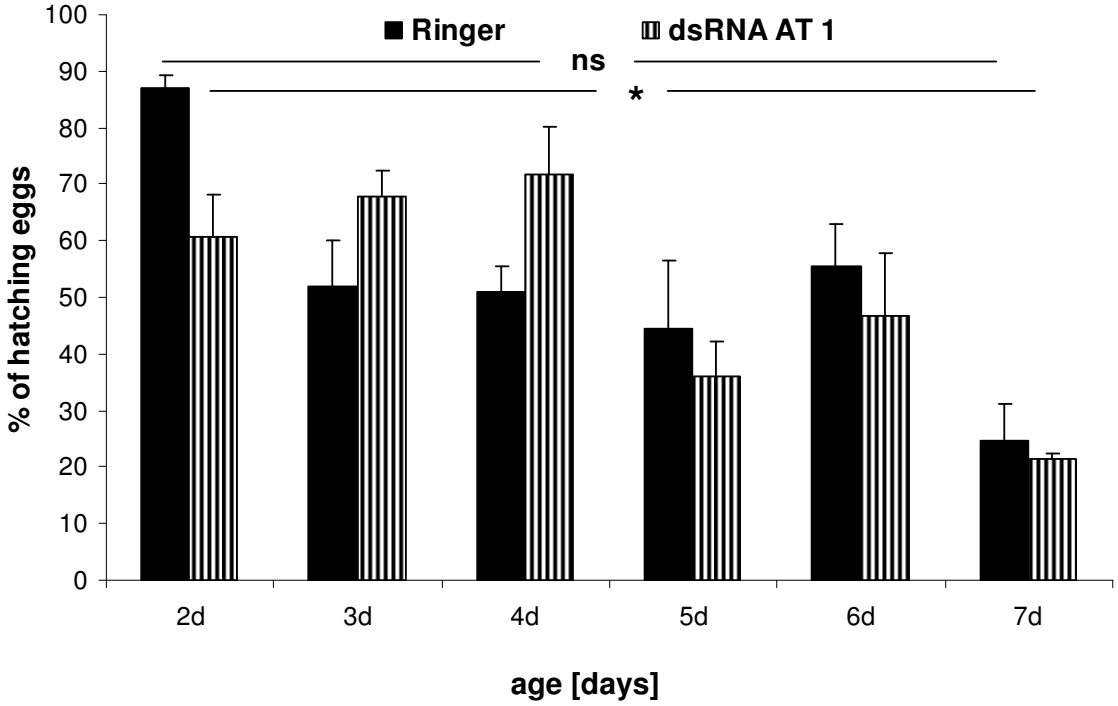


Figure (25): Effect of *AT 1* gene silencing on hatching of eggs laid by mated females of *S. frugiperda*. Freshly emerged females were injected with either 2 µl noctuid Ringer or 1.5 µg dsRNA AT 1 in 2 µl noctuid Ringer. Males were added to females in a 1: 1 ratio. 10% sucrose, water, and filter paper were supplied for oviposition. The daily laid eggs from 2 to 7 day old females were counted and eggs were transferred to incubation containers. Eggs were observed daily for hatching of first instar larvae. Noctuid Ringer n = 8, dsRNA AT 1 n = 8. Means ± SEM. Mann-Whitney *U*-test, asterisks indicate significant differences between treatments, ** P < 0.05*; ns, not significant.

3.1.14 Spermatophores deposited by males

The switch from virgin to ovipositional behaviour of mated females is mediated by the presence of sperm and associated testicular fluids in the bursa copulatrix. The number of deposited spermatophores in the bursa copulatrix of mated females reflects the age of the female and the number of matings **(Figure 26)**. First spermatophores were found in the bursa copulatrix of 4 day old females (younger females were not dissected) and the number of spermatophores increased by one daily. Number of spermatophores was not affected by *AT 1* gene silencing of the female.

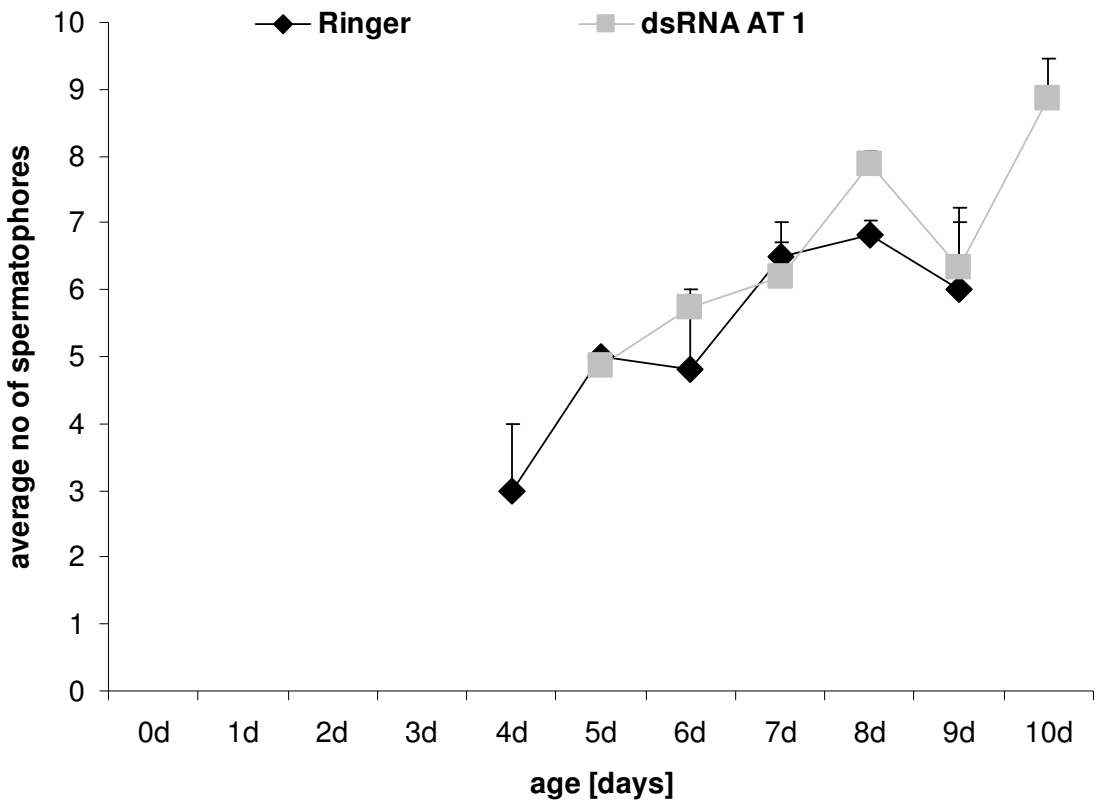


Figure (26): Effect of *AT 1* gene silencing of females of *S. frugiperda* on number of spermatophores deposited by the male into the bursa copulatrix of the female. Freshly emerged females were injected with either 1.5 µg dsRNA AT 1 in 2 µl noctuid Ringer or with 2 µl noctuid Ringer into the third abdominal segment. Untreated males were added to treated females in a ratio of 1: 1. Animals were reared in the presence of 10% sucrose and water. Females of certain age were dissected under binocular microscope. The spermatophores isolated from the bursa copulatrix were counted. Noctuid Ringer n = 10, dsRNA AT 1 n = 10. Means ± S.E.M.

In the following experiment, freshly emerged females and males of *S. frugiperda* were injected with either 1.5 µg dsRNA AT 1 in 2 µl noctuid Ringer or with 2 µl noctuid Ringer into the abdomen and animals were reared under optimal conditions. The number of spermatophores deposited from the male into the bursa copulatrix of the female again increased with age but there were no differences between *AT 1* gene silenced couples and Ringer control couples **(Figure 27)**.

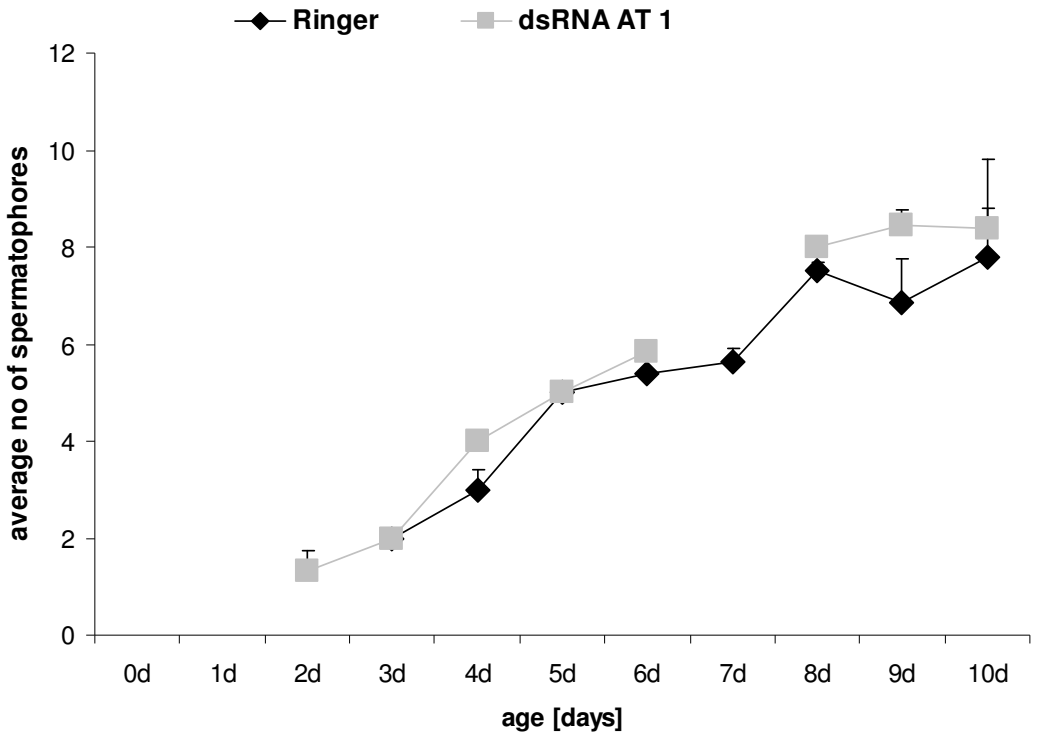


Figure (27): Effect of *AT 1* gene silencing of females and males of *S. frugiperda* on the number of spermatophores deposited in the bursa copulatrix of the females. Freshly emerged females and males were injected with either 1.5 µg dsRNA AT 1 in 2 µl noctuid Ringer or 2 µl noctuid Ringer into the third abdominal segment. Treated males were added to treated females in a ratio of 1: 1. Animals were reared in the presence of 10% sucrose and water. The isolated spermatophores were counted. Noctuid Ringer n = 10, dsRNA AT 1 n = 10. Means ± SEM.

3.2 *In vivo* gene silencing of *AT 1* in males of *S. frugiperda* and effects on hormone level, fertility and reproduction of females mated with treated males

3.2.1 JH in the hemolymph of virgin males

The JH III titer in the hemolymph of virgin males of *S. frugiperda* was measured by LC-MS. 2 day old males showed a concentration of 35.6 ± 11.9 pg / µl. The titer increased to 54.7 ± 14.9 pg / µl in 4 day old males, stayed more or less constant in 6 day old males (40.8 ± 9 pg/ µl), but decreased towards older males (7 day old: 11.3 ± 11.6 pg /

µl). Besides JH III the hemolymph of the males contained considerable amounts of JH II and JH III, which, however, did not change with age except on day 7 of adult life. The total amount of JH, therefore, mainly reflects changes in JH III. *AT 1* gene silencing induced a significant increase in JH II in 2 day old males, but on a low absolute level (2.3 ± 0.5 pg / µl compared with 5.1 ± 1 pg / µl, *P < 0.01* in the control). Moreover, it caused a drastic reduction of JH III on day 4 (12.7 ± 2.2 pg / µl vs. 54.7 ± 14.9 pg / µl, *P < 0.005*) and day 6 (16.2 ± 5.5 pg / µl vs. 40.8 ± 9 pg / µl, *P < 0.05*) which is also seen in total JH concentration **(Figure 28)**.

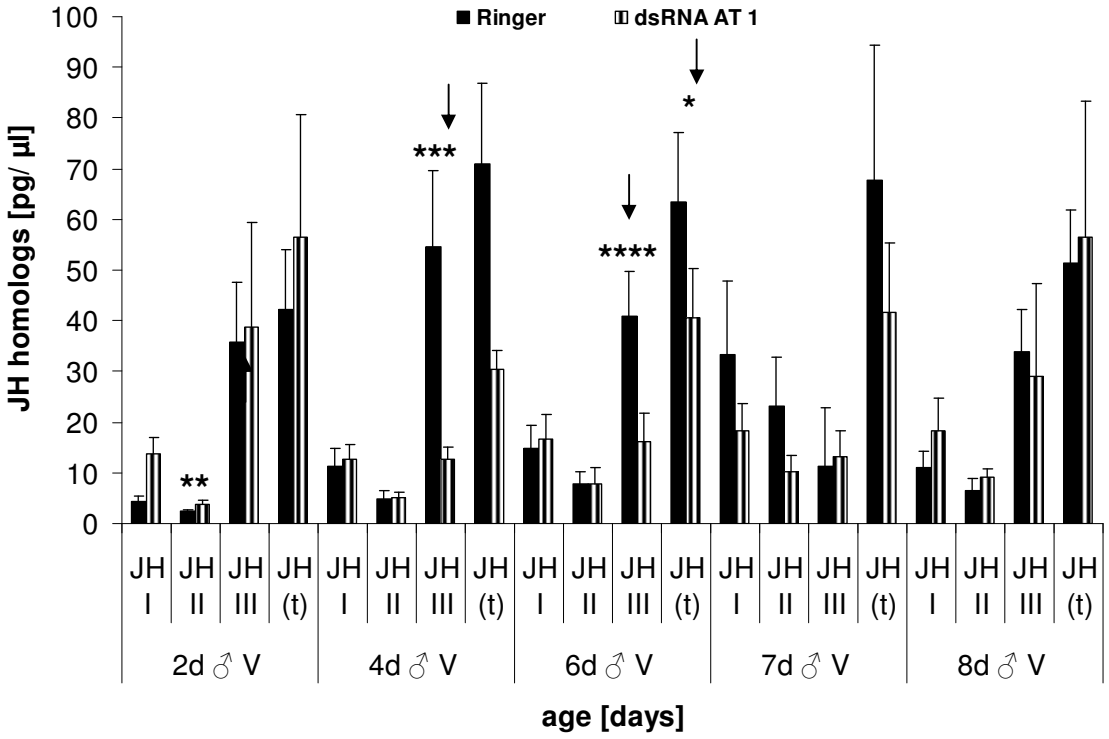


Figure (28): Effect of *AT 1* gene silencing on the titer of the JH homologs in the hemolymph of 2 to 8 day (d) old virgin (V) males of *S. frugiperda*. Freshly emerged males were injected with either 2 µl noctuid Ringer or 1.5 µg dsRNA AT 1 in 2 µl noctuid Ringer, and water and 10% sucrose were supplied. JH titers were measured by LC-MS. Means ± SEM, n = 5-7 (R), 6 (dsRNA AT 1). Mann-Whitney *U*-test, asterisks indicate significant differences between treatments, ** P < 0.05, ** P < 0.01, *** P < 0.005, **** P < 0.001.*

3.2.2 Ecdysteroids in the hemolymph of virgin males

The presence of free ecdysteroids in the hemolymph of virgin 2 to 8 day old males was measured by LC-MS. Concentrations of ecdysone and 20-hydroxyecdysone (20E) in the hemolymph were low in animals of all ages, except for ecdysone in 8 day old males. *AT 1* gene knockdown did not significantly affect the titers of free ecdysteroids in the hemolymph **(Figure 29)**.

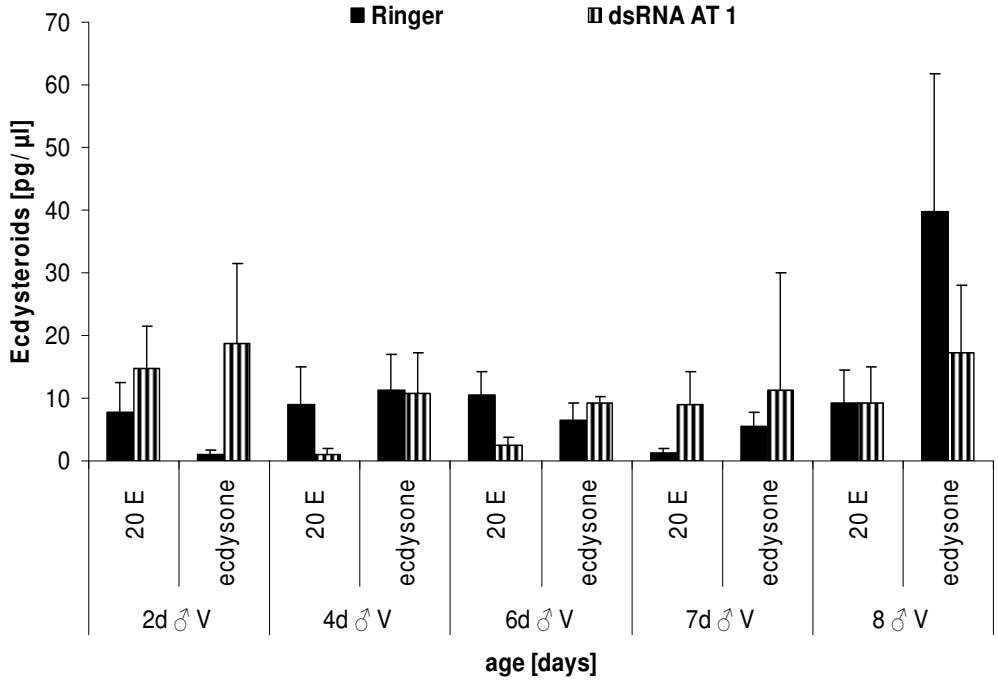


Figure (29): Effect of *AT 1* gene silencing on the titer of free ecdysteroids ecdysone and 20-hydroxyecdysone (20E) in the hemolymph of 2 to 8 day (d) old virgin (V) males of *S. frugiperda.* Freshly emerged males were injected with either 2 µl noctuid Ringer or 1.5 µg dsRNA AT 1 in 2 µl noctuid Ringer. Water and 10% sucrose were supplied. Ecdysone and 20-hydroxyecdysone (20E) measured by LC-MS. Means ± SEM, n = 5 - 7 (R), 6 (dsRNA AT 1). Mann-Whitney *U*-test, no significant differences between treatments.

3.2.3 JH titers in accessory glands of virgin and mated males

JH, mainly JH I and JH II, were detectable in male accessory glands (AG) of newly eclosed virgin males. Concentrations were low at the day of emergence, but significantly increased thereafter (*P<0.001*). Silencing of the *AT 1* gene in virgin males

significantly reduced the amount of JH I in the AG of 2 day old virgin males (12266 ± 3.485 pg JH I / AG compared to 26145 ± 4937 pg JH I / AG, *P < 0.05* in the control) **(Figure 30)**.

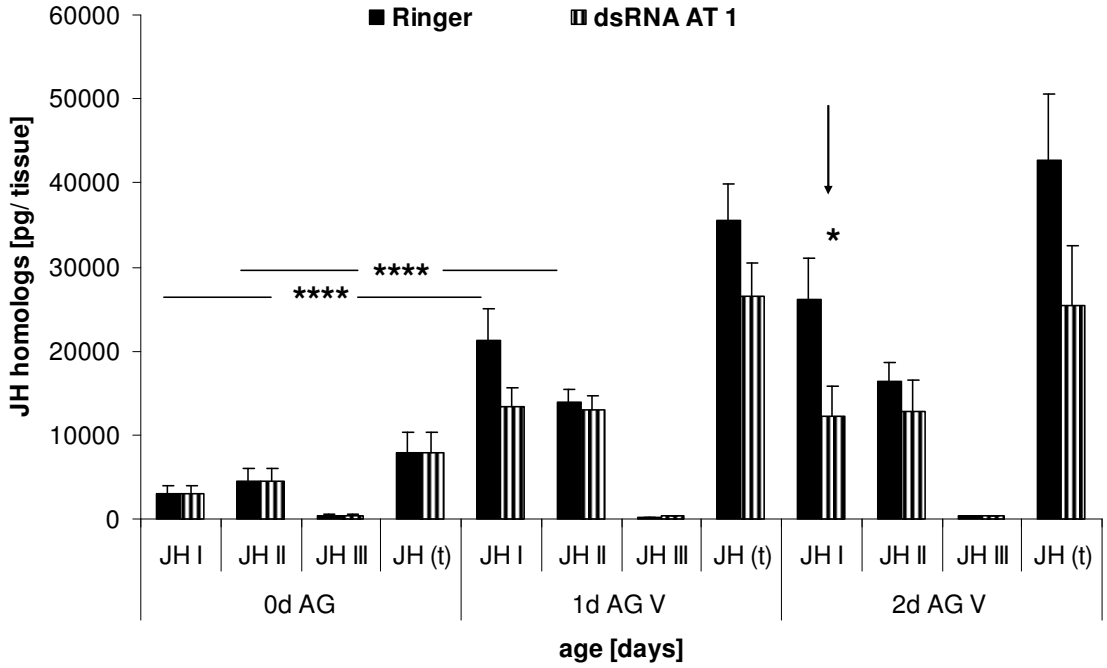


Figure (30): Effect of *AT 1* gene silencing on the amount of JH homologs in male accessory glands (AG) of virgin (V) males of *S. frugiperda*. Newly emerged males were injected with either 2 µl noctuid Ringer or 1.5 µg of dsRNA AT 1 in 2 µl noctuid Ringer. Males were kept separated in boxes, and supplied with water and 10% sucrose. JH homologs were extracted from tissues and measured using LC-MS. The AG were analysed from newly eclosed males (0 d AG), from 1 day old virgin males (1 d AG V), and from 2 day old virgin males (2 d AG V). n = 12 for Ringer and dsRNA AT 1. Means ± SEM. Mann-Whitney *U*-test, asterisks indicate significant differences, ** P < 0.05, **** P < 0.001.*

Coupling of a male with a female led to a transfer of JH from the male accessory gland to the female bursa copulatrix, mainly of JH I and JH II. Therefore, after mating, the JH titer strongly decreased in the AG of control males (4000 pg / AG compared to about 40000 pg / AG in virgin males, see Figure 30). AT 1 RNA interference did not affect the amount of JH homologs in the AG, except for JH III in the glands of 1 day old mated males (215.6 ± 94.1 pg / AG vs. 65.9 ± 25.6 pg / AG, *P < 0.005* in the control) **(Figure 31)**.

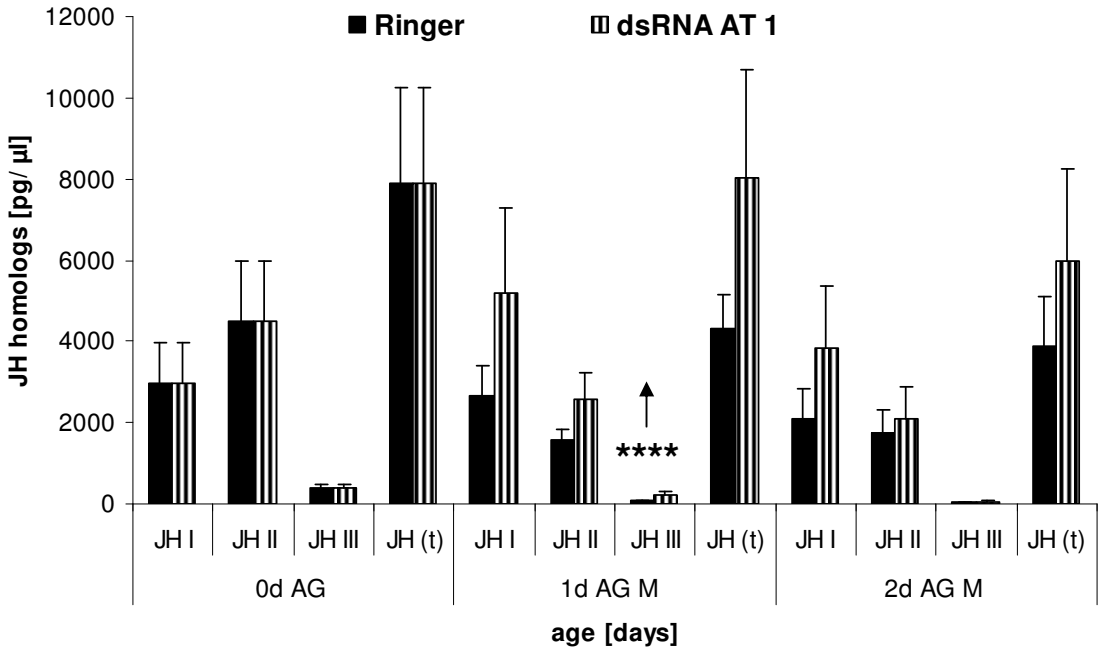


Figure (31): Effect of *AT 1* gene silencing on the amount of JH homologs in male accessory glands (AG) of mated (M) males of *S. frugiperda*. Newly emerged males were injected with either 2 µl noctuid Ringer or 1.5 µg of dsRNA AT 1 in 2 µl noctuid Ringer. Females were added in 1: 1 ratio and animals were supplied with water and 10% sucrose. JH homologs were extracted from tissues and measured using LC-MS. The AG were analysed from newly eclosed males (0 d AG), 1 day old mated males (1 d AG M), and 2 day old mated males (2 d AG M). n = 12 for Ringer and dsRNA AT 1. Means ± SEM. Mann-Whitney *U*-test, asterisks indicate significant differences between treatments, ***** P < 0.001.*

3.2.4 Material transferred from male to female by mating

A high amount of JH, mainly JH I and JH II, was found in the male accessory reproductive gland (AG) of the Ringer controls prior to copulation (about 26000 pg JH I / tissue; 2 d AG V), but the animals were depleted of JH I and JH II after mating (less than 2700 pg JH I / tissue; 2 d AG M) (*P<0.001*) **(Figure 32)**. *AT 1* gene silencing significantly reduced the amount of JH I in the AG of 2 day old virgin males (12266 ± 3485 p / AG compared to 26145 ± 4937 pg / AG in the control, *P < 0.05*) **(Figure 32)**.

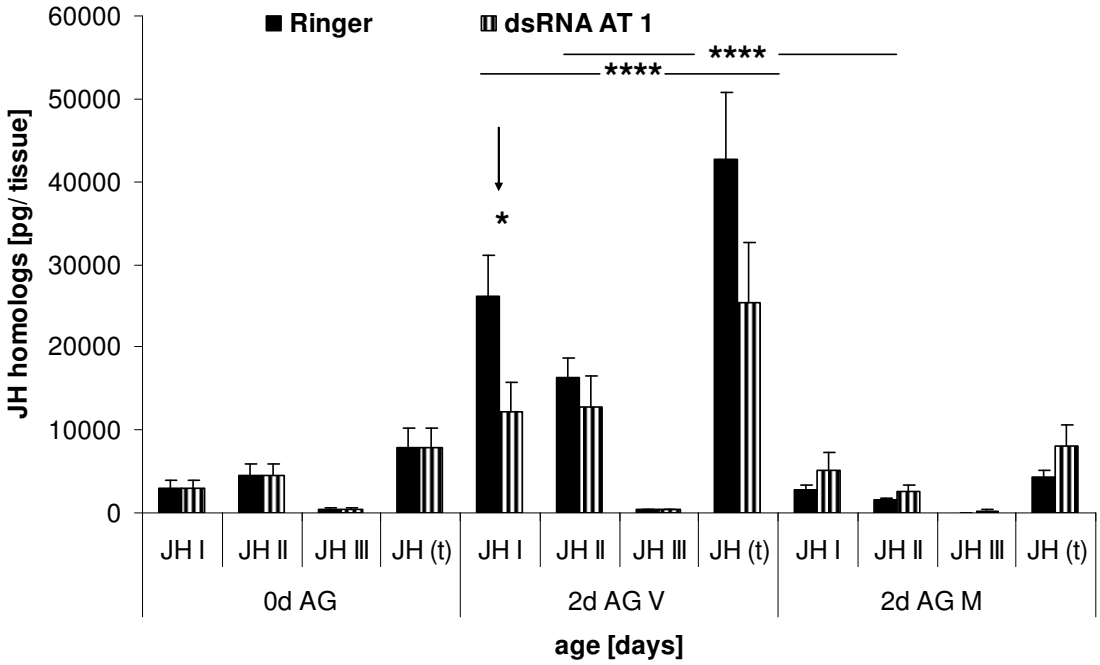


Figure (32): Effect of *AT 1* gene silencing on the amount of JH homologs in virgin (V) male accessory glands (AG) and AG of same age mated (M) males of *S. frugiperda*. Newly emerged males were injected with either 2 µl noctuid Ringer or 1.5 µg of dsRNA AT 1 in 2 µl noctuid Ringer. Females were added in 1: 1 ratio in the case of mating and animals were supplied with water and 10% sucrose. JH homologs were extracted and measured using LC-MS. The AG were analysed from newly eclosed males (0 d AG), 2 day old virgin males (2 d AG V), and from same age mated males (2 d AG M). n = 12 for Ringer and dsRNA AT 1. Means ± SEM. Mann-Whitney *U*-test, ** P < 0.05, **** P < 0.001.*

In the bursa copulatrix (B) of the female almost no JH was detected in virgin animals **(Figure 33)**, but then JH I, JH II, and JH III were transferred from the male accessory gland by the spermatophore through mating. The same changes in the amount of JH were observed for mating with *AT 1* gene silenced males, with about 25000 pg / tissue of total JH. The transferred JH disappeared from the bursa copulatrix 24 to 48 hours after mating (853 ± 352 pg JH (t) / tissue in the Ringer control and 3297 ± 2526 pg JH (t) / tissue in AT 1 treated animals.

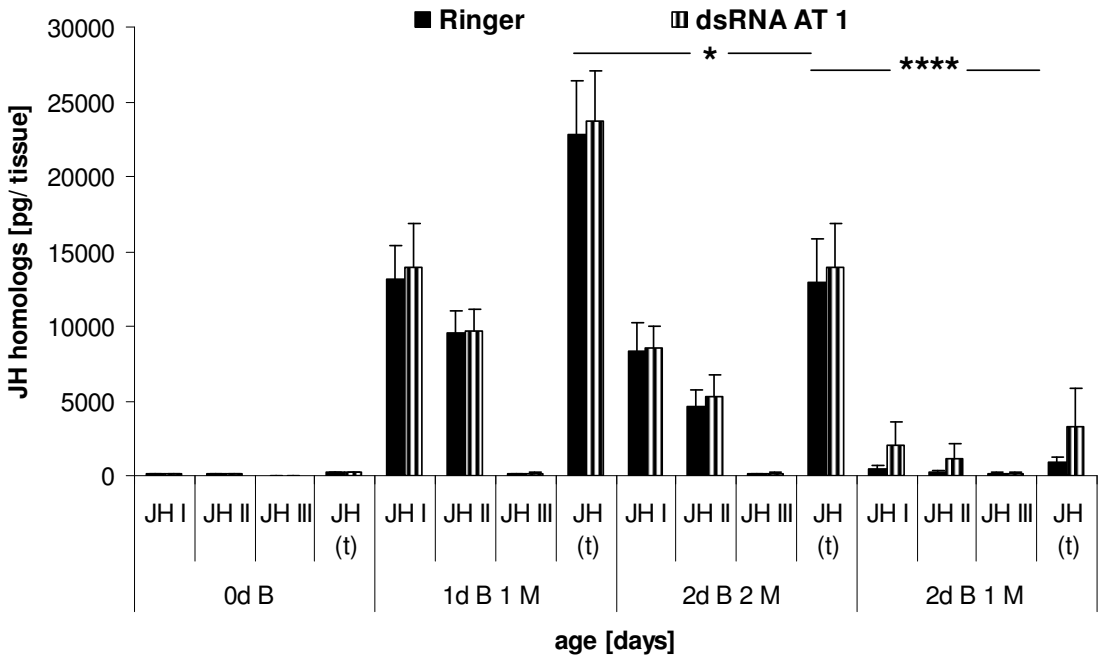


Figure (33): Effect of *AT 1* gene silencing on JH homologs transferred from male accessory glands of *S. frugiperda* to the bursa copulatrix (B) of females during mating. Newly emerged males were injected with either 2 µl noctuid Ringer or 1.5 µg of dsRNA AT 1 in 2 µl noctuid Ringer in the third abdominal segment. Untreated females were added in 1: 1 ratio to the males on day 1 after ecdysis. Pairs were kept in boxes in presence of water and 10% sucrose. The isolated bursa copulatrix were homogenized and the amounts of JH homologs were measured using LC-MS. The bursa copulatrix was analysed from newly eclosed females, 1 day old females mated once (1 d B 1 M), from 2 day old females mated twice (2 d B 2 M) and from 2 day old females mated once on day 1 (2 d B 1 M). n = 12 for Ringer and dsRNA AT 1. Means ± SEM. Mann-Whitney *U*-test, asterisks indicate significant differences, ** P < 0.05, **** P < 0.001*.

3.2.5 Hemolymph JH titers of females mated with *AT 1* gene silenced males

In mated females of S*. frugiperda* the JH homologs depleted from the bursa copulatrix 24 to 48 hours after mating (especially JH I and JH II) seem to have been released into the hemolymph. Concentrations of JH I and JH II in the hemolymph increased from day 2 to day 4 and this effect was especially pronounced in females mated with *AT 1* gene silenced males (2 d 1 M females have 13.5 ± 5.4 pg JH I / µl and 12.9 ± 1.2 pg JH II / µl, females 4 d 3 M exhibited 66.3 ± 14 pg JH I / µl, *P < 0.001* and 76.5 ± 14.6 pg JH II / µl*, P < 0.001*) whereas, no differences were found between the same age control females.

AT 1 gene silencing of males induced elevation of untreated 4 day old females hemolymph JH (66.3 ± 14 pg JH I / µl and 76.52 ± 14.6 pg JH II / µl compared to 24.2 ± 2.9 pg JH I / µl, *P < 0.001* and 23.2 ± 2.6 pg JH II / µl, *P < 0.005* in the Ringer controls) **(Figure 34)**.

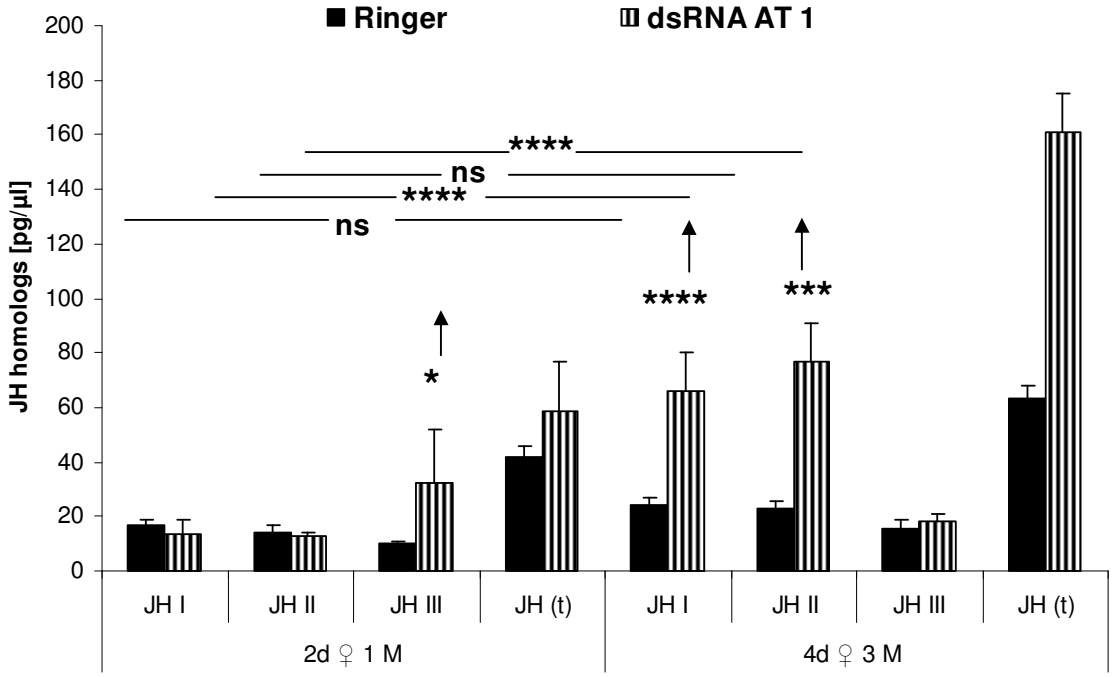


Figure (34): Effect of *AT 1* gene silencing in males on the hemolymph JH titer of untreated females of *S. frugiperda* after mating. Newly emerged males were injected with either 2 µl noctuid Ringer or 1.5 µg of dsRNA AT 1 in 2 µl noctuid Ringer. Untreated females were added in a ratio of 1: 1 to the treated males. Water, 10% sucrose, and filter paper were supplied for egg deposition. JH hemolymph titers were determined 1 day after mating in 2 day old females (2 d ♀ 1 M) and 3 day after mating in 4 d old females after 3 successive matings (4 d ♀ 3 M). The JH titers were measured using LC-MS. n = 10 for Ringer and dsRNA AT 1. Means ± SEM. Mann-Whitney *U*-test, asterisks indicate significant differences between treatments, ** P < 0.05, *** P < 0.005, **** P < 0.001;* ns, not significant.

3.2.6 Hemolymph ecdysteroid titers of females mated with *AT 1* gene silenced males

Mated females contain high concentrations of free ecdysteroids (ecdysone, E; 20-hydroxyecdysone, 20E) in the hemolymph already at day 2 after emergence (see Figure 21). These high concentrations of ecdysone and 20E were significantly reduced when females had been mated with *AT 1* gene silenced males (78.3 ± 42 pg 20E / µl vs. 24.9

± 7.7 pg 20E / µl, *P < 0.05* and 59.6 ± 32.3 pg E / µl vs. 15.5 ± 5.4 pg E / µl, P < 0.005) **(Figure 35)**. In 4 day old females, however, a significant increase in the concentration of 20E in the hemolymph was observed when females had been mated daily with *AT 1* gene silenced males (35.3 ± 4.1 pg 20E / µl vs. 16.5 ± 3.7 pg 20E / µl, *P < 0.005*).

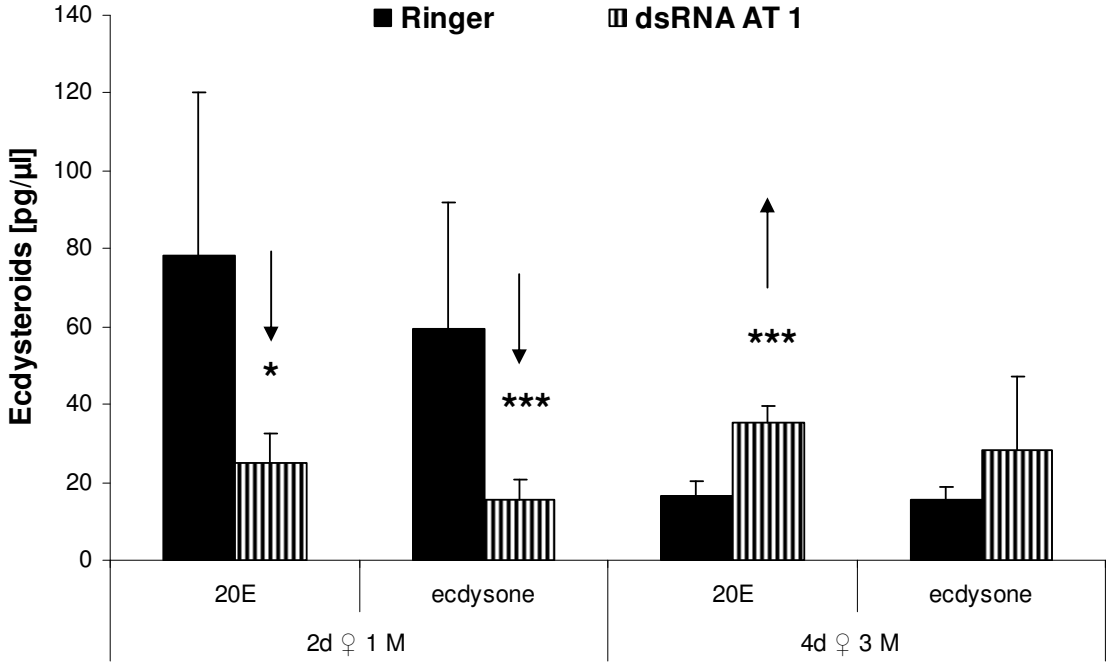


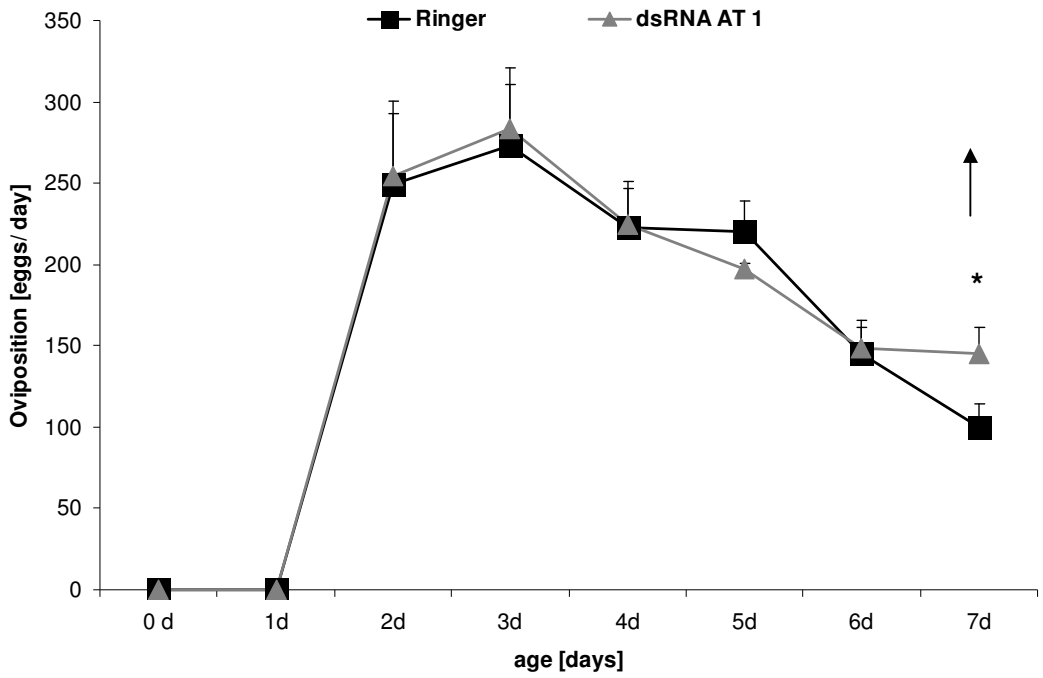
Figure (35): Effect of *AT 1* gene silencing in males on the hemolymph ecdysteroid titers of untreated females of *S. frugiperda* after mating. Newly emerged males were injected with either 2 µl noctuid Ringer or 1.5 µg of dsRNA AT 1 in 2 µl noctuid Ringer. Untreated females were added in a ratio of 1: 1 to the treated males. Water, 10% sucrose, and filter paper were supplied for egg deposition. Free ecdysteroids (ecdysone; 20-hydroxyecdysone, 20E) in the hemolymph were determined on day 1 after mating in 2 day old females (2 d ♀ 1 M), and in 4 day old females after three successive matings (4 d ♀ 3 M). Ecdysteroids were measured using LC-MS. n = 10 for Ringer and dsRNA AT 1. Means ± SEM. Mann-Whitney *U*-test, asterisks indicate significant differences between treatments, ** P < 0.05, *** P < 0.005.*

3.2.7 Reproduction of females mated with *AT 1* gene silenced males

Mated females started to lay eggs on day 2 after of adult life and reached a maximum of more than 200 eggs per day on day 3. Oviposition rate decreased in older females (see also Figure 22 A). *AT 1* gene silencing of the males and coupling with untreated females

did not affect the timing and amount of egg laying **(Figure 36 A)** except on day 7 of adult life, where females mated with *AT 1* gene silenced males laid significantly more eggs than the control females (144.8 ± 16.4 eggs / day vs. 99.7 ± 15 eggs / day, $P < 0.05$). Females mated with *AT 1* gene silenced males laid a total amount of 1252.2 ± 107.8 eggs / female, which is not significantly different from females mated with Ringer injected control males (1210.7 ± 98.8 eggs / female) **(Figure 36B)**.

(A)



(B)

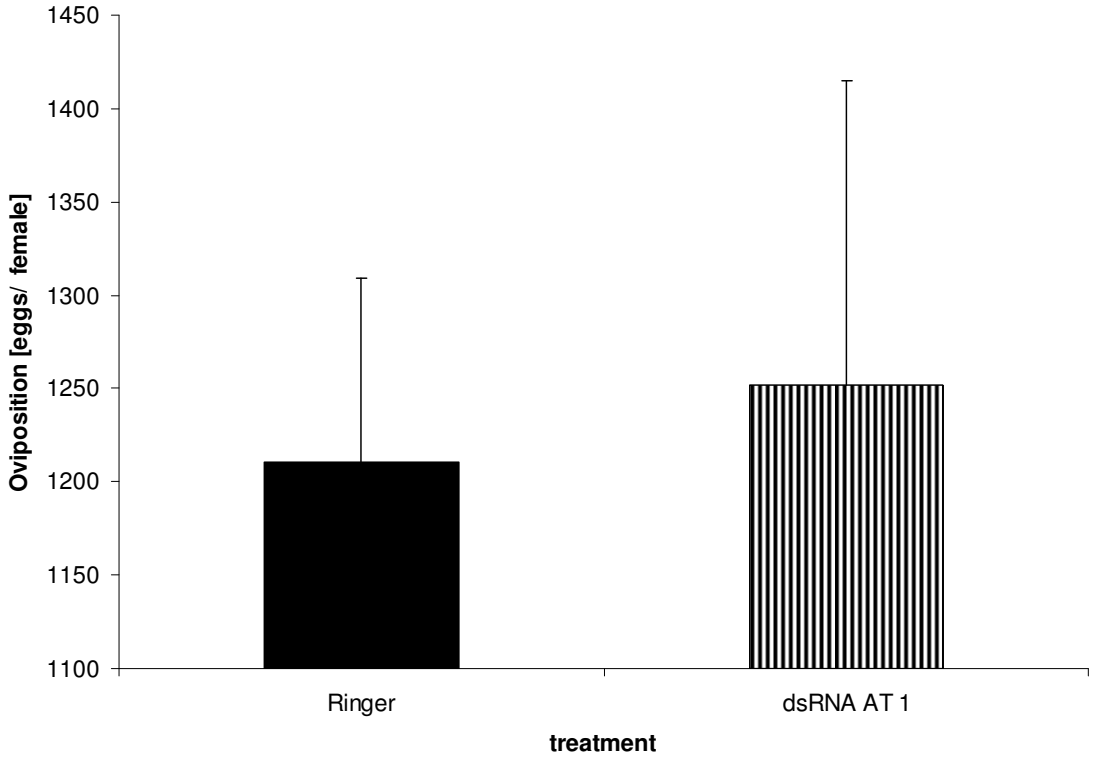


Figure (36): Effect of *AT 1* gene silencing in males on the profile of egg laying (A) and on accumulated egg deposition (B) of *S. frugiperda* females mated with such males. Freshly moulted adult males were injected with either 2 µl noctuid Ringer or 1.5 µl dsRNA AT 1 in 2 µl noctuid Ringer. Untreated females were added in 1: 1 ratio to the treated males. Animals were kept paired in boxes, and water, 10% sucrose, and filter paper were supplied for egg deposition. Daily laid eggs were counted. Means ± SEM; n Ringer = 25, dsRNA AT 1 = 26). Mann-Whitney *U*-test, asterisks indicate significant differences between treatments, ** P < 0.05*.

3.2.8 Ovary weight of females mated with *AT 1* gene silenced males, weight of oviposited eggs and hatching

The ovary weight of 7 day old females mated with *AT 1* gene silenced males (67.4 ± 19.7 mg) did not differ significantly from the Ringer control (46.4 ± 10.7 mg) **(Figure 37).**

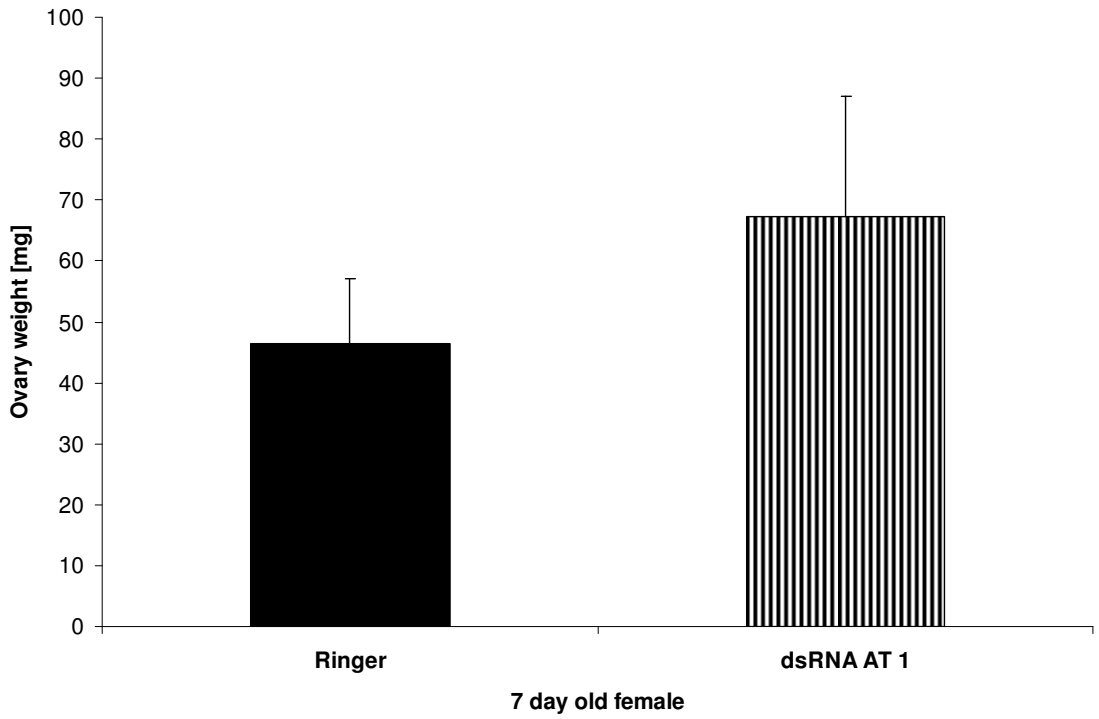


Figure (37): Effect of *AT 1* gene silencing in males on the ovary weight of females (day 7 of adult life) mated with such treated males. Freshly moulted males were injected with either 2 µl noctuid Ringer or 1.5 µg dsRNA AT 1 in 2 µl noctuid Ringer. Untreated females were added to the males in 1: 1 ratio. Animals were kept in boxes, in the presence of water, 10% sucrose, and filter paper for egg deposition. Ovaries from 7 day old mated females were dissected and weighed using precise balance. Means ± SEM. Noctuid Ringer n = 8, dsRNA AT1 n = 8. Mann-Whitney *U*-test, no significant differences between treatments.

The weight of eggs laid by females mated with Ringer injected control males of *S. frugiperda* was low for young males (day 2 of adult life) but increased in older females. On day 2 100 eggs weight an average of 9.1 ± 3.8 mg, but more that 20 mg on day 4 (21.2 ± 4.1 mg) ($P < 0.05$). Weight of eggs laid by 2 to 7 day old females mated with *AT 1* gene silenced males did not significantly differ from the Ringer controls, but the increase in weight from day 2 to day 3 was significantly different ($P<0.05$) **(Figure 38)**.

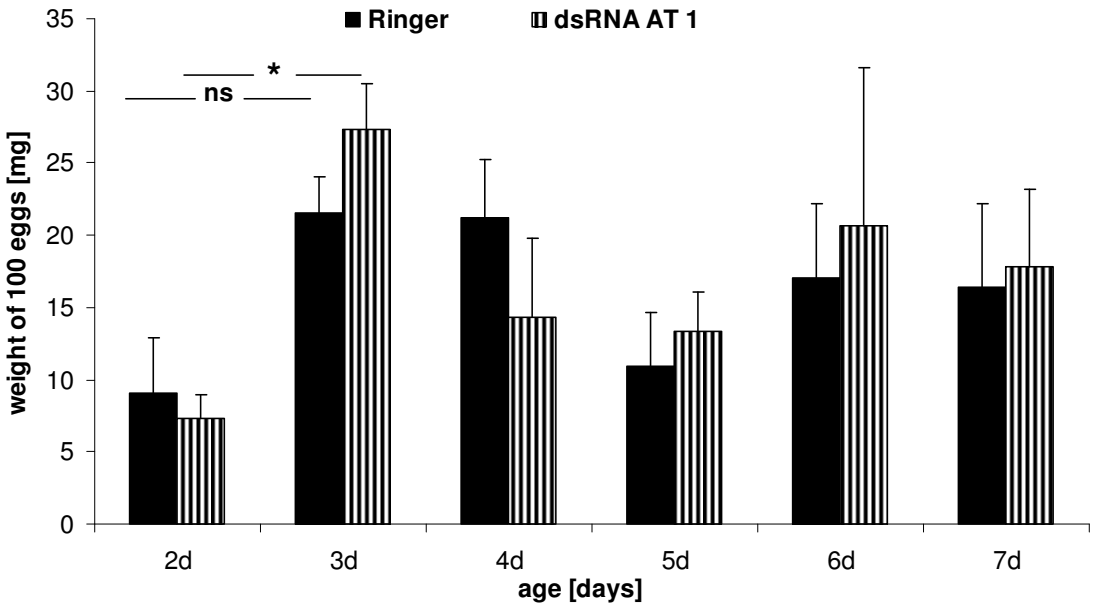


Figure (38): Effect of *AT 1* gene silencing on weight of laid eggs by untreated females mated with in *AT 1* gene silenced males. Freshly emerged males were injected with either 2 µl noctuid Ringer or 1.5 µg dsRNA AT 1 in 2 µl noctuid Ringer. Untreated females were added to the males in 1: 1 ratio. Water, 10% sucrose, and filter paper for egg deposition were supplied. Daily laid eggs were counted on days 2 to 7 of adult life. Eggs of certain days were weighed in portion of 100 using precise balance. Noctuid Ringer n = 8, dsRNA AT 1 n = 8. Means ± SEM. Mann-Whitney *U*-test, ** P < 0.05;* ns, not significant.

The percentage of larval hatching from eggs deposited by females mated with Ringer injected males was high for eggs from young females (2 day old 56.5 ± 16.2%, 3 day old 68.4 ± 13 %), but then declined (4 day old 31.5 ± 4.4 %, 5 day old 36.14 ± 3.3 %, 6 day old 34.7 ± 7.3 %, and 7 day old 44 ± 8 %). There is a significant difference between controls at day 3 (68.4 ± 13 %) and day 4 (31.5 ± 4.4 %, *P < 0.05*), but no significant difference between *AT 1* gene silenced day 3 (59 ± 3.6 mg) and day 4 eggs (43.9 ± 11.6 %) (*P < 0.05*). The percentage of hatched eggs laid by 2 to 7 day old females mated with *AT 1* gene silenced males did not differ from the respective Ringer controls **(Figure 39)**.

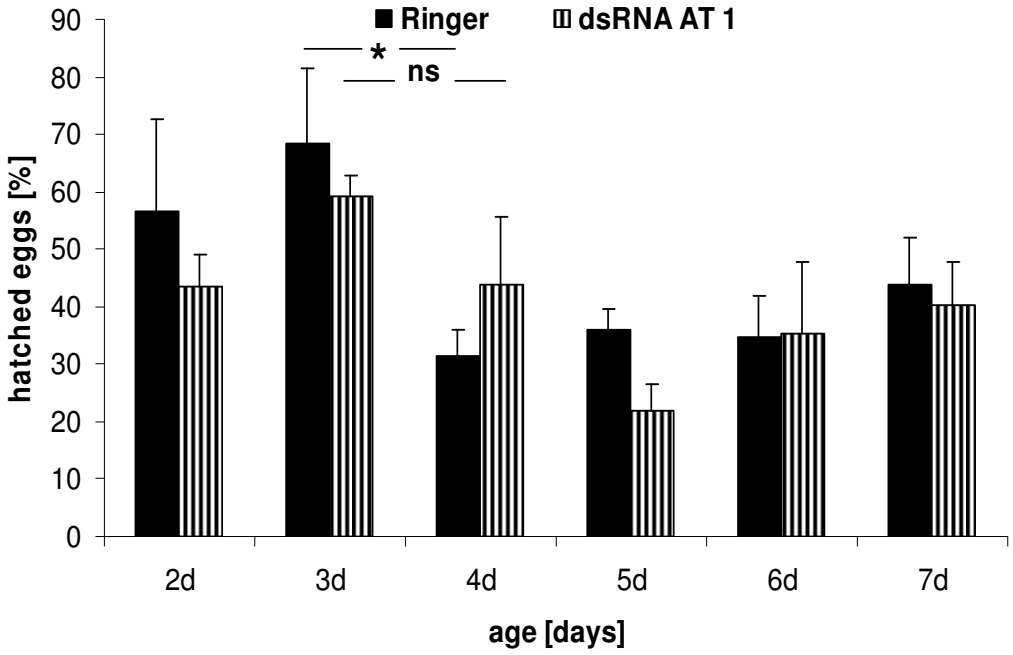


Figure (39): Effect of *AT 1* gene silencing of males on the hatch rate for eggs laid by untreated females mated with *AT 1* gene silenced males. Freshly ecdysed males were injected with either 2 µl noctuid Ringer or 1.5 µg dsRNA AT1 in 2 µl noctuid Ringer. Untreated females were added to the males in 1: 1 ratio. Water, 10% sucrose, and filter paper for egg deposition were supplied. Daily laid eggs were counted and transferred to incubation containers. Eggs were observed daily for hatching of first instar larvae. Means ± SEM). Noctuid Ringer n = 8, dsRNA AT 1 n = 8. Mann-Whitney *U*-test, asterisks indicate significant differences, ** P < 0.05;* ns, not significant.

3.2.9 Spermatophores deposited by males

The number of spermatophores dissected from the bursa copulatrix of mated females reflects the number of matings. Males of *S. frugiperda* deposit an average of one spermatophore per 24 hours. The number of spermatophores transferred to the bursa copulatrix of the females increased with age, but there was no difference between *AT 1* gene silenced males and Ringer controls **(Figure 40)**.

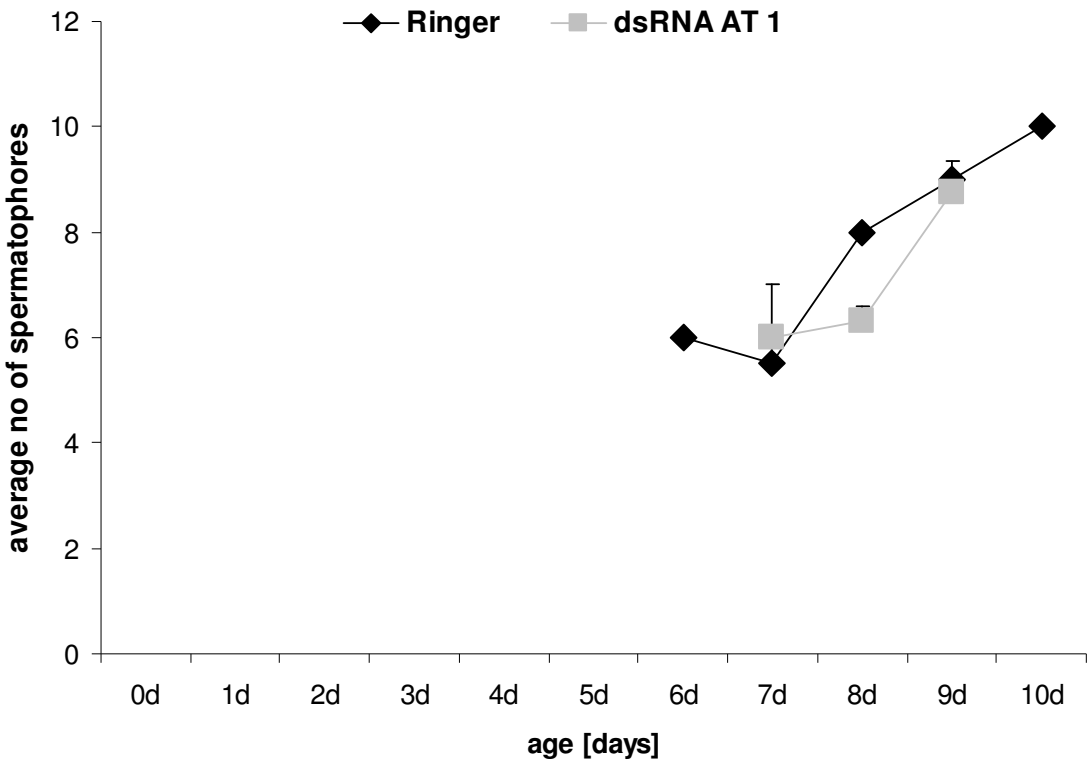


Figure (40): Effect of *AT 1* gene silencing of males of *S. frugiperda* on the number of spermatophores from the bursa copulatrix of females mated with such treated males. Freshly emerged adult males were injected with either 1.5 µg dsRNA AT 1 in 2 µl noctuid Ringer or 2 µl noctuid Ringer into the third abdominal segment at the day of moulting. Untreated females were added to the males in a ratio of 1: 1. Animals were kept in boxes, in the presence of 10% sucrose and water. The isolated spermatophores were counted in females of certain age. Noctuid Ringer n = 10, dsRNA AT 1 n = 10. Means ± SEM.

3.3 *In vivo* gene silencing of allatostatin *type A* in males of *S. frugiperda* and effects on material transferred from the male to the female by mating

Former experiments on injection of dsRNA targeted against type-A allatostatin into adult females and males of *S. frugiperda* had resulted in a significant and specific knockdown of the respective mRNA in brain, ovaries, and gut (Meyering-Vos et al., 2006; M. Meyering-Vos, unpublished) and are not shown here.

3.3.1 JH titers in accessory glands of virgin and mated males

JH, mainly JH I and JH II, were found in the accessory glands (AG) of newly eclosed males. Concentrations were low at the day of emergence (see Figure 30) but increased thereafter. Silencing of the *AS A-type* gene in virgin males led to a drastic increase in the amount of JH I (and total JH) in the AG of 1 day old virgin males (56334 ± 4363 pg JH (t) / AG compared to 35557 ± 4363 pg JH (t) / AG, $P < 0.05$ in the control and 40778 ± 5443 pg JH I / AG compared to 21312 ± 3655 pg JH I / AG, $P < 0.005$ for the control) **(Figure 41)**. On day 2 after ecdysis JH concentrations in the AG of virgin *AS type A* gene silenced males were not different from the control.

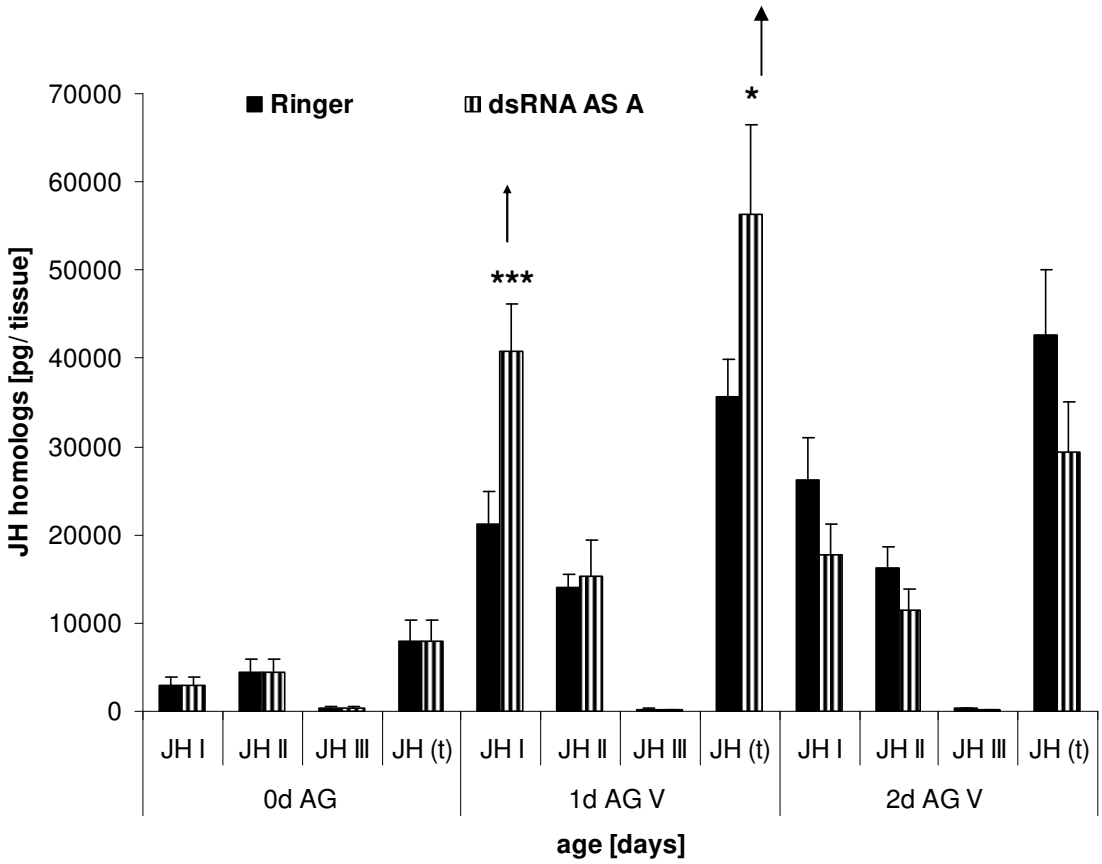


Figure (41): Effect of *AS A-type* gene silencing on the amount of JH homologs in male accessory glands (AG) of virgin (V) males of *S. frugiperda*. Newly eclosed males were injected with either 2 µl noctuid Ringer or 1.5 µg of dsRNA *AS A-type* in 2 µl noctuid Ringer. Males were kept separated in boxes, and supplied with water and 10% sucrose. JH homologs were extracted from tissues and measured using LC-MS. The AG were analysed from newly eclosed males (0 d AG), 1 day old virgin males (1 d AG V), and 2 day old virgin males (2 d AG V). n = 12 for Ringer and dsRNA AS A-type. Means ± SEM. Mann-Whitney *U*-test, asterisks indicate significant differences between treatments, ** P < 0.05, *** P < 0.005.*

Coupling of a male with a female led to a transfer of JH from the male accessory gland to the female bursa copulatrix (see 3.2.3). Therefore, after mating, the JH titer strongly decreased in the AG of control males, but also in *AS A-type* gene silenced males. A gap in mating on day 2 resulted in a significant reloading of the AG with JH I and JH II. Another mating at day 2 again led to almost complete depletion of the AG from JH*; P < 0.001*). *AS type A* gene silencing of the males did not affect the changes in JH concentrations in the accessory glands **(Figure 42)**.

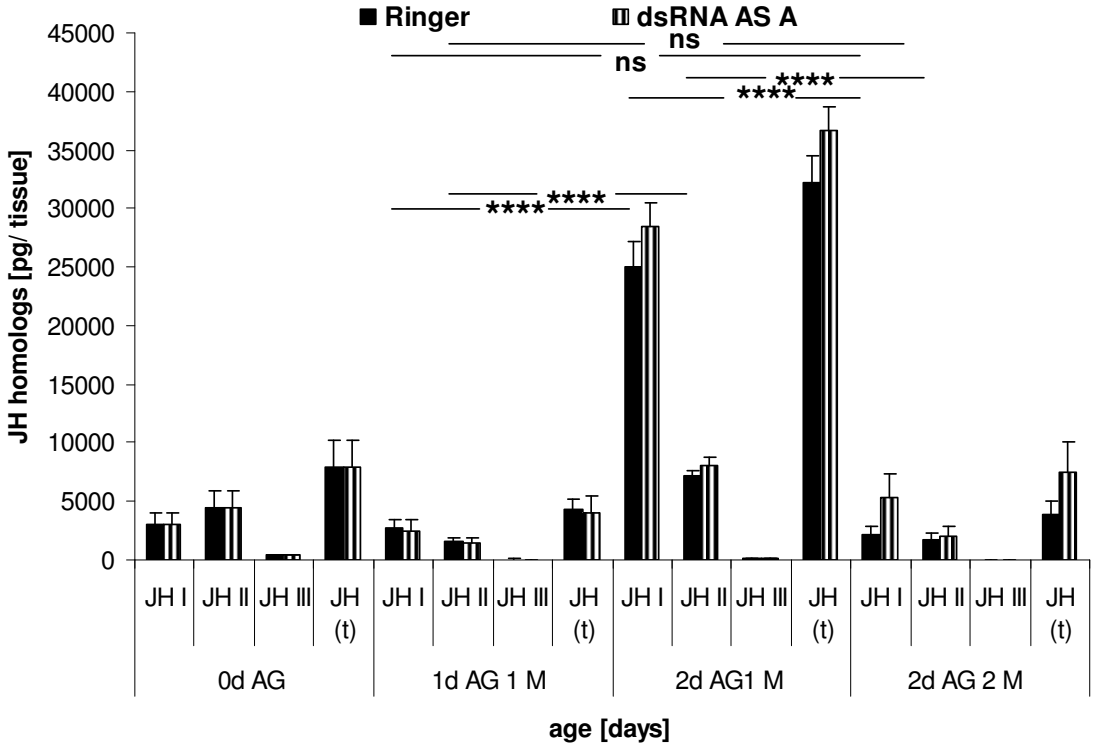


Figure (42): Effect of *AS A-type* gene silencing on the amount of JH homologs in male accessory glands (AG) of mated (M) *S. frugiperda* males. Newly eclosed males were injected with either 2 µl noctuid Ringer or 1.5 µg of dsRNA *AS-A type* in 2 µl noctuid Ringer. Untreated females were added to the males in 1: 1 ratio. Water and 10% sucrose were supplied. The isolated tissues were homogenized and the JH concentrations measured using LC-MS. The AG were analysed from 1 day old males mated once (1d AG 1 M), from 2 day old males mated once on day 1 (2 d AG 1 M) and from 2 day old males mated twice (2 d AG 2 M). Data for 0 d AG are from Figure 41. n = 12 for Ringer and dsRNA AS A-type. Means ± SEM. Mann-Whitney *U*-test, asterisks indicate significant differences, ***** P < 0.001;* ns, not significant.

3.3.2 Material transferred from male to female by mating

Freshly emerged males contain rather low amounts of JH, mainly JH I and JH II, in their accessory glands (AG) (see also Figure 32). In virgin males, the amount of JH I and JH II in the AG increased dramatically from the day of moulting until day 2 of adult life (0 d AG 2971.1 ± 999.4 pg JH I / AG compared to 2 d AG V 26145.1 ± 4937.5 pg JH I / AG, *P < 0.001* and 0 d AG 4516 ± 1447.5 pg JH II / AG compared to 2 d AG V 16293 ± 2369 pg JH II / AG, *P < 0.001*), but dropped again after mating (2 d AG 2 M 2676 ± 722 pg JH I / AG, *P < 0.001* and 2 d AG 2 M 1564 ± 261 pg JH II / AG, *P < 0.001*). *AS type A* gene

silencing of the males immediately after ecdysis did not affect the concentrations of JH homologs in virgin and mated males, respectively **(Figure 43)**.

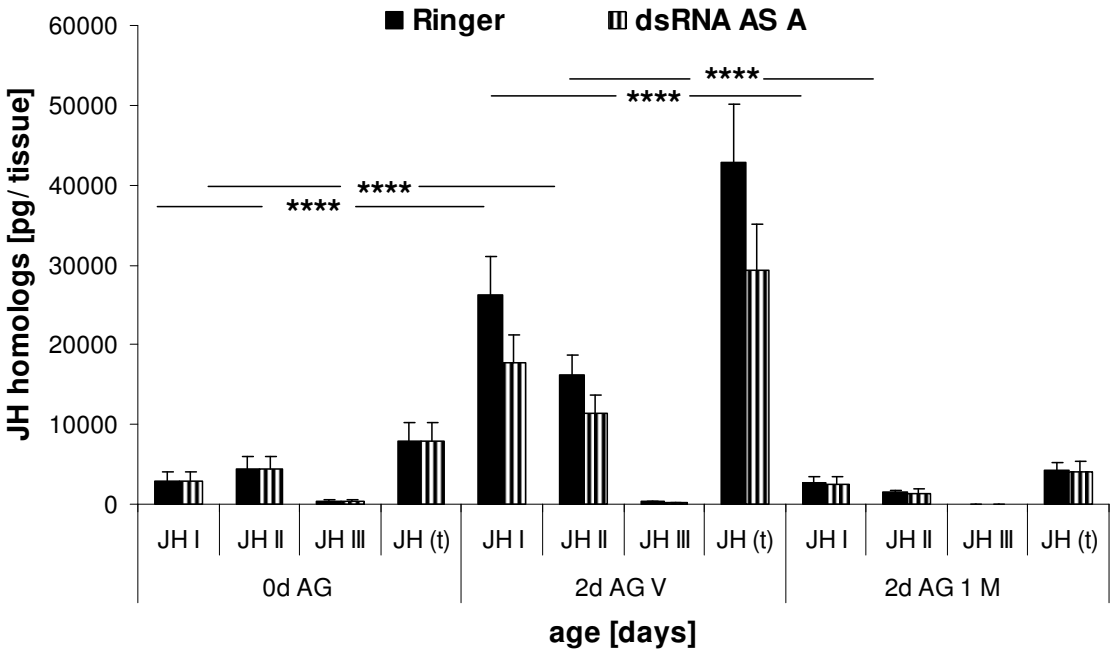


Figure (43): Effect of *AS A-type* gene silencing on the amount of JH homologs in virgin (V) male accessory glands (AG) and in the AG of same age mated (M) males of *S. frugiperda*. Newly eclosed males were injected with either 2 µl noctuid Ringer or 1.5 µg of dsRNA AS A-type in 2 µl noctuid Ringer into the third abdominal segment. Untreated females were added to the males in 1: 1 ratio. Animals were kept paired in boxes, in presence of water and 10% sucrose. JH concentrations were measured using LC-MS. The AG were analysed from newly eclosed males (0 d AG), from 2 day old virgin males (2 d AG V), and from 2 day old males mated once on day 1 (2 d AG 1 M). n = 12 for Ringer and dsRNA AS A-type. Means ± SEM. Mann-Whitney *U*-test, asterisks indicate significant differences, ***** P < 0.001*.

Newly emerged females of *S. frugiperda* do not have measurable amounts of JH in their bursa copulatrix (0 d B) (see also Fig. 33). Mating with a male leads to a significant increase in JH I and JH II in the bursa copulatrix, in one as well as in two day old females (*P < 0.001*) **(Figure 44)**. A gap in mating for 24 hours resulted in an almost complete depletion of JH from the bursa (*P < 0.001*). Females mated with *AS A- type* gene silenced males exhibited almost the same JH concentrations in the bursa copulatrix as those mated with Ringer injected males.

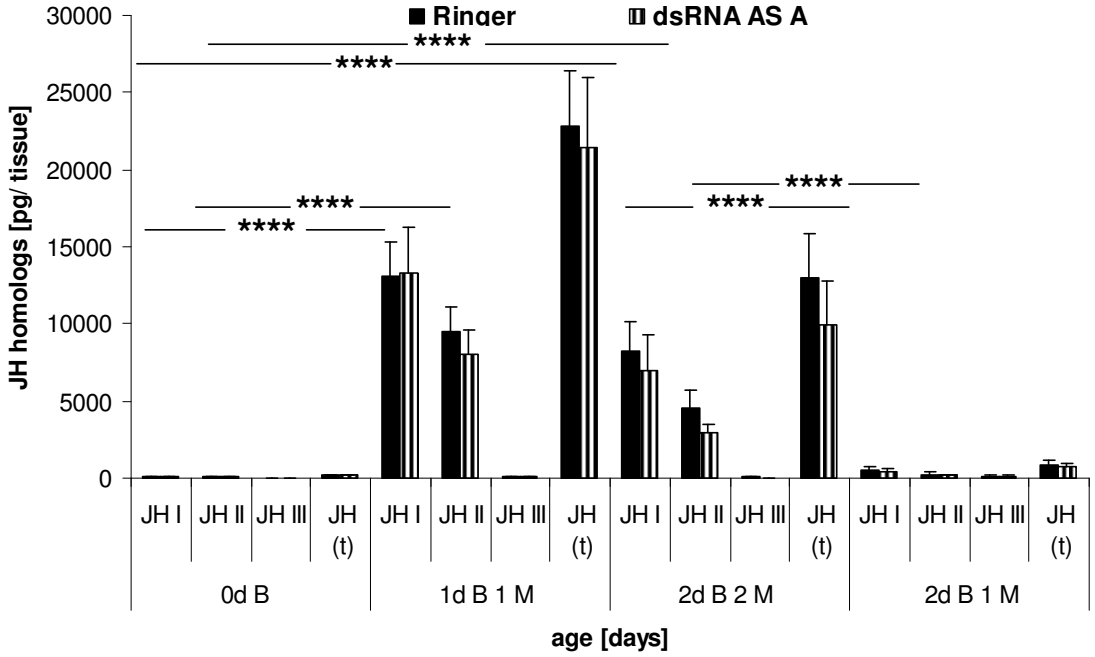


Figure (44): Effect of *AS A-type* gene silencing in males on JH homologs transferred from male accessory glands (AG) of *S. frugiperda* to the bursa copulatrix (B) of females during mating. Newly eclosed males were injected with either 2 µl noctuid Ringer or 1.5 µg of dsRNA AS A-type in 2 µl noctuid Ringer. Untreated females were added in 1: 1 ratio to the males on day 1 after ecdysis. Pairs were kept in boxes in presence of water and 10% sucrose. The JH concentrations were measured using LC-MS. The tissues were analysed from newly eclosed females (0 d B), from 1 day old females mated once (1 d B 1 M), from 2 day old females mated twice (2 d B 2 M), and from 2 day old females mated once on day 1 (2 d B 1 M). n = 12 for Ringer and dsRNA AS A-type. Means ± SEM. Mann-Whitney *U*-test, asterisks indicate significant differences, ***** P < 0.001.*

3.3.3 Hemolymph JH titers of females mated with *AS type A* gene silenced males

The JH homologs depleted from the bursa copulatrix 24 to 48 hours after mating again seem to have been released into the hemolymph of the females. Young virgin females do not contain significant amounts of JH I and JH II in their hemolymph (see Fig. 28). JH I and JH II titers in the hemolymph increased in mated young females (day 2) compared to virgin animals, and once more from day 2 to day 4 of adult life parallel to the number of matings (but differences not significant). *AS type A* gene knockdown in males did not affect the JH titer in the hemolymph of females mated with such *AS A* gene silenced males compared to Ringer injected controls.

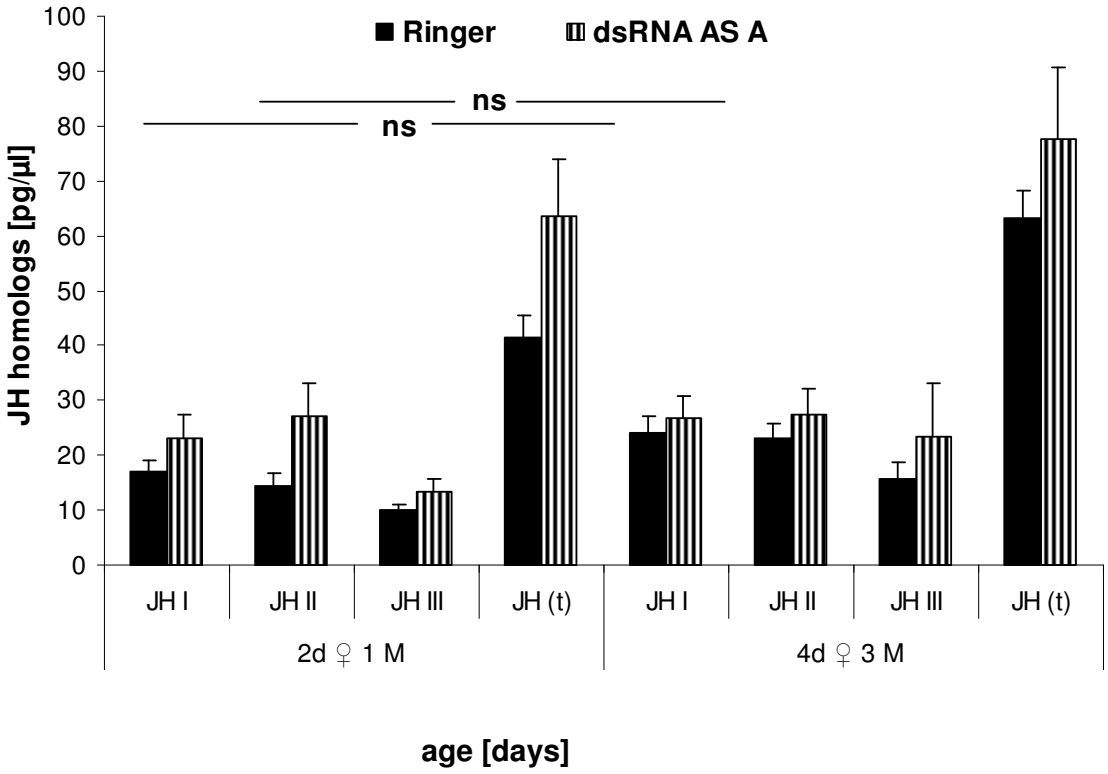


Figure (45): Effect of *AS A-type* gene silencing in males on hemolymph JH titer of untreated females of *S. frugiperda* mated with such treated males. Newly eclosed males were injected with either 2 µl noctuid Ringer or 1.5 µg of dsRNA AS A-type in 2 µl noctuid Ringer into the third abdominal segment. Untreated females were added to the males in a ratio of 1: 1. Animals were kept paired in boxes, supplied with water, 10% sucrose, and filter paper for oviposition. The JH titers were measured using LC-MS. Hemolymph samples were collected from 2 day old females once mated 2 d ♀ 1 M) and from 4 day old females 3-times mated 4 d ♀ 3 M). n = 8 - 10 for Ringer and dsRNA AS A-type. Means ± SEM. Mann-Whitney *U*-test, no significant differences between treatments; ns, not significant.

3.3.4 Hemolymph ecdysteroid titers of females mated with *AS type A* gene silenced males

Mated females contained high concentrations of ecdysone and 20-hydroxyecdysone (20E) in the haemolymph after one mating on day 2 after ecdysis (see Figure 35). These high concentrations of free ecdysteroids were reduced (significant reduction for ecdysone) when females had been mated with *AS A-type* gene silenced males (19.5 ± 4.2 pg / µl vs. 59.6 ± 32.3 pg / µl, *P < 0.05*) **(Figure 46)**. In 4 day old females, however, a significant increase in the concentration of 20E in the hemolymph was observed when

females had been multiply mated with *AS A-type* gene silenced males (31.1 ± 3.9 pg / µl vs. 16.5 ± 3.7 pg / µl, *P < 0.05*).

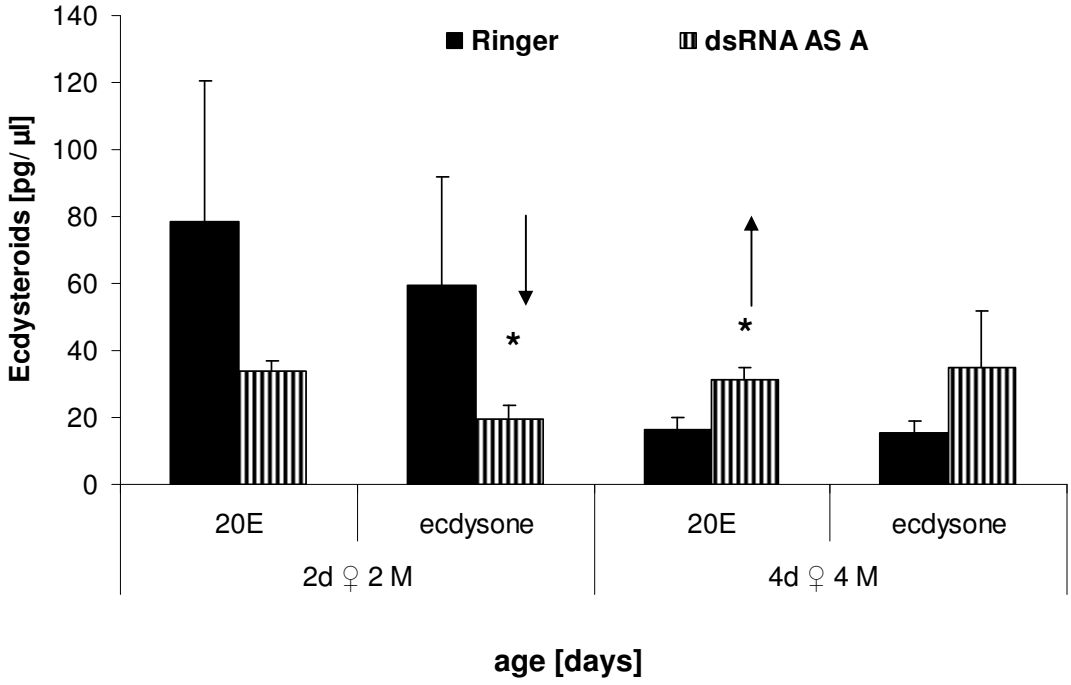


Figure (46): Effect of *AS A-type* gene silencing in males on hemolymph ecdysteroid titers of untreated females of *S. frugiperda* after coupling. Newly eclosed males were injected with either 2 µl noctuid Ringer or 1.5 µg of dsRNA AS A-type in 2 µl noctuid Ringer. Untreated females were added to the males in a ratio 1: 1. Animals were kept paired in boxes; water, 10% sucrose, and filter paper for egg deposition were supplied. Free ecdysteroids (ecdysone; 20-hydroxyecdysone, 20E) were measured using LC-MS. Hemolymph samples were collected from 2 day old females twice mated and from 4 day old females 4-times mated. n = 8 - 10 for Ringer and dsRNA AS A-type. Means ± SEM. Mann-Whitney *U*-test, asterisks indicate significant differences between treatments, ** P < 0.05.*

4 Discussion

4.1 Effect of *AT 1* gene silencing on gene expression in *S. frugiperda*

Neuropeptides represent the most ancient hormonal signals in the metazoa. They are processed from precursor polypeptides by proteolysis and other posttranslational modifications (Broeck, 2001). More than 200 different peptides belonging to a number of distinct neuropeptide families have been characterized from various insect species (Audsley et al., 1992; Brown et al., 1999; Hoffmann et al., 1999; Nässel et al, 2000; Nässel, 2000; Nässel, 2002; Nässel and Homberg, 2006). From the fall armyworm, *S. frugiperda*, genes of four allatoregulating neuropeptides, *AT 1* or *Manse-AT*, *Manse-AS, AS A-type* and *Spofr- AT 2,* were cloned (Abdel-latief et al., 2003, 2004a; 2004c). So far, only one of these peptides has been extracted from the brain of the moths (Manse-AT) (Oeh et al., 2000), but the functions of Manse-AS and Manse-AT on the biosynthesis of JH were studied *in vitro* (Oeh et al., 2000) with synthetic peptides, and that of Manse-AS, Spofr-AT 2 and A-type allatostatins also *in vivo* using RNA interference (Meyering-Vos et al., 2006; Griebler et al., 2008). The efficiency of RNA interference in the Lepidoptera was multiply recorded (Rajagopal et al., 2002; Eleftherianos et al., 2006; Terenius et al., 2011; Swevers et al., 2011). For example, Meyering-Vos et al. (2006) demonstrated the effectiveness and specificity of gene silencing of the *AS A-type* gene in *S. frugiperda* by injections of dsRNA targeted against the A-type allatostatin (FGLamides) into larval and adult armyworms, and Griebler et al. (2008) by injection as well as by feeding of dsRNA targeted against Spofr (Manse)-AS and Spofr-AT 2, respectively, to larvae and adults of *S. frugiperda*.

RT-PCR is a sensitive, precise and efficient technique for identifying and quantifying mRNA levels (Ririe et al., 1997; Auboeuf and Vidal, 1997; Fasco, 1997; Bustin, 2000; Livak and Schmittgen, 2001). In *S. frugiperda*, the *AT 1* gene is expressed in brain, digestive tract, and reproductive organs (restricted to the follicle cells between the oocytes) of adults in a time- and tissue-specific manner (Abdel-latief et al., 2004a). Its expression in various tissues let expect pleiotropy in function that means a myoregulatory role in the gut besides its effect on JH biosynthesis in the CA, and perhaps also a role in embryonic development (Abdel-latief et al., 2004a).

In order to find out the role of AT 1 peptide in adults of *S. frugiperda*, we used the RNAi technique to suppress its mRNA expression in newly eclosed penultimate larvae and in adult females and males. We evaluated the effectiveness of this technique by silencing the *AT 1* gene and quantifying the levels of mRNA in the brain and ovary of 2 day old virgin females. For knockdown of mRNA expression, we injected dsRNA targeted against AT 1 into freshly ecdysed animals and 2 days later we quantified the mRNA by an optimized, sensitive, and specific real-time reverse-transcriptase polymerase chain reaction (RT-PCR). The level of mRNA, following *AT 1* gene silencing, and utilizing absolute as well as relative quantification, was significantly lowered in comparison to control injections with noctuid Ringer, but not zero. Injections of dsRNA targeted against a control gene (sulfakinin of the cricket *G. bimaculatus*) did not at all affect AT 1 mRNA expression. These results prove that the effectiveness of the RNAi technique is specific and that only the target gene is affected. Griebler et al. (2008) have shown that silencing of *Spofr-AT 2* and *Manse-AS* genes in the brain and reproductive tissue of adults and penultimate instar larvae of *S. frugiperda* by RNA interference were highly tissue-specific and affected the amounts of JH and ecdysteroids in the hemolymph of the animals, consequently altering the lifecycle of the animals as well as its reproduction rate.

In the following, therefore, we studied the effects of *AT 1* gene silencing on larval and adult development of the fall armyworm. Since we did not find any significant changes, for example on larval weight gain or the circadian rhythm of adult emergence, we did not conduct control expression studies on brains of larvae. The same is true for adult males, where our studies did not show any effects of *AT 1* gene silencing on the JH transfer from the male to the female through coupling. The efficiency of *AS type A* gene silencing in various tissues of adult females and males of *S. frugiperda* had been shown in previous work by Meyering-Vos et al. (2006).

4.2 Effects of *AT 1* gene silencing on life cycle characteristics of *S. frugiperda*

In most of our gene silencing experiments we compared the results following gene knockdown with those for Ringer injected controls and not for untreated animals, because the injections *per se* had significant influences on most of the life cycle parameters measured here.

4.2.1 Mortality

In this study, penultimate and last instar larvae and adults of *S. frugiperda* were injected with dsRNA AT 1 or noctuid Ringer at the day of their moulting. The injected penultimate instar larvae of *S. frugiperda* normally moulted to the last larval stage and no body weight changes compared to the Ringer injected controls were observed. Concentrations of the JH homologs JH I, II, and III (larvae mainly contain JH III and only traces of JH I and JH II) as well as concentrations of free ecdysteroids in the hemolymph (ecdysone, 20-hydroxyecdysone) also did not differ between treated and control animals. Therefore, it is not surprising that no differences in the food consumption and mortality of these larvae was observed (results are not shown). In contrary, injections of dsRNA AT 1 into last instar larvae led to a significant increase in the hemolymph JH III titer as well as in the concentration of 20-hydroxyecdysone at the end of the larval stage, but mortality and food intake of the animals remained unaffected.

Griebler et al. (2008) demonstrated that injection of dsRNA into larvae of *S. frugiperda* resulted in generally lower mortality than into adult animals, but that in both developmental stages the mortality rate strictly depended on the amount of injected dsRNA. Mortality increased at injections of 2 µg dsRNA upwards, but we used not more than 1.5 µg in our experiments.

Injection of AT 1 peptide into penultimate instar larvae of *S. frugiperda* drastically reduced their weight gain and increased mortality (Oeh et al., 2001). Injections of AT 1 peptide into last instar larvae also reduced their weight gain and increased mortality in a dose-dependent manner. However, injections of Manse-AS peptide into larvae of both larval stages hardly affected growth, development and mortality of the animals.

Adult virgin females lived for about 14 days, whereas untreated ones showed some earlier mortality than treated ones. Adult males lived 6 to 8 days and mated females 7 to 10 days. Injection of AT 1 peptide into adult (mated) females shortened their lifespan and decreased the number oviposited eggs (Oeh et al., 2001).

4.2.2 Larval development

Larval development is a complex process, inducing behavioural changes, altering of gene levels, cellular division and growth, commitment and metamorphosis, associated by a wide range of oscillations in JH and ecdysteroids titers in the hemolymph (for review see Nijhout, 1994; Riddford, 1996; Gäde et al., 1997).

Ecdysteroids are released from the prothoracic glands, while juvenile hormones are synthesized in the corpora allata, both of them are under neurohormonal control. In summary, JH allows larval moulting in response to ecdysteroids. High JH titers in the hemolymph prevent the switching of gene expression necessary for metamorphosis. A high JH-esterase (JHE) peak is found, when larvae have reached their critical weight for pupation, possibly to remove any remaining traces of JH (Browder, 2001). Therefore, JH influences early actions of 20E which then results in the preservation of the juvenile "*status quo"* (Riddford, 1996). Moreover, 20E deactivates the prothoracic glands in the early fifth instar of lepidopterans by feedback inhibition and this effect is mediated by JH (Takaki and Sakurai, 2003). Treatment of last instar larvae with JH analogs stimulated supernumerary larval moult and metamorphosis was delayed (Kremen and Nijhout, 1998).

Our quantification of JH titers in the hemolymph from penultimate instar larvae of *S. frugiperda* showed that JH III is the predominant JH homolog followed by JH II, whereas JH I was nearly undetectable. Higher amounts of JH are present in the younger larval stages, but JH is absent prior to the moult from pupa to adult. The hemoylmph JH titer dropped from the penultimate larval stage L5/1 to the last day of the last larval stage L6/3, which well agrees with earlier JH measurements by Griebler et al. (2008). Another increase in the JH concentration of the hemolymph during the prepupal phase was somewhat higher (and earlier) in our experiments than described by Griebler et al. (2008), but goes in line with a drastic increase in the concentration of free ecdysteroids in the hemolymph of these animals.

AT 1 gene silencing at the beginning of the penultimate larval stage had no effect on hemolymph titers of JH and ecdysteroids in the 5^{th} and 6^{th} larval stages. Consequently, the larvae moulted as usual and developed normally; their body weight showed no differences compared with the Ringer control. In contrast, injection of AT 1 peptide twice daily into *S. frugiperda* larvae caused significant reduction of body weight in the last larvae stage (Oeh et al., 2001), whereas in *vivo* injection of AT 1 peptide into sixth instar larvae of *L. oleracea* had no effect on larval development and food consumption (Audsely et al., 2001).

The AT 1 preprohormone is expressed highly in the brains of the 5^{th} larval stage, but low in L6 larvae and during prepupation (Abdel-latief et al., 2004a). The overall abundance of the AT 1 mRNA, and also of (Spofr) Manse-AS mRNA, differs markedly between developmental stages and tissues of *S. frugiperda* (Abdel-latief et

al., 2004a). The time-dependent changes of *Spofr-AS* gene expression in larvae and adult female brains (with fairly constant rates of AT 1 expression) correspond to previous measurements of the rate of JH biosynthesis by CA in *vitro* (Range et al., 2002). The expression of Manse-AS in the brain should be low (and *AT 1* gene expression high) when JH biosynthesis is high and *vice versa* (Abdel-latief et al., 2004a). Thus, the changes in AT 1 mRNA confirm the JH titers measured by Westerlund (2004) and Griebler et al. (2008), which were high in the L5 stage and almost diminished in L6/3, but JH III titer was elevated again in L6/4 and prepupa. Riddiford (1972) demonstrated that in holometabolous insects the JH titer is high again at the time of last larval ecdysis (beginning of wandering phase, time of pupation), and then declines to a very low or undetectable level in the pupa.

In contrast to AT 1, Spofr-AS or Manse-AS expression remained high until the 6th larval instar but was low during pupation (Abdel-latief et al., 2004a). Consistently, in case of *Manse-AS* gene silencing in L5 larvae, the transcript level was reduced in brain and gut of last instar larvae and this suppression led to an increased JH titer in the animals. As a result of the elevated hormone titer, the last larval stage was prolonged. In prepupae, the JH titer decreased, but the animals pupated and molted normally (Griebler et al., 2008). These results may show that in the last larval stage of *S. frugiperda* expression of AS C-type (Manse-AS) is more important for the regulation of the JH titer in the hemolymph, and therefore for moulting at the appropriate time, than the *AT 1* gene expression. The fluctuation and interaction of these two neuropeptides during larval development support their dual role in an 'on-off' mechanism of controlling JH biosynthesis (McNeil and Tobe, 2001).

4.2.3 Metamorphosis

In the Lepidoptera, larval–pupal transformation is achieved by precise changes in the hemolymph ecdysteroid titer (Sukura et al., 1998) and some neuropeptide hormones such as myoinhibitory peptide I (MIP I) and crustacean cardioactive peptide (CCAP) as well (Davis et al., 2003). Metamorphosis is initiated when last instar larvae reach a critical weight, followed by a sudden drop in the hemolymph JH titer 24 h later. Thereby, the brain will get competent to release PTTH during the succeeding photophase (Nijhout and Williams, 1974a; Riddiford and Truman, 1978). The subsequent commitment is induced by a small impulse of ecdysteroids in the absence of JH. The commitment pulse triggers the wandering behaviour of the last

instar larva and enhances cells to subsequent pupal development. Ismail et al. (2000) have shown that JH acid and ecdysteroids are required for inducing the change from larval to pupal development of the Verson's gland, dermal glands in *M. sexta*, derived from epidermal cells. JH acid, therefore, seems to be also an essential metamorphic hormone in lepidopterans. Functional failure in these processes led to diapause of mature larvae (Nijhout and Williams, 1974b). Larval allatectomy caused a one day earlier pupation and adult differentiation of the eyes and forewings (Kiguchi and Riddford, 1978). The reversed happened when a JH analog (ZR 512) was applied. Pupal commitment was delayed in a dose-dependent manner (Rountree and Bollenbacher, 1986).

In our studies on *S. frugiperda,* commitment occurred on day 5 after moulting to the last larval stage and most of the animals pupated on day 7. The *AT 1* gene is expressed in the 6th larval stage of *S. frugiperda*, whereas expression is low in prepupae (PP) and in 10 day old pupae (Abdel-latief et al., 2004a). When L6/1 larvae were *AT 1* gene silenced in this work, we measured some differences in the hemolymph titer of JH I and JH III in L6/4 larvae compared with the controls. There was a reduction in JH I but a drastic stimulation of JH III, and that means in total JH, in synchrony with a peak of ecdysteroids in L6/4 and in the early prepupa (PP1). Whisenton et al. (1985) suggested that 20E has a stimulatory effect on JH I and JH III biosynthesis in the CA, but only when glands form a complex with the brain-corpora cardiaca. Thus from this literature, it appears that the increase in CA activity is elicited in response to the commitment peak of 20-hydroxecdysone, acting indirectly via the brain-corpora cardiaca (CC) and resulting in a post commitment increase in JH titer that is important for the moult to the pupa. This re-elevation of JH is important to protect the developing pupa from immature adult characteristics. *AT 1* gene silencing in L6/1 *S. frugiperda* larvae in this work accelerated the commitment impulse for one day (maximum at day 4), as well as the time for pupation (maximum at day 6). Abdel-latief et al. (2004a) observed an increase in *AT 1* gene expression towards the pupal phase of *S. frugiperda*, which agrees with the increase in JH during pupal moult. Larvae which pupated without JH indeed showed adult characteristics (Cymborowski and Stolarz, 1979). Sparks et al. (1983) suggested that this increase in JH induces a second peak of JHE activity prior to pupation which keeps JH low or absent during the following pupal phase.

From these results and the corresponding literature we conclude that AT 1 peptide is required for proper timing of JH release in last instar larvae of *S. frugiperda*, which is crucial for transformation to the pupa. However, *AT 1* gene silencing of the larvae did not affect the time of emergence of the adults, which occurred at day 9 after pupation for the females and at day 10 for the males in both gene silenced and control animals.

4.2.4 Adult moulting

Juvenile hormone (JH) is a key hormone in regulation of the insect life cycle maintaining the larval state during moults (Riddiford, 2008). However, absence of JH is observed during the pupal stage (*Bombyx mori*; Kinjoh et al., 2007) and lepidostatin-1, a type-A allatostatin, disappeared early in metamorphosis (*M. sexta;* Davis et al., 1997).

Ecdysteroids in the absence of JH activate two classes of genes: one responsible for the adult phenotype and the other permitting a modification in the respond to ecdysteroids (Wang et al., 1995), thus inducing the histolysis of nearly all larval tissues and the differentiation of the adult structures. Baker et al. (1987) did not find a sexual dimorphism in timing or magnitude of JH, JH acid, and ecdysteroid titers or JH esterase activity during larval-adult development of *M. sexta*, but in our experiments, females of *S. fugiperda* emerged one day earlier than the males. *AT 1* gene silencing of newly eclosed 6th instar larvae did not affect the duration of the pupal stage in both sexes.

Our results show a clear circadian rhythm of adult eclosion in females and males of *S. frugiperda*. In both sexes, adult emergence was maximal in the early scotophase.

Adult emergence rhythm under (semi)natural conditions has been reported previously (De et al., 2012). *D. melanogaster* flies showed a robust rhythm in adult emergence, which is coupled either to humidity/temperature or to light cycles and shows seasonal variations. Physiological circadian rhythms in insects have been well documented in relation to hormone production, particularly hormones controlling postembryonic development (Lazzari and Insausti, 2008). Hormones such as the PTTH, ecdysteroids and JH form a key component of the circadian system and are produced in a rhythmic fashion.

AT 1 gene silencing of 6th instar larvae of *S. frugiperda* did not abolish the circadian rhythm of adult emergence, but the maximum of eclosion was shifted to the late

photophase. However, similar results were obtained by mere Ringer injection (controls) and we assume that the injection stress might be responsible for the slight time-shift in adult emergence.

4.2.5 Mating and reproduction

Gonadotropic hormones (JH and 20E) control vitellogenesis, oogenesis and egg production in adult insects and dopamine may mediate their interplay (Gruntenko and Rauschenbach, 2008). In the Lepidoptera, differences exist in the type of hormones (juvenile hormones or ecdysteroids) controlling vitellogenesis, depending on their time of action (last larval instar, pupa, adult stage) (Ramaswamy et al., 1997).

Many endogenous and exogenous factors affect the reproduction of moths (Ramaswamy et al., 1997). The lepidopteran female mating system varies from strict monoandry to strong polyandry. In the polyandric *S. frugiperda,* vitellogenesis takes place after adult emergence and relies on JH as gonadotropic signal, while both ecdysteroids and JH are crucial for yolk protein synthesis and egg maturation in other species.

Several mechanisms have been suggested for the occurrence of polyandry. In the case of polyandry, males transfer nutrients in addition to the sperm with their spermatophore to the female. An additional reason for polyandry is that virgin females lack their gonadotropic signal and eggs that have been produced already will be reabsorbed. During copulation, the male either triggers a neural or humoral response in the female, thus stimulating release of the endogenous gonadotropic signal JH, or JH itself is transferred from the male to the female during mating, as it is in *S. frugiperda*. Females gain from multiple mating in terms of increased lifetime offspring production (Arnqvist and Nilsson, 2000), and the transfer of additional sperm will increase the genetic variability.

In our experiments, the virgin females laid about half as much eggs as mated once. The switch from a virgin female to a mated female is mediated by the sperm and the seminal fluid in its bursa copulatrix that can fertilize the eggs. Moreover, various other substances, including JH, are transferred from the male to the female with sperm during copulation (Edward et al., 1995). However, the amount of JH present in a mated insect female not only depends on the amount of JH transferred from the male to the female, but the CA activity in the female depends on both internal and environmental factors, such as the age and developmental stage of the animal,

photoperiod, feeding, or nutritional status (Li et al., 2003). For instance, long day conditions induced the production of CA stimulatory substances (allatotropin) in the median neurosecretory cells of the brain, resulting in high activity of the CA under long day conditions (*Schistocerca gregaria*; Pratt and Tobe, 1974), whereas under short day conditions the rate of JH biosynthesis gradually decreased and became undetectable just prior to a diapause (*Leptinotarsa decemlineata*; De Kort et al., 1987).

In this study, we observed *S. frugiperda* female offspring production including time of copulation, mating intervals, number of deposited spermatophores, and the nature of the fluid transferred from the male accessory reproductive glands to the bursa copulatrix of the female during mating. Since JH represents a major component of the transferred seminal fluid, we measured whether gene silencing of either the allatotropin (AT) 1 preprohormone or the type A allatostatin preprohormone affects the reproduction rate of the females.

Animals were reared under constant temperature and photoperiodic conditions similar to those encountered in summer in their natural habitat. Adult females started feeding activities shortly before the first scotophase, and first mating was observed about 24 hours after emergence and lasted for 1.5 to 2 hours. *AT 1* gene silencing in the females retarded first mating for about 1 hour. Females mated each 24 $\pm$ 3 hours, which was confirmed by counting the spermatophores in the bursa copulatrix. *AT 1* gene silencing of females and males did not affect the number of spermatophores transferred to the female. However, gene silencing against *Manse-AS* and *Spofr-AT 2* of males reduced the number of spermatophores transferred to the bursa copulatrix of the females compared to controls (Griebler, 2009). First oviposition occurred in the succeeding scotophase about 45 to 55 hours after moulting and 24 hours after mating. Mated females started some earlier with egg laying than virgin animals. Moreover, mated females laid about twice the number of eggs than virgin females, although the oviposition period was about 2 days longer for virgin than for mated animals. *AT 1* gene silencing in freshly ecdysed females had no effect on the number of eggs deposited by virgin animals, whereas the number of laid eggs was significantly increased in mated females. S. Müller (2012, unpublished) has recently demonstrated that the type of adult food my affect the rhythm of egg deposition.

The fresh weight of the eggs was higher when eggs had been deposited from older females (6 to 8 days after ecdysis) than from younger ones. These heavier eggs,

however, showed lower hatching rates than those deposited by younger females. Such a phenomenon that eggs from older females are more often infertile had been observed in several other insect species and may be a result of increasing reactive oxygen species (ROS) in older females.

Zeng et al. (1997) demonstrated that a higher JH titer in mated females is the main reason for high vitellogenin and egg production in the moth *H. virescens*, but applications of JH to virgin females could not mimic mating. In another moth, *Cydia pomonella*, an increase in choriogenesis was induced by JH treatment (Webb et al., 1999). In *M. sexta*, egg development was stimulated by mating as well as by an adequate food supply (Sasaki and Riddiford, 1984).

Since vitellogenesis and egg production in *S. frugiperda* rely on the JH titer of the females (Range et al., 2002), we measured the amount of JH I, II, and III in the hemolymph of adult females, both in *AT 1* gene silenced and in Ringer injected controls, on certain days. JH III biosynthesis *in vitro* (Range et al., 2002) as well as JH titers were low in newly eclosed adult females, increased as oocytes developed and became maximal at the time of oviposition, but were lower again in older females. Although JH III was the predominant homolog also in adult females, titers of JH I and JH II were much higher than in the larvae. Absolute amounts of JH were much higher in the hemolymph of mated females than in unmated animals, but the variation of the JH titer in time was not different between the two experimental groups. *AT 1* gene silencing of freshly ecdysed adult females led, as expected, to a significant decrease in the concentration of JH III in the hemolymph of young (2 day old) mated females, but an increase in older animals (day 7). In virgin females, such an *AT 1* gene silencing effect was not observed before days 6/7 after ecdysis, whereas in older animals (day 8), again an increase in JH (JH II in this case) was observed. Consequently, *AT 1* gene silenced females started egg deposition earlier than controls. The differences in JH concentrations between virgin and mated females together with the changes in the circadian rhythm of emergence following *AT 1* gene suppression, can explain, at least in part, the differences in egg oviposition of the females from our experimental groups when we assume that JH is the major gonadotropic hormone in *S. frugiperda*.

In the following chapter, the role of JH during the reproductive period of female *S. frugiperda* as well as the regulation of JH biosynthesis by allatoregulating neuropeptides during this developmental period will be discussed in more details.

4.3 Effect of *AT 1* gene silencing on *S. frugiperda* female reproduction

Vitellogenesis and egg retention are independent physiological processes of egg laying. Yolk proteins and the cDNA of yolk proteins were isolated from many insect species and the gene expression in the fat body and ovary was quantified (Chen et al., 2012).

Vitellogenesis, the process of yolk protein (YP) synthesis and yolk uptake into the oocytes, is controlled by JH and ecdysteroids interacting with membrane receptors of the follicle cells (Koeppe et al., 1985; Davey et al., 1993). In most cases JH alone is not sufficient to up-regulate the expression of the *Vg* genes, but ecdysteroids and nutrients are necessary (Ma et al., 1988; Panaitof and Scott, 2006). In the Diptera, a peak of ecdysone is noticed during vitellogenesis (Grau et al., 1995).

The ovaries are the major source of circulating ecdysteroids, but at least in some cases the ovaries can also synthesize JH III from farnesoic acid (Bellés et al., 1987, Borovsky et al., 1994a; Borovsky et al., 1994b; Romana et al., 1995). A model system of the relationship between JH production and oocyte growth and maturation was presented by Stay and co-workers (Stay and Tobe, 1978; Tobe, 1980; Stay et al., 1983). In cockroaches and many other insect species, the young developmental stages of the ovary are directly responsible for an increase in JH biosynthesis by the CA (Ranking and Stay, 1984). JH, circulating in the hemolymph, synthesized by the CA, is required for oocyte maturation and for the production of vitellogenins in the fat body and their uptake into the growing ovaries. All developmental changes seem to be affected by nutritional signals and may dependent on a mating stimulus (Engelmann, 1979*;* Hatle et al., 2000; Li et al., 2003b). The action of JH will depend on the presence of the respective allatoregulating neuropeptides (Yin and Stoffolano, 1997). An imbalance in the gonadotropins may lead to reproductive defects such as oviposition arrest or degradation of vitellogenic oocytes (Gruntenko et al., 2005; Gruntenko and Rauschenbach, 2008).

In the order of the Lepidoptera some differences exist in the timing and the type of hormones involved in vitellogenesis (see above). In *S. frugiperda*, a single female-

specific protein, likely to be the *S. frugiperda* vitellogenin (Vg), appeared approximately 5 h after adult eclosion in the hemolymph of virgin females (Sorge et al., 2000). This protein was also present in egg extracts, but absent in male hemolymph. Vitellogenic oocytes became visible 36 to 48 h after emergence and egg deposition began on day 3 of adult life. Vitellogenesis strictly depended on JH, but was modified by the presence of free ecdysteroids.

In this work, we measured juvenile hormone and ecdysteroid titers in the hemoymph of 2, 4, 6, 7 and 8 day old virgin and mated *AT 1* gene silenced and control females of *S. frugiperda*. In the hemolymph of virgin and mated females JH III was the predominant homolog followed by JH II, whereas JH I was hardly detectable. In virgin females, hemolymph JH titers were low at emergence, but increased thereafter and reached a maximum on day 2, the time of first egg laying. *AT 1* gene silencing in virgin females had no effect on the total number of deposited eggs. This is in line with the observation that no clear effect of the gene suppression on hemolymph JH in young virgin females was measured, whereas the amount of free ecdysteroids was increased on day 4 and day 8 after emergence. After mating, JH titers increased significantly. 48 hours after emergence JH titer was 3-times higher than that in 2 day old virgin females, i.e. mating stimulated JH production, resulting in a significant increase in egg production. Thus, mating provided correct stimuli for enhanced oogenesis and egg laying, and this was correlated with a drastic elevation in the hemolymph JH titer. Similar results were obtained by Edwards et al. (1995), who have demonstrated that virgin females of *L. oleracea* exhibit much lower JH titers than mated females. Therefore, one would expect that the AT 1 peptide, which was shown to have allatotropic action *in vitro* on JH biosynthesis in the CA, would be important in increasing the JH titer and egg production in adult females.

Consistently, in young adult mated females (day 2) of *S. frugiperda, AT 1* gene silencing induced a significant reduction of JH III to nearly a quarter of that in mated control females, whereas 20-hydroxyecdysone was elevated. Surprisingly, however, total oviposition significantly increased compared to controls, about 3-fold to the number of eggs deposited by virgin untreated females, and twice to that of mated controls. The profile of deposited eggs shows that most of this increase in egg deposition occurred between days 2 and 5 of adult life. In 7 day old females another significant peak of egg laying coincided with an elevation of hemolymph JH III and ecdysteroids, and may be a result of “rejuvenation”.

In the absence of a continuous polyandric mating signal and its action as allatotropic enhancer on the CA, the *AT 1* gene silenced virgin females laid the same amount of unfertile eggs as virgin controls. This is consistent with the results of Griebler et al. (2008) who showed that after silencing of the *Manse-AS* gene, neither the hemolymph JH titer nor the number of deposited eggs were changed. In contrast, mated *S. frugiperda* females which had been *Manse- AS* or *Spofr-AT2* gene silenced (Griebler, 2009), had a significantly elevated hemolymph JH III titer, a declined amount of 20-hydroxyecdysone in the hemolymph, and in the case of *AS A-type* gene silencing, the number of deposited eggs was lowered (Meyering-Vos et al., 2006). In general, mating increases egg production by altering the degree of inhibition of the corpus allatum by the brain and making the CA more sensitive for allatoregulating neuropeptides, although the nature of mating stimulus and the route by which the stimulus reaches the brain remain uncertain (Davey, 1997). Also Park et al. (1998b) showed that an allatotropic effect on JH biosynthesis by the CA is present in mated females of *Heliothis virescens*, but absent in virgins.

In the present study as well as in the experiments done by Meyering-Vos et al. (2006), following suppression of the allatoregulatory neuropeptide genes, an unexpected manner of egg deposition has been observed despite the expected manner of hemolymph JH III changes. Although the 2 day old mated *AT 1* gene silenced females exhibited a lower amount of JH III in the hemolymph, they deposited a much higher number of eggs during the early oviposition period. In the study of Meyering-Vos et al. (2006) suppression of the *AS A-type* gene resulted in an elevated JH III titer in the hemolymph, but number of laid eggs was significantly reduced. From these results we can conclude that Spofr-AT 1 and *S. frugiperda* A-type allatostatins both act as allatoregulating neuropeptides in mated adult females, but that egg production- and especially oviposition- does not necessarily correlate with the titer of JH in the hemolymph of the animals. The increased number of deposited eggs from *AT 1* gene silenced females seems not to be accomplished by an increase of egg mass production: *AT 1* gene silenced females laid smaller eggs with increasing age.

Since we believe that a transfer of JH during mating, combined with the allatotropic effect of mating, is involved in stimulating egg production and egg maturation in mated *S. frugiperda*, we studied the effect of *AT 1* and *AS- type A* gene silencing of

freshly ecdysed adult males on the JH transfer during copulation, as well as on egg maturation and egg deposition in females mated with such treated males.

4.4 Effect of *AT 1* gene silencing in *S. frugiperda* males

4.4.1 Male accessory glands

The storage of juvenile hormone (JH) in the male abdomen is a phenomenon known from some species of moths; JH stored in male accessory sex glands (AG) may be transferred to the female during copulation, but the physiological significance of the JH transfer remains unclear. In females that exhibit last male sperm precedence, the transferred hormone will promote endogenous JH synthesis and egg development in the female, whereas the hormone acts as a bioactive substance in species, which the first male to mate use (Pszczolkowski et al., 2006).

In *H. virescens*, mated females exhibited much higher hemolymph JH titers accomplished by higher Vg and egg production (enhancement of fecundity) than virgin females (Zeng et al., 1997; Park et al., 1998b). On the other side, males without AG and/or testes (without sperm) were unable to enhance egg maturation in the female (Park et al., 1998a). Testes produced ecdysteroids, but gonadectomized males developed normally, indicating that ecdysteroids from the testes are not essential for general development (*H. virescens;* Loeb et al., 1984).

Studies on the effect of allatoregulating neuropeptides on JH biosynthesis and ecdysteroid titers in adult males are scarce. In this work, the potential effects of *AT 1* and *AS A-type* gene silencing on amounts of hemolymph JH and ecdysteroid titers and on the amounts of JH in male accessory glands and females bursa couplatrix of *S. frugiperda* were measured using LC-MS. We did not find any ecdysteroids in both the male accessory glands and the spermatophores (unpublished results). However, immediately after adult emergence, *S. frugiperda* male accessory glands contained considerable amounts of JH I and JH II, whereas JH III was close to detection limit. Concentrations of JH I and JH II increased to very high levels in the AG of 1 day and 2 day old virgin males (up to 50 ng per tissue), whereas mating drastically reduced the amount of JH in the glands (less than 10 ng per tissue). In contrast to the AG, the hemoylmph of virgin adult males contained only low amounts of JH I and JH II, but considerable quantity of JH III. Total JH concentration in the hemolymph increased from the day of emergence to a maximum between days 4 and 8 of adult life. The

different pattern of JH homologs in the AG compared to the hemolymph let expect a *de novo* synthesis of JH in the glands, but this has not been proved so far for *S. frugiperda* males. Borovsky et al. (1994) have shown that male mosquitoes synthesize JH I and JH III in their accessory glands (see above) and speculated that these hormones will be transferred with the sperm into the females. Tian et al. (2010) confirmed the synthesis of JH III in the male accessory glands of another insect order, the longhorned beetle*, Apronia germari*, as well as its transport to female ovaries during copulation. Our results on *S. frugiperda* may suggest that JH synthesized by the CA of males is used for internal regulation of adult development, whereas JH synthesized by the AG is transferred through the spermatophore to the female.

AT 1 gene silencing of freshly ecdysed males drastically reduced the concentration of JH (mainly JH III) in the hemolymph of the animals, but also reduced the amount of JH I in the AG of virgin males. *AS A-type* gene silencing, on the other hand, resulted in a significant increase in the amount of JH (again, mainly JH I) in the AG of virgin males. In conclusion, our results suggest that allatoregulating neuropeptides may control JH biosynthesis in both, the CA and the AG *of S. frugiperda* males. Since AS-type A peptides had no allatostatic effect on JH biosynthesis of *S. fugiperda* CA in *vitro* (Oeh et al., 2000), the allatostatic effect of this neuropeptide *in vivo* may be indirect, for example through changes in the activity of juvenile hormone esterase (JHE) and/or juvenile hormone epoxide hydrolase.

Previously, we had shown in our laboratory that gene silencing of *Manse-AS* in young males of *S. frugiperda* resulted in a significant reduction in the number of spermatophores transferred to the bursa copulatrix of the female (M. Meyering-Vos, unpublished results), but *AT 1* gene silencing did not affect spermatophore production and spermatophore transfer (this work).

4.4.2 JH transfer from males to females during mating

It is well known that mating generally causes a reduction of receptivity and increases oviposition in mature insect females, triggered by material transferred (JH and sex peptide) during copulation (Ramaswamy et al., 1990; Moshitzky et al., 1996; Dubrovsky et al., 2002).

Polyandrous moths develop eggs exclusively with JH as gonadotropin, and egg production is stimulated by feeding and mating (Ramaswamy et al., 1990). Mated

females of *Choristoneura fumiferana* and *C. rosaeana* moths had higher hemolymph JH titers than virgins of the same age, which however was not a result of transferred JH during copulation (Cusson et al., 1999). Ramawasmy et al. (1997) presented three mechanisms, which may be responsible for higher JH levels in mated females as compared to virgin female moths (Shu et al., 1998): (1) increase in JH biosynthesis by the CA of mated females by stretching of the bursa copulatrix as a result of spermatophore insertion as shown for *M. sexta* (Sasaki and Riddiford, 1984), (2) the transfer of JH from the male to the female during copulation as in *H. virescens* (Ramaswamy et al., 2000), and (3) reduced hemolymph JHE activity as reported for *Trichoplusia ni* (Venkatesh et al., 1988).

In the present study, juvenile hormones transferred from the male to the female of *S. frugiperda* were quantified. The young virgin female bursa copulatrix did not contain any JH, whilst after mating the bursa copulatrix did contain JH levels comparable to those depleted from the male AG during copulation. The transferred JH (almost exclusively JH I and II) disappeared again 24 h after mating from the female bursa copulatrix. These results confirm the previous findings of Park et al. (1998b) who also showed that the virgin female bursa copulatrix did not contain any JH, whereas after coupling, the gland had JH at levels comparable to those observed in virgin male AG. The depletion of JH I and II from the bursa copulatrix of *S. frugiperda* about 1 day after mating and the concurrent appearance of JH I and JH II in the hemolymph of the mated females suggests that at least part of the transferred JH has been released into the hemolymph of the female.

To evaluate any effects of AT 1 and AS A-type peptides on the transfer of JH from the male to the female, we again used the RNAi technique to suppress the respective genes. Following gene suppression, we measured the amounts of JH in the male AG tissues of virgin and mated males as well as in the female bursa copulatrix immediately after mating. In another study conducted in our research group, the effects of two other allatoregulating neuropeptides, *Manse-AS (AS C-type)* and *Spofr –AT 2* gene silencing on the transfer of JH were studied (Griebler, 2009; M. Meyering-Vos, unpublished results). *AT 1* gene silencing of freshly ecdysed males resulted in a slightly lower amount of JH I in the AG, whereas no differences in the amounts of JH I to JH III in the bursa copulatrix of the female were observed. *AS-type A* gene silencing of freshly ecdysed males resulted in some higher amount of JH I in the AG, but again, no significant differences in the amount of JH I to JH III in the

bursa copulatrix of the female were observed. In conclusion, both allatoregulating neuropeptides, AT 1 and AS A-type, seem to have no (direct) effect on the synthesis of JH in the male AG as well as on its transfer into the female. However, in spite of the unchanged male-female transfer of JH between *AT 1* gene silenced males and Ringer injected controls, females mated with *AT 1* gene silenced males showed a significant increase in their JH hemolymph titer 1 to 3 days post mating compared to the controls. Moreover, concentrations of free ecdysteroids in the hemolymph of females, mated with *AT 1* gene silenced males, were significantly decreased one day after mating, but elevated three days after mating. The observed changes in hormone concentrations suggest the existence of another mating-induced control of JH biosynthesis in the female, which is mediated by the allatotropin concentration in the male. However, these changes in female hemolymph hormone titers did only slightly affect ovarian development and egg deposition rate of those females, and only in older animals.

The previous studies in our laboratory on the effect of *Manse-AS* gene silencing on male-female JH transfer through coupling had demonstrated a significant increase in the amount of JH I and JH II in the male AG following gene knockdown, which is also transferred into the bursa copulatrix of the female by mating (M. Meyering-Vos, unpublished results). *Spofr-AT 2* gene silencing, on the other hand, led to a drastic decrease in the amount of JH I in the male AG in connection with a significantly diminished transfer of JH into the female bursa copulatrix through mating. In both cases, however, such mated females laid a significant lower number of eggs than the control animals. These results suggest that Manse-AS or type C allatostatin acts as a true allatostatin in adult males of *S. frigiperda*, and Sporfr-AT 2 as a true allatotropin.

4.5 Conclusions

Juvenile hormones (JH) have a juvenoid function during insect larval development, but can act gonadotropic in adult insects. In lepidopteran species with ovarian development starting after adult emergence, juvenile hormones act as true gonadotropins. The synthesis of the juvenile hormone homologs JH I to JH III in the corpora allata (CA) is controlled by allatoregulating neuropeptides, which either stimulate (allatotropins) or inhibit (allatostatins) CA activity. So far, two types of allatostatins (Spofr-AS type C or Manse-AS and Spofr-AS type A or FGLamides) and two types of allatotropins (Spofr-AT 1 or Manse-AT and Spofr-AT 2) have been

identified from the fall armyworm, *S. frugiperda*. Most of these peptides are pleiotropic in function and their specific role in certain developmental stages is still questionable.

The present work characterizes the biological functioning of Spofr-AT 1 and Spofr-AS type A in penultimate and last instar larvae and in adults of the fall armyworm by using RNA interference for specific gene silencing. Injection of dsRNA constructs targeted against the Spofr-AT 1 and Spofr-AS A preprohormones into the hemocoel of the animals induced systemic effects with a specific suppression in gene expression of the respective mRNA in brains and ovaries. *AT 1* gene silencing in penultimate and last instar larvae accelerated preadult development and animals pupated one day earlier than the controls. Spofr-AT 1, therefore, acts as a true allatotropin in the caterpillar. Unmated females have low amounts of JH in their hemolymph and produce a low number of unfertile eggs. Mating induces oogenesis, egg development and oviposition rate, which goes in line with a drastic increase in the hemolymph JH titer. By measuring the amount of JH in the accessory glands of males before and after mating, as well as the amount of JH in the bursa copulatrix of the female before and after mating, we could demonstrate a transfer of JH (mainly JH I and II) from the male to the female during coupling. This transfer of JH during mating was only hardly affected by *AT 1* and *AS A-type* gene silencing in freshly ecdysed males. Former studies in our laboratory had shown that gene silencing of the Spofr-AS C-type preprohormone in young males led to a significant increase in the amount of JH transferred from the male to the female during mating, whereas *Spofr-AT 2* gene silencing had the opposite effect (Griebler et al., 2008; Griebler 2009; unpublished data). From these results we conclude that Spofr-AS C (C-type allatostatin) acts as a true allatostatin in larvae and adults of S*. frugiperda*, whereas A-type allatostatins come up with other functions. Spofr-AT 1 acts as true allatotropin in larvae and adults, but its function may be substituted, at least in part, by Spofr-AT 2.

5 Summary

The fall armyworm, *Spodoptera frugiperda*, is an agricultural important pest species. In former studies, four allatoregulating neuropeptides had been cloned from *S. frugiperda*, AT 1 or Manse-AT, Manse-AS, AS A-type and Spofr- AT 2, but only one has been extracted from the brain of the moths, Manse-AT. The functioning of the pleiotropic allatoregulating neuropeptides in larvae and adult armyworms is still unresolved.

In the present study, we analysed the development of penultimate and last instar larvae, metamorphosis, mating behaviour, and fertility of the moths of *S. frugiperda*, regarding to the role of allatoregulating neuropeptides, with special emphasis on Spofr-AT 1 (*Manduca sexta* allatotropin) and AS A-type (FGLamides) allatostatins in these processes.

RNA interference (RNAi) has been proved to induce a systemic and specific gene knockdown in larvae and adults of *S. frugiperda*, and has been used for the analysis of gene functions. Juvenile hormone (JH) and ecdysteroid titers (ecdysone, E and 20-hydroxyecdysone, 20E) in the hemolymph of the animals as well as the amounts of JH in the male accessory glands and in the female bursa copulatrix were measured by liquid chromatography-mass spectrometry (LC-MS).

AT 1 gene knockdown in freshly ecdysed last instar larvae induced acceleration of prepupal commitment and pupation for about 24 hours compared to Ringer treated controls, whereas duration of the pupal stage was not affected. Adult emergence showed a clear circadian rhythm with maximal number of ecdysing moths at the end of the photophase and beginning of scotophase.

The juvenile hormone titer in the hemolymph of penultimate (L5) and last instar larvae (L6) dropped from a high value at L5/3 to a low value at L6/3, but increased again towards pupation (L6/4 and PP 1). JH III was the main JH homolog in all larvae and only traces of JH I and JH II were detected. *AT 1* gene silencing at the beginning of the last larval stage led to a significant increase in the amount of JH III and 20E in the hemolymph of wandering larva (L6/4). *AT 1* gene knockdown did not affect the increase in body weight during larval development.

In female adult moths, mating led to a significant increase in hemolymph JH titers compared to virgin animals. Besides JH III the hemolymph of adult females contains considerable amounts of JH I and JH II. Correspondingly to changing JH titers, mated females laid about twice number of eggs than virgin animals. *AT 1* gene silencing immediately after emergence decreased the amount of JH but increased the titer of free ecdysteroids in the hemoylmph of virgin animals, whereas the number of produced eggs was only slightly affected. In contrast, *AT 1* gene silencing in combination with mating led to a significant increase in egg production and oviposition. Egg hatching rates were high for eggs laid by young females, but low for eggs from older females.

Quantification of the amount of JH in the male accessory reproductive glands as well as in the female bursa copulatrix following mating clearly confirmed a transfer of JH, mainly JH I and JH II, from the male to the female during coupling. About 24 hours after mating, JH disappeared from the female bursa copulatrix and could be found in the hemolymph. From these results we conclude that JH from the males acts as true gonadotropin in females of *S. frugiperda*. *AT 1* gene silencing in freshly emerged males had only slight effects of the transfer of JH from the male to the female during mating, and the number of eggs laid by females mated with such treated males was not affected.

Injection of dsRNA targeted against the preprohormone of the A-type allatostatins (FGLamides) into freshly emerged males also did not affect the amount of JH transferred from the male to the female during coupling.

6 Zusammenfassung

Der Heerwurm, *Spodoptera frugiperda,* stellt einen wichtigen Schädling in der Landwirtschaft dar. In früheren Untersuchungen haben wir vier allatoregulierende Neuropeptid-Präprohormone kloniert, AT 1 oder Manse-AT, Manse-AS, AS vom Typ A und Spofr-AT 2. Nur eines der Peptide (Manse-AT) konnte aus dem Oberschlundganglion der Falter extrahiert werden. Die Funktionen der pleiotropen allatoregulierenden Neuropeptide bei Larven und adulten Faltern sind weitgehend unklar.

In der vorliegenden Arbeit wurde die Entwicklung im vorletzten und letzten Larvenstadium, die Metamorphose, sowie das Paarungsverhalten und die Fertilität der adulten Falter bezüglich der Rolle allatoregulierender Neuropeptide untersucht, mit besonderer Berücksichtigung von Spofr-AT 1 (*Manduca sexta* Allatotropin) und Allatostatinen vom Typ A (FGLamide) auf diese Prozesse.

RNA Interferenz (RNAi) wurde als Methode mit systemischem Effekt und spezifischer Wirkung bei Larven und Adulten evaluiert und zur Untersuchung der Funktionen allatoregulierender Neuropeptide eingesetzt. Juvenilhormone (JH) und freie Ecdysteroide (Ecdyson, E und 20-Hydroxyecdyson, 20E) in der Hämolymphe der Tiere sowie in den männlichen Akzessorischen Drüsen und in der Bursa copulatrix der Weibchen wurden mittels Flüssigkeitschromatographie-Massenspektrometrie (LC-MS) quantifiziert.

Suppression der Expression von AT 1 im letzten Larvenstadium beschleunigte den Übergang vom letzten Larvenstadium zur Puppenentwicklung sowie die Verpuppung um 24 Stunden gegenüber Kontrolltieren. Die Dauer des Puppenstadiums wurde hingegen nicht beeinflusst. Die adulten Tiere schlüpften in einem deutlichen Tagesrhythmus, mit höchsten Schlupfraten zum Ende der Lichtphase und zu Beginn der Dunkelphase.

Der JH-Tier in der Hämolymphe von Larven des vorletzten (L5) und letzten (L6) Larvenstadiums fällt von einem hohen Wert am 3. Tag des 5. Larvenstadiums (L5/3) kontinuierlich bis zum 3. Tag des 6. Larvenstadiums (L6/3) ab, steigt zur Wanderphase und Präpuppe hin aber wieder an (L6/4 und PP1). JH III stellt das

wichtigste JH-Homologe in den Larven dar, JH I und JH II treten nur in Spuren auf. AT 1 Knockdown zu Beginn des letzten Larvenstadiums führte zu einem signifikanten Anstieg an JH III und 20E in der Hämolymphe der Wanderlarve (L6/4), beeinflusste aber nicht das Wachstum der Larven.

Bei adulten Weibchen führte Verpaarung zu einem signifikanten Anstieg im JH-Titer der Hämolymphe im Vergleich zu unverpaarten Tieren. Neben JH III findet man in verpaarten Tieren beträchtliche Mengen an JH I und JH II. Entsprechend den Veränderungen im JH-Titer legen verpaarte Weibchen etwa doppelt so viele Eier ab wie unverpaarte Tiere. *AT 1* Geninaktivierung unmittelbar nach der Adulthäutung führte bei unverpaarten Weibchen zu einem Abfall im JH-Titer der Hämolymphe, aber zu einem Anstieg im Gehalt an freien Ecdysteroiden. Die Eiproduktion wurde nicht beeinflusst. Bei verpaarten Tieren hatte *AT 1* Gensuppression hingegen einen signifikanten Anstieg bei der Eiablage zur Folge. Aus von jungen Weibchen abgelegten Eiern schlüpften signifikant mehr Erstlarven als aus Eiern, die von älteren Weibchen stammten.

Aus der Menge an JH in den männlichen Akzessorischen Drüsen und in der weiblichen Bursa copulatrix vor und nach der Verpaarung kann ein Transfer von JH I und JH II vom Männchen zum Weibchen bei der Kopula abgeleitet werden. Etwa 24 Stunden nach der Verpaarung verschwand JH wieder aus der Bursa copulatrix und tauchte in der Hämolymphe der Weibchen auf. Aus diesen Ergebnissen kann gefolgert werden, dass JH aus den Männchen als echtes Gonadotropin bei den Weibchen fungiert.

AT 1 und *AS-Typ A* (FGLamide) Genstillegung bei frisch geschlüpften Männchen beeinflussten den JH-Transfer vom Männchen zum Weibchen nur geringfügig und hatte keinen Einfluss auf die Eiproduktion und –ablage der Weibchen.

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8 Appendix

8.1 Sequences of isoform A cDNA of allatotropins of *S. frugiperda*

Figure (A1): **Spofr-AT sequence data**. Nucleotide and deduced amino acids sequences of *S. frugiperda*. AT1 isoform A cDNA. The sequences are numbered at the right.
The Spofr-AT peptide amino acids sequence is shown in bold type. Possible proteolytic cleavage sites are in boxes and the glycine residue required for amidation is underlined. The polyadenylation signal is shown in bold type and underlined; --- represents the stop codon. A signal peptide cleavage site is indicated by a downward arrow (Abdel-latief et al., 2003). The used primers AT5prf and AT5prR are marked in red.

```
5'-  TAATACGACTCACTATAG GGC  TTC  AAG  AAT  GTC  GAG  ATG  ATG  42
                           G    F    K    N    V    E    M    M    7

     ACC  GCC  AGG  GGA  TTC  GGC  AAG  CGG  GAC  AGG  CCA  CAC  ACT   82
      T    A    R    G    F    G    K    R    D    R    P    H    T   20

     CGG  GCT  GAG  CTT  TAC  GGT  TTG  GAC  AAC  TTC  TGG  GAG  ATG   121
       R    A    E    L    Y    G    L    D    N    F    W    E    M   33

     CTG  GAG  GCT  ACA  CCT  GAG  AGG  GAA  GGA  CAG  GAG  AAT  GAT   160
       L    E    A    T    P    E    R    E    G    Q    E    N    D   46

     GAG  AAG  ACT  TTG  GAA  AGC  ATT  CCT  TTG  GAC  TGG  TTC  GTG   199
       E    K    T    L    E    S    I    P    L    D    W    F    V   59

     AAC  GAG  ATG  CTG  AAC  AAT  CCA  GAT  TTC  GCG  CGA  TCT  GTT   238
       N    E    M    L    N    N    P    D    F    A    R    S    V   72

     GTC  CGC  CCTATAGTGAGTCGTATTA     `3                              262
       V    R                                                          74
```

Figure (A2): Nucleotide and amino acid sequences used for transcript creation of Spofr-AT isoform A fragments.
The fragment was derived from Spofr-AT isoform A cDNA (s. Fig. 15; Abdel-latief *et al.*, 2003). The sequences are numbered at the right. The used primer T7-ATF7 and T7-ATr9 are marked in blue. The T7 minimal sequences are shown in bold type.

8.2 Sequences for allatostatin A cDNA from ***S. frugiperda***

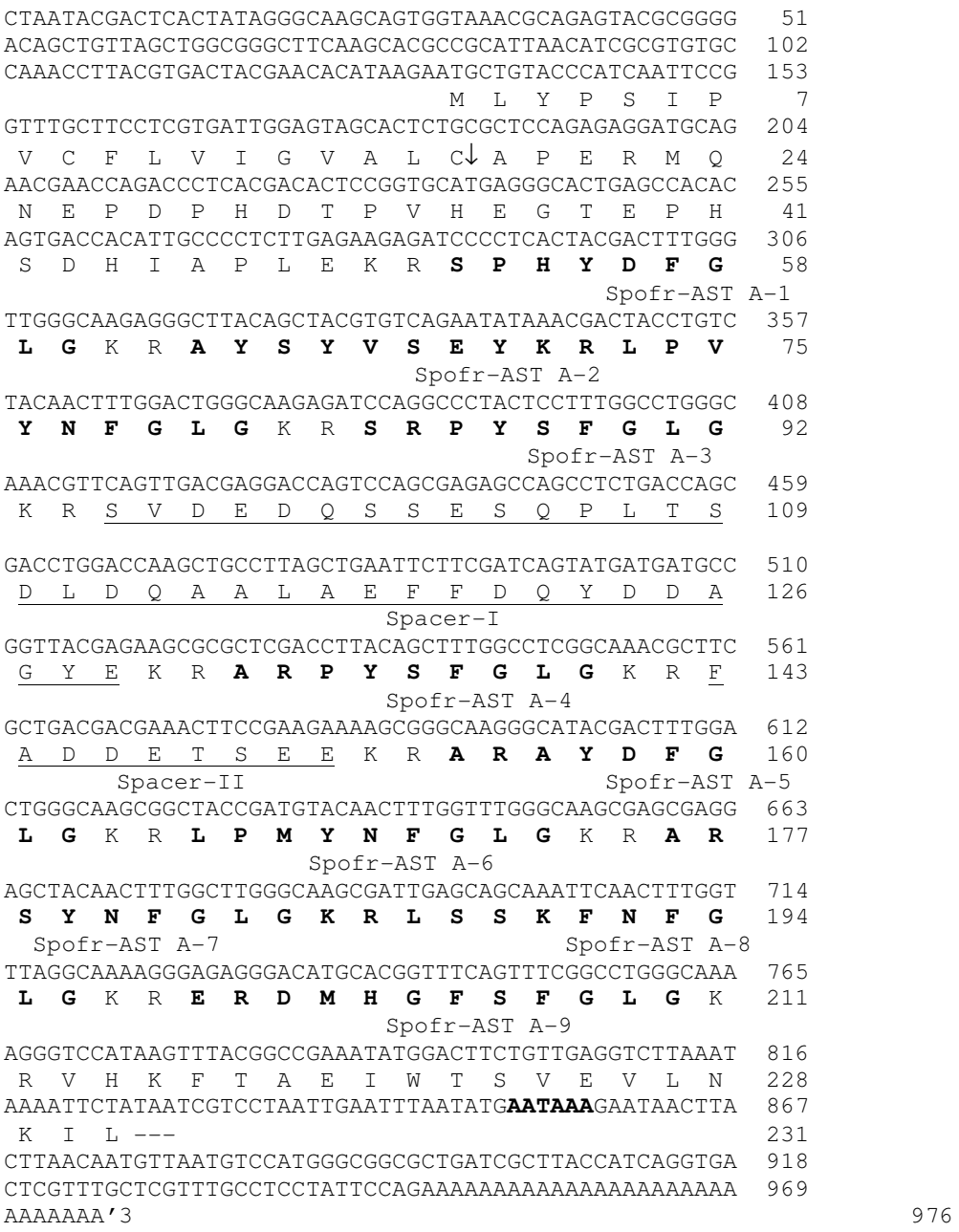

Figure (A3): Nucleotide and amino acid sequences of allatostatin A cDNA from *S. frugiperda*. The sequences are numbered at the right (Abdel-latief *et al*., 2004b).

9 List of Abbreviations

AT 1	*Manduca sexta* allatotropin
Amp	ampicillin
AS	allatostatin
AG	accessory sex gland
AT	allatotropin
Bp	base pair
β-actin	beta-actin
C-	carbon
℃	Celsius degree
CA	corpora allata
$CaCl_2$	calcium chloride
CC	corpora cardiaca
cDNA	complementary DNA
cm	centimeter
cAMP	cyclic adenosine monophosophate
ct/C_T	cycle threshold
d	day
0 d	freshly moulted animal, day 0
DEPC	diethyl pyrocarbonat
dGTP	2'-desoxyguanosin 5'-triphosphat
Dippu-AST	*Diploptera punctata* allatostatin
DNA	2' – deoxy ribonucleic acid
DNase	deoxy ribonuclease
dATP	2'-desoxyadenosin 5'-triphosphat
dCTP	2'-desoxycytidin 5'-triphosphat
dNTP	mixture of dATP, dCTP, dGTP, dTTP
dsDNA	double strand deoxyribonucleic acid
dsRNA	double strand 2`-ribonucleic acid
dTTP	2'-desoxy thymidin 5'-triphosphat
dUTP	2'-desoxyuridin 5'-triphosphat
E 75A	putative JH receptor
E. coli	*Escherichia coli*

EDTA	ethylen diamine tetraacetic acid
Ec	ecdysteroid
20E	20-hydroxyecdysone
EtOH	ethanol
FGLa	type A allatostatins (Phe-Gly-Leu-amides)
FLRFa	Phe-Leu-Arg-Phe-amides
g	gram
GAL4-EcR	DNA binding domain of ecdysone receptor
h	hour
HAc	acetic acid
HCl	hydrochloride
H_2O	water
IGR	insect growth regulator
IPTG	isopropyl-β-D-thiogalactosid
JH	juvenile hormone
JHBP	juvenile hormone binding protein
JHE	juvenile hormone esterase
JHEH	JH epoxide hydrolase
KAc	potassium acetate
KCl	potassium chloride
V	volt
kV	kilo volt
KH_2PO_4	potassium dihydrogen phosphate
l	liter
L6	larva in the 6th larval stage
LB	Luria-Bertani medium
LC-MS	liquid chromatography-mass spectrometry
LiCl	lithium chloride
M	molar
Manse-AS	*Manduca sexta* allatostatin
MeOH	methanol
mg	milligram
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate

Milli-Q-H_2O	Millipore water
min	minute
MIP	myoinhibitory peptide
ml	milliliter
mm	millimeter
mM	millimolar
mRNA	messenger RNA
n	number of replications
N_2	(liquid) nitrogen
Na^+	sodium ion
NaCl	sodium chloride
$(NH_4)_2SO_4$	ammonium sulphate
ng	nanogram
nm	nanometer
ns	not significant
nt	nucleotide
NTC	non template control
OD	optical density
Oligo (dT)	oligonucleotide from deoxy thymidine nucleotides
Ov	ovary
p.a.	pro analysis
PBAN	pheromone biosynthesis activating neuropeptide
PCR	polymerase chain reaction
pg	picogram
pH	potential hydrogen
pmol	picomol
PP	prepuppae stage
PTGS	post transcriptional gene silencing
PTSP	prothoracicostatic peptides
PTTH	prothoracicotropic peptide
RISC	RNA-inducing silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease

RT	reverse transcription or room temperature or real- time
RT-PCR	reverse transcriptase polymerase chain reaction
sec	second
SEM	standard error from means
siRNA	small interfering RNA
SK	sulfakinin
SOB	Super Optimal Broth
SOC	SOB + glucose
Spofr	*Spodoptera frugiperda*
ssRNA	single strand RNA
SYBR Green	I 2-{2-[3-Dimethylamino-propyl)-propylamino]-1-phenyl-1*H* chinolin-4-ylidenmethyl}-3-methyl-benzothiazol-3-ium-
T	temperature
T7	bacteriophage endonuclase restriction enzyme
Tab.	table
Taq	*Thermus aquaticus*
TBE	Tris-borate-EDTA
Tris	Tris-(hydroxymethyl) aminomethan
Tris-HCl	Tris-(hydroxymethyl) aminomethan- hydrochloride
U	unit
UK	United Kingdom
UV	ultra violet
UV/VIS	visualized/ultraviolet
USA	United States of America
USP	ultra spiracle
V	volume
VE	de-ionized water
Vg	vitellogenin
X	number of PCR cycles
X-Gal	5-bromo-4-chloro-3-indoyl-β-D galactopyranosid
µg	microgram
µl	microliter
µM	micromolar
%	percent

% (v/v)	volume percent per volume
% (w/v)	weight percent per volume
∞	infinity
♀	female
♂	male

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