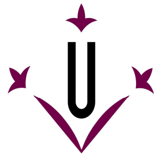

Universitat de Lleida

Hormonal regulation of the larval development of *Sesamia nonagrioides*.

Meritxell Pérez Hedo

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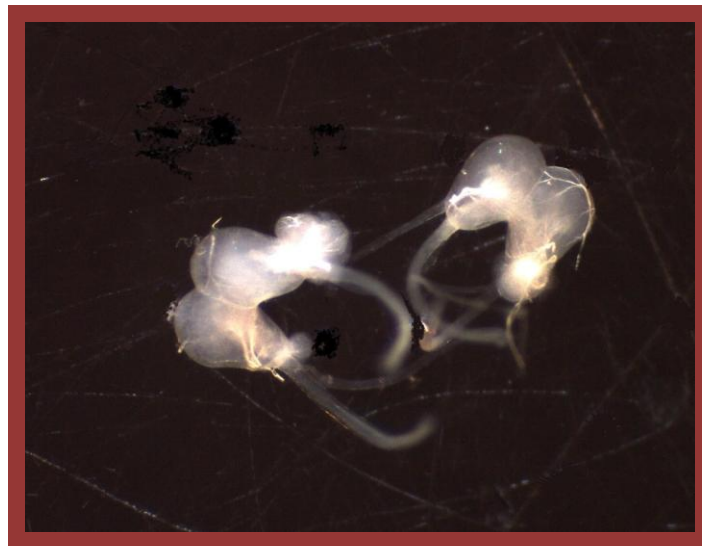

Hormonal regulation of the larval development of *Sesamia nonagrioides*.

A dissertation submitted to obtain the PhD degree in Agronomy by

Meritxell Pérez Hedo

Supervisor: Dr. Matilde Eizaguirre Altuna

PhD Thesis

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ABSTRACT

The results of this Thesis highlight the role of brain in the control of larval development in *Sesamia nonagrioides*. Thus, *S. nonagrioides* is the first lepidopteran found to develop from larvae to adult without brain. Our data show that molts depend on the release of ecdysteroids by prothoracic glands (PGs) but they can occur without prothoracicotropic hormone (PTTH) from the brain.

While all decapitated larvae (larvae without brain nor corpora allata, (CA)) pupate, the debrained larvae (larvae with no brain but with CA) often undergo several successive molts; first molt could be to larva or pupa, depending on the age at which the larva has been debrained and on the photoperiod under which the larvae have developed: many of the L6 larvae debrained 1 day after molting, molted to larvae independently of the photoperiod conditions of development, long (LD) or short (SD) day, but 5 days later all larvae developed under LD conditions pupated whereas larvae of the same age developd under SD conditions molted to larvae. Brain implants slightly accelerate pupal molts but do not alter the timing of larval molts. *S. nonagrioides* does not seem to have any alternative source of juvenile hormone (JH) to the CA. Decapitated larvae did not show noticeable amounts of JH while debrained larvae showed detectable concentrations of JH still 10 days after the surgical manipulation but the brain implants do not activate CA, apparently being neural the activation of these factors. The brain might be responsible for larval stage maintenance by neural inhibition of pupation; when the larvae of any age were deprived of their brain, the majority pupated. In the same way, the brain might be also responsible of diapause maintenance by neural inhibition of pupation; consequently, when the larvae were deprived of their brain (maintaining or not their CA) differences between diapausing and non-diapausing larvae disappeared.

The level of ecdysteroids in the decapitated larvae increased ten days after manipulation, approximately the time needed to pupate in these larvae in absence of JH, but the removal of PGs prevented molting, proving that the presence of PGs is essential for the molting process. The PGs of *S. nonagrioides* larvae can function without brain stimulation and PTTH could be released by a source outside the head; in *S. nonagrioide*s we have identified the PTTH mRNA and an alternative PTTH source in the gut. The qPCR confirmed that the PTTH gene of *S. nonagrioides* is strongly expressed in the brain of the 6th instar with a maximum on day 5 and a minimum in prepupa, but the level of PTTH expression was also detected in the gut of intact and even more in decapitated larvae with a maximum expression in prepupa.

Most decapitated larvae molt to pupa with no sign of adult development while the majority of debrained pupae suffer metamorphosis to adult thus suggesting that pupal-adult transformation depends on an unknown factor present in the debrained but not in the decapitated larvae. In *S. nonagrioides* JH applied topically not only did not inhibit the pupal-adult metamorphosis but could have favored it while the application of an ecdysteroids agonist to the pupae had no effect on the adult development.

Ingestion by *S. nonagrioides* larvae of sub-lethal amounts of Cry1Ab protein contained in maize leaves or the diet produced a prolonged development accompanied by an increase in the number of molts before pupating only in the larvae reared under LD conditions but not in the larvae reared under SD conditions. These results are due to an increase of the level of JH in the hemolymph in the non-diapausing larvae fed with Bt maize leaves or with Bt protein in the diet; on the contrary, in diapausing (SD) larvae the possible low increase of JH due to the Bt toxin ingested was not detected. In addition, the effect of Bt toxin on the ecdysteroids titer in non diapausing larvae was to suppress the increase of the hormone necessary for the pupation of and thus delaying pupation in the treated larvae. These responses may be considered as a defense mechanism allowing some larvae to molt and to survive to the toxin ingestion.

RESUMEN

Los resultados de esta Tesis ponen de manifiesto el papel del cerebro en el control del desarrollo larvario de *Sesamia nonagrioides*. Así, *S. nonagrioides* es el primer lepidóptero en el que se demuestra que el desarrollo de larva a adulto puede producirse sin la presencia del cerebro. Nuestros datos demuestran que aunque las mudas dependen de la liberación de ecdisteroides por las glándulas protorácicas (GPs), estas pueden ser activadas sin hormona protoracicotrópica (PTTH) del cerebro.

Mientras que las larvas decapitadas (larvas sin cerebro ni corpora allata, (CA)) pupan, las larvas descerebradas, de las que se ha extraído el cerebro pero mantienen el CA, sufren a menudo varias mudas sucesivas, la primera a otra larva o pupa según la edad a la que la larva ha sido desprovista del cerebro y según el fotoperiodo bajo el cual la larva se había desarrollado. Muchas larvas desprovistas del cerebro un día después de la muda a sexto estadio mudan a larva independientemente de las condiciones de fotoperiodo, día largo (DL) o corto (DC), recibidas durante su desarrollo, mientras que 5 días después todas las larvas desarrolladas en día largo puparán y las de la misma edad pero desarrolladas en día corto mudaran a larva. Los implantes de cerebro aceleran las mudas a pupa pero no alteran las mudas a larva. *S. nonagrioides* no parece tener ninguna fuente de hormona juvenil (HJ) alternativa a los CA. Las larvas decapitadas no mostraron cantidades significativas de HJ mientras que las desprovistas de cerebro mostraron cantidades de HJ detectables incluso 10 días después de la manipulación quirúrgica indicando así que los CA siguieron liberando HJ en ausencia del cerebro. Los implantes de cerebro no activaron los CA por lo que su activación es aparentemente neural. El cerebro debe ser el responsable del mantenimiento del estado larvario por inhibición neural de la pupación ya que cuando se extrajo el cerebro a larvas de diferentes edades la mayoría puparon. De manera similar, el cerebro también debe ser el responsable del mantenimiento de la diapausa ya que cuando las larvas fueron desprovistas del cerebro (manteniendo o no los CA) las diferencias entre larvas dipausantes o no diapausantes desaparecieron.

El nivel de ecdisteroides en las larvas decapitadas aumentó 10 días después de la extracción del cerebro, este tiempo es aproximadamente el tiempo que las larvas de último estadio larvario necesitan para pupar. La extracción de las GPs impidió la muda, lo que demuestra que su presencia es esencial para que tenga lugar el proceso de la muda. Ante la evidencia de que las GPs de las larvas de *S. nonagrioides* podían activarse sin la estimulación por hormona PTTH del cerebro se buscaron fuentes alternativas de la hormona y se identificó PTTH mRNA en el intestino de la larva. La qPCR confirmó que el gen de la PTTH en *S. nonagrioides* se expresa de forma elevada en el cerebro de la larva de 6º estadio con un máximo el 5º día del mismo y un mínimo en la prepupa, pero la expresión de la PTTH se detectó también en el intestino de las larvas intactas y mucho más en las decapitadas con máxima expresión en el periodo de prepupa.

De forma general se asume que la metamorfosis pupa-adulto en los insectos se produce en ausencia de HJ y en el caso de *S. nonagrioides* la mayoría de las larvas decapitadas mudan a pupa sin mostrar posteriormente ningún indicio de desarrollo a adulto mientras que la mayoría de pupas desprovistas de cerebro pero que mantiene su CA sufren metamorfosis a adulto. Este hecho sugirió que la transformación de pupa a adulto en esta especie depende de algún factor presente en las larvas descerebradas pero no en las decapitadas. En *S. nonagrioides* la HJ aplicada tópicamente no solamente no inhibió la metamorfosis de pupa a adulto sino que la favoreció mientras que la aplicación de un agonista de ecdisteroides a las pupas no tuvo efecto sobre el desarrollo a adulto.

La ingestión por las larvas de *S. nonagrioides* de cantidades subletales de la proteína Cry1Ab contenida en hoja de maíz o en dieta produjo un prolongamiento de su desarrollo acompañado de un aumento en el número de mudas larvarias antes de pupar, pero sólo en las larvas desarrolladas en condiciones de DL, no en las desarrolladas en condiciones de DC. Estos resultados son consecuencia del aumento de HJ en la hemolinfa de las larvas no diapausantes alimentadas con hoja de maíz Bt o con la proteína Bt añadida. Sin embargo, no se detectó el posible ligero aumento de HJ causado por la ingestión de la proteína Bt en las larvas diapausantes (desarrolladas en DC). Otro efecto de la ingestión de la proteína Bt en las larvas no-diapausantes fue suprimir el aumento en la concentración de ecdisteroides necesario para la pupación que por tanto se retrasó en las larvas tratadas. Estas respuestas pueden ser consideradas como un mecanismo de defensa que permite que algunas larvas puedan mudar y así sobrevivir a la ingestión de la toxina.

RESUM

Els resultats d'aquesta Tesi posen de manifest el paper del cervell en el control del desenvolupament larvari de *Sesamia nonagrioides*. Així, *S. nonagrioides* és el primer lepidòpter on s'ha trobat que es pot desenvolupar de larva a adult sense cervell. Les nostres dades mostren que les mudes depenen de l'alliberament d'ecdisteroides per les glàndules protoràciques (GPs) però poden tenir lloc sense la hormona protoracicotròpica (PTTH) del cervell.

Mentre que les larves decapitades (larves sense cervell ni corpora allata, (CA)) pupen, les larves de les que s'ha privat del cervell (però s'ha mantingut el CA) sofreixen sovint varies mudes successives; la primera muda pot ser de larva o pupa segons l'edat a la que la larva ha estat privada del cervell i el fotoperíode al qual la larva s'ha desenvolupat. Moltes de les larves L6 privades del cervell un dia després de la muda van mudar a larva independentment de les condicions de fotoperíode durant el desenvolupament, de dia llarg (DL) o curt (DC), mentre que 5 dies després totes les larves desenvolupades en DL van pupar i les de la mateixa edat però desenvolupades en DC van mudar a larva. Els implants de cervell acceleren les mudes a pupa però no alteren les mudes a larva. *S. nonagrioides* no sembla tenir cap font alternativa als CA de hormona juvenil (HJ). Les larves decapitades no van mostrar quantitats significades de HJ mentre que les larves privades de cervell van mostrar concentracions que es poden detectar fins i tot 10 dies després de la manipulació quirúrgica, però els implants de cervell no activen els CA essent aparentment neural l'activació d'aquests factors. El cervell podria ser el responsable del manteniment de l'estat larvari per inhibició neural de la pupació; quan les larves d'edat variada van ser privades del cervell, la majoria van pupar. De manera similar, el cervell també podria ser el responsable del manteniment de la diapausa per inhibició de la pupació, conseqüentment, quan les larves van ser privades del cervell (tot mantenint o no els CA) les diferencies entre larves dipausants o no diapausants van desaparèixer.

El nivell d'ecdisteroides en les larves decapitades augmentà 10 dies després de la manipulació, aproximadament el temps necessari per a pupar en aquelles larves en absència de HJ, encara que l'extracció de les GPs va impedir la muda, i això probà que la presencia de GPs és essencial per al procés de muda. Les GPs en les larves de *S. nonagrioides* poden funcionar sense estimulació del cervell i la PTTH pot ser alliberada per una font de fora del cap; a *S. nonagrioides* hem identificat la PTTH mRNA i una font alternativa en l'intestí. La qPCR confirmà que el gen de la PTTH a *S. nonagrioides* s'expressa molt al cervell de l'instar 6è amb un màxim al dia 5è i un mínim en la prepupa, però el nivell d'expressió de la PTTH es va detectar també en l'intestí de les larves intactes i encara més en el de las decapitades amb una expressió màxima en la prepupa.

La més gran part de les larves decapitades muden a pupa sense cap senyal de desenvolupament d'adult mentre que la majoria de pupes provades de cervell sofreixen metamorfosi a adult, lo que suggereix que la transformació de pupa a adult depèn d'un factor desconegut present en les larves privades de cervell

però no en les decapitades. En *S. nonagrioides* la HJ aplicada tòpicament no solament no va inhibir la metamorfosi de pupa a adult però la pogué haver afavorit mentre que l'aplicació d'un agonista d'ecdisteroides a les pupes no va tenir cap efecte en el desenvolupament d'adult.

La ingestió per les larves de *S. nonagrioides* de quantitats subletals de la proteïna Cry1Ab continguda en les fulles de panís o en la dieta va produir un perllongament del seu desenvolupament acompanyat d'un augment en el nombre de mudes abans de pupar però només en les larves criades en condicions de DL, no en les criades en condicions DC. Aquests resultats són deguts a un augment del nivell de HJ en la hemolimfa de les larves no diapausants (DL) alimentades amb fulles de panís Bt o amb la proteïna Bt afegida a la dieta; pel contrari, no es va detectar el possible lleuger increment de HJ causat per la ingestió de la proteïna Bt en les larves diapausants (DC). A més, l'efecte de la proteïna Bt en la concentració d'ecdisteroides en les larves no diapausants va ser suprimir l'augment de la hormona que era necessari per la pupació i per tant va retardar la pupació en les larves tractades. Aquestes respostes poden ser considerades com un mecanisme de defensa que permet que algunes larves puguin mudar i sobreviure a la ingestió de la toxina.

CHAPTER 1

1. General introduction

GENERAL INTRODUCTION

The Mediterranean Corn Borer *Sesamia nonagrioides* (Lefèbvre) is a Lepidoptera of the Noctuidae family that feeds mainly on maize, sugar cane and sorghum.

The first record of the occurrence of *S. nonagrioides* as a maize pest in Spain dates back to 1902 in Asturias (Delgado de Torres, 1929). Since then, it has been considered one of the most important pests of maize in Spain (Anglade, 1972; Castañera, 1986). It is found in almost all the Mediterranean countries, from the northern border at the 45º parallel to the southern border north of Africa (Anglade, 1972; Larue, 1984). Damage caused by larvae of the borer, depends on the phenological stage of the attacked maize, the population density and the duration of damage, usually causing losses of yield between 5 and 15% (Brookes, 2002). The larva is the only stage that causes damage; it feeds on the medulla of the cornstalk causing a circulatory disorder in the vascular system of the plant, so the plant cannot assimilate the nutrients necessary for its development. Growth and maturation of the plant may be affected and the wind could break the cornstalk at the site affected by the larva (Delgado de Torres, 1929; Anglade, 1972). In addition, sometimes the borer larvae can attack the corncob, favoring the entry of pathogens such as *Fusarium* (Sobek & Munkvold, 1999), which enhance the development of mycotoxins in grains, reducing their quality.

S. nonagrioides is a multivoltine species, in the area of this study (northeastern Spain, Lleida) it has two complete generations and one incomplete third generation (Eizaguirre *et al*., 2002). The importance of this third generation depends on the percentage of second-generation larvae that enter diapause. The diapause is a hormonally regulated state of low metabolic activity with altered or reduced activity (Tauber and Tauber, 1981). This state is determined genetically and it is a response to a series of stimuli that create adverse conditions for the insect. The most important diapause-inducing factor in *S. nonagrioides* is the short photoperiod, but it may be modified by the temperature or the phenology of the maize (Eizaguirre & Albajes, 1992). Photoperiod induces diapause during the first and second instars (Eizaguirre *et al*. 1994) but the larvae continue to develop during diapause over the winter in the stubbles of maize. Furthermore, induction of diapause in this species is related to increased levels of juvenile hormone (JH) in the hemolymph (Eizaguirre *et al*. 1998; Eizaguirre *et al*. 2005a). During diapause the larvae continue to maintain JH at a titer that allows retention of larvae characters during the stationary molts that continue to occur throughout diapause (Chippendale, 1977). The diapausing larvae feed, move and molt with an indeterminate number of supernumerary molts (Fantinou *et al*., 1995, Gadenne *et al*., 1997), while non-diapausing larvae mostly molt to pupae after the sixth instar. Overwintering larvae pupate in the spring and first generation adults emerge from late March to May (López *et al*., 2001). They mate in the fields where they spent the winter (Eizaguirre *et al.,* 2002), and the males disperse in a radius up to 400 meters (Eizaguirre *et al*., 2004). The females of this species emit pheromones to attract

the male (Albajes *et al*., 2002). Mated females lay their eggs between the stem and the lower leaves of the maize plant and the neonate larvae quickly bore into the cornstalk where they complete their lifecycle, emerging only to disperse to nearby plants. This endophytic behavior of *S. nonagrioides* makes this pest difficult to control through the application of conventional chemical methods and hinders the action of some natural enemies of this species such as the microbial agent *Beauveria bassiana* (Balsamo) and the most common parasitoid of larvae, the dipterous *Lydella thompsoni* (Herting). Alternatives to chemical control such as the use of synthetic pheromones in mating disruption techniques (Albajes *et al*., 2002), cultural control (crop rotation and destruction of overwintering larvae, to reduce the first generation adults) and the use of transgenic plants expressing insecticidal proteins are strategies that improve the intrinsic resistance of the plant against the insect.

Transgenic maize has incorporated the insecticidal capacity of *Bacillus thuringiensis* (Bt) bacteria and it is very effective against *S. nonagrioides* (Gonzalez-Cabrera *et al*., 2006). The area of GM maize grown in Spain was 76,057 hectares in 2009 which represents the 22% of the total maize area in the country (www.mapya.es). Until 2005, the transgenic maize allowed contained Event 176 unauthorized today, and Event MON810. Now the only Bt maize event allowed in EU is Event MON810. Both events (MON810 and 176) express one unique Bt toxin, the Cry1Ab. There are two types of delta-endotoxins: Cry proteins and Cyt proteins. The first classification of endotoxins was based on specific insecticidal genes which were grouped into six classes of Cry genes and two classes of Cyt genes (Höften & Whiteley, 1989). Due to the discovery of homologous genes with different insecticidal activity, as well as the existence of dual genes, a new nomenclature was proposed based on the similarity of the primary sequence (Crickmore *et al*., 1998). Cry toxins constitute a set of proteins sprayed as a biological insecticide or introduced into transgenic plants. They are considered relatively harmless to humans, vertebrates, non-target insects and plants, and are completely biodegradable (Flexner *et al*., 1986; Farinós *et al*., 2008). One feature that distinguishes these Cry proteins is their high selectivity for their target insect (Bravo *et al*., 2005). The mechanism of action of Bt protein is based on different steps, first the crystals enter the midgut through estomodeum practically intact. The protoxin in crystal form is insoluble in most chemical conditions but does dissolve in highly alkaline pH. Dissolved protoxins are processed by specific digestive proteases of the insect and the toxin can cross the peritrophic membrane and bind to specific receptors on the epithelial membrane. Cry proteins produce small pores in the apical membrane of columnar cells of the midgut causing an osmotic imbalance which causes cell lysis (Knowles, 1994; Bravo *et al*., 2005). Bt maize is highly effective for controlling *S. nonagrioide* usually killing 100% of the larvae that feed on it. However, there is the possibility that some developing larvae fed sporadically on transgenic maize will not die. Eizaguirre *et al.* (2005b) observed that young larvae fed on a semi-synthetic diet of maize containing sublethal amounts of a commercial preparation of the Cry1Ab protein survived showing alterations in larval development and a higher sensitivity to the

critical photoperiod for diapause induction. These results suggested the possibility that the feeding of the *S. nonagrioides* larvae with sublethal quantities of Bt could have changed the hormonal balance of the larvae.

To understand more fully the changes on the endocrine system that occur due to different external factors such as the feeding with sub-lethal amounts of Bt, the normal function and the brain control over the endocrine glands must be well understood. Important structures include the corpora allata (CA), which produces JH and prothoracic glands (PGs), the main source of ecdysteroids in developing insects. The great majority of insect hormones are neurosecretory products that control a broad diversity of physiological and developmental processes. The insect brain contains the greatest number and diversity of neurosecretory cells and thus, it is the principal neuroendocrine organ of the insects. The brain and its associated glands and neurohemal organs together form an integrated control, synthesis, and release system for hormornes from the head region called the *Brain-retrocerebral neuroendocrine complex*, that includes Brain-corpora cardiaca-CA (Nijhout, 1994). The main hormones responsible for the development of insects are JH and ecdysteroids. The primary role of JH during larva life is to prevent metamorphosis. This action is manifested at every molt. JH must be present at the outset of the ecdysteroid peak for the molt in order for the cells to retain their larval characteristics (Riddiford, 1994). Also, JH functions include regulation of metamorphosis, caste determination, behavior, diapause, reproduction and various polyphenisms (Nijhout, 1994). 20-hydroxyecdysone (20E) controls ecdysis and metamorphosis of arthropods. The prothoracicotropic hormone (PTTH), a brain neuropeptide hormone, drives the prothoracic glands to release ecdysone which is converted to the more hormonally active 20E by the fat body (Bollenbacher *et al.*, 1977; Rybczynski *et al.*, 2009).

The objective of this thesis was first to determine the role of the brain in the control of *S. nonagroides* larvae development and diapause and to determine the role of JH and ecdysteroids production (Chapters 2.1 and 2.2). Other objective of this thesis was to determine if *S. nonagrioides* larvae have another source of PTTH other than the brain and to verify whether molting depends on the presence of the prothoracic glands (Chapter 3). Finally, the last objective of this thesis was to determine the effect of the Bt protein on hormone levels in the larvae of *S. nonagrioides* and the consequences on their development as a response to this kind of stress.

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CHAPTER 2

Role of the brain in larval development of *S. nonagrioides*:

2.1 Brain-independent development in the moth *Sesamia nonagrioides*.

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Brain-independent development in the moth *Sesamia nonagrioides*.

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Abstract

The caterpillars of *Sesamia nonagrioides* developing under long-day (LD) photoperiod pupate in the 5th or 6th instar whereas under short day (SD) conditions they enter diapause and undergo several extra larval molts. The diapause is terminated within 1–3 instars upon transfer of SD larvae to the LD conditions. Brain removal from the 6th instar larvae promotes pupation followed by imaginal development; however, one third of the SD larvae and 12% of the LD larvae debrained at the start of the instar first undergo 1–2 larval molts. The incidence of larval molts is enhanced by the brain implants. Exclusively pupal molts occur in the LD larvae debrained late in the 6th instar. Decapitation elicits pupation in both LD and SD larvae, except for some of the 4th and 5th and rarely 6th instar that are induced to a fast larval molt. The pupation of decapitated larvae is reverted to a larval molt by application of a juvenile hormone (JH) agonist. No molts occur in abdomens isolated from the head and thorax prior to the wandering stage. Abdomens isolated later undergo a larval (SD insects) or a pupal (LD insects) molt. Taken together the data reveal that in *S. nonagrioides* (1) several larval molts followed by a pupal and imaginal molt can occur without brain; (2) an unknown head factor outside the brain is needed for the pupal–adult molt; (3) brain exerts both stimulatory and inhibitory effect on the corpora allata (CA); (4) larval molts induced in CA absence suggest considerable JH persistence.

Keywords: Brain; Diapause; Ecdysteroids; Juvenile hormone; Molting.

1. Introduction

Since the time of Kopeć (1922) it has repeatedly been demonstrated that the development of insects is governed by the brain. Numerous studies performed in Lepidoptera showed that brain removal before a critical period prevented molting. Williams (1947) discovered in the silkmoth *Hyalophora cecropia* that brain translated environmental changes in photoperiod into a neurohormonal signal that stimulated molting hormone (= ecdysteroids) production from the prothoracic glands (PG). Brain neurosecretion was identified as prothoracicotropic hormone, PTTH (Kataoka et al., 1987). PG regulation proved more

complex (Gilbert et al., 2002, Yamanaka et al., 2006 and Watanabe et al., 2007) but pivotal role of brain is obvious from the cessation of development in the decapitated or debrained larvae.

The switch from larval development to metamorphosis is primarily controlled by juvenile hormone (JH) secretion from the corpora allata (Wigglesworth, 1934). Corpora allata (CA) of Lepidoptera were found to be hormonally regulated by the brain (Granger and Sehnal, 1974 and Sehnal and Rembold, 1985). The role of brain in CA regulation was examined in a number of species and several allatotropic and allatostatic factors were isolated (Audsley et al., 2008). JH titer in many caterpillars is further regulated by a specific esterase that occurs in the last larval instar and renders JH inactive by cleaving off the methyl ester group (Hammock, 1985).

The Mediterranean corn borer, *Sesamia nonagrioides*, is a noctuid that produces 2–4 generations per season. Fully grown larvae of the late summer generation bore into the maize hypocotyl for overwintering and their pupation is postponed until next spring (Galichet, 1982). The hibernation was recognized as diapause induced by the short photoperiod (Eizaguirre and Albajes, 1992) and regulated by JH (Eizaguirre et al., 1998). In larvae that develop under long day conditions, JH titer in the hemolymph drops from about 20 nM in the 4th and 5th instar to undetectable level in the 6th instar when the insects pupate (Eizaguirre et al., 2005). Under the short day conditions, the JH titer is about 60 nM in the 4th and 5th instars and up to 20 nM in the 6th and subsequent extra larval instars that characterize the diapausing insects. The titer of total ecdysteroids in the 4th and 5th instars is similar in the diapause-destined and the non-diapausing larvae; in the 6th instar the titer raises to a small peak of 0.2 μg/ml hemolymph on day 5 and to a larger peak on days 7–9. This second peak reaches 0.6 μg/ml in the pupating larvae but less than 0.3 μg/ml in the diapausing ones (Eizaguirre et al., 2007).

Effects of hormone agonists administered to the larvae of *S. nonagrioides* suggested that JH and ecdysteroids affect each other's concentration in a way that gears subsequent development to perfect realization of one of the three programs: larval growth, diapause, and pupation, respectively. Mixed modes, indicating a failure of the regulatory mechanisms, are limited to the development of larval–pupal intermediates after application of JH agonist in the second third of the last larval instar (Eizaguirre et al., 2005). The stability of developmental programs indicated that a higher control center – presumably the brain – integrates environmental signals such as the photoperiod with those from the internal milieu (hormone titers, body size, etc.), “selects” the mode of development, and sends appropriate commands to the endocrine glands. This paper is the first attempt to elucidate regulatory mechanisms that ensure perfect realization of the chosen developmental program in this species. The tuning of regulatory centers to realization of a certain program (larval, diapause, or pupation) is called here “programming”. It should be distinguished from the developmental programming of the target tissues that is referred to as “commitment” (Riddiford, 1976).

2. Materials and methods

2.1. Insects and their rearing

The culture of *S. nonagrioides* was established from insects collected in central Catalonia and reared on a semi-artificial diet at 25 °C as described by Eizaguirre et al. (1994). The age of larvae was specified in respect to the instar and the number of days after ecdysis; for example, L5d6 denotes larvae of the 5th instar, 6 days after the ecdysis. Larvae kept under the long day (LD) photoperiod of 16:8 h light:dark cycle pupated in the 5th (about 20%) or the 6th larval instar. Those going to pupate in the 5th instar were recognized and discarded on day 4 of this instar when the remaining larvae showed clear signs of larval apolysis. After ecdysis into the 6th instar the LD larvae fed for about 6 days (maximal weight was reached on day 5), wandered on day 7, underwent pupal apolysis on days 8–9, and ecdysed to pupae one day later. By contrast, larvae grown since hatching under the short day (SD) photoperiod of 12:12 h light:dark cycle never pupated in the 5th or 6th instar but entered diapause characterized by up to ten extra larval molts occurring in irregular intervals (Eizaguirre et al., 1994, Fantinou et al., 1996 and Gadenne et al., 1997). Our experiments included alternation of the two photoperiods in L6d2. This age was chosen because treatments with JH agonist indicated that the tissues of LD larvae could still realize a perfect larval molt. The commitment for pupal molt was detected in some body regions in 40% of the L6d4 larvae (Eizaguirre et al., 2005).

2.2. Brain removal and implantation

The role of brain was examined by means of decerebration and brain re-implantation in the L6d1 and L6d5 larvae. We assumed, and the experiments confirmed, that the newly ecdysed L6d1 were developmentally flexible, while the L6d5 larvae, which experienced a small ecdysteroid peak (Eizaguirre et al., 2007), were partially committed to a certain type of molt. Larvae were anaesthetized by submerging in water for 2–3 h, blotted on absorbent paper, and fixed with dorsal side up in a paraffin-lined Petri dish by means of pins. Integument between the head capsule and the pronotum was stretched and cut with a scalpel. The head was bent forward and the partly exposed brain was removed with a pair of forceps's. Inspection of the dissected brain confirmed that the corpora cardiaca–corpora allata (CC–CA) complex remained in situ. Some of the debrained larvae received implants of brains taken from donors of the same age and either of the same or of the opposite photoperiodic experience than the recipients. Brains (without CC–CA) were dissected under insect saline from the cut-off heads and within 30 min implanted (two brains per specimen) into recipient larvae through a V-shaped slit in the integument on the dorso-lateral side of the abdomen. Following insertion of the implant, the V-flap of the integument was placed back, the wound was wiped, and the insects were kept individually and without food in a refrigerator for 2 h before return to appropriate rearing conditions. The debrained larvae without or with the implants did not feed but crawled when touched. They were kept in vials supplied with a small amount of diet to maintain humidity.

In the sham operated insects we verified that injuries mimicking the brain removal and brain implantations did not alter the type of development determined by the photoperiod. The implantation procedure (anesthesia in water, injury similar to the brain removal, insertion of an inert implant, and recovery in refrigerator) performed at L6d1 extended the length of the intermolt period by about one day. The extension was shorter in the L6d2 and L6d5 insects.

2.3. Decapitated larvae and isolated larval abdomens

Anaesthetized larvae were ligated with a thin cotton thread either behind the head or across the mesothorax. The body part anterior to the ligature was cut off. Body constriction behind the head yielded decapitated larvae that lacked the major neuroendocrine center brain–CC–CA but contained the prothoracic glands (PG). Neither of these organs was present in the isolated abdomens obtained by ligating the larvae across mesothorax. Both the decapitated larvae and the isolated abdomens were kept in vials supplied with a small amount of diet that provided air humidity. The insects were motionless but responded to tactile stimuli by body wriggling. The molts of decapitated larvae and isolated abdomens usually advanced until the stage of molting fluid resorption but the exuvia was fully shed off in a few insects only.

2.4. Application of juvenile hormone agonist

Juvenile hormone agonist methoprene (ethyl 3,7-dimethyl-11-methoxydodeca-2,4-dienoate) was obtained from the Zoecon Corporation (Palo Alto, CA) as >97% trans, trans, S isomer in 1994 and thereafter stored in a freezer. Tests with *Galleria mellonella* larvae showed that it had retained biological activity and was therefore used in the present study. L6d1 larvae of *S. nonagrioides* were decapitated and one day later treated topically with 2 µl acetone containing 1, 0.1, 0.01, 0.001, and 0 µg methoprene. One group of larvae treated with 0.01 µg received this dose again 3 and 6 days later (the last treatment was not applied to larvae that had already molted).

2.5. Statistical evaluation of the results

Experimental insects were checked daily. Mortality due to surgical manipulations occurred within 1–3 days and never exceeded 10%. Dead larvae were not considered in treatment evaluations and are not included in the "total number of insects" shown in the tables and figures. When the number of individuals permitted, the time to the first larval ecdysis or to the pupal apolyses was analyzed by one-, two- or three-way ANOVA, depending on the number of factors considered. LSD test was used to compare durations of the intermolt periods.

3. Results

3.1. Photoperiodic determination of metamorphosis versus diapause

About 20% of larvae grown since hatching under LD conditions pupated in the 5th instar and were discarded. All remaining LD larvae molted to the 6th larval instar and pupated about 9 days later (Fig. 1A and column 'Permanent LD' in Table 1). By contrast, the insects grown since hatching under SD conditions entered diapause and underwent at least four and some of them more than eight extra larval molts (Fig. 1B) in long and irregular intervals (Table 1, 'Permanent SD') before they either pupated or perished. The stability of developmental programming of the LD larvae was tested by transferring them at L6d2 to the SD conditions (Fig. 1C and column 'LD, transfer to SD' in Table 1). The programming for metamorphosis was retained in all insects and the timing of pupal molt was not affected by the transfer ($F = 0.06$, $P = 0.81$, DF = 1,40). The dependence of diapause programming on the photoperiod was similarly examined by

Molt	Permanent LD	LD, transfer to SD	Permanent SD	SD, transfer to LD
L6–PUPA	9.2 ± 0.4 (20/20)	9.3 ± 1.3 (22/22)	(0/9)	21.2 ± 4.2 (6/17)
L7–PUPA	–	–	(0/9)	14.2 ± 2.5 (10/17)
L8–PUPA	–	–	(0/9)	12.0 ± 0.0 (1/17)
L9–PUPA	–	–	(0/9)	–
L10–PUPA	–	–	16.0 ± 0.0 (1/9)	–
L11–PUPA	–	–	32.0 ± 0.0 (1/9)	–
L12–PUPA	–	–	(0/9)	–
L13–PUPA	–	–	22.0 ± 0.0 (1/9)	–
L6–L7	–	–	8.0 ± 1.8 (9/9)	14.5 ± 2.3 (11/17)
L7–L8	–	–	14.6 ± 4.5 (9/9)	17. 0 ± 0.0 (1/17)
L8–L9	–	–	16.3 ± 6.0 (9/9)	–
L9–L10	–	–	18.9 ± 6.8 (8/9)	–
L10–L11	–	–	18.7 ± 4.2 (6/9)	–
L11–L12	–	–	20.0 ± 4.0 (5/9)	–
L12–L13	–	–	8.0 ± 7.1 (2/9)	–
L13–L14	–	–	10.0 ± 0.0 (1/9)	–

Results of statistical analysis by two-way ANOVA (manipulation and age of the larvae) are provided in the text.

Table 1. Duration (mean ± S.D. in days) of the 6th (L6) and subsequent instars before pupal or additional larval molts in larvae exposed to the LD or SD photoperiod since hatching either permanently or transferred to the opposite photoperiod at L6d2. Ecdysed/total numbers of insects are given in parentheses.

transferring L6d2 SD larvae to the LD conditions. This treatment changed the programming from diapause to metamorphosis in all insects but at different rate. About 35% of the original SD larvae pupated in the 6th, 60% in the 7th, and the remainder in the 8th instar (Fig. 1D). Pupal molts were delayed (Table 1, 'SD, transfer to LD') by comparison with the pupation timing in the LD insects ($F = 129.91$, $P < 0.001$, DF = 2,45 for pupation in the 6th, and $F = 15.81$, $P < 0.001$, DF = 1,14 in the 7th instar). These results reveal that diapause programming requires persistent SD conditions and that its change upon transfer to LD conditions takes 1–3 instars.

3.2. Effects of brain removal

To examine the role of brain in the control of development, the SD and LD larvae were debrained on days 1 or 5 of the 6th instar. Surprisingly, brain removal never prevented further development but caused a dramatic change of its course. Decerebration terminated diapause and elicited pupation in nearly 70% SD larvae of both age groups (Fig. 2). The change from larval to pupal molt was associated with instar extension, especially in the L6d5 insects (Table 2). The remaining debrained SD larvae underwent an extra larval molt and pupated but a few accomplished up to 3 extra molts and then perished. In the LD

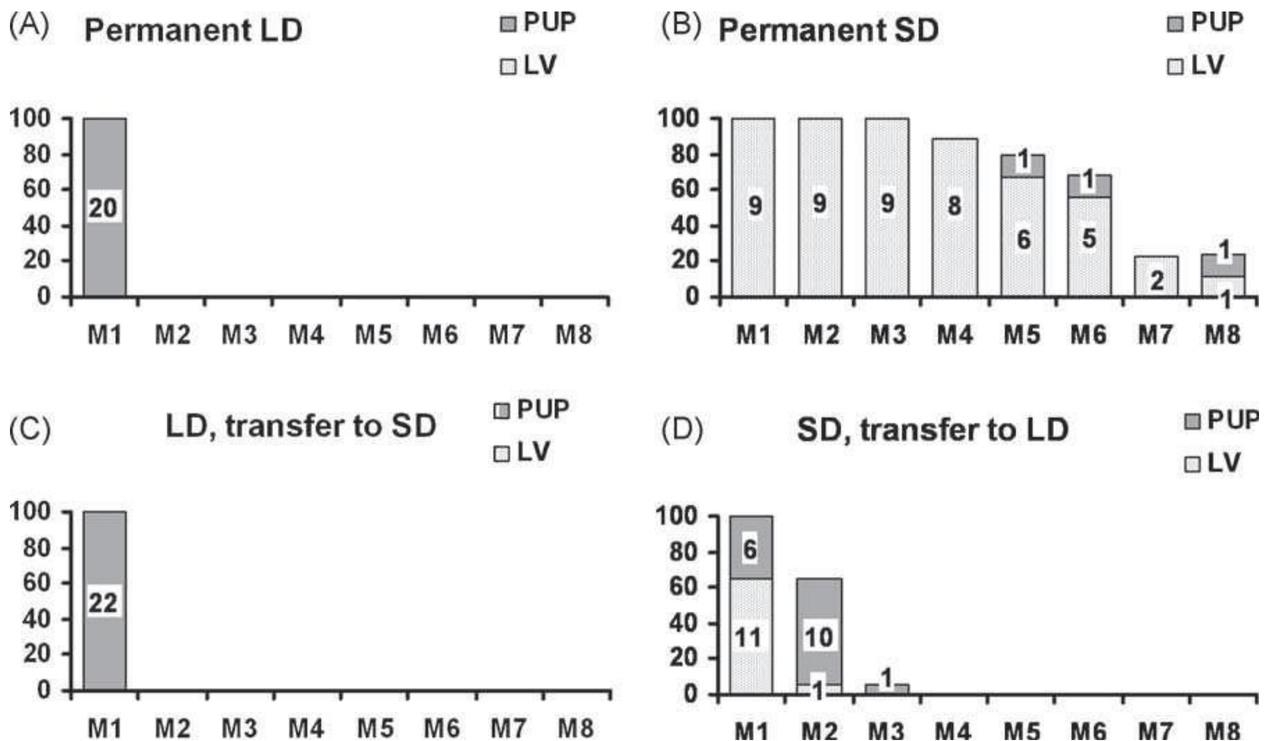

Fig. 1. Percent larval (LV) and pupal (PUP) molts in larvae kept since hatching under the LD or SD photoperiod (A and B, respectively) or transferred to opposite photoperiod at L6d2 (C and D, respectively). The molts are numbered from the start of the experiment (M1 = molt to the 7th instar). Figures in the columns show the numbers of molted individuals (100% = total initial numbers).

larvae, decerebration performed at L6d5 did not alter pupation programming whereas 12% of the debrained L6d1 LD larvae underwent an extra larval molt. Five of them died afterwards but one pupated in the 7th instar. The molts of debrained LD larvae occurred about 10 days later than the pupal molts of the intact LD larvae (cf. Table 1).

pupation in the debrained L6d5 LD larvae (Fig. 2). Larval molts induced in debrained L6d1 larvae by the brain implants seemed to occur earlier than without the implants but the difference was insignificant (Table 2). Pupal molts were accelerated by the implants in both groups of SD larvae and in the L6d1 LD larvae ($F = 7.08$,

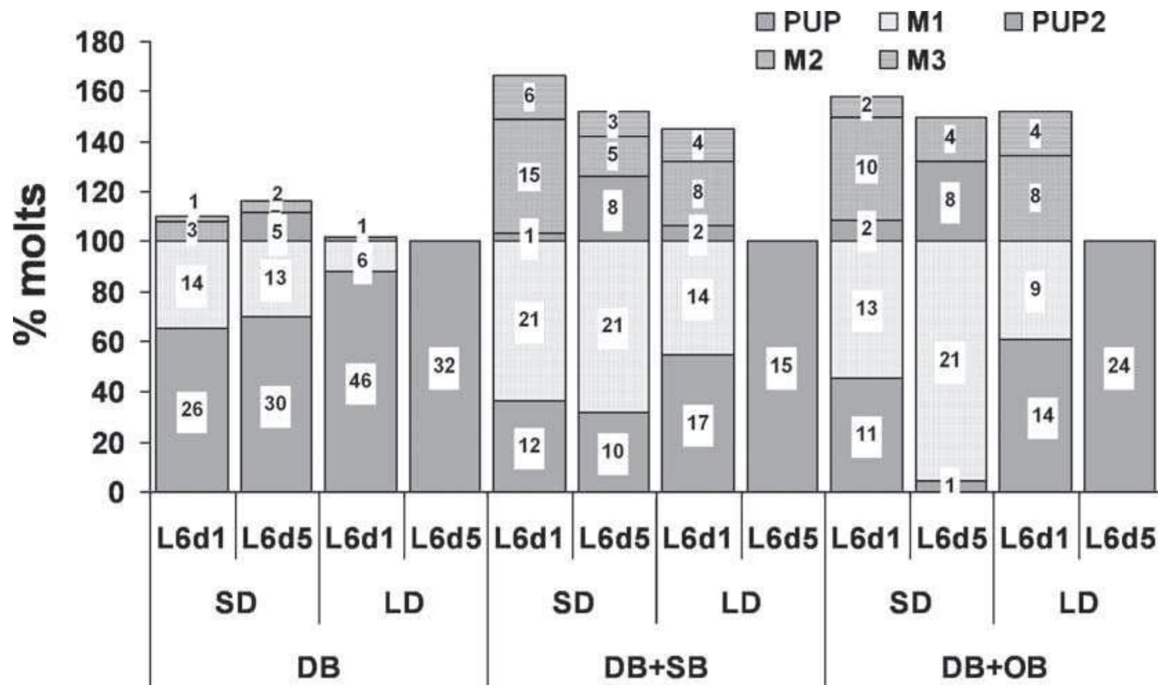

Fig. 2. Percent molts in the SD and LD larvae that were debrained (DB) or debrained and implanted with 2 brains at L6d1 or L6d5. Implants were taken from larvae of the same age and either the same (DB + SB) or the opposite (DB + OB) photoperiodic experience. Nearly all insects accomplished one molt either to larva (M1) or pupa (PUP). Some M1 larvae molted again to larvae (M2, M3) and eventually to pupae (PUP2). Figures in the columns show the numbers of molted individuals (100% = total initial numbers).

The effect of brain removal was altered when the debrained insects received implants of two brains (without CC–CA) taken from larvae of the same age (L6d1 and L6d5, respectively). Brain donors were reared either under identical or under opposite (short day versus long day) photoperiodic conditions but this had no effect on implant activity. All types of implants increased the incidence of larval molts to 70–90% in both age groups of debrained SD larvae and to 45% in the debrained L6d1 LD larvae, but never prevented

$P = 0.002$). The photoperiod and the interaction manipulation/photoperiod were not significant ($F = 1.12$, $P = 0.293$ and $F = 0.96$, $P = 3.89$, respectively). Brain implants had no effect on pupation timing in the debrained L6d5 LD larvae (Table 2).

Pupae that originated from the debrained larvae continued to develop into adults (Fig. 3). The length of pupal–imaginal development varied between 12 and 16 days, i.e. it was longer than the normal pupal instar of 10 days. The time of molt

completion was difficult to assess because most insects failed to escape from the pupal exuvia. Some of them did and produced normally looking mobile imagoes. The incidence of successful completion of metamorphosis was in some groups increased by the brain implants but this effect was not consistent. The development of debrained larvae until the adult stage demonstrated that the entire metamorphosis of *S. nonagrioides* can be accomplished without the brain.

CA was eliminated by decapitation that was performed in larvae of different age (Fig. 4). Surprisingly, 20–60% of both SD and LD insects decapitated in the 4th and 5th instars underwent a rapid, perfectly larval molt in spite of CA absence (Fig. 4). Our interpretation was that JH present in the larvae of this age (Eizaguirre et al., 2005) persisted after ligature in sufficient concentration until the larval molt commitment was completed. Larval molts occasionally occurred after decapitation of the 6th instar larvae (maximally in 12% and usually in less than 2% of insects decapitated on days 1, 3, and 5, respectively), possibly reflecting differences in the body content of JH at the time of decapitation. It must be

Treatment	**SD larvae**		**LD larvae**	
	L6d1	**L6d5**	**L6d1**	**L6d5**
Time to larval ecdysis				
Debrained	15.0 ± 6.0 (14/40) a	13.1 ± 5.0 (13/43) a	18.2 ± 5.9 (6/52) a	(0/32)
Debr + SB*	12.5 ± 3.2 (21/33) a	13.9 ± 2.7 (21/31) a	13.3 ± 5.2 (14/31) a	(0/15)
Debr + OB*	11.8 ± 1.8 (13/24) a	13.8 ± 1.8 (21/22) a	11.2 ± 1.5 (9/23) a	(0/24)
Decapitated	(0)	(0)	(0)	(0/24)
Time to pupal ecdysis				
Debrained	18.9 ± 4.1 (26/40) a	23.2 ± 4.4 (30/43) a	19.9 ± 6.1 (46/52) a	14.7 ± 4.0 (32) a
Debr + SB*	17.5 ± 3.8 (12/33) ab	18.1 ± 3.2 (10/31) bc	15.9 ± 2.5 (17/31) b	14.9 ± 3.5 (15) a
Debr + OB*	15.2 ± 2.3 (11/24) b	15.4 ± 1.4 (1/22) c	16.0 ± 2.6 (14/23) b	15.5 ± 3.4 (24) a
Decapitated	13.2 ± 1.9 (40/40) c	18.4 ± 2.5(26/26) b	14.0 ± 2.4 (35/35) b	16.9 ± 2.0 (24) a

* These insects received 1 day after decerebration implant of 2 brains taken from donors of the same age as the recipient. The donors and recipients were reared under identical (Debr + SB) or under opposite (Debr-OB) photoperiods (LD versus SD). Values marked with different letters within a column differ at 0.05% probability.

Table 2. Effects of decerebration, decerebration combined with brain implants*, and decapitation of the L6d1 and L6d5 SD and LD larvae, respectively, on the nature and timing (mean ± S.D. in days) of the next ecdysis. Molted/total numbers of insects are provided in parentheses.

3.3. Development of decapitated larvae

The occurrence of larval molts in debrained larvae indicated that their development was affected by JH secreted from the CA. The effect of

mentioned that most molted larvae were trapped in the old exuvia and suffocated. A few (insect numbers above the 100% line in Fig. 4) were able to escape from the exuvia and developed to pupae that were in some cases tiny but morphologically normal.

Most decapitated larvae produced the pupae directly (Fig. 4). They had to be extracted from the exuvia with the aid of forceps but afterwards they survived for a fortnight without any sign of adult development. This contrasted with the adult development of pupae that ecdysed from the

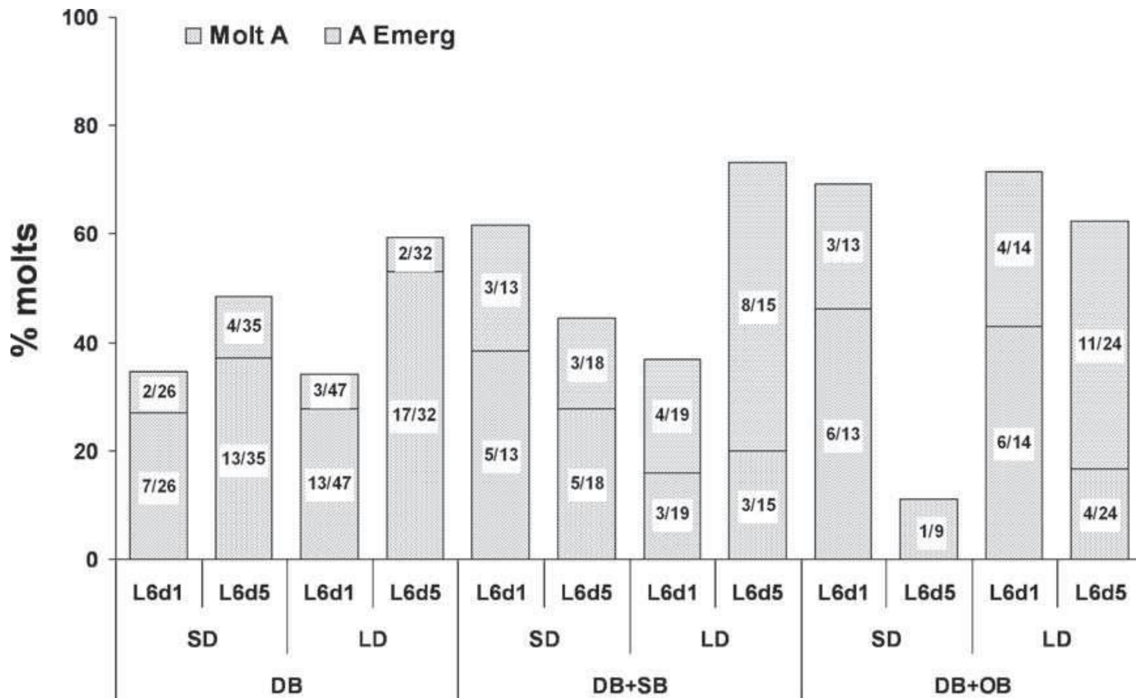

Fig. 3. Percent of debrained larvae that accomplished full metamorphosis; some adults were trapped in the pupal exuvia (Molt A), others successfully emerged to viable imagoes (A Emerg). Figures in the columns show the numbers of insects reaching the adult stage/initial numbers of operated larvae (= 100%).

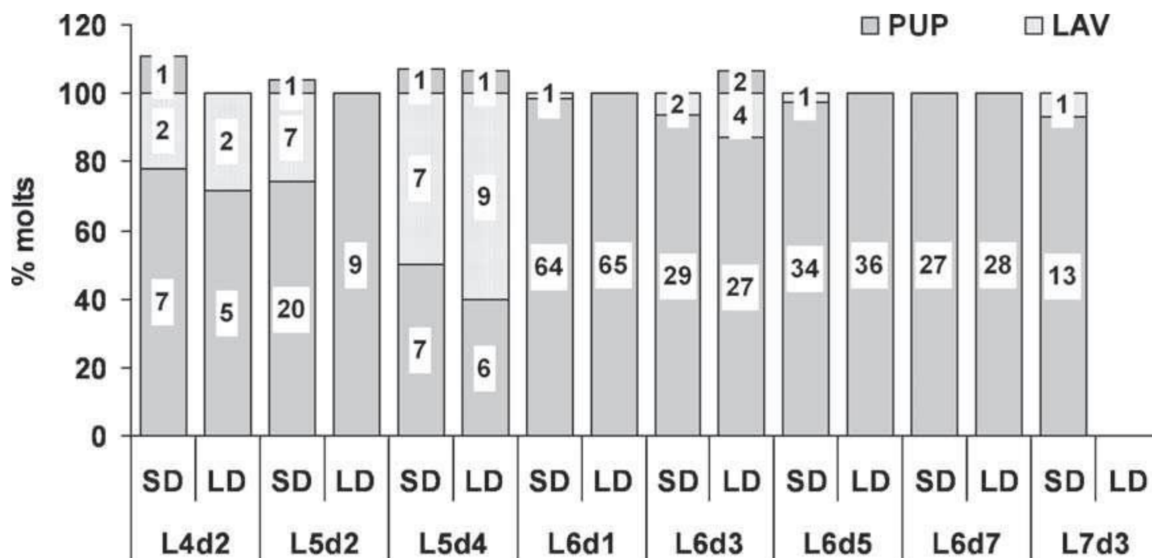

Fig. 4. Percent pupal (PUP) and larval (LAV) molts in the SD and LD larvae decapitated at indicated days of the 4th to the 7th instars (L4d2 to L7d3). Pupal molts (PUP2) above the 100% line represent successful second molt after decapitation (first was a larval molt). Figures in the columns show the numbers of molted insects (100% = total initial numbers).

debrained larvae (Fig. 3). Consistent absence of adult development in pupae obtained from the decapitated insects suggested that the pupal–adult transformation did not depend on the brain but on an unknown factor that was present in the decerebrate and not in the decapitated larvae.

after treatments that typically caused a pupal molt. The long interecdysial period resembled molt periodicity in diapause.

The length of time between decapitation and pupation depended on the day of decapitation

Time of decapitation	SD larvae		LD larvae	
	Larval molt	Pupal molt	Larval molt	Pupal molt
L4d2	5.0 ± 0.0 (2/9) b	10.4 ± 2.1 (7/9) c	5.5 ± 2.1 (2/7)	10.4 ± 1.7 (5/7) cd
L5d2	5.9 ± 1.9 (7/27) b	11.2 ± 2.2 (20/27) c	(0/9)	10.4 ± 1.2 (9/9) d
L5d4	5.6 ± 0.5 (7/14) b	12.4 ± 4.2 (7/14) bc	5.2 ± 0.6 (9/15)	14.3 ± 2.7 (6/15) ab
L6d1	21.0± 0.0 (1/25) a	10.0 ± 1.0 (24/25) c	(0/30)	10.9 ± 2.2 (30/30) d
L6d3	5.5 ± 2.1 (2/31) b	13.9 ± 3.2 (29/31) b	6.0 ± 0.0 (4/31)	13.8 ± 2.1 (27/31) b
L6d5	6.0 ± 0.0 (1/9) b	15.4 ± 1.7 (8/9) b	(0/12)	15.8 ± 2.1 (12/12) a
L6d7	(0/27)	18.0 ± 3.6 (27/27) aA	(0/28)	12.7 ± 3.7 (28/28) bc B
L7d3	21.0 ± 0.0 (1/14) a	14.8 ± 3.6 (13/14) b	No insects molt to the 7th instar	

Different low case letters in a column and upper case letters in a row indicate that the compared instar lengths were statistically different. LSD test for SD larval molts: $F = 39.4$, $P < 0.000$; SD pupal molts: $F = 7.6$, $P < 0.001$; LD larval molts: $F = 1.6$, $P > 0.05$; LD pupal molts: $F = 8.53$, $P < 0.001$).

Table 3. Instar length (mean ± S.D. in days) in the SD and LD larvae decapitated at indicated days of the 4th to 7th instar (L4d2 to L7d3). Molted/total numbers of insects are given in parentheses.

The larval molts of decapitated larvae were usually much faster than the pupal molts. The average time from decapitation to a larval ecdysis varied between 1 day in the L6d5 and 3.9 days in the L5d2 SD larvae. In the LD larvae, larval molts occurred in 1.2–3.5 days after decapitation. It should be emphasized that the total lengths of the larval–larval intermolt periods (Table 3) were in these cases similar to the length of the 5th instar of the intact larvae (4–5 days). By contrast, the interecdysial period was extended to 21 days in two SD larvae that underwent larval molts after decapitation at L6d1 and L7d3, respectively, i.e.

($F = 4.8$, $P = 0.0001$) but not on the photoperiod ($F = 0.9$, $P = 0.3451$). In both SD and LD insects that pupated after decapitation performed within the first two days of the 4th, 5th, and 6th instar, respectively, the length of the interecdysial period was close to 10 days, similar to the normal 6th instar of the LD insects (Table 1). The instar length was extended when decapitation was done on days 3–5 of the 6th instar of either SD or LD larvae. Significant effect of the photoperiod ($F = 7.95$, $P = 0.000$) was found only in the L6d7 larvae. All insects decapitated at this time pupated but the molt occurred after 18 days in the SD, and after

12.7 days in the LD insects (Table 3). We interpret such molt delays as indications that the developmental programming initiated by decapitation differed from the original one and the change required time.

The timing of molts induced by decapitation resembled the development of intact insects rather than the periodicity of molts in the debrained larvae (cf. Table 2 and Table 3). The length of time from decapitation to a larval molt was in most cases similar to the duration of the normal 5th instar, and the timing of pupation in the decapitated L4d2, L5d2, and L6d1 corresponded to the 6th instar length of the LD insects. A delay of larval molts resembling the molting pace of the diapausing larvae occurred after decapitation only exceptionally. Both the larval and pupal molts occurred in the decapitated larvae faster (Table 3) than in the debrained larvae (Table 2). Since the debrained, but not the decapitated larvae, contain JH (Perez et al., 2009), secretion of this hormone from the CA present in the debrained larvae might be responsible for the molt delay. This assumption was verified by treating decapitated larvae with the JH agonist methoprene.

3.4. Decapitated larvae treated with methoprene

L6d1 SD and LD larvae were decapitated and next day topically treated with methoprene. Controls receiving only solvent invariably pupated whereas some of the insects exposed to the methoprene underwent a larval molt (Fig. 5). Administration of 0.001 µg methoprene per specimen caused larval molting only exceptionally in one SD larva. Doses 0.01, 0.1, and 1 µg induced larval molt in 67%, 64%, and 75% of the SD, and in 50%, 59%, and 50% of the LD larvae, respectively. SD larvae were more prone to undergo larval molts than the LD larvae. The proportion of larval versus pupal molts was

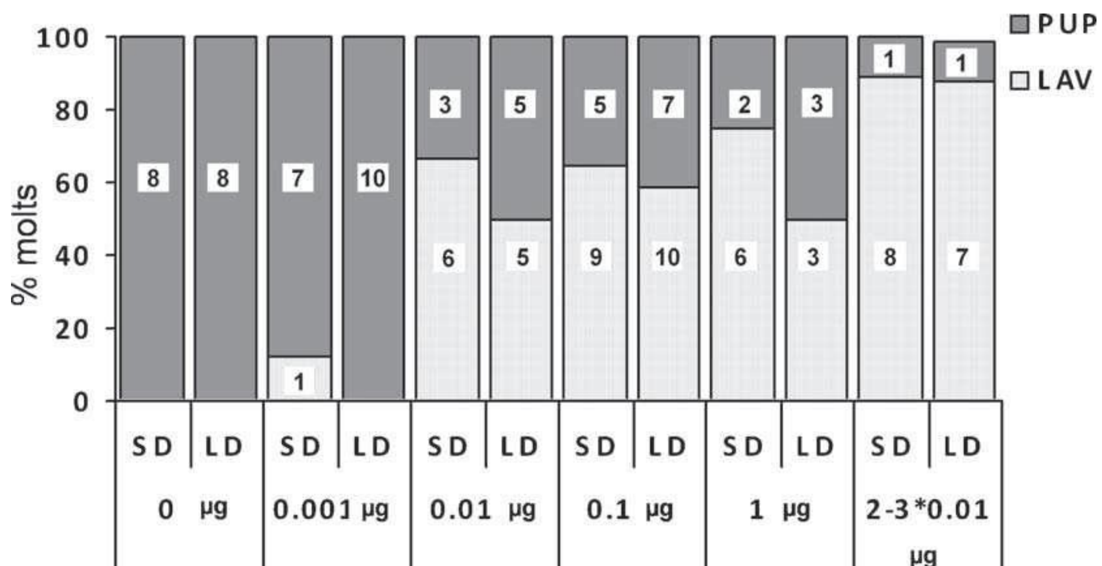

Fig. 5. Percent larval (LAV) and pupal (PUP) molts in the SD and LD larvae decapitated at L6d1 and treated 1 day later with indicated amounts of the juvenoid methoprene dissolved in 2 µl acetone (the controls received acetone alone). Last two columns represent larvae treated with 0.01 µg methoprene on days 1, 4, and 7 (insects molting on days 6 or 7 did not receive the last treatment). Only the first molt after treatment was recorded. Figures in the columns show the numbers individuals that molted to larvae and pupae, respectively (sum = 100%).

markedly increased in insects treated twice or thrice with 0.01 µg methoprene (Fig. 5), suggesting importance of methoprene persistence. The dose of 0.02 µg methoprene applied in two 0.01 µg portions was more effective than a single application of 1 µg. repeated 0.01 µg application in both SD ($t = 0.005$) and LD ($t = 0.000$) insects.

3.5. Molting of isolated larval abdomens

Isolated abdomens were prepared in six age groups (L5d1, L6d1, L6d3, L6d5, L6D7 and L6d9)

Methoprene dose	**SD larvae**		**LD larvae**	
	Larval molt	**Pupal molt**	**Larval molt**	**Pupal molt**
Intact larvae*	15.8 ± 3.6 (20) b	(0/20)	(0/20)	9.2 ± 0.9 (20/20) b
0 µg	(0/8)	9.4 ± 0.8 (8/8) b	(0/8)	9.4 ± 0.8 (8/8) b
0.001 µg	18.0 ± 0.0 (1/8) a	9.6 ± 0.0 (7/8) b	(0/10)	10.3 ± 1.6 (10/10) b
0.01 µg	14.5 ± 2.7 (6/9) a	15.7 ± 4.2 (3/9) a	8.2 ± 5.6 (5/10)	13.6 ± 4.7 (5/10) a
0.1 µg	14.0 ± 3.9 (9/14) a	10.1 ± 0.0 (5/14) b	16.7 ± 2.8 (10/17)	10.6 ± 1.3 (7/17) b
1 µg	13.3 ± 2.2 (6/8) ab	11.0 ± 0.0 (2/8) b	18.7 ± 4.2 (3/6)	10.0 ± 2.1 (3/6) b
2–3 × 0.01 µg **	10.4 ± 2.5 (8/9) b	8.0 ± 0.0 (1/9) b	10.9 ± 1.9 (7/8)	9.0 ± 0.0 (1/8) b

Values marked with different letters within a column are different at 0.05% probability.
* Data taken from Eizaguirre et al. (2007).
** Treatments were done every 3rd day; insects ecdysing before day 9 were treated only twice.

Table 4. Type of molt and the instar length (mean ± S.D.) in the SD and LD larvae decapitated at L6d1 and treated with methoprene in 2 µl acetone on the next day (the controls received acetone alone). Molted/total numbers of insects are given in parentheses.

Larval molts induced in the decapitated larvae with methoprene were in most cases slower than the pupal molts (Table 4). The timing of larval molts (the single larval molt induced with the 0.001 µg dose was not considered) depended on the dose ($F = 6.3$, $P = 0.001$) but not on the photoperiod ($F = 0.26$, $P = 0.609$). The interaction dose × photoperiod was significant for the SD larvae ($F = 9.76$, $P = 0.0003$). A clear difference between the SD and LD insects was found when the data on the larval molt timing in the 0.1 and 1 µg methoprene treatments were combined; the length of time to the larval molt in these two treatments was significantly longer than after the of the SD and LD larvae. Each group included atleast 10 abdomens. All abdomens survived for 10–15 days but none from the L5d1, L6d1, L6d3, and L6d5 larvae showed signs of molting. The photoperiod was irrelevant. A considerable portion of abdomens of the L6d7 and L6d9 larvae underwent either a larval or a pupal molt (Fig. 6), suggesting that enough ecdysteroids were produced prior to abdomen isolation. The frequency of molting was similar in the SD and LD insects but the former molted in 2–8 days exclusively to the larval, and the latter in 1–7 days exclusively to the pupal abdomens. These results showed that a few larvae could molt without the

head and prothorax since day 7, and more than half of them since day 9 of the 6th instar.

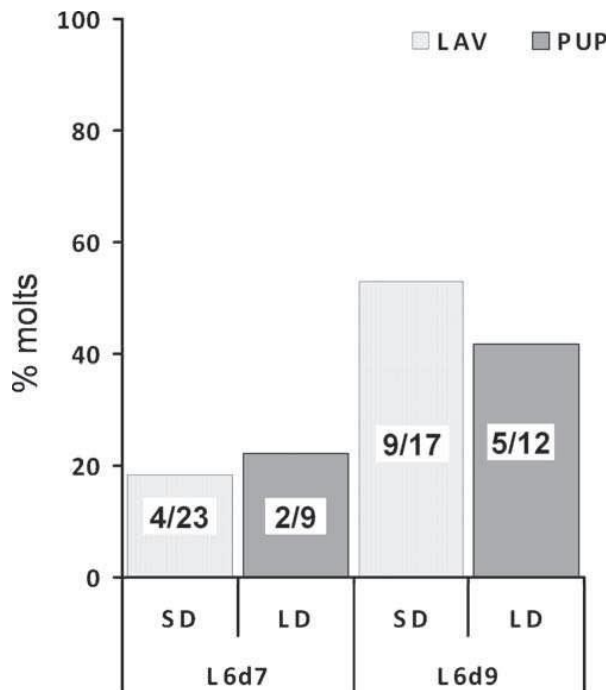

Fig. 6. Percent larval (LAV) and pupal (PUP) molts in abdomens isolated from the SD and LD larvae on days 7 or 9 of the 6th instar. The remainder to 100% represents abdomens that died between 15 and 25 days after isolation without any sign of molting. Figures in the columns show the numbers of molted/total (= 100%) individuals.

4. Discussion

4.1. Development programming in S. nonagrioides

S. nonagrioides larvae can undergo larval development, pupation, or diapause. The rate of larval development, which proceeds until the 6th instar (in 20% of insects only until the 5th instar) and is characterized by larval molts in short intervals (4–5 days in the 5th instar), is independent of the photoperiod that determines development programming in the 6th and later instars. While the larvae developing under LD conditions pupate in the 6th instar, those kept continuously under the SD conditions enter diapause characterized by larval molts in longer intervals (8–20 days in the set of insects shown in Table 1). The programming for pupation is not altered by transferring 6th instar larvae from the LD to the SD conditions, but diapause programming is changed to pupation by moving SD larvae to the LD conditions (Fig. 1). This switch of programming needs time (Table 1) and is often completed only in the 7th or 8th instar (Fig. 1). Surgeries performed in this study were done in attempt to detect roles of the cephalic regulatory centers in development programming. We examined conditions that elicit a switch from the original molt programming to the other molt type. Since the type of development depends on the titers of ecdysteroids and JH, the nature and timing of molts occurring after the surgical treatments indicate which cephalic centers are involved in the control of CA and PG.

4.2. S. nonagrioides can develop without brain

In the Lepidoptera studied so far, presence of brain for a certain period of time in each larval and usually also in the pupal instar was found indispensable for the molt induction. Brain is required as a source of the prothoracicotropic hormone (PTTH) that stimulates ecdysteroid secretion from the PG (Gilbert et al., 2002). The “brain critical period”, i.e. the length of time for which brain must be present, often occupies most of the last larval instar and its termination coincides with increased level of ecdysteroid secretion. PTTH release is a common mechanism for the termination of pupal diapause (Denlinger et al., 2005). Molt dependence on the PG presence (“PG critical period”) extends beyond the brain

critical period and is lost during the molt-inducing surge of ecdysteroid secretion.

S. nonagrioides is to our knowledge the first lepidopteran found to develop from larva to adult after brain removal (Fig. 2 and Fig. 3). In the 6th instar larvae, PG presence is required until apolysis which begins in L6d7; the L6d9 abdomens (stage of advanced apolysis characterized by accumulation of the molting fluid) accomplish either a larval (in the SD larvae) or a pupal (in the LD larvae) molt in about 50% cases (Fig. 6). These data show that the molt-inducing surge of ecdysteroids depends in *S. nonagrioides* on the PG presence, similarly to other caterpillars, but PG can function without PTTH from the brain. PG activation without brain observed in other lepidopterans was usually slow and supported just one molt. For example, the PG of decapitated *G. mellonella* larvae secreted a molt-inducing surge of ecdysteroids after 30–40 days but after brain implantation in 10–12 days (Sehnal et al., 1981). Similar slow PG activation in the brain absence was reported for several other lepidopterans such as *H. cecropia* pupae (McDaniel, 1979). Existence of autocrine PG stimulation was proven in explanted larval PG of *Bombyx mori* (Gu, 2007).

The debrained larvae of *S. nonagrioides* often undergo several successive molts (Fig. 2 and Fig. 3). The first of them is either larval or pupal, depending on the photoperiodic experience of the larva and its age at the time of decerebration. Brain implants slightly accelerate the pupal molts but do not alter the timing of larval molts (Table 2). We do not know if PG activation in the brain absence is autonomous or is controlled by other hormones than the brain-derived PTTH. PG stimulation by the suboesophageal and inhibition by the thoracic ganglia was inferred from the surgical experiments in *G. mellonella* larvae (Malá et al., 1977) and recently proved by the isolation of regulatory substances in *B. mori*. Stimulatory activity was demonstrated in the diapause hormone and related peptides that are secreted from the suboesophageal ganglion (Watanabe et al., 2007) and inhibitory activity was found in several neuropeptides which are delivered to the PG by axonal transport from the thoracic ganglia (Yamanaka et al., 2006). Two inhibitory factors, which were found in the brain (Hua et al., 1999 and Yamanaka et al., 2005), belong to peptide families that may occur in different parts of the nervous system.

There are indications that both inhibitory and stimulatory factors occur in the head of *S. nonagrioides* outside the brain. The delayed molts of debrained larvae by comparison with the decapitated larvae (cf. Table 3 and Table 2) indicate that decapitation, but not decerebration, removes a molt-inhibiting influence. On the other hand, decapitation deprives insects of a stimulant that drives the pupal–adult molt. The debrained larvae contain this hypothetical regulator and are therefore able to accomplish one or more larval molts followed by complete metamorphosis. The decapitated larvae readily pupate, sometimes after an induced larval molt, but their development does not continue beyond the pupal stage.

4.3. Regulation of JH production

JH titer in the hemolymph drops to undetectable level in the pupating 6th instar LD larvae but it is

maintained at about 20 pmol in the diapausing SD larvae that undergo larval molts (Eizaguirre et al., 2005). The JH decline in LD larvae is prevented by decerebration at L6d1 that causes some insects to switch from pupation to a larval molt (Fig. 2). These L6d1 LD larvae contain in average 11 pmol JH II (the major JH type in this species) on day 5, and 6 pmol on day 10 after the brain removal (Perez et al., 2009). Similar values were measured in the SD larvae debrained at L6d1 or L6d5; JH II titer was slightly enhanced in insects that received implants of 2 brains. By contrast, in the debrained L6d5 LD larvae, which always pupate (Fig. 2), the JH II titer dropped from 2 pmol on day 5 to undetectable level on day 10 and this decline was not affected by the brain implants. No JH was detected in the decapitated larvae, consistently with the observation that the decapitated L6d1 and L6d5 larvae from either SD or LD conditions molt nearly exclusively to pupae (Fig. 4).

The JH measurements and the development of debrained and decapitated larvae confirm that JH secretion from CA, which are absent in the decapitated larvae, is indispensable for the larval development. The enhancement of JH titer in the debrained 6th instar larvae suggests that the brain in situ restrains CA function. The incidence of larval molts in debrained larvae indicates that the inhibition is mild in the SD and stronger in the LD insects. Prior to their full inhibition, CA respond to the brain implants by increased JH secretion that leads to a higher incidence of larval molts. The action of implanted brains must be humoral because they have no nervous connection to the CA. The brains of LD larvae, which exert inhibitory influence in situ (CA of the L6d5 LD larvae seem to be fully inhibited), stimulate CA when implanted into the debrained SD larvae or debrained L6d1 LD larvae. A plausible explanation of these opposite effects is that the inhibition is accomplished via nerves (and therefore executed only when the brain remains in situ), whereas stimulation is humoral. Both stimulatory and inhibitory peptides have been detected in lepidopteran brain and some of them also in the CC–CA complex and the stomatogastric ganglia (Audsley et al., 2008). Our results indicate existence of both allatostatic and allatotropic brain factors in *S. nonagrioides* but the development of debrained larvae through perfect larval, pupal, and imaginal molts demonstrates that crucial CA regulation may occur without the brain.

4.4. The puzzle of larval molts in corpora allata absence

Decapitation depriving insects of the whole complex brain–CC–CA elicits fast larval molts when performed in the 4th or 5th larval instar (Fig. 4), i.e. at times of high JH titer (Eizaguirre et al., 2005). One can assume that these insects were committed to the larval molts at the time of decapitation. However, this explanation hardly applies to larvae that were decapitated within the first 3 days of the 6th or 7th instar and also occasionally undergo larval molt. The LD L6d3 larvae contain a low JH titer (Eizaguirre et al., 2005)-how it can rise to a level supporting larval development in CA absence? Furthermore, one larva decapitated at L6d1 and another one decapitated at L7d3 underwent larval molts after more than 20 days, suggesting a very long JH

persistence. Unusual JH sustainability is also indicated by the larval molts of isolated abdomens (Fig. 6). We propose that maintenance of a JH titer promoting larval development is due to a low rate of JH degradation by JH esterase that was demonstrated for *S. nonagrioides* (Schafellner et al., 2008). We also do not exclude that either JH or JH acid is sequestered and stored somewhere in the body for a later use. JH storage based on JH acid esterification was discovered in the sex accessory glands of *H. cecropia* males (Dahm et al., 1976). Recent analysis of JH titer regulation concluded that the role of JH sequestration by binding proteins is greatly underestimated (Nijhout and Reed, 2008).

There is a discrepancy between larval development of the isolated abdomens of SD larvae and the pupal development of most decapitated SD larvae. We assume that ecdysteroid titre, which is higher before pupation than before larval molt (Eizaguirre et al., 2007), plays here a role. The larval commitment of SD larvae is reverted to pupal one in the decapitated larvae that possess PG and can produce more ecdysteroids, whereas isolated abdomens without PG cannot. In addition, JH can persist for a few days during which molt occurs in the isolated abdomens but not for 18 days needed for molting in the decapitated SD larvae.

Acknowledgments

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CHAPTER 2

Role of the brain in larval development of *S. nonagrioides*:

2.2 Control of larval-pupal-adult molt in the moth *Sesamia nonagrioides* by juvenile hormone and ecdysteroids

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Control of larval-pupal-adult molt in the moth *Sesamia nonagrioides* by juvenile hormone and ecdysteroids

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Abstract

Sesamia nonagrioides (Lepidoptera: Noctuidae) larvae reared under long day (LD; 16L:8D) conditions pupate after 5 or 6 larval instars, whereas under short day (SD; 12L:12D) conditions undergo up to 12 molts before pupating. This extended period of repeated molting is maintained by high levels of juvenile hormone (JH). Previous work demonstrated that both LD and SD larvae decapitated in the 6th instar pupate but their further development is halted. By contrast, about one third of SD larvae from which only the brain has been removed, undergo first a larval molt, then pupate and subsequently developed to the adult stage. Debrained LD larvae molt to larvae exceptionally but regularly pupate and produce adults. Results of the present work demonstrate that the prothoracic glands (PGs) and the corpora allata (CA) of debrained larvae continue to produce ecdysteroids and JHs, respectively. JH absence in the decapitated larvae is consistent with the notion that CA are the only source of this hormone. Completion of the larval-pupal-adult development in debrained insects demonstrates that brain is not essential regulator of *S. nonagrioides* development. Implanted brains, however, may induce several larval molts in debrained recipient larvae irrespectively of the photoperiodic conditions. Brain might be responsible for maintaining the diapauses stage by neural inhibition of pupation. JH application to headless pupae increased the number of insects undergoing adult differentiation. In a similar way, JH is also probably important for the adult differentiation in the brainless pupae. Application of ecdysteroid agonist RH 2485 to headless pupae does not seem to elicit ecdysteroid secretion according to a pattern needed for normal adult development.

Key words: *Sesamia nonagrioides*; Juvenile hormone; Ecdysteroids; Brain; Insect development.

1. Introduction

The Mediterranean corn borer, *Sesamia nonagrioides*, is one of the most important corn pests in the Mediterranean Basin. Under long day (LD) conditions it displays a typical lepidopteran life cycle with 5 to 6 larval instars followed by the pupal stage. Under short day (SD) conditions it overwinters as diapausing larva continuing to feed, move and molt (Fantinou et al., 1995). Short photoperiod is the primary diapause-inducing

factor but its effect is modified by temperature and phenology of the host plant (Eizaguirre and Albajes, 1992; Eizaguirre et al., 1994). Pupation of the LD larvae after the fifth (L5) or sixth (L6) larval instar is associated with a decline of the juvenile hormone (JH) titer, while the diapausing SD larvae maintain an elevated JH level that obviously prevents pupation in the fully developed larvae of the sixths and later (supernumerary) larval instars (Eizaguirre et al., 2005). SD larvae contain in their hemolymph a surprisingly high activity of JH esterase (JHE) (Schafellner et al., 2008) that in other Lepidoptera causes a crucial decline of JH titer (Hammock, 1985; Roe and Venkatesch, 1990) followed by a pupation-inducing surge of ecdysteroids (Wolfgang and Riddiford, 1986). In SD *S. nonagrioides,* an increase in ecdysteroid levels in the L6 SD larvae leads to a supernumerary larval molt (Eizaguirre et al., 2007) in spite of high JHE level.

Numerous studies demonstrated that insect development is governed by the brain and that removal of the larval brain before a critical period prevents molting (Sakurai, 1983; Bollenbacher et al., 1987; Nijhout, 1994). Nevertheless, *S. nonagrioides* larvae pupate when deprived of the brain-corpora cardiac-corpora allata complex (Br-CC-CA) by decapitation (Pérez-Hedo et al., 2010a). Debrained larvae, which retain the retrocerebral endocrine glands CC-CA, undergo one to several larval molts that are followed by a pupal and then by an imaginal molt. Since brainless pupae obtained from the debrained larvae can develop into adults whereas headless pupae derived from the decapitated larvae stop their development after the pupal ecdysis, an unknown head factor from a source other than brain seems to be needed for the initiation of pupal-adult metamorphosis. Using surgical manipulations, we have probed the role of brain in the control of larval CA and prothoracic glands (PGs) under the SD and LD photoperiods and examined involvement of these hormones in the stimulation of pupal-adult development.

2. Material and methods

2.1 Insects and their rearing

Larvae of the Mediterranean corn borer, *Sesamia nonagrioides* (Lepidoptera, Noctuidae), collected in the fields of central Catalonia were used to establish a laboratory culture of this species. Insects were reared on a semi-synthetic diet at 25ºC and high humidity (>60%) (Eizaguirre and Albajes, 1992) and maintained under the LD (16:8 h light:dark) or SD (12:12 h light:dark) photoperiodic conditions. All experiments were performed using L6 larvae.

2.2 Surgical procedures

Before surgical manipulations the larvae were anaesthetized by submersion in water for 2-3 hours. Larvae of the first or fifth day after ecdysis to L6 (L6d1 and L6d5, respectively) were decapitated (DC) or debrained (DB) as previously described (Pérez-Hedo et al., 2010a). DB larvae were deprived of the brain only, whereas DC larvae lacked also the retrocerebral glands CC-CA, the suboesophageal ganglion, part of the stomatogastric nervous system, and other head tissues. Both the DC and DB larvae retained the

PGs. Some larvae received implants of two brains from donors of the same age and reared under the same (DB+SB) or under the opposite photoperiod (DB+OB) (Pérez-Hedo et al., 2010a).

2.3 Hemolymph collection

Five or ten days after a surgery, hemolymph was withdrawn from the larvae by clipping off a proleg with microscissors and gently expressing hemolymph into a graduated glass micropipette. At least 25 µl were collected; 20 µl for the JH and 5 µl for the ecdysteroids analyses. A minimum of 6 insects were used for each of the 32 treatments analyzed (2 photoperiodic rearing conditions, 4 surgical operations done, 2 ages for each surgical operations and 2 days of sample extractions).

2.4 JHII quantification

The JH II content was determined according to Rembold and Lackner (1985) using GC-MS with SIM, with several modifications (Eizaguirre et al., 2005). All organic solvents were purchased from Merck (analytical grade or nanograde purity) and the JH II standard was obtained from SciTech (Prague, Czech Republic).

2.5 20-hydroxyecdysone quantification

Ecdysteroids were quantified by a competitive enzyme linked immunosorbent assay (ELISA) based on the protocol of Kingan (1989). Each sample of 5 µl hemolymph was vortexed with 495 µl 80% methanol:water (v/v) and the mixture was stored at -20ºC. To extract ecdysteroids, the mixture was centrifuged (12,000 rpm for 10 min), the supernatant collected and the precipitate re-extracted with 200 µl 80% methanol. The supernatants were pooled and evaporated in a vacuum centrifuge. The residue was dissolved in 500 µl 80% methanol. EIA plates (Easy Wash, Costar®3369) were incubated overnight at room temperature with 90 µl PBS (10 mM Na_2HPO4, 0.15 M NaCl) containing 5 µg of goat anti-rabbit IgG (Fc fragment specific; Jackson Immuno Research). The wells were emptied, filled with 300 µl AB solution (25 mM Na_2HPO_4, 0.15 M NaCl, 1mM Na_2EDTA, 0.1% BSA, 0.002% NaN_3) for 1 h, and washed 3 times with PBS containing 0.05% Tween-20. The assay was performed by adding dilutions of the samples or standards (1.25 fmol to 5000 fmol 20E, Sigma Chemical Co. MO, USA) in 50 µl PBS per well. Subsequently, 50 µl AB solution with anti-ecdysone antibody diluted to 1/100,000 and 50 µl AB with a 20E-Horse Radish Peroxidase (HRP) conjugate diluted to 1/40,000 were added to each well. The 20E-HRP was purchased from Dr. Kingan (University of California, Riverside). Solutions were discarded from the wells after an overnight incubation at 4ºC and replaced with 100 µl tetramethylbenzidine (Sigma). Plates were then incubated for 15 min in the dark with gentle shaking. The color reaction was stopped by adding 1M H_3PO4 (100 µl/well). Absorbance was read at 450 nm with a spectrophotometer. Ecdysteroid content was expressed in equivalents of 20-hydroxyecdysone (20E), which is the major ecdysteroid in the hemolymph of *S. nonagrioides*.

2.6 Administration of JH II, methoprene or the ecdysteroid agonist (RH-2485)

JH II was obtained from SciTech® (Prague, Czech Republic) and the JH agonist methoprene was purchased from Sigma®. The non-steroidal ecdysteroid agonist, RH-2485 (95% purity), was obtained from Rohm and Haas Co. (Spring House, PA, USA). All hormonal agonists were applied in 1 μl acetone to the decapitated LD L6d5 insects on day 2 or 4 after their ecdysis to pupae. These headless pupae each received 0.1 μg JH II or methoprene and 0.05 or 5 μg RH-2485, respectively. At least 20 headless pupae were taken for each treatment. Acetone alone served as a control treatment. Treated pupae were observed daily to detect molting and the progress of pupal-adult transformation. Individuals were classified 15 days after the treatment as follows: a) no sign of molt (NM), b) secretion of indifferent cuticle without clear pupal or adult features (IC), c) secretion of pupal-like cuticle (PC), and d) secretion of a cuticle with scales (CS). The percentage of each type was statistically analyzed.

2.7 Statistical analysis

A four-way ANOVA (type or manipulation, photoperiod under which the larvae developed, day of manipulation, and day of the hemolymph sampling) was used to compare the JH II and ecdysteroid titers (SAS package, 2001). When significant differences between treatments were detected, Duncan's test was applied to compare the means. The action of hormone agonists on headless pupae was evaluated in respect to the percentage of molting insects and the type of secreted cuticle. Data were analyzed by the chi-square test. Level of significance was set at $\alpha \leq 0.05$.

3. Results

3.1 JH II concentration in larvae deprived of the brain

JH II titer depended on four factors: the type or surgical manipulation (DC, DB, DB+SB, DB+OB), the photoperiod under which the larvae developed (LD or SD), the day of manipulation (L6d1 or L6d5), and the day of hemolymph collection (5 or 10 days after surgery). Since the effects of all four factors on the JH II titer were significant without mutual interactions among them, Fig. 1 shows the effect of each factor separately. The columns in Fig. 1 present means of all JH titer measurements done in insects examined in respect to the factor in question. The hemolymph JH II titers were extremely different between the DC and DB larvae and data on all four surgical manipulations also revealed significant differences ($F = 10.04$, $P < 0.0001$, d.f. = 3; 168). Five or ten days after the surgery, DC larvae (lacking CC-CA) displayed no detectable JH II in the hemolymph. By contrast, DB larvae with or without the implant of exogenous brain contained JH II in the hemolymph, indicating that their CA were active five and ten days after the brain removal (Fig. 1A). Cumulative JH II titer data showed significant effects of the photoperiod, age of larvae, and time of sample collection after surgery. Larvae reared under the LD photoperiod showed in average a lower JH II titers than those reared under the SD photoperiod ($F = 10.04$, $P = 0.0016$, d.f. = 1; 168) (Fig. 1B). Also, larvae analyzed on L6d1 showed a higher level of JH II

than those of L6d5 (F = 11.26, P = 0.0010, d.f. = 1; 168) (Fig. 1C). Finally, JH II was still detectable in the hemolymph ten days after decerebration but at a significantly lower level than five days after the surgery (F = 10.83, P = 0.0012, d.f.= 1; 168) (Fig. 1D).

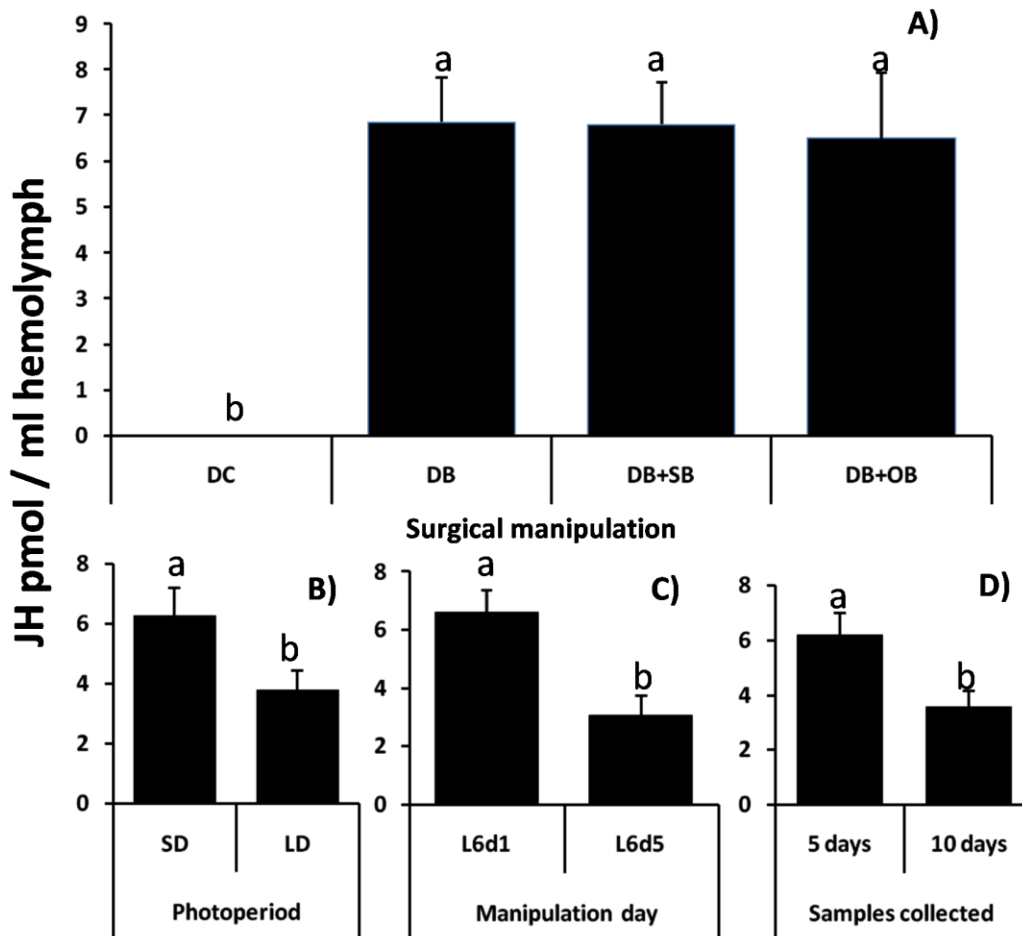

Fig. 1. Mean JH II titers in larvae subjected to diverse treatments that included the following factors: A) Surgical manipulation: decapitated (DC), debrained (DB), debrained and implanted with 2 brains from larvae of the same age and the same (DB+SB) or the opposite (DB+OB) photoperiodic experience. B) Photoperiodic rearing conditions: long (LD) or short (SD) day. C) Manipulation day: L6d1 and L6d5. D) Day of sample collection: 5 or 10 days after manipulation. Different letters above the columns indicate significant differences ($P \leq 0.05$) in JH titer in respect to indicated alternatives of analyzed factors.

3.2 Ecdysteroids concentration in debrained larvae

Ecdysteroid analyses were done in the same hemolymph samples as the JH measurements. The results revealed no significance differences in the ecdysteroid content due to photoperiod (F = 0.68, P = 0.4115, d.f.= 1; 173) or the age of larvae (L6d1 or L6d5) at the time of decapitation or brain removal (F = 0.77, P = 0.3808, d.f.= 1; 173). The influence of the type of surgical manipulation and of the day of sample collection on the ecdysteroids concentration is shown in Fig. 2 - four-way statistical analysis revealed interaction between these two factors (F = 10.76, P < 0.0001, d.f.= 3; 173). Decapitated larvae exhibited a significant ecdysteroid titer increase between days 5 and 10. By contrast, no significant difference in

ecdysteroid titer was found between days 5 and 10 in the debrained larvae, including those that received brain implants.

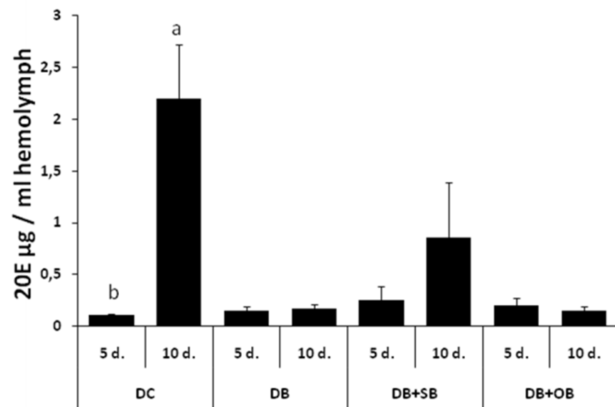

Fig. 2. Ecdysteroid (20E) concentration in the hemolymph of L6d1+L6d5 larvae decapitated (DC), debrained (DB), debrained and implanted with 2 brains from larvae of the same age and either the same (DB+SB) or the opposite (DB+OB) photoperiod. Different letters above the columns indicate significant differences in ecdysteroid concentration between samples collected 5 or 10 days after the surgery.

3.3 Ecdysteroid and JH II levels in debrained larvae

Fig. 3 shows variations in JH II and ecdysteroids after various surgical manipulations. No JH II was detected 5 or 10 days after decapitation, while all debrained larvae examined 5 days after the brain removal contained this hormone. The results demonstrate that CA are active in the debrained larvae but that their activity declines between days 5 and 10 post-surgery. JH II absence in the decapitated larvae indicates that there is no alternative source of JH. SD larvae contained in average more JH II than the LD larvae; no JH at all was detected 10 days after the brain removal from the L6d5 LD larvae. The levels of ecdysteroids measured 5 days after any treatment were very low, except for the LD L6d5 debrained larvae. In the decapitated larvae, ecdysteroids were low 5 days and high 10 days after the surgery, indicating an increase in PG activity between these two days. Interestingly, ecdysteroid titer remained low in the debrained larvae – the apparent increase observed after some brain implantations (Fig. 3B) proved insignificant. As noted previously, implanted brains had no influence on the level of either JH or ecdysteroids.

3.4 Molt induction in headless pupae by JH II or methoprene

No signs of adult development were noted in headless pupae that were obtained from the decapitated L6 larvae. However, topical application of acetone induced some of the pupae to undergo apolysis followed by secretion of a new cuticle. The old pupal cuticle was digested to different degree and could be removed. The new cuticle often bore some scales which are a typical adult feature. Fig. 4 shows that the rate of molting was much higher ($P \leq 0.001$) when the headless pupae were treated on day 2 after pupation with 0.1 µg JH II or methoprene. More than 90% of molted insects had some scales except for those molting after the JH treatment in which scales occurred in less than 80% cases. JH and methoprene similarly promoted molting of headless pupae when applied 4 days after their pupation ($P \leq 0.001$). The percentage of pupae that died without any sign of molt after methoprene application was similar to the control group ($P = 0.064$) but different in the group treated with JH II ($P \leq 0.001$). The incidence of molt induction after treatments on days 2 and 4 after the pupal ecdysis was similar with acetone ($P = 0.127$) as well as with JH II or methoprene ($P = 0.214$).

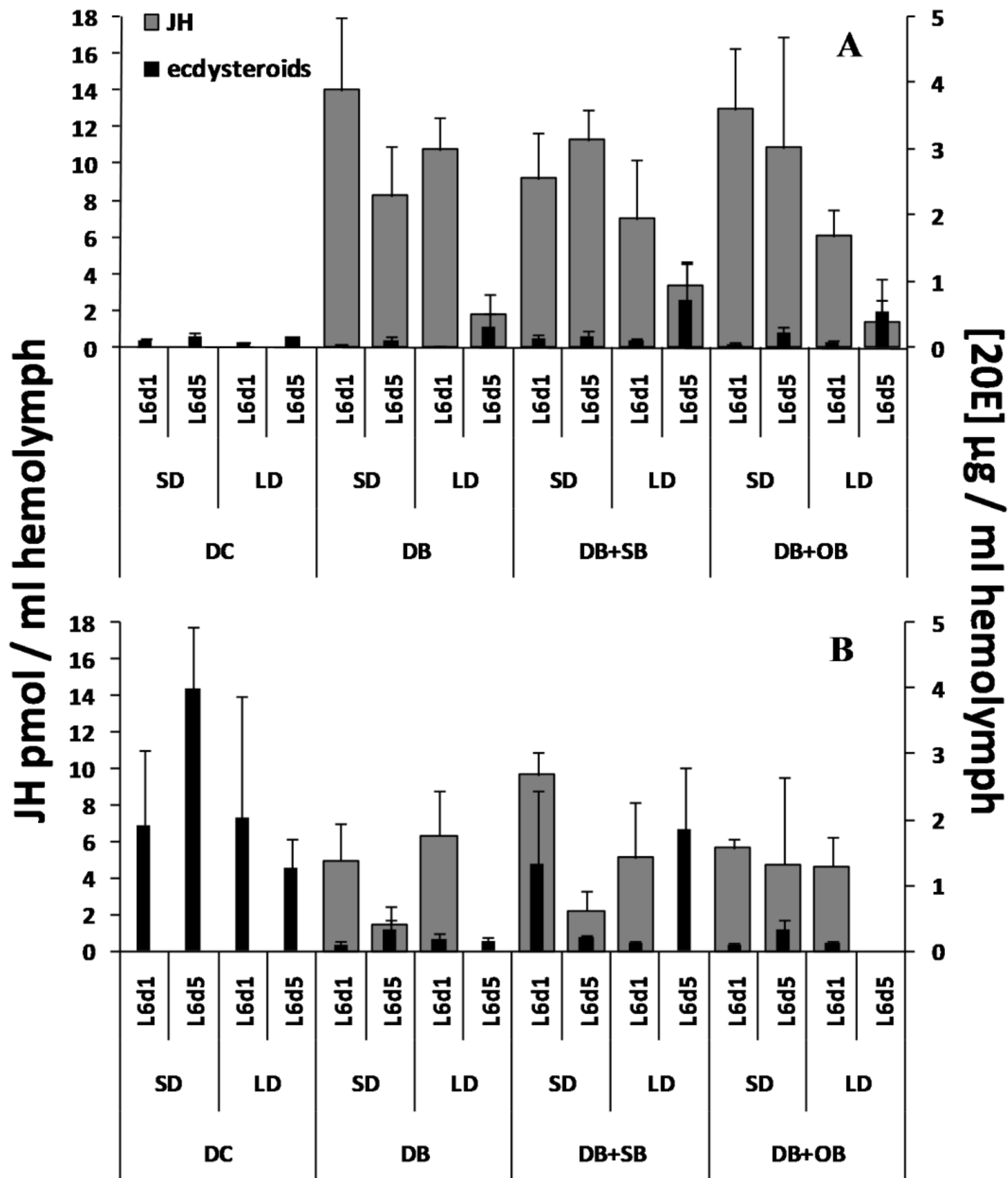

Fig. 3. Relationship between the JH and ecdysteroid titers in larvae reared under LD and SD photoperiods, subjected to a surgery at L6d1 or L6d5, and analyzed for the hormone titers 5 days (A) or 10 days later (B). Surgeries: decapitated larvae (DC), debrained larvae (DB), debrained larva implanted with 2 brains from larvae of the same age and photoperiod exposure (DB+SB), debrained larva implanted with 2 brains from larvae of the same age and opposite photoperiod exposure (DB+OB).

3.5 Influence of the ecdysteroid agonist on pupal-adult metamorphosis

The incidence of molting in headless pupae treated with 0.05 or 5 µg RH-2485 was significantly higher than in the controls treated with acetone ($P = 0.030$ and $P = 0.002$, respectively). Three types of newly secreted cuticle were distinguished: IC, indifferent cuticle (possibly procuticle) without clear pupal or adult features; PC, brownish and corrugated pupal-like cuticle; CS, cuticle with scales that are typical for adult moths. The dose of

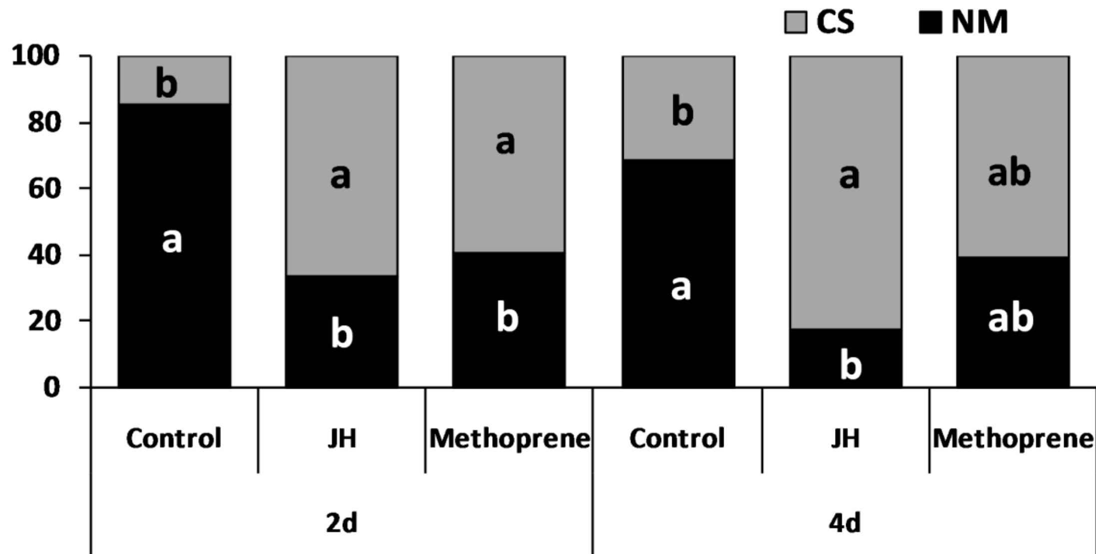

Fig. 4. Effects of JH II, methoprene and acetone (control) treatments on molt induction in headless pupae. Treatments were done 2 or 4 days after pupation and the percentages of non-molting (NM) and molting insects (CS) were recorded. Molting was mostly associated with adult differentiation manifested by the presence of scales. Different letters in the columns indicate statistical differences in the effect of the treatments (P ≤ 0.05).

0.05 µg RH-2485 applied 2 days after the pupal ecdysis increased the percentage of insects secreting either indifferent cuticle or the cuticle with scales, whereas high dose of 5 µg RH-2485 mostly caused secretion of an indifferent cuticle and in a few cases of a cuticle with pupal features (Fig. 5). The secretion of pupal-like cuticle was also induced in about 15% of insects treated with

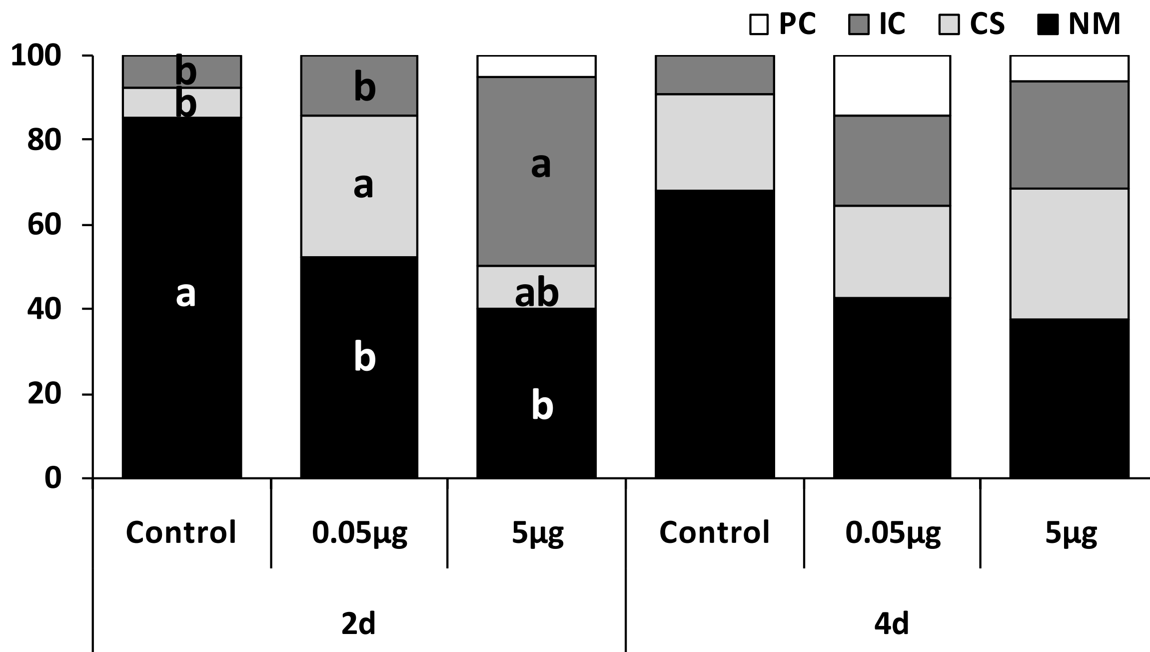

Fig. 5. Effect of RH-2485 on the decapitated insects treated 2 or 4 days after pupation. Percentages of individuals displaying no molting (NM), secretion of indifferent cuticle (IC), secretion of cuticle with scales (CS), and secretion of a second pupal cuticle (PC). Different letters in the columns indicate statistical differences in the effect of the treatments (P ≤ 0.05). At least 20 headless pupae were used for each treatment

0.05 µg RH-2485 4 days after the pupal ecdysis. The rate of scale occurrence after application of 0.05 µg RH-2485 on day 2 was higher than in the controls (P = 0.022) but similar after the treatment with 5 µg (P = 0.752). Application of either dose on day 4 elicited secretion of a cuticle with scales in similar percentage of insects as did acetone application. On the other hand, secretion of a pupal-like cuticle occurred only after the treatment with RH-2485. Two doses of this agent applied on day 4 exhibited no difference in ecdysis initiation (P = 0.602), secretion of a cuticle with scales (P = 0.568) or with pupal texture (P = 0.155).

Discussion

S. nonagrioides larvae that develop under LD photoperiodic conditions pupate after 5 or 6 instars while those that develop under SD conditions enter diapause and undergo several extra larval molts (Eizaguirre et al., 1994). The transformation of LD larvae to pupa is initiated by a burst of ecdysteroids when JH titer drops to undetectable level. Diapausing larvae display a significantly higher JH titer and low, irregular fluctuations in ecdysteroids (Eizaguirre et al., 2005, Eizaguirre et al., 2007). Pérez-Hedo et al., (2010a) demonstrated that decapitation of larvae of different age elicited pupation while debrained larvae that maintained their CA, usually underwent one to several larval molts before pupating.

Our present results demonstrate that JH becomes undetectable in both LD and SD in 5 days after their decapitation. By contrast, decapitated larvae contained ecdysteroids and their level increased between days 5 and 10 after decapitation. This increase in ecdysteroids could be related to the peak that elicits larval-pupal apolysis in the last larval instar of Lepidoptera (Sehnal, 1989). In our previous work (Pérez-Hedo et al., 2010a), debrained larvae ecdysed to pupae slightly later than the decapitated ones, suggesting that the increase of ecdysteroids occurred later. We speculate that the activity of PG was accelerated in the decapitated larvae in response to the lack of JH. The maintenance of sufficient JH in the hemolymph of debrained larvae could suppress PG activity (Safranek et al., 1980). The major PG stimulant in Lepidoptera, the brain-derived prothoracicotrophic hormone (PTTH), cannot be involved in either debrained or decapitated larvae. We showed that PTTH can probably be derived also from the midgut (Pérez-Hedo et al., 2010b) but there are no data on possible effects of decapitation and decerebration on this PTTH source or on other possible mechanisms of PG activation (Marchal et al., 2010).

In our previous work we demonstrated that brain implants initiated molting in debrained hosts (Pérez-Hedo et al., 2010a), yet, brain implants caused no appreciable change in JH titers (Fig. 3) thus leading to the conclusion that implanted brains did not activate CA of the host. Similarly, implanted brains from either SD or LD donors did not affect photoperiodic determination of the host development (unpublished observation). The results strongly suggest that diapause is initiated only when the brain and its neural connections remain intact; differences between diapausing and non-diapausing larvae disappear after the brain removal. In course of the 6th instar of the intact larvae, however, CA activity declines in the LD

insects but not in the SD insects. The difference between LD and SD larvae is maintained after decerebration at L6d5 (Fig. 3), possibly due to maintenance of a pre-programmed CA activity or to a very low rate of JH degradation (Schaffelner et al., 2008) due to stress caused by the surgery. In *Drosophila* (Gruntenko et al., 2000) and *Manduca sexta* (Sparks et al., 1983), stress was shown to reduce JHE activity and thereby the rate of JH decay. Tauchman et al. (2007) working with *M. sexta*, showed that the hemolymph JH binding protein (hJHBP), a protein responsible for transporting JH to target tissues, was significantly reduced in response to stressors and can lead to increased JH bioavailability.

Pérez-Hedo et al. (2010a) observed that debrained larvae of *S. nonagrioides* completed metamorphosis to the adult stage, whereas decapitated larvae stopped their development after the pupal ecdysis. We proposed that an unknown head factor was necessary for induction of the pupal-adult molt. The most obvious difference between the decapitated and debrained larvae was the absence of CA and JH in the decapitated animals. To examine significance of this difference, we applied JH II or methoprene to the decapitated insects 2 or 4 days after their pupal molt. The agents were applied in acetone that alone induced molting in some of the headless pupae. Apparent stimulations of the endocrine system by acetone were reported in other insects. For example, applications of acetone and other organic solvents terminated pupal diapause in the flesh fly *Sarcophaga crassipalpis* (Žďárek and Denlinger, 1975), possibly by activating the PGs. In the cockroach *Naphoeta cinerea,* acetone administration to the last instar larvae induced extra larval molts (Radwan and Sehnal, 1974), obviously by stimulating JH secretion from the CA. Headless pupae of *S. nonagrioides* which were induced to molt by acetone treatment produced either an indifferent cuticle or a cuticle with scales which are a typical feature of adult cuticle. The incidence of molt significantly increased after the treatment with JH II or methoprene, and most insects produced cuticle with scales on the thorax, abdomen and wings. This result is similar to the report by Safranek and Williams (1987) who showed that a treatment of *M. sexta* pupae with a JH agonist accelerated the initiation of adult development. They suggested that JH provided an ecdysiotropic stimulus even in the absence of the brain. Both JH II and methoprene stimulated molting in the headless pupae of *S. nonagrioides* and some of the insects appeared as normal adult moths. A plausible explanation of this result is that the agents were applied too late to inhibit imaginal differentiation but molt induction or acceleration was possible (Sehnal, 1976).

Ecdysteroids appear to promote adult development during the early stages of the pupa but are inhibitory during later stages (Schwartz and Truman, 1983). Sakurai et al. (1991) demonstrated that in intact *M. sexta* pupae, an elevated ecdysteroid titer was required to elicit adult development. In our study, the application of ecdysteroid agonist to decapitated pupae of *S. nonagrioides* had no effect on the adult development. The absence of an effect may stem from a lack of function for ecdysteroids in the

control of adult development similar to that seen in pupae of *Mamestra configurata* (Bodnaryk, 1986).

Our data show that CA and PGs continue to produce their hormones in brain absence. The role of brain in PGs regulation in *S. nonagriodes* is probably minor because molts occur in brain absence and are not accelerated by brain implants. Brain regulation of CA seems to be mainly inhibitory: the measurements of JH titer in the intact and the debrained and decapitated larvae show that brain programmed by long photoperiod inhibits JH production in L6d5 and probably earlier in the 6th instar. While the role of brain is reduced by comparison with other Lepidoptera, interactions between JH and ecdysteroids and the respective glands seem to be of great importance. It is clear that PGs are activated when subjected to low concentrations of JH and this mechanism is probably of crucial importance.

Acknowledgements

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CHAPTER 3

Role of the PTTH hormone in *S. nonagrioides*: 3. Gene encoding the prothoracicotropic hormone of a moth is expressed in the brain and gut.

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Gene encoding the prothoracicotropic hormone of a moth is expressed in the brain and gut.

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Abstract

The molts of lepidopteran insects are typically controlled by the brain-derived prothoracicotropic hormone (PTTH) that stimulates ecdysteroidogenesis in the prothoracic glands (PGs). We report here that the larvae and pupae of the moth *Sesamia nonagrioides* can molt without brain (PGs must be present), suggesting that there might be a secondary source of PTTH. We addressed this issue by characterizing spatial and temporal expression patterns of the *PTTH* gene. To this end we identified a major part of the corresponding cDNA. Protein deduced from this cDNA fragment consisted of 128 amino acids and showed 48-85% homology with the matching regions of PTTHs known from other Lepidoptera. Quantification of *PTTH* expression in major body organs of the last instar larvae revealed high expression in the brain (fading in post-feeding larvae) and considerable expression in the gut (with a maximum in post-feeding larvae). The content of *PTTH* message in the gut was enhanced after decapitation. It is concluded that the molts of *S. nonagrioides* larvae are driven by *PTTH* gene expression in the gut.

Keywords: PTTH; Brain; Gut; Ecdysteroids; Insect development.

1. Introduction

It is generally accepted that the molting process in caterpillars is initiated when the brain-derived prothoracicotropic hormone (PTTH) stimulates ecdysteroid biosynthesis in the prothoracic glands (PGs) (Gilbert et al., 2000). The PTTH of Lepidoptera was first identified in *Bombyx mori* (Kataoka et al., 1987, Kawakami et al., 1990) and later in the representatives of Saturnidae (Sauman and Reppert, 1996; Sehnal et al., 2002), Sphingidae (Shionoya et al., 2003), and Noctuidae (Xu et al., 2003; Xu and Denlinger, 2003; Wei et al 2005; Xu et al., 2007) as a glycoprotein secreted by two pairs of neurosecretory brain cells.

Sesamia nonagrioides is a noctuid that completes larval development in a variable number of instars, dependent on the photoperiod. Larvae reared at 25ºC under long day conditions (16:8 h light:darkness) pupate after five (mainly the males) or six (mainly the females) larval instars while

those grown under short day conditions enter diapauses and can undergo more than five supernumerary larval molts after the 6th instar (Eizaguirre and Albajes, 1992). Our recent work (Pérez-Hedo et al., 2010) demonstrated that the debrained or decapitated last instar larvae molted - some of them more than once - in spite of the brain absence. The removal of PGs by body ligation applied across the mesothorax prevented molting, proving that the presence of PGs was essential. These simple experiments revealed that the function of PGs in the caterpillars of *S. nonagrioides* was independent of the brain, in contrast to all Lepidoptera examined so far. It is possible that the PGs are regulated by another neuropeptide, for example by the diapause hormone whose prothoracicotropic activity was revealed in *B. mori* (Watanabe et al., 2007), or that PTTH is derived from a source outside the head (Sakurai et al., 1991). This paper examines the latter possibility.

The first step of the present work was to verify molt independence of the brain and dependence on the PGs in *S. nonagrioides* caterpillars of different ages. The second step was to identify *PTTH* cDNA and to examine expression of the *PTTH* gene in different larval tissues at several time points.

2. Material and methods

2.1. Insects and their rearing

The larvae of *S. nonagrioides* (Lepidoptera: Noctuidae) came from a culture maintained in the Laboratory of Entomology of the UdL-IRTA research center. After every 3-4 generations, the culture was boosted by insects collected in the maize fields of central Catalonia. The larvae were reared individually at 25±0.5 ºC and 16:8 h (light:dark) photoperiod on a semiartificial diet (Eizaguirre and Albajes, 1992). Their age was measured in days after the preceding ecdysis; for example, L6d0 marks newly ecdysed larvae of the 6th instar, L6d1 larvae 24 h after ecdysis, etc.

2.2. Decapitated larvae and isolated abdomens.

To confirm the importance of brain and PGs, the larvae were ligated behind the head or across the mesothorax, respectively, and the anterior body part was cut off. The head-ligated (decapitated) larvae were deprived of the brain and other head organs but retained their PGs which are located in prothorax, whereas isolated abdomens obtained by ligation across the mesothorax lacked both the brain and the PGs. The ligations were carried out with larvae a half day after ecdysis into the 4th, 5th, and 6th instar, and at days 1, 5, 7 and 9 (prepupae) of the 6th instar, respectively. The operated insects were kept for 5 h at 6ºC to reduce mobility during the initial phase of wound healing. Afterwards they were returned to the standard rearing conditions in vials supplied with small amounts of food that sustained sufficient air humidity. The insects were checked daily for 3 weeks. At least 10 larvae were used for each treatment.

2.3. RNA isolation

The following tissues were dissected under insect saline (Novák, 1966) at days 2, 5, 7, and 9 after

ecdysis into the 6th instar: brain (Br), subesophageal ganglion (SbG), thoracic ganglia (ThG), abdominal ganglia (AbG), whole body except for the central nervous system and the digestive tract (carcass, Car), whole body except the brain (LwB), fat body (Ft), and gut (Gut). Dissected tissues were quickly immersed in liquid nitrogen and then held at -80° C until use. Total RNA was isolated with the acid phenol method (Chomczynski and Sacchi, 1987), precipitated with isopropanol and re-suspended in RNase-free diethylpyrocarbonate-treated water. The RNA was quantified and its quality assessed by absorbance measurements at λ260/ λ280 nm with the Nanodrop ND-1000 spectrophotometer. Total RNA (1 µg) was treated with Turbo DNA-free DNase (AMBION, Austin, TX) according to the manufacturer's protocol in order to eliminate any traces of genomic DNA.

2.4. cDNA synthesis and PCR

The first-strand cDNA was synthesized from 1 µg brain RNA with random hexamer primers and the SuperScriptTM III First-Strand Synthesis System kit (Invitrogen, Carlsbad, CA, USA) following the recommended protocol. Primers PTTH-F1 and PTTH-R1 (Table 1), which were designed from the conserved regions of *PTTH* cDNAs known so far (Fig. 1), were employed to amplify the *PTTH* cDNA of *S. nonagrioides*. A set of specific primers PTTF-F2 and PTTH-R2 (Table 1) based on the identified cDNA sequence was used in subsequent PCR reactions carried out in the PTC-100 thermocycler (MJ Research, Waltham, MA, USA). The reaction mixtures of 25 µl contained 1x buffer, 200 µM dNTP mix, 2.0 Mm MgCl2, 400 nM of each primer, 1U of Taq polymerase (BIOTOOLS, Madrid, Spain) and 0.5 µl of the cDNA solution. The initial denaturing step of 3 min at 95°C was followed by 35 cycles of 20 s at 95ºC, 1.30 min at 65°C with a -0.2°C change per cycle, and 1.30 min 72ºC; the reaction was concluded with 5 min at 72ºC. PCR product (about 390 bp) was separated by electrophoresis in 1.2 % agarose gel and subsequently extracted with the QIAquick PCR purification kit (QIAGEN, Düsseldorf, German).

Primer name	**Sequence 5' → 3'**	**lenght**	**Tºm**	**Amplicon size**
PTTH-F1	CCGTTAGTTTGTGTGATAGTATG	23	50.8	387 bp
PTTH-R1	CAGGTGCAGGGAGGATCCGG	20	65.8	
PTTH-F2	TTAGTCCCAAGGGTGATGGC	20	58.4	309 bp
PTTH-R2	TGGCTTGATTATGATGCTTCC	21	57.9	
qPTTH_Fw	AGATTGGCTCGAGACAGTGAATT	23	65.0	83 bp
qPTTH_Rv	GGATCAGGTTGAATGGAATCCAT	23	66.8	
qrRNA_Fw	ATTACGCTGTTATCCCTAAGGTAA	24	60.6	112 bp
qrRNA_Rv	GGTGACAGAAAAATATGGAGAACTT	25	62.2	

Table 1. Primers used for amplification and quantification of *S. nonagrioides PTTH* cDNA and rRNA.

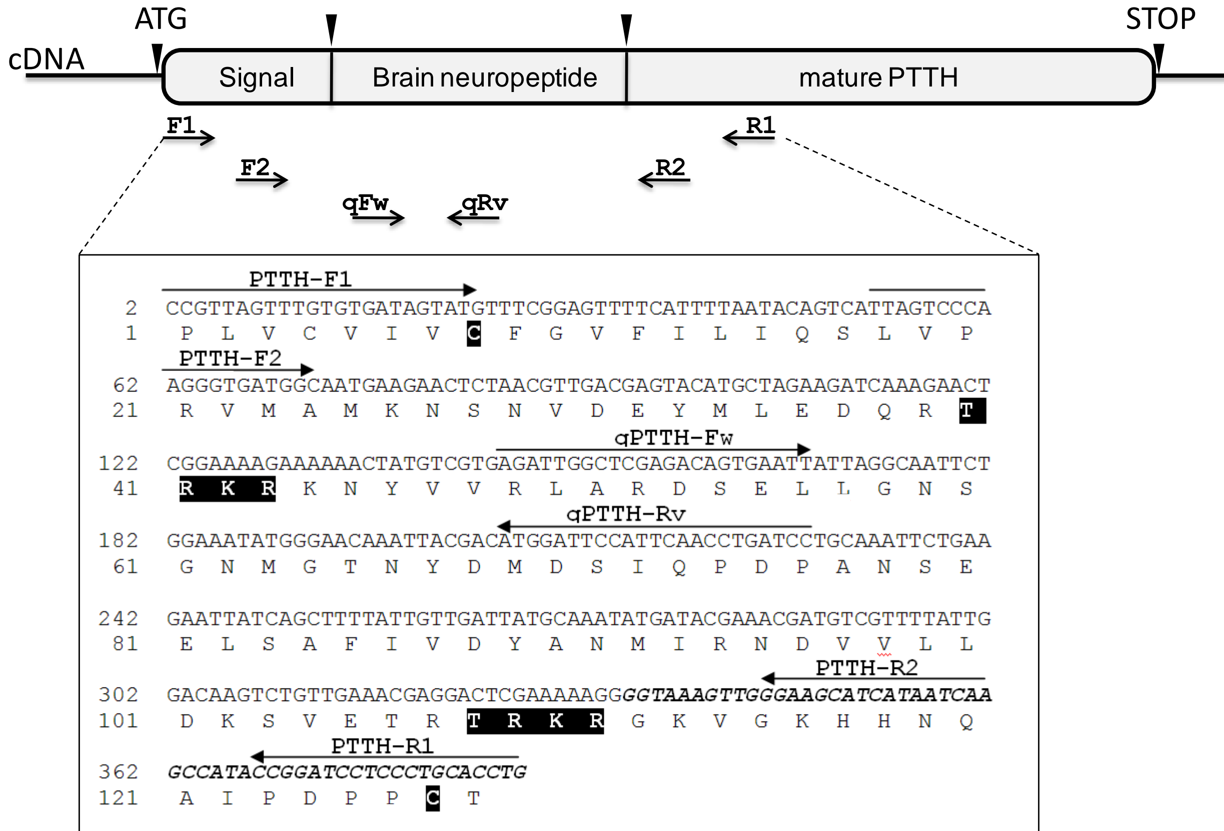

Fig. 1. Schematic organization of the *PTTH* cDNA in Lepidoptera (full arrowheads mark cleavage sites in the encoded preprohormone), the positions of primers used in the present study, and the sequence of 387 nt identified in *S. nonagrioides*. The deduced amino acid sequence of *S. nonagrioides* PTTH preprohormone includes conserved positions of proteolytic domains and cysteine residues (highlighted black).

2.5. Sequencing and phylogenic analysis

PCR products were sequenced with the PTTH-F1 and PTTH-R1 primers (Table 1) using the BigDye Terminator Sequencing kit v3.1 (Applied Biosystems, Foster City, CA, USA) and the ABI-3130 capillary electrophoresis system. Sequence data were edited with the Sequencing software (Applied Biosystems) and aligned with the cDNA sequence of other Lepidoptera using the ClustalW program (Chenna et al., 2003). The Neighbor-Joining method was employed for the construction of a phylogenetic tree (Tamura et al., 2007), validating the clustering structure with a bootstrapping test (10000 replicates).

2.6. Quantitative RT-PCR (qPCR) analysis

Real-time qPCR assays included end-point RT PCR and were run in the ABI-7500 device (Applied Biosystems). Sesamia-specific PTTH primers (qPTTH-Fw and qPTTH-Rv; Table 1) were designed from the identified cDNA sequence with the Primer Express v2.0 software (Applied Biosystems). The *16S rRNA* was quantified in parallel as an internal control used to normalize expression of the *PTTH* gene; the primers (Table 1) corresponded to conserved regions of the 16S rRNA in Lepidoptera. The qPCR was done with 5 µl mixtures containing 200 nM of the *PTTH* or *rRNA* primers and 1x SYBR Green Master mix

(Applied Biosystems) and subjected to the following thermal profile: 10 min at 95°C, 40 cycles of 15 s at 93°C and 1 min at 60°C, followed by quick denaturation at 95ºC plus a slow 5 min ramp to 30ºC. Three independent reactions were run with each tissue sample. The ΔΔCt method (Yuan et al., 2006) was used to normalize the PCR results and to quantify the mRNA contents.

2.7. Statistical analysis

Analysis of variance was run using SAS/STAT user´s guide version 9.1 (SAS Institute, Cary, NC, USA) to test differences among the treatments. The comparisons were made using the Duncan method. The t- tests between treatments were run only when the ANOVA F-test suggested significant differences.

3. Results

3.1. Development of decapitated larvae and isolated abdomens

Decapitated larvae molted despite the absence of brain as a PTTH source. Decapitation also removed the corpora allata, which are the source of juvenile hormone (JH) preventing metamorphosis (Goodman and Granger, 2008). Due to JH elimination, all decapitated larvae molted to headless pupae (Table 2); those decapitated in the 4th and 5th instar thereby skipped 2 and 1 normal larval molts, respectively. Since the decapitated larvae could not feed, the size of headless pupae depended on the time of decapitation: they were very tiny after decapitation in the 4th and somewhat larger after decapitation in the 5th instar, about half the normal size after decapitation at L6d0.5 and L6d1, and practically normal after decapitation at L6d5 and L6d7. In spite of differences in the age and size of larvae, the length of time from decapitation to pupal ecdysis was about 10 days in all age groups mentioned above. It was similar to the length of the last (6th) larval instar in intact larvae, suggesting that decapitation performed at L6d7 or earlier induced development that normally begins shortly after ecdysis into the 6th instar and is terminated by the molt to pupa.

By contrast, head removal in the L6d9 larvae induced pupal molt in just 5.2 days (Table 2), indicating that the molt-inducing ecdysteroid secretion from the PGs began prior to ligation. This conclusion was consistent with the development of isolated larval abdomens deprived of PGs. The abdomens isolated prior to L6d7 never developed, whereas some of those isolated at this time accomplished pupal molt in a very short time (Table 2). The rate of pupation increased in abdomens isolated later, suggesting that the PGs released enough ecdysteroids for pupation in 33%, 50%, 57%, and 75% of the L6d7, L6d8, L6d9, and L6d10 insects, respectively. The timing of PG activity at the end of the 6th instar was apparently independent of the brain because decapitation at L6d1 had insignificant effect on the rate of pupation after PG removal by ligation at L6d9 (Table 2). The discrepance between the development of abdomens isolated on L6d7 in the intact larvae (33% pupating) and those isolated in larvae that had been decapitated at L6d1 (0% pupation) was probably due to reduced rate of development in the decapitated larvae.

Age at ligation	**Decapitated**		**Isolated abdomens**		**Decapitated L6d1, then isolated abdomens**	
	% molts	Days to ecdysis	% molts	Days to ecdysis	% molts	Days to ecdysis
L4d0.5	100 (14)	8.5±0.3 b	0 (10)	-	-	-
L5d0.5	100 (10)	10.1±0.5 ab	0 (10)	-	-	-
L6d0.5	100 (8)	11.0±0.5 a	0 (10)	-	-	-
L6d1	100 (8)	9.3± 0.4 ab	0 (10)	-	-	-
L6d2	-	-	-	-	0 (10)	-
L6d5	100 (12)	10.8±0.4 a	0 (15)	-	0 (10)	-
L6d6	-	-	0 (6)	-	-	-
L6d7	100 (12)	9.3±0.5 ab	33 (15)	3.0±0.7 d	0 (7)	-
L6d8	-	-	50 (8)	2.0±0.5 d	-	-
L6d9	100 (10)	5.2±0.6 c	57 (21)	2.1±0.3 d	57 (7)	1.5±0.4 d
L6d10	100 (10)	-	75 (8)	0.8±0.3 d	-	-

* Values marked with different letters differ from one another at P<0.05 level (LSD test).

Table 2. The incidence (in %, total number of insects is given in parentheses) and the timing of pupal ecdysis (days, mean ± SE) in the decapitated larvae and in the isolated larval abdomens.

3.2. Characterization of the PTTH cDNA

A cDNA fragment of 387 bp (GenBank Accession No. FJ717680), which was amplified with the primer pair PTTH-F1 and PTTH-R1 (Table 1), encoded 128 amino acids (translation frame +2). The deduced peptide sequence overlapped with known PTTHs in a region that included a signal peptide, an intercalated peptide of unknown function, and the N-terminal portion of a mature PTTH (Fig. 1). The identified cDNA lacked a short 5' region with the start codon and a 3' region that would encode about 90 amino acids of the carboxyl end of the PTTH. Since the identified cDNA sequence left no doubt that it was derived from the *PTTH* gene, it was sufficient for our study of this gene expression and no attempt was made to identify the missing parts of the gene.

Amino acid sequence alignment (Fig. 2) of homologous regions of PTTHs from 11 lepidopteran species revealed high identity scores between *S. nonagrioides* and other noctuids: *Spodoptera exigua* (85%), *Heliothis virescens* (84%), *Helicoverpa armigera* (84%), *Helicoverpa Zea* (83%), and *Helicoverpa assulta* (82%). The homology with the PTTHs of Bombycoidea was much lower: 48% in case of *B. mori* (Bombycidae), 56% in *Manduca sexta* (Sphingidae), and 49-57% in three examined species of Saturdinae. Consistent with these data, phylogenetic analysis clustered all PTTH sequences of Noctuidae in a single branch (Fig. 3). It also revealed considerable differences between the three families of Bombycoidea and relatedness of PTTHs among Saturniidae.

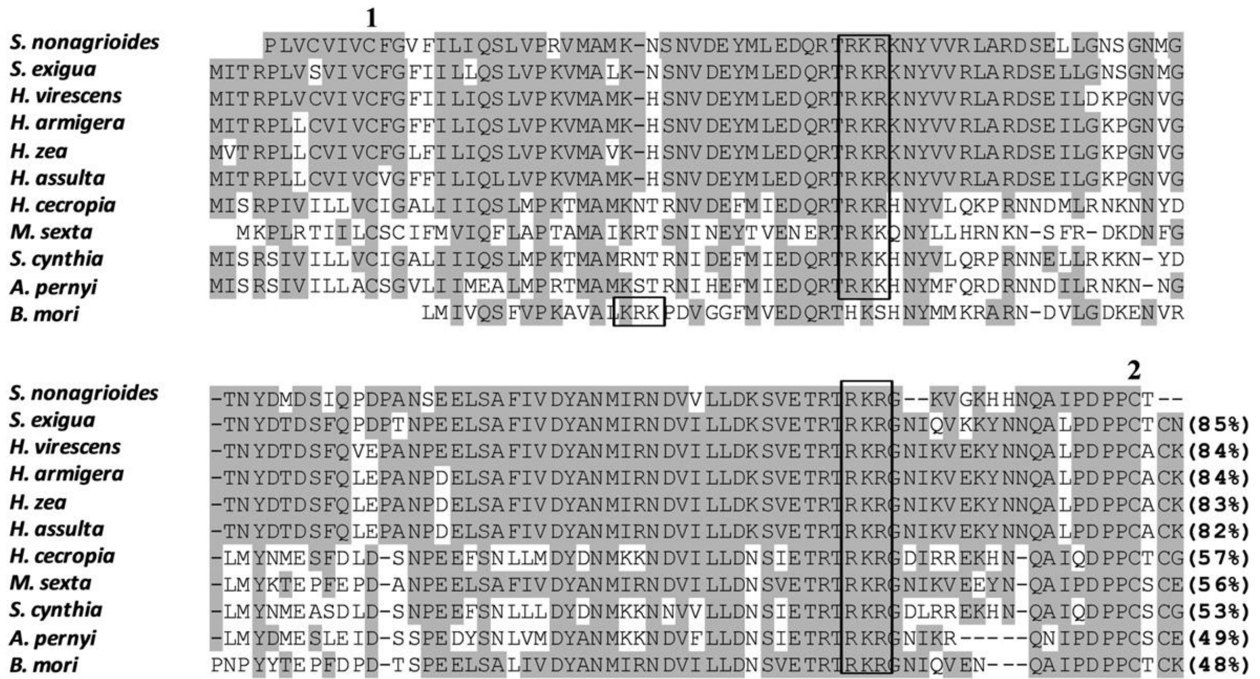

Fig. 2. Amino acid sequence deduced from the *PTTH* gene region identified in *Sesamia nonagrioides* (GenBank Accession No. FJ717680) and homologous parts of the PTTHs known from other Lepidoptera: *Spodoptera exigua* (AY628763), *Heliothis virescens* (AY172671), *Helicoverpa armigera* (AY286543), *Helicoverpa zea* (AY172670), *Helicoverpa assulta* (AY780526), *Hyalophora cecropia* (AF288695), *Manduca sexta* (AY007724), *Samia cynthia ricini* (L25668), *Antheraea pernyi* (U62535), and *Bombyx mori* (D90082). Shaded areas indicate sequence conservation higher than 50%. Potential proteolytic cleavage sites are framed and the Cys residues are numbered. The percentages of conserved amino acid positions between *S. nonagrioides* and the other species are shown in parentheses.

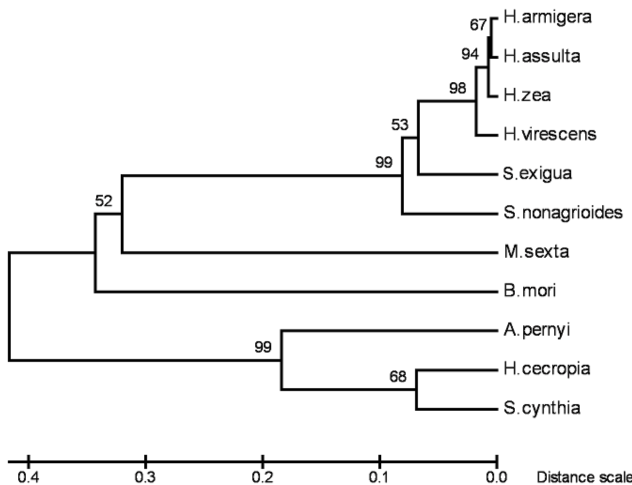

Fig. 3. Phylogenetic tree of lepidopteran PTTH protein sequences constructed with the Neighbor-Joining method that compares amino acid sequences between all combinations of the species pairs. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10000 replicates) is shown above the branches. Species distances in the chart reflect sequence similarities.

3.3. Tissue- and stage-specific expression of the PTTH gene

The expression pattern of the *PTTH* gene was examined by RT-PCR in a variety of tissues dissected from the last instar larvae of four ages. Consistent generation of a PCR product of expected size suggested strongly that it was derived from the *PTTH* mRNA. The sequencing of PCR product amplified from the gut RNA confirmed that it corresponded to the sequence identified in the brain RNA pool (Fig. 2).

As expected, the presence of *PTTH* mRNA was consistently detected in the brain samples (Br), being very strong at L6d2 and L6d5 and weaker at L6d7 and L6d9 (Fig. 4). Strong signal occurred also in the gut of prepupae (= L6d9) and in the samples comprising prepupal bodies without the

brain (LwB). Gut samples or whole body samples without brain analyzed at days 2, 5, and 7 of the 6th instar produced none or only small amounts of the PCR product. Very weak *PTTH* signal was in some cases detected in the subesophageal ganglion, thoracic ganglia, abdominal ganglia, fat body, and the carcass that contained these organs (Fig. 4).

PTTH expression in the gut and ventral ganglia (except the suboesophageal ganglion that could not be analyzed in the decapitated larvae) was examined by quantitative PCR also in larvae that were decapitated a half day after ecdysis into the 6th instar. Intact L6d2 larvae were used for comparison. The results revealed high expression of the *PTTH* gene in the gut and weak in the ventral ganglia of the intact L6d2 larvae and low expression in both tissues of the decapitated larvae on days 2, 5, and 7 (Fig. 5). However, the content of *PTTH* transcript was high in the L6d9 decapitated larvae (= prepupae, column GutDcd9 in Fig. 5) that were close to pupal ecdysis which occurred in most decapitated larvae on day 10. No clear *PTTH* expression was detected in the ventral ganglia of decapitated larvae (Fig. 5).

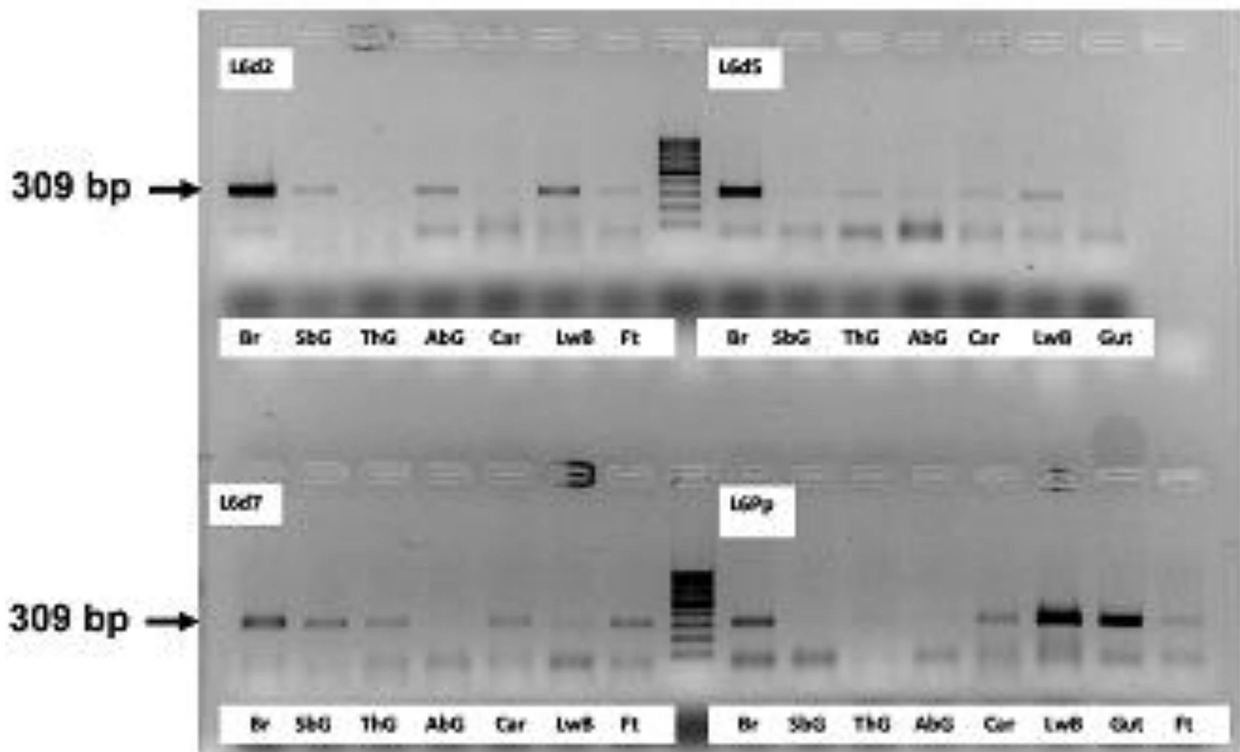

Fig. 4. RT-PCR analysis of the *PTTH* gene expression in the brain (Br), subesophageal ganglion (SbG), thoracic ganglia (ThG), abdominal ganglia (AbG), carcass after the brain and gut removal (Car), larva without brain (LwB), the fat body (Ft), and the gut (Gut) obtained from *S. nonagrioides* on days 2, 5, 7, and 9 (prepupae) of the 6th larval instar (L6d2, L6d5, L6d7, and L6Pp, respectively). The band corresponding to the PTTH-specific product (309-bp) is indicated. The lower band corresponds to primer-dimers.

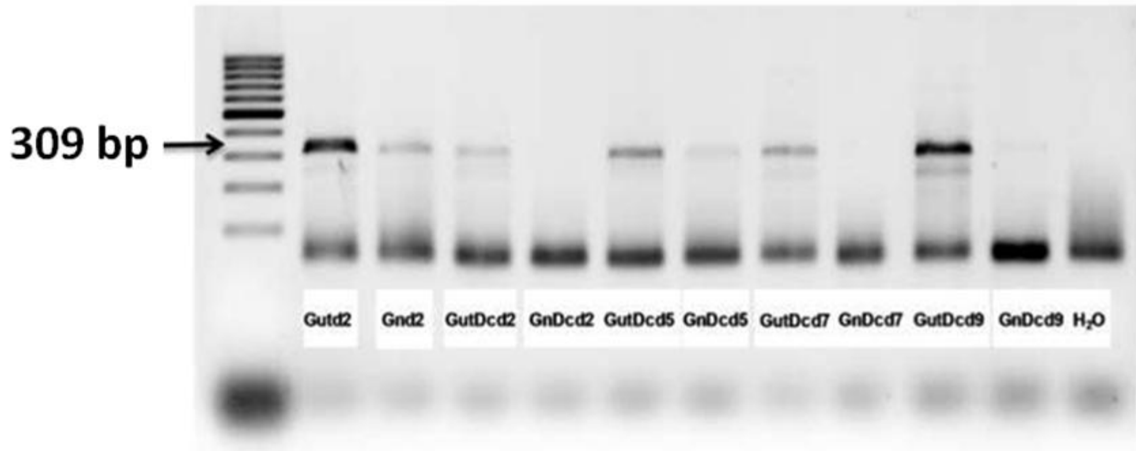

Fig. 5. Quantification of the *PTTH* transcript in the gut (Gut) and ventral ganglia (Gn) by the end-point quantitative RT-PCR. Samples were taken from the intact 6th instar larvae on day 2 (Gutd2 and Gnd2, respectively) and from larvae decapitated on day 1 and analyzed on days 2, 5, 7, and 9 of the 6th instar (samples marked Dcd2, Dcd5, Dcd7, and Dcd9, respectively). The PTTH-specific product (309-bp) is indicated. The lower band corresponds to primer-dimers.

3.4. Quantitative analysis of PTTH gene expression

The *PTTH* cDNA amplified by qPCR in the brain and gut RNA extracts was separated by electrophoresis and quantified by densitometry. Established values were normalized against the contents of *16S rRNA*. Maximal *PTTH* expression, which was detected in the brain of the L6d5 larvae, was scored as 1000. The cDNA amounts measured at other times and in other tissues were expressed relative to this value. As shown in Fig. 6, the *PTTH* transcript content relative to the rRNA content was in general higher in the brain than in the gut. The brain and gut also differed by developmental changes in the transcript content. The *PTTH* gene expression in the brain was maximal in the feeding larvae (L6d2 and L6d5), declined in the post-feeding larvae (L6d7) and was low in the prepupae (L6d9). Gene expression in the gut was considerable in early last larval instar (L6d2), negligible during the rest of the feeding

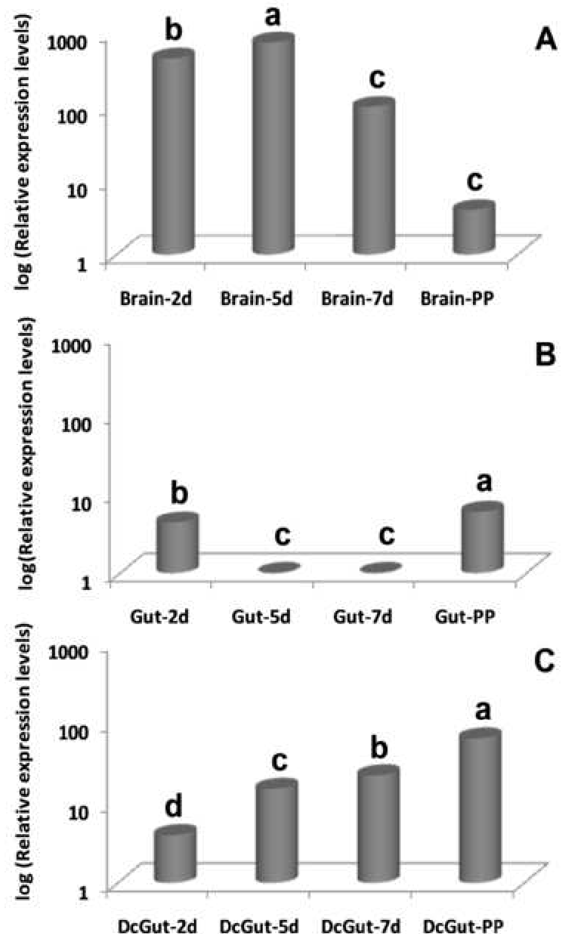

Fig. 6. Relative levels of *PTTH* gene expression in the brain (panel A) and the gut of intact larvae (panel B), and in the gut of larvae decapitated on day 1 of the 6th instar (panel C). The time of analysis is given in days of the 6th instar (day 2, 5, 7, and 9 (prepupae), respectively). Columns marked with different letters represent values different at more than 95% probability level.

period, and elevated again in the prepupae. Decapitation markedly enhanced the content of *PTTH* message in the gut of the L6d5 and older larvae. The value established in L6d9 (prepupae) was about 10 times higher than the content found in the brain of intact insects of this age.

4. Discussion

The results of our experiments with isolated larval abdomens of *S. nonagrioides* are consistent with the profile of ecdysteroid titer changes in this species. The titer rises to a small transient peak at L6d5 and to a molt-inducing surge at L6d7 (Eizaguirre et al., 2007), i.e. at the time when PGs removal by ligation no longer prevents pupation in about one third of the insects (Table 2). These data are in accord with the paradigm of the hormonal regulation of lepidopteran development: PGs must be present until enough ecdysteroids are secreted to induce molting, i.e. at least until L6d7 in *S. nonagrioides*. Unlike in other Lepidoptera, however, PG function in this species is independent of the brain stimulation because debrained larvae undergo one to several molts and can develop until the adult stage (Pérez-Hedo et al., 2010). The decapitated larvae, which are deprived of all head organs, including the corpora allata, source of juvenile hormone, invariably undergo precocious pupation (Table 2). Larvae decapitated at the start of the 4th, 5th, or 6th instar all molt to pupae in about 10 days. This timing is similar to the length of the 6th (normally last) larval instar of the intact larvae, suggesting that the time pattern of ecdysteroid secretion does not require any regulator from the head. This conclusion is supported by the finding that the development of both intact and decapitated larvae becomes independent of PG presence at L6d7, when PG removal by ligation does not prevent pupation in some of the resulting isolated abdomens (Table 2).

The molts of decapitated larvae to perfect pupae in due time indicate existence of a PGs control by a center outside the head. Sakurai et al. (1991) conceded the presence of alternative PTTH sources and suggested that they begin to operate (or are up-regulated) when the major source, the brain, is removed. In search of alternative PTTH sources we identified PTTH homologue in *S. nonagrioides*. Using PCR with primers designed on Lepidoptera-conserved PTTH sequences we amplified 387 nt of a cDNA that encodes a peptide homologous to more than half of the PTTH preprohormone of other Lepidoptera. The homology is 82-85% with the PTTHs of Noctuidae (Xu et al., 2003, Wei et al., 2005, Xu et al., 2007) and 48-57% with the PTTHs of the bombycoid species (Kawakami et al., 1990; Sauman et al., 1996; Sehnal et al., 2002; Shionoya et al., 2003). The homology includes positions of two proteolytic cleavage sites that are crucial for the preprohormone processing (Ishibashi et al., 1994).

The cDNA in question was amplified in the RNA extracts from the brain and gut. A PCR product of proper size was occasionally obtained also from the subesophageal ganglion, thoracic ganglia, abdominal ganglia, fat body, and body preparations containing these organs. Similar expression of the *PTTH* gene in different larval tissues was previously reported for other Noctuidae: Wei et al. (2005) detected *PTTH* transcript in the gut of

H. armigera and Xu et al. (2007) found weak expression in the subesophageal ganglion, thoracic ganglia, and abdominal ganglia but not in the gut and fat body of *S. exigua*. We assume that the low levels of *PTTH* cDNAs are due to transcription “leakage” (Spellman and Rubin, 2002) and do not represent functional gene expression.

The qPCR confirmed that the *PTTH* gene of *S. nonagrioides* is strongly expressed in the brain of the 6th instar larvae with a maximum on day 5 and a minimum in prepupae (Fig. 6). This pattern is consistent with reports on the PTTH titer changes in the hemolymph of *B. mori* (Shirai et al., 1993; Mizoguchi et al., 2002). The high level of *PTTH* gene expression on day 5 of the last instar larva of *S. nonagrioides* can be related to the transient increase of ecdysteroid titer at this time (Eizaguirre et al., 2007).

A consistent level of PTTH expression was also detected in the gut of the intact and even more in the decapitated larvae of *S. nonagrioides*. Quantitative PCR is a very reliable quantification technique, which gives better indication of differences between samples than the end-point PCR. The differences between the two methods and the choice of the normalising control gene (see below) explain discrepancies between Figs. 5 and 6 in the estimated expression levels. We propose that both brain and gut produce PTTH but at different rates and according to different developmental patterns. Brain PTTH is apparently derived from two pairs of protocerebral neurosecretory cells that have been identified in all examined lepidopterans, for example *B. mori* (Agui et al., 1979) and *Antheraea pernyi* (Sauman and Reppert, 1996). The source of PTTH in the gut is unknown. Žitňan et al. (1993) detected PTTH in the stomatogastric ganglia of *Galleria mellonella* larvae by immunocytochemistry. Gelman et al. (1991, 1993) reported on the presence of an ecdysteroidogenic factor localized in the hindgut of *Ostrinia nubilalis* and *M. sexta* larvae but the chemical nature of the factor has not been elucidated.

It must be emphasized that the level of *PTTH* gene expression in the gut of *S. nonagrioides* is probably higher than indicated by our figures. The gut includes a large proportion of secretory cells which contain more ribosomes than most other cell types, for example the brain cells. Gut cells of the feeding intact larvae are likely to possess more ribosomes than the cells of the starving decapitated larvae. These differences in the content of rRNA relative to the amount of *PTTH* mRNA are a source of a serious bias in qPCR based on equally large samples of total RNA and relying on the adjustments of measured values to the 16S rRNA content. In spite of this shortcoming, the measurements clearly demonstrate differences in the time-course of PTTH expression in the gut of intact and decapitated animals. The relative level of PTTH mRNA in the gut of intact animal showed two peaks of expression, around day 2 and in the prepupae stage. By contrast, the relative level of expression increased exponentially from day 2 to prepupae in the gut of the decapitated larvae to reach a higher level in the prepupae than found in the brain of the intact insects of the same age. Considering the great difference in the size of the two organs, gut may be a more important source of PTTH than the brain. The rate of PTTH gene expression in the gut seems to increase after

decapitation and renders the whole system independent of the brain presence.

Several PG stimulators other than PTTH were found in Lepidoptera (Marchal et al., 2010). They include an unidentified factor from the brain (Dedos et al., 1998) and FXPRL-peptides such as the diapause hormone from the suboesophageal ganglion (Zhang et al., 2004). Such regulators could not replace the brain-derived PTTH in our assays because their sources were eliminated by decapitation. However, a gene encoding FXPRL-peptides is also weakly expressed in the thoracic ganglia (Xu and Denlinger, 2004) and we cannot exclude participation of these peptides in the PGs regulation. Mizoguchi et al. (2002) showed that the prothoracic and mesothoracic ganglia can inhibit PGs activity by a neuropeptide delivered to the glands by axonal transport. The prothoracic ganglion is located close to the suboesophageal ganglion and both are removed by decapitation. This alone might cause some PG stimulation in our assays. Finally, Gu (2007) demonstrated in *B. mori* that PGs themselves can produce an autocrine activator that was not considered in our study. However, in spite of the possible existence of a number of subtle PG regulators, *S. nonagrioides* is the only lepidopteran shown to undergo larval molts and metamorphosis without the brain and to express a *PTTH* gene in the gut.

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CHAPTER 4

Sub-lethal effects of Bt toxin on the larval development of *S. nonagrioides*:

4. Modification of hormonal balance in larvae of the corn borer *Sesamia nonagrioides* due to *Bacillus thuringiensis* protein ingestion.

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Modification of hormonal balance in larvae of the corn borer *Sesamia nonagrioides* due to *Bacillus thuringiensis* protein ingestion

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Abstract

Bacillus thuringiensis (Bt) maize is highly efficient against the corn borer *Sesamia nonagrioides* (Lefèbvre) when the larvae complete their development in the transgenic plants. However, when the larvae feed on Bt plants only for a part of their development or when they ingest sub-lethal amounts of Bt toxins, a number of individuals may survive. In previous papers, we observed a prolonged development and precocious diapause induction in larvae of *S. nonagrioides* fed on a diet with sub-lethal amounts of a commercial preparation of Bt containing the Cry1Ab protein. Effects were similar to those observed in larvae treated with juvenile hormone (JH). The present paper demonstrates that non-diapausing developed larvae of *S. nonagrioides* that survive feeding on Bt maize or on a diet with Bt toxin increase their JH level and decrease the ecdysteroid levels in the hemolymph, and this leads to a longer larval development and more larval molts. This response may be considered as a defence mechanism that allows some larvae to survive toxin ingestion; it is similar to the response observed in other larvae to insecticidal toxins or viruses and cannot be specifically attributed to Bt toxin. Changes in the hormone levels in diapausing larvae were undetectable, probably because these changes were masked by the higher level of JH in the hemolymph of diapausing larvae and because of lack of ecdysteroid titer increase, a phenomenon that is usually observed a few days before pupation in non-diapausing larvae. These results should be taken into account in the establishment of non-Bt refuges to prevent development of Bt-resistance in the corn borer populations.

Key word: Sesamia nonagrioides; Bt corn; Juvenile hormone, Ecdysteroids, Development, Maize.

Introduction

The corn borer *Sesamia nonagrioides* (Lefèbvre) (Lepidoptera: Noctuidae) is a major pest of maize in the Mediterranean area, reducing crop yield significantly (Castañera, 1986). Its endophytic behavior, with larval and pupal development taking place inside the maize stalk, reduces the effectiveness of conventional chemical control methods (Alfaro, 1972). The number of generations per year varies according to the geographical area in which it develops

(Anglade, 1972). In the area of this study (Lleida, NE Iberian Peninsula) it has two complete generations and an incomplete third one (Eizaguirre et al., 2002). The importance of the third generation depends on the percentage of second-generation larvae that enter diapause. The most important diapause-inducing factor is the short photoperiod, but diapause induction may be modified by the temperature or the phenology of the maize (Eizaguirre & Albajes, 1992). The photoperiod-sensitive larval instars of *S. nonagrioides* are the first and second ones (Eizaguirre et al. 1994a) but it overwinters as diapausing developed larvae in the stubbles of the maize. Diapausing larvae reared under short-day photoperiodic conditions (SD) feed, move and molt (Fantinou et al., 1995). While the non-diapausing larvae reared under long-day photoperiodic conditions (LD) and 25°C temperature mostly molt to pupae after the fifth or sixth larval instar, those diapausing subject to a SD photoperiod may have thirteen larval molts (Eizaguirre et al., 1994b). Transgenic maize that has incorporated the insecticidal capacity of *Bacillus thuringiensis* (Bt), the so-called Bt maize, is very effective against *S. nonagrioides* (Gonzalez-Cabrera *et al*., 2006). The only Bt maize grown in the EU contains event MON810, but until 2005 the transgenic maize allowed was based on event 176, which is now unauthorized. Both of them express the production of one single Bt toxin, Cry1Ab. The area of GM maize grown in Spain was 76057 hectares in 2009, representing 22% of the total maize area (www.mapya.es). The introduction of transgenic maize may lead to major changes in maize phytophagous insect populations and in their ecophysiology which need to be studied. Bt maize is highly effective for controlling maize borers, and most of the larvae that feed on it die. However, as *S. nonagrioides* larvae may move to adjacent plants from the native one in mature instars (Eizaguirre *et al*., 2004), they can be exposed to sublethal amounts of Bt toxin and survive in certain circumstances. This is the case when Bt maize fields contain a certain percentage of non-transformed seeds, when a mixture of transgenic and non-transgenic seeds are mixed as a strategy to delay development of Bt-resistance, or when Bt maize fields are surrounded by strips of non-Bt maize to reduce gene flow or to comply with a 20% requirement of non-Bt refuges.

Eizaguirre *et al*. (2005a) observed that young larvae fed on a semi-synthetic diet with sublethal amounts of a commercial preparation in which Cry1Ab was the prevalent Bt toxin showed alterations in larval development and earlier induction of diapause; these effects were hypothesized to be caused by an effect of the Bt toxin on the endocrine system regulating the development. The role of juvenile hormone (JH) in the induction and development of diapausing larvae of *S. nonagrioides* was described by Eizaguirre *et al*. (2005b), who found that the levels of JH are higher in diapausing than in non-diapausing fourth (L4) and fifth instar (L5). In the sixth instar (L6) the JH concentration decreases along the instar in non-diapausing larvae, being undetectable the last days of the instar whilst diapausing L6 larvae maintain a more constant JH level, above 20 pmol/ml (Eizaguirre *et al*., 2005b). However, levels of ecdysteroid are similar in diapausing and non-diapausing L4 and L5 larvae. The ecdysteroids level is maintained in diapausing

L6 larvae around 0.15 μg/ml hemolymph whilst in non-diapausing L6 larvae the level increases sharply to 0.6 μg/ml hemolymph the last days of the instar allowing thus the methamorphosis to pupa (Eizaguirre *et al.*, 2007). JH analog applications topically to L6 larvae induce at least a larval molt and malformations in the pupa whereas the application of the analog in the diet induce in L6 non-diapausing larvae a state similar to the diapause with larvae incapable of pupating and having several supernumerary larval molts (Eizaguirre et al 2005b).

The aim of this study was to investigate the implication of the hormonal system in the sublethal effects of the Bt protein on *S. nonagrioides* development. To this end, larvae of the species reared under diapausing and non-diapausing conditions were submitted to the Bt toxin supplied in Bt maize leaves to simulate different field exposure durations, or in the diet to test different Bt toxin doses, and a number of parameters of development and juvenile hormone and ecdysteroid titers in the larvae were measured in each experimental condition.

Material and Methods

Insects and rearing

The larvae of *S. nonagrioides* came from the culture maintained in the entomology laboratory at the UdL-IRTA research center, reared on a semi-artificial diet described by Eizaguirre & Albajes (1992). Culture was renewed every three or four generations with insects collected in the field in the area of Lleida. After the eggs hatched, the larvae were individualized in plastic boxes 5 cm in diameter and 3.2 cm tall, with a piece of diet in each box. All larvae developed at the same temperature 25ºC (±0.5ºC) and 55% RH, but in two different photoperiod conditions. Half of the larvae were held in a short-day photoperiod (SD), 12:12 h (light:dark) inducing diapause and the other half in a long-day photoperiod (LD), 16:8 h (light:dark) for continuous development and pupation of the larvae.

Bioassays with leaf

Larvae reared under LD or SD conditions were fed with semi-artificial diet until the first day of the 6th instar (L6d1). From this day they were fed with maize leaves of two maize varieties for 2, 3, 4, 5, 6 or 7 days to simulate different exposure durations to Bt plants when developed larvae move from the plant on which they have developed to adjacent Bt plants. After feeding with maize leaves they were fed again with the semi-artificial diet. The maize varieties assayed were the Bt-transgenic PR33P67 containing the transformation event MON810 and their corresponding near isogenic PR33P66 (non-Bt), both of the Pioneer Hi-Bred company. Plants were grown in a greenhouse at 23°C and a photoperiod of 13:11 h (light: dark). Maize leaf pieces were cut when the plants were at the V5-V6 phenological stage. The number of larvae per group was always higher than 25. On the 7th day of L6, the hemolymph of 6 larvae of each experimental group was collected for hormonal analysis. The rest of the larvae were periodically observed and the number and duration of the next larval instars were recorded.

Bioassays with toxin in the diet

In this assay 1-day-old larvae of the fourth (L4d1), fifth (L5d1) and sixth (L6d1) instar developed under LD photoperiodic conditions were fed with a semi-artificial diet with different sub-lethal amounts (0, 0.35, 0.9 and 2 mg/kg diet) of active Cry1Ab toxin with trypsin, sent by Dr. Juan Ferré (Department of Genetics, Faculty of Biological Sciences, University of Valencia, Spain). Treatment was offered to the larvae until the molt to pupa or the death of the larvae. The diet used was a modification of the semi-artificial corn diet described by Eizaguirre & Albajes (1992), and consisted of 13 g of maize flour, 6 g of wheat germ, 6 g of yeast, 0.9 g of ascorbic acid, 0.19 g of benzoic acid, 0.15 g of methyl p-hydroxybenzoate, 3.9 g of agar, and 150 ml of water. The toxin was added to the diet when the temperature decreased to 40ºC. The number of larvae per group was always higher than 20. On day 5 of L6, the hemolymph of 6 larvae for each experimental group was collected for hormone analysis. The rest of the larvae were periodically observed and the mortality, number and duration of the next larval instars were recorded.

Hemolymph collection

Hemolymph was extracted from live larvae by cutting a proleg with microscissors. Twenty-five µl for JH analysis and 5 µl for ecdysteroid analysis were collected in a graduated glass micro-pipette. According to the JH measurements done in a previous work (Eizaguirre *et al*, 2005b) and with our experience on duration of larval development of the larvae fed on maize leaves, hemolymph extractions were done when the JH level of the L6 larvae was suppose to be low, the 5th day and the 7th day of the 6th larval instar (L6d5, L6d7) for larvae feed in diet or in maize leaves respectively.

JHII quantification

The extraction was performed following the protocol described by Westerlund & Hoffmann (2004), with some modifications. Hemolymph was collected in a vial with methanol-isooctane (1:1 v/v) and methoprene as an internal standard. This mixture was vortexed and stored in a freezer at –80ºC until sample preparation. Upon thawing of the sample, the hemolymph-solvent solution was vortexed for 20 s and allowed to stand at room temperature for 30 min. Then, the whole sample was centrifuged at 8500 *g* for 15 min and the isooctane phase was transferred to a new glass vial. The remaining methanol phase was vortexed again, centrifuged at 10000 *g* for 30 min and combined with the isooctane phase in the same vial. The extracts were stored at –80ºC or concentrated under nitrogen flow down to 100 µL for immediate analysis. JHII, the predominant hormone in *S. nonagrioides* (Eizaguirre *et al* 2005b), was measured. Five-point calibration curves, as standard, were obtained with methanol and by spiking sample blank extract free from JHII to cover a range in both cases of 1-100 ng/mL, with 18 ng/mL of methoprene as internal standard. For having blank extracts free from JHII, L6d1 larvae were decapitated according to Pérez-Hedo *et al.* (2010) and the hemolymph was extracted 5 days after decapitation. The instrumental parameters used was a Waters Acquity UPLC coupled with a

QqQ-MS TQD, that is, a triple quadrupole mass spectrometer using ESI, APCI and APPI interfaces, and the system was operated under Masslynx 4.1 software. The chromatographic separation was carried out at 28ºC in the isocratic mode using methanol/waters (80+20 v/v) as mobile phase. The injection volume was 15 µL in partial loop with needle overfill. A reverse-phase C18 UPLC column (Acquity BEH C18 2.1*100mm 1.7µ) was used at a flow rate of 400 µL/min. A total separation time of 6 min was used.

20-hydroxyecdysone quantification

20-hydroxyecdysone (20E), which is the majority ecdysteroid in the hemolymph of *S. nonagrioides* larvae, was analyzed. Hemolymph was collected in a vial with 80% methanol (1:100 v / v). This mixture was vortexed and stored in a freezer at –20ºC until extraction. The precipitated material was spun down (12000 rpm for 10 min) and re-extracted with 200 µl 80% methanol. The extract was taken to a clean tube and the solvent was evaporated in a vacuum centrifuge. The residue was dissolved in 500 µl 80% methanol and stored in a freezer at –20ºC until analysis. Ecdysteroids were quantified by competitive ELISA, following with some modification the protocol described by Kingan (1989). All wells of the EIA plates (Easy Wash, Costar®3369) were coated with 90 µl goat anti-rabbit IgG, Fc fragment specific (Jackson Inmuno Research) to a concentration of 5 µg/90µl, and incubated overnight at room temperature. We then emptied the plates and added 300 µl blocker: AB (25mM Na2HPO4, 0.15 M NaCl and 1mM Na2EDTA)/0.1%BSA containing 0.002% sodium azide. After 1 h, wells were washed 3 times with PBS (10mM Na2HPO4, 0.15 M NaCl) containing 0.05% Tween-20. The immunological reaction was then achieved by adding dilutions of the samples or standard at a decreasing concentration from 5000fM to 1.25 fM (20-Hidroxyecdysone, Sigma) in a volume of 50 µl/well. Subsequently, 50 µl of anti-ecdysone diluted at 1/100000 in AB/BSA and 50 µl of 20E-HRP conjugate (sent by Dr. Kingan, University of California, Riverside) diluted at 1/40000 in AB/BSA were added to each well. After overnight incubation at 4ºC, the contents were discarded, plates were washed and 100 µl of tetramethylbenzidine (Sigma) was added. Finally, the plates were incubated for 15 min in the dark with gentle shaking. Color reaction was stopped by adding 100 µl/well of 1M H_3PO_4. Absorbance was read at 450 nm with a spectrophotometer.

Statistical analysis

Two- or three-way ANOVA tests were carried out using the SAS package (2001). In cases of significant differences between treatments, the LSD test was used to compare means. The comparison of the proportion of dead larvae between treatments for the total mortality was performed using the chi-square test.

Results

Effect of Bt toxin in maize leaves on larvae of S. nonagrioides

Effects on the larval development

Feeding with Bt maize leaves modified the development of the larvae of *S. nonagrioides* (Figures 1 and 2). The number of molts of the larvae developed in SD photoperiod was significantly higher than that of those developed in LD photoperiod (F=1732.69, P<0.0001, d.f =1; 349) (Figure 1A). The larvae fed with Bt maize leaves molted more times than those fed with non-Bt maize independently of the photoperiod under which the larvae developed (F=15.09, P=0.0001, d.f.=1;349) (Figure 1B).

Regarding the duration of the development, as there was an interaction between the effect of the photoperiod and the variety (Bt *vs*. non-Bt) of maize leaves provided to the larvae (F=3.81, P=0.0023, d.f.=1; 313), the two photoperiod conditions were studied separately. Ingestion of maize leaves with the Bt protein affected the duration of the development only in the larvae reared under the LD photoperiodic conditions (F=27.92, P<0.0001, d.f.=1; 171) (Figure 2A); those fed with Bt maize needed more time to pupate than those fed with non-Bt maize, though this difference was not found in larvae developed under SD photoperiodic conditions (F=0.02, P=0.8800, d.f.=1; 148) (Figure 2B).

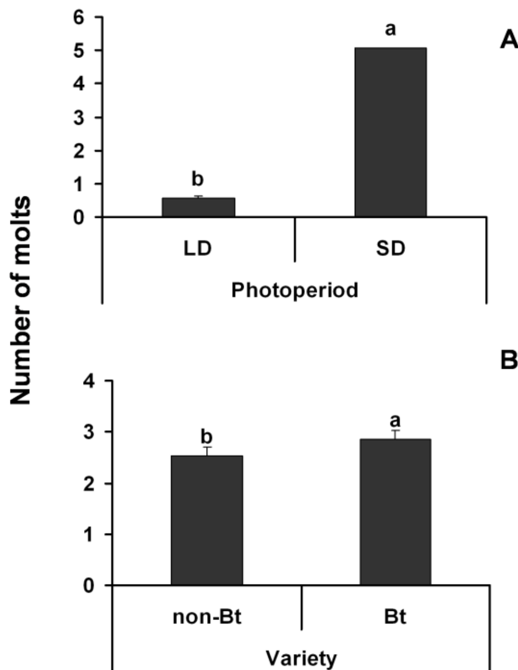

FIG. 1. Number of supernumerary molts in the larvae reared under non-diapausing, long day (LD) and diapausing, short day (SD) photoperiodic conditions and fed with Bt and non-Bt maize leaves. A) Effect of the photoperiod on the number of supernumerary molts. B) Effect of the variety (Bt and non-Bt) on the number of supernumerary molts. Different letters above the columns indicate significant differences between treatments.

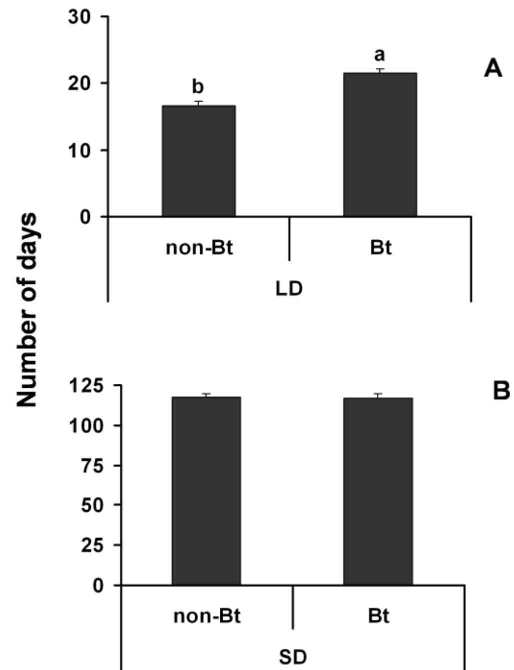

FIG. 2. Effect of the variety (Bt *vs*. non-Bt) on the time, number of days, to pupation in L6 larvae reared under non-diapausing, long day (LD) and diapausing, short day (SD) photoperiodic conditions and fed with Bt and non-Bt maize leaves. A) Larvae reared in long-day conditions. B) Larvae reared in short-day conditions. Different letters above the columns indicate significant differences between treatments.

Effect on the concentration of JHII and ecdysteroids in the larval hemolymph

The effect of feeding on Bt leaves on the concentration of JH is shown in Figure 3. The three-way statistical analysis showed an interaction between the photoperiodic conditions and the variety of maize leaves provided to the larvae; therefore, the variety (Bt and non-Bt maize) and the treatment (number of days fed with maize leaf) were analyzed within each photoperiodic condition. JH titer depended on the photoperiod under which the larvae were reared: SD larvae showed a higher JH titer (F=11.80, P=0.0009, d.f.=1; 79) than LD larvae (Fig. 3). Feeding with Bt maize leaves increased the concentration of JH in the hemolymph of the larvae developed under LD photoperiod (F=3.88, P=0.0500, d.f.=1; 36) (Figure 3A), but this increase was not detected in the larvae developed under SD or diapausing conditions (F=2.86, P=0.0990, d.f.=1; 39) (Figure 3B), probably because the larvae developed under SD condition usually had a much higher JH concentration in the hemolymph that masked the effect of the feeding. The increase in JH concentration in larvae feeding on Bt maize leaves and subject to LD conditions could explain the longer larval development and the higher number of molts of the larvae shown in the previous results (Figs. 1 and 2). There was no difference in the concentration of JH in the hemolymph according to the number of days (3, 4, 5, 6, 7) in which the larvae were fed with maize leaves and subjected to either SD (F=0.98, P=0.4280, d.f.=4; 39) or LD conditions (F=0.41, P=0.7979, d.f.=1; 76).

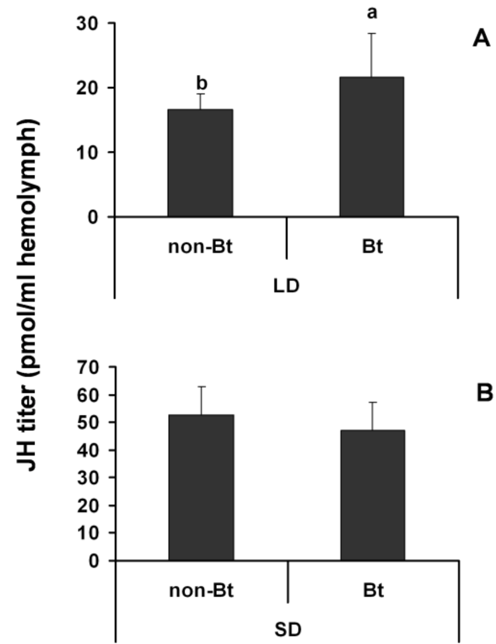

FIG. 3. Effect of Bt toxin in maize leaves on the concentration of JH in the larval hemolymph. Larvae were fed on Bt maize or its corresponding isogenic non-Bt and subjected to non-diapausing, long day (LD) (A), or diapausing short day (SD) (B) photoperiod conditions Different letters above the columns indicate significant differences between treatments.

With regard to the ecdysteroid concentration in the hemolymph, the three-way statistical analysis showed again a significant interaction among the three factors (F=2.46, P=0.0371, d.f.=5; 119). Thus, the variety (Bt and non-Bt maize) and the treatment (number of days fed with maize leaves) were analyzed within each photoperiod. In larvae fed with the non-Bt variety, those subjected to LD photoperiod conditions showed a significantly higher concentration of ecdysteroids in the hemolymph than those subjected to SD photoperiod conditions (F=6.31, P=0.0150, d.f.=1; 55) (Figure 4A) but in the larvae fed with Bt variety this photoperiod effect was not found (F=1.29, P=0.2610, d.f.=1; 64) (Figure 4B). Feeding with Bt maize leaves prevented the increase in molting hormone in the LD larvae

necessary for pupation and therefore prolonged their development, reinforcing the above results (Figures 1and 2).

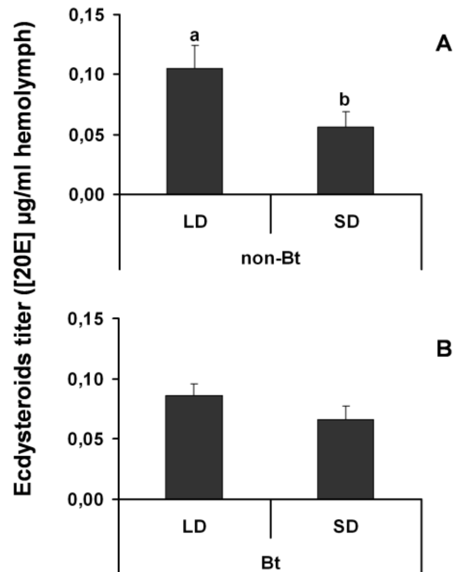

FIG. 4. Effect of Bt toxin in maize leaves on the concentration of 20-hydroxyecdisone in the larval hemolymph. Larvae were fed on isogenic non-Bt (A) or its corresponding Bt (B) maize and subjected to non-diapausing, long day (LD) or diapausing, short day (SD) photoperiod conditions. Different letters above the columns indicate differences between treatments.

Effect of Bt toxin in the diet on larvae of S. nonagrioides

Effects on the larval development

Because the effect of feeding *S. nonagrioides* larvae with Bt maize leaves was not detected in the larvae reared under SD photoperiod conditions in the previous experiments, the effect of Bt toxin in the diet was only tested in the larvae reared under LD photoperiod conditions. Figure 5 compares the mortality resulting from feeding *S. nonagrioides* larvae of different ages L4, L5 and L6 with different concentrations of Bt protein (0, 0.35, 0.9 and 2 mg/kg diet). As expected, younger larvae fed on a Bt diet showed higher mortality than older ones. The great majority of larvae died before any molt although some of them died after a larval molt. The mortality of all experimental groups of larvae of different ages and fed with different concentrations of Bt protein, less L6 larvae fed with the lowest concentration of Bt (0.35 mg/kg diet), was significantly higher than the mortality of control larvae (fed with diet without protein) ($P \leq 0.001$). In L4 or L6 larvae there were no differences in the mortality according to the concentration of protein in the diet (P=0.151 and P=0.229 respectively) but the mortality of L5 larvae fed with the highest concentration of Bt protein was higher than the mortality of those fed with the lowest concentration (P=0.002). Although there were no differences in the mortality between the larvae fed with Bt protein in L6, the mortality increased as the Bt protein concentration increased, and the highest concentration (2 mg/kg diet) caused death in 50% of the treated larvae (LC50).

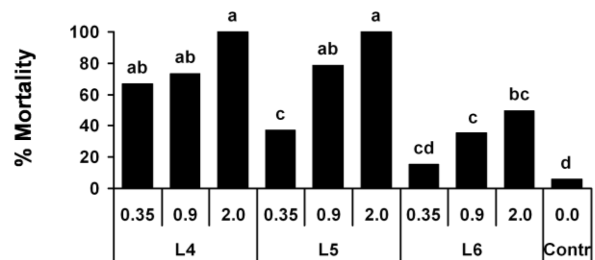

FIG. 5. Percentage of mortality in the larvae fed from the first day of the 4th, 5th or 6th instar (L4, L5, and L6 respectively) with diet with different concentrations (0, 0.35, 0.9 and 2 mg/kg) of Bt toxin. Different letters above the columns indicate differences in the total mortality resulting from the treatments.

All L4 and L5 larvae fed with 2 mg/kg of Bt protein died; the rest of the surviving L4, L5 and L6 larvae fed with different concentrations of Bt protein molted more times than the larvae fed on

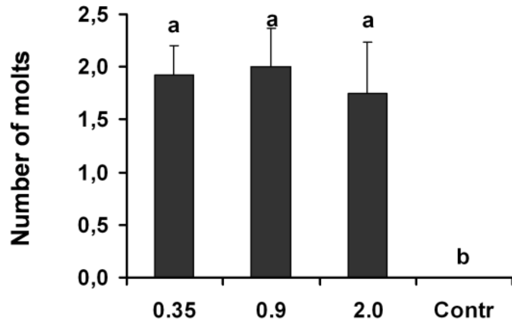

FIG. 6. Effect of different concentrations (0, 0.35, 0.9 and 2 mg/kg) of Bt protein in the diet on the number of supernumerary molts. For each concentration, results of larvae fed from L4d1, L5d1 and L6d1 with the Bt diet are pooled. Different letters above the columns indicate differences in the number of supernumerary molts.

untreated control diet (F=13.86, P≤0.001, d.f.=3; 36), and showed at least 1.5 supernumerary molts after L6 instar, while all the larvae fed with control diet pupated without any supernumerary molt (Figure 6). The age of the larvae (L4, L5 or L6) did not influence the number of supernumerary molts (F=0.56, P=0.5748, d.f.=2; 36).

Table 1 shows the days that the larvae of the different ages fed with Bt toxin diet needed to reach a first molt to larva, the percentage that

		days to molt	**% molt**	**days to pupa**
L4	**0.35 mg/Kg**	9.0±1.1 a	55.5 b	48.6±10.5 a
	0.9 mg/Kg	11.0±1.7 a	40.0 b	43.0±2.5 a
	2 mg/Kg	10.0± - a	11.1 b	-
	0 mg/kg Control	***5.0±0.69 b***	***100 a***	***11.0±0.7 b***
L5	**0.35 mg/Kg**	11.8±1.3 a	66.6 b	40.5±8.5 a
	0.9 mg/Kg	14.6±2.6 a	28.5 b	49.0± - a
	2 mg/Kg	-	-	-
	0 mg/kg Control	***5.2±0.37 b***	***100 a***	***10.6±0.4 b***
L6	**0.35 mg/Kg**	16.2±2.2 a	75.0 a	31.6±5.9 ab
	0.9 mg/Kg	17.0±1.6 a	55.5 a	48.0±9.2 a
	2 mg/Kg	21.2±2.6 a	66.6 a	54.0± - a
	0 mg/kg Control	***-***	***-***	***11.0±0.33 b***

TABLE 1. Days (average ± standard error) that the larvae of the different ages fed with different concentrations Bt toxin in the diet needed to molt (first column), percentage that molted at least once before pupating (second column) and days (average ± standard error) that they needed to pupate from L6d1 (third column). Within each age, different letters in each column indicate significant differences in the number of days needed to molt or to pupate or in the percentage of larvae that molted before pupating.

molted at least once before pupation and the number of days from L6d1 needed to pupate. Statistical analysis was carried out within each instar. Feeding with Bt protein diet significantly lengthened the development of the L4 and L5 larvae. Instead of pupating, L6 larvae fed with a Bt diet molted to larvae and the duration of this supernumerary larval instar was also longer than the duration of the last instar before pupating in the control larvae. Furthermore, feeding with the toxin increased the number of days needed to pupate in all instars and for all concentrations of protein that had not caused the total mortality of the larvae, except when L6 were fed with the lower Bt protein concentration diet (0.35 mg/kg diet). The mortality of the larvae before the first molt was the reason for the low percentage of molts, especially in diets with the highest concentration of Bt protein.

Effect on the concentration of JHII in the larval hemolymph

As there were no differences in the JH titer in the hemolymph between the larvae fed from the first day of the fifth (L5d1) or sixth (L6d1) instar with diet with different Bt protein concentrations (F=0.07, P=0.7965, d.f.=1; 21), Figure 7 shows the concentration of JH in the hemolymph on the fifth day of the sixth instar (L6d5) of those larvae fed with different concentrations of the Bt toxin from L5d1 or L6d1 pooled. All larvae fed with different concentrations of Bt protein showed a higher JH concentration in the hemolymph than control larvae (fed with no Bt protein) (F=6.26, P=0.0033, d.f.=3; 21).

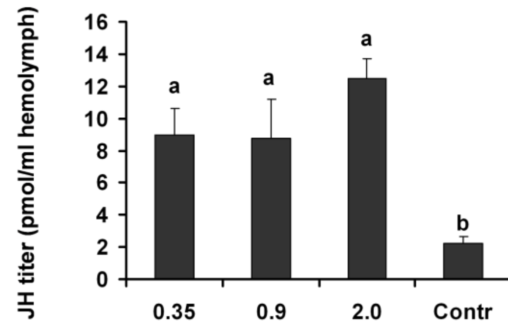

FIG. 7. Titer of JH in the hemolymph on the fifth day of the sixth instar (L6d5) of larvae fed with different concentrations of Bt toxin (0, 0.35, 0.9 and 2 mg/kg). For each concentration, results of larvae fed from L5d1 and L6d1 with the Bt diet are pooled. Different letters above the columns indicate differences in the JH titer in the larval hemolymph.

Discussion

Bt maize is highly efficient against corn borers when the larvae complete their development in the transgenic plants (Gonzalez-Cabrera *et al*., 2006; Huang *et al*., 2006). However, it is possible that some of the larvae that feed on Bt plants for only part of their development or that ingest sub-lethal amounts of Bt toxin may survive. The different ways in which larvae feeding on Bt plants ingest sub-lethal amounts of the toxin has been one of the main subjects of research on the effect of the toxin on development and the consequences for Bt-resistance management, not only in Bt maize but also in Bt cotton (Lopez *et al*., 2010). The ingestion of sub-lethal amounts of Bt toxin in the field may occur in different ways: the surviving larvae that have developed on a Bt plant may move to another plant that does not express the toxin and complete their development (Li *et al*., 2006); some plants express a lower level of toxin (Horner *et al*., 2003; Nguyen & Jehle, 2007); the larvae may feed on a part of the plant with lower toxin expression than other parts (Basavaraja *et al*., 2007); and the

expression of the toxin may decrease at the end of the crop season (Li *et al*., 2007a). Several authors have demonstrated that some phytophagous insects can discriminate between plants or diets with or without Bt toxin, thus avoiding the food with the toxin (Bowling *et al*., 2007; Chen *et al*., 2008; Li *et al*., 2007b; Lei *et al*., 2009) or they ingest a lower amount of food with the toxin (Li *et al*., 2006). Moreover, the susceptibility of the larvae to the Bt toxin decreases at older ages, so amounts of toxin that produce total mortality of young larvae may only be sublethal for developed larvae (Henneberry *et al*., 2001).

S. nonagrioides overwinters as developed diapausing larvae in the stubbles of maize (Alfaro, 1972). Diapausing larvae (SD) have a higher level of JH in the hemolymph than non-diapausing larvae (LD) (Eizaguirre *et al*., 1998). Previous studies have demonstrated that larvae fed on sublethal amounts of commercial Bt toxin (Dipel) in the diet anticipate diapause induction (Eizaguirre *et al*., 2005a) in a similar way to the effect produced by JHA ingestion (Eizaguirre *et al*., 2005b). Since other studies have demonstrated that some larvae move from the plant where they were born to the adjacent ones (Eizaguirre *et al*., 2004), the possibility that in some circumstances larvae developed in non-Bt maize moved to a close Bt maize ingesting sublethal amounts of the Bt protein was considered. Therefore, it was decided to study how feeding on sub-lethal amounts of the Bt toxin may affect the development and the hormonal balance of larvae with a high level of JH in the hemolymph (the case of diapausing larvae) or a low level of JH in the hemolymph (the case of non-diapausing larvae).

The effect of the Bt provided in diet has been similar, although stronger, to the Bt provided in maize leaves. Ingestion of sub-lethal amounts of Cry1Ab protein in diet or leaves has produced a prolonged development in mature and younger larvae under LD conditions. The extension of the development time is one of the most common effects studied in all the works done with larvae fed on different GM crops: maize (Dutton *et al*., 2005; Huang *et al*., 2006; Chilcutt *et al*., 2007; Obonyo *et al*., 2008), cotton (Muhammad *et al*., 2009), rice (Cai *et al*., 2008), and potatoes (Hussein *et al*., 2005). In *S. nonagrioides* this extended development time has been accompanied by an increase in the number of molts that the larvae have suffered, so the larvae needed more instars to pupate as occurs when a JH analog is applied to the larvae (Eizaguirre *et al* 2005b). This effect, molting increase, has been studied little in relation to Bt plant ingestion but it has been studied widely in relation to the ingestion of sub-lethal amounts of several insecticides that produce extended development (Wang *et al*., 2009) and increased number of molts (Reynolds *et al*., 2009). In the case of diapausing larvae, although the number of molts increased due to feeding on Bt toxin, the duration of development did not increase. In a similar way to other species (Giles *et al*., 2000; Storer *et al*., 2001; Binning & Rise, 2002; Huang *et al*., 2006; Wu *et al*., 2009), mortality of *S. nonagrioides* mature larvae is lower than that of younger ones, so the LC50 for developed larvae produces 100% mortality in the L4 larvae. This fact may also be related, according

to Keller *et al*. (1996), to an increase in the proteolytic activity in the developed larvae.

Ingestion of Bt toxin increased the level of JH in the hemolymph of non-diapausing *S. nonagrioides* larvae fed with Bt maize leaves or with the highest amount of Bt protein in the diet. This effect of the Bt protein may be the cause of the extended larval developmental time and the increase in molts in non-diapausing larvae, while in diapausing larvae the high titer of JH in the hemolymph may have masked the effect of the Bt toxin. Thus, if JH is present during a critical period, no developmental switch takes places, and the current developmental state is maintained (Nijhout, 1994) as also occurred with the JH analog application to the *S. nonagriodes* larvae (Eizaguirre *et al* 2005b). The JH level increase could be due to several factors that should be studied more thoroughly, but it could be considered as a response to the stress caused by the ingestion of the toxin. A similar effect was observed when the ecdysteroid agonist RH2485 was added to the diet of *S. nonagrioides* the level of JH in the hemolymph and the number of molts increased (Eizaguirre *et al*., 2007) and when *Manduca sexta* L. was fed with bisacylhydrazunes (RH5849) or tebufenozide (RH5992) (Reynolds *et al*., 2009). The increase in the JH level could also be due to the stress produced by the reduced feeding of larvae on Bt diets (Prutz & Dettner, 2005; Rao & Rao, 2008), which results in lower pupal weight than that of larvae fed on non-Bt diet and than larval starvation, as pointed out by Riddiford (1980), Cymborowski *et al*. (1982), and Tauchman *et al.* (2007). This increase in the concentration of JH in the hemolymph could also explain the extended developmental time of some parasitoids that feed on caterpillars fed on sublethal amounts of Bt toxin and show no traces of Bt in their hemolymph (Sharma *et al*., 2008). On the other hand, some parasitoids induce their host to increase the JH level in the hemolymph as a response to parasitization, which could also be considered as a stress factor for the host (Schafellner *et al*., 2004).

Preceded by an increase of ecdysteroids, the developed L6d7 non-diapausing *S. nonagrioides* larvae will pupate in few days while the diapausing larvae, without that ecdysteroids increase, will molt to another larval instar. These differences were reflected in the hemolymph titer of ecdysteroids in the larvae fed with non-Bt maize, which was higher in non-diapausing than in diapausing larvae (Eizaguirre *et al*., 2007). However, when larvae were fed with Bt maize the differences disappeared and the titer of ecdysteroids did not increase in the non-diapausing larvae, thus delaying pupation. Although Chen & Gu (2006) report that the production of ecdysteroids can be increased in response to external stress factors such as starvation, Adel & Abdel-Hakim (2007) and Josephrajkumar *et al*. (1999) report that ingestion of different toxins or viruses by several Lepidoptera larvae (Palli *et al*., 2000) resulted in decreased ecdysteroid titer. As observed in the present results, the increase in JH concentration together with the decrease in ecdysteroid concentration favors longer larval development and a higher number of larval molts, and may allow some developed larvae to recover from the ingestion of the toxin as occurs with *H. zea* and *H. virescens* (Ali & Lutrell, 2009) and to pupate to produce viable adults. This finding

could have important implications for resistance management. In this regard, Lopez *et al*. (2010) showed for *O. nubilalis* that greater larval delays of resistant larvae feeding on Bt maize could lead to temporal isolation from adults emerging from refuge maize (Peck *et al*., 1999), as has also been noted for *S. nonagrioides* (Eizaguirre *et al*., 2005a) and for the western corn rootworm (*Diabrotica virgifera virgifera* Le Conte) when adult emergence timing from separate non-Bt refuges and refuges within a seed mixture are compared (Murphy *et al*. 2010).

In summary, implication of the hormonal system in the prolonged development of *S. nonagrioides* larvae fed on sublethal amounts of *B. thuringensis,* as it had been hypothesized in a previous work, has been confirmed. Non-diapausing developed larvae of *S. nonagrioides* that survive feeding on Bt maize increase their JH level maintaining low their ecdysteroid levels, resulting in longer larval development and more larval molts. This response may be considered as a defense mechanism that allows some larvae to survive the toxin; and it is similar to the response to insecticidal toxins or viruses that has been observed in other species and cannot be specifically attributed to Bt toxins. Diapausing developed larvae have a higher level of JH in the hemolymph and maintain a low ecdysteroids level sufficient to molt but not for pupating, so possible changes in hormone levels are masked and undetectable.

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CHAPTER 5

5. General discussion

GENERAL DISCUSSION

Sesamia nonagrioides larvae can undergo larval development, pupation, or diapause. The rate of larval development, which proceeds until the 6th instar (in 20% of insects only until the 5th instar) and is characterized by larval molts in short intervals (4–5 days in the 5th instar), is independent of the photoperiod that determines development programming in the 6th and later instars. While the larvae developing under long day (LD) conditions pupate in the 6th instar (L6), those kept continuously under short day (SD) conditions enter diapause characterized by larval molts in longer intervals. The results of this thesis highlight the role of brain in the control of larval development in *S. nonagrioides*. Thus, *S. nonagrioides* is the first lepidopteran found to develop from larvae to adult without brain. From a general point of view, insect molting and metamorphosis are governed by the ecdysteroids and juvenile hormone (JH) orchestrating the molting process and JH determining the nature of the molt (Riddiford *et al*., 2003). Ecdysteroids synthesis at this time is stimulated by a brain neurosecretion that was identified as prothoracicotropic hormone PTTH (Bollenbacher & Granger, 1985; Kataoka *et al*., 1987). However, our data show that the molts are dependent on the release of ecdysteroids by prothoracic glands (PGs) but in *S. nonagrioides* larvae they can occur without PTTH from the brain.

While all decapitated larvae pupate the debrained larvae often undergo several successive molts; the first molt could be to larva or pupa, depending on the age at which the larva has been debrained and on the photoperiod under which the larvae have developed: many L6 larvae debrained 1 day after the molt, molted to larvae independently of the photoperiodic conditions of development, LD or SD, but 5 days later all larvae developed under LD conditions pupated but some larvae of the same age developed under SD conditions molted to larvae. Brain implants slightly accelerate the pupal molts but do not altered the timing of larval molts. We did not know if PG activation in the brain absence was autonomous or was controlled by other hormones than the brain-derived PTTH. PGs stimulation by the suboesophageal and inhibition by the thoracic ganglia was inferred from the surgical experiments in *Galeria mellonella* larvae (Malá *et al.*, 1977) and recently proved by the isolation of regulatory substances in *Bombix mori*.

The JH measurements and the development of the debrained and decapitated larvae confirmed that JH secretion from corpora allata (CA), absent in the decapitated larvae, is indispensable for the larval development and that *S. nonagrioides* do not show any alternative source of JH II to the CA. Decapitated larvae did not show noticeable amounts of JH while debrained larvae showed detectable concentrations of JH still 10 days after the surgical manipulation due to the CA activity or to a low rate of JH degradation by JH esterase (Schaffelner *et al*., 2008). HJ concentration was greatly reduced in the larvae that were manipulated the fifth day of L6, showing that the brain implants failed to activate the CA. The activity of CA is governed by allatropins and allatostatins factors from the brain which stimulate and inhibit it respectively and which are perfectly conserved among all Lepidoptera species (Abdel-Latief *et al*.,

2003; Li *et al*., 2005). Our results confirm that *S. nonagrioides* might have both allatostatics and allatotropics brain factors but implanted brains do not activate the CA of the host by humoral action, apparently the activity of these factors must be neural. The brain might be responsible for larval stage maintenance by neural inhibition of pupation; when the larvae of any age were deprived of their brain, the majority pupated. In the same way, the brain might be also responsible of diapause maintenance by neural inhibition of pupation; consequently, when the larvae were deprived of their brain (maintaining or not their CA) differences between diapausing and non-diapausing larvae disappeared. The importance of JH in larval maintenance (Eizaguirre *et al.*, 2005) was confirmed applying a JH analog to decapitated larvae, thus these larvae instead of pupate molted often to another larval instar.

Decapitated larvae showed a detectable level of ecdysteroids, which increased ten days after manipulation, approximately the time needed to pupate in these larvae. Decapitated larvae lack of brain and the CA, source of JH which prevent metamorphosis (Goodman & Granger, 2008), thus the PGs were strongly activated al low levels of JH in the hemolymph. The removal of PGs by body ligation applied across mesothorax prevented molting, proving that the presence of PGs is essential for the molting process. The PGs of *S. nonagrioides* larvae can function without brain stimulation and the PTTH could be derivate from a source outside the head, in this sense some authors have identified several PG stimulators other than PTTH (Marchal *et al*., 2010). In *S. nonagriode*s we identified an alternative PTTH source in the gut. For this, the PTTH mRNA was identified and using PCR with primers designed on Lepidoptera-conserved PTTH sequences it was amplified 387 nt of a cDNA, which showed 48-85% homology with the matching regions of PTTH known from other Lepidoptera. The qPCR corfirmed that the PTTH gene of *S. nonagrioides* is strongly expressed in the brain of the 6th instar with a maximum on day 5 and a minimum in prepupa, but the level of PTTH expression was also detected in the gut of intact and even more in decapitated larvae with a maximum expression in prepupa. The rate of PTTH gene expression in the gut seems to increase after decapitation and renders the whole system independent of the brain presence.

Most decapitated larvae molt to pupa with any sign of adult development while the majority of debrained pupae suffer metamorphosis to adult thus suggesting that the pupal-adult transformation did not depend on the brain but on an unknown factor that was present in the debrained and not in the decapitated larvae. On this basis, the influence of exogenous HJ and ecdysteroids on pupa of decapitated larvae was studied. Previous works in Lepidoptera signaled that CA had to be inactive for allowing the adult differentiation (Williams, 1961; Safranek & Williams, 1987; Shu *et al*., 1997). However, in *S. nonagrioides* JH applied topically not only did not inhibit the pupal-adult metamorphosis but could have favored it while the application of an ecdysteroids agonist to the pupa had no effect on the adult development. The lack of effect of the ecdysteroid agonist treatment could be due to that the PGs are not active during the pupal-adult metamorphosis (Bodnaryk, 1986) or because decapitated pupae (keeping their

prothoracic glands) already produced enough ecdysteroids and the application did not increase their effect.

The last part of this thesis studies the response of an stress on the main hormonal centers; the possible mechanism of adaptation by these responses were considered, as well as some mechanisms protecting the ontogenetic program from disturbances caused by a stress-induced release of hormones (Chernysh, 1991). Stress applied in this experiment was provide by feeding the *S. nonagrioides* larvae with sublethal concentrations of *Bacillus thuringiensis* (Bt) in a similar way that can occur in the field in transgenic corn. Ingestion of sub-lethal amounts of Cry1Ab protein in maize leaves or diet produced a prolonged development accompanied by an increase in the number of molts before pupating only in the larvae reared under LD conditions but no in the larvae reared under SD conditions. These results are due to an increase of the level of JH in the hemolymph of non-diapausing larvae fed with Bt maize leaves or with Bt protein in the diet; while in diapausing larvae the possible small increase of JH due to the Bt toxin ingested may have been masked by the normal high level of JH of these larvae. However, the effect of Bt toxin on the ecdysteroids titer was to suppress the increase of the hormone necessary for pupation (Eizaguirre *et al*., 2007) in non-diapausing larvae thus delaying pupation in the treated larvae. These responses may be considered as a defense mechanism that allows some larvae to molt and to survive the toxin and it is similar to the response to insecticidal toxins or viruses that has been observed in other species (Josephrajkumar *et al*., 1999; Palli *et al.,* 2000; Adel & Abdel-Hakim,2007; Reynolds *et al*., 2009).

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CHAPTER 6

6. Conclusions

CONCLUSIONS

Role of the brain in larval development of S. nonagrioides

- Diapause programming maintenance requires persistent short day conditions.
- Brain is not necessary for larval-pupal and pupal-adult molts.
- An unknown head factor outside the brain, present in the debrained and not in the decapitated larvae, is needed for pupal-adult molt.
- Brain is necessary for the diapause maintenance.
- Prothoracic glands can function without brain prothoracicotropic hormone release.
- Prothoracic glands are necessary for molting.
- There is no alternative source of juvenile hormone other than the corpora allata.
- The maintenance of juvenile hormone 10 days after depriving the larvae of the brain could be due to the corpora allata activity or to the lack of juvenile hormone degradation.
- A rapid increase in the activity of the prothoracic glands in decapitated larvae could be the response to a prolonged lack of juvenile hormone titer in the hemolymph.
- Juvenile hormone does not inhibit the pupal-adult metamorphosis rather it facilitates it.

PTTH hormone in S. nonagrioides

- The developmental pattern of ecdysteroids secretion does not require a regulator from the head because larvae whether decapitated at the start of the 4th, 5th or 6th instar always molt to pupae in about 10 days.
- Prothoracicotropic hormone of *S. nonagrioides* larvae has been isolated and sequenced.
- Prothoracicotropic hormone expression has been detected not only in the brain but also, and for the first time in a Lepidopteran gut.
- The prothoracicotropic hormone in the gut follows in different developmental pattern than the brain.

Sub-lethal effects of Bt toxin on the larval development of S. nonagrioides

- Feeding Bt toxin, in whether in the diet or maize, increases the juvenile hormone titer and suppresses the increase in ecdysteroids levels in the hemolymph of non-diapausing larvae.
- The effects of the feeding Bt toxin to diapausing larvae are undetectable probably because these larvae maintain a high level of juvenile hormone and sufficient ecdysteroids to molt than masked them.
- The variation of juvenile hormone and ecdysteroids levels in non-diapause larvae prolongs development and increases the number of molts and can be related to a defense mechanism against the toxin.

CONCLUSIONES

El papel del cerebro en el desarrollo larval de S. nonagrioides

- El mantenimiento de diapausa requiere una persistencia en condiciones de día corto.
- El cerebro no es necesario para que se produzca una muda de larva a pupa o de pupa a adulto.
- Un factor desconocido de la cabeza, presente en las larvas descerebradas y no en las larvas decapitadas, es necesario para la muda de pupa a adulto.
- El cerebro es necesario para mantener la diapausa.
- Las glándulas protorácicas pueden funcionar sin hormona protoracicotrópica proveniente del cerebro.
- Las glándulas protorácicas son necesarias para que se produzca cualquier tipo de muda.
- No existe otra fuente de hormona juvenil alternativa a los corpora allata.
- El mantenimiento de hormona juvenil 10 días después de privar a las larvas del cerebro podría ser debido a una continua actividad de los corpora allata o a una ausencia de degradación de la hormona juvenil.
- Un rápido incremento en la actividad de las glándulas protorácicas de las larvas decapitadas podría ser una respuesta a una prolongada ausencia de concentración de hormona juvenil en la hemolinfa.
- La hormona juvenil no inhibe la metamorfosis de pupa a adulto sino que la favorece.

Hormona protoracicotrópica en S. nonagrioides

- Las glándulas protorácicas no requieren ningún regulador que provenga de la cabeza porque las larvas decapitadas al inicio del 4º, 5º o 6º estadio sufrieron una muda a pupa en unos 10 días.
- La hormona protoracicotrópica fue aislada y secuenciada.
- La expresión de la hormona protoracicotrópica se ha detectado no sólo en el cerebro, sino también y por primera vez en el tubo digestivo de un Lepidóptero.
- La hormona protoracicotrópica en el tubo digestivo sigue un modelo diferente de desarrollo que en el cerebro.

Efectos subletales de la toxina Bt sobre el desarrollo larval de S. nonagrioides

- La alimentación con toxina Bt en hoja de maíz o en dieta aumenta la concentración de hormona juvenil y suprime el aumento de ecdisteroides en la hemolinfa de las larvas no diapausantes.
- Los efectos de la alimentación con toxina Bt en las larvas diapausantes son indetectables, probablemente porque estas larvas mantienen una elevada concentración de hormona juvenil y suficiente concentración de ecdisteroides en la hemolinfa que los pueden enmascarar.
- La variación en la concentración de hormona juvenil y ecdisteroides en las larvas no diapausantes prolonga su desarrollo y aumenta el número de mudas de larvas desarrolladas, y puede estar relacionado como un mecanismo de defensa contra la toxina.

CONCLUSIONS

El paper del cervell en el desenvolupament larval de S. nonagrioides

- El manteniment de diapausa requereix una persistència en condicions de dia curt.
- El cervell no és necessari perquè es produeixi una muda de larva a pupa o de pupa a adult.
- Un factor desconegut del cap, present a les larves sense cervell i no en les larves decapitades, és necessari per a la muda de pupa a adult.
- El cervell és necessari per mantenir la diapausa.
- Les glàndules protoràciques poden funcionar sense hormona protoracicotròpica provinent del cervell.
- Les glàndules protoràciques són necessàries perquè es produeixi qualsevol tipus de muda.
- No hi ha una altra font d'hormona juvenil alternativa als corpora allata.
- El manteniment d'hormona juvenil 10 dies després de privar a les larves del cervell podria ser causa d'una contínua activitat dels corpora allata o una absència de degradació de l'hormona juvenil.
- Un ràpid increment en l'activitat de les glàndules protoràciques de les larves decapitades podria ser una resposta a una prolongada absència de concentració d'hormona juvenil en la hemolinfa.
- L'hormona juvenil no inhibeix la metamorfosi de pupa a adult sinó que la afavoreix.

Hormona protoracicotròpica a S. nonagrioides

- Les glàndules protoràciques no requereix cap regulador que provingui del cap perquè les larves decapitades a l'inici del 4t, 5è o 6è estadi van patir una muda a pupa en uns 10 dies.
- L'hormona protoracicotròpica va ser aïllada i seqüenciada.
- L'expressió de l'hormona protoracicotròpica s'ha detectat no només en el cervell, sinó també i per primera vegada al tub digestiu d’un Lepidòpter.
- L'hormona protoracicotròpica en el tub digestiu segueix un model diferent de desenvolupament que al cervell.

Efectes subletals de la toxina Bt sobre el desenvolupament larval de S.nonagrioides

- L'alimentació amb toxina Bt en fulla de panís o en dieta augmenta la concentració d'hormona juvenil i suprimeix l'augment d'ecdisteroids en l'hemolimfa de les larves no diapausants.

- Els efectes de l'alimentació amb toxina Bt en les larves diapausants són indetectables, probablement perquè aquestes larves mantenen una elevada concentració d'hormona juvenil i suficient concentració de d'ecdisteroids en l'hemolimfa que els poden emmascarar.

- La variació en la concentració d'hormona juvenil i ecdisteroids en les larves no diapausants prolonga el seu desenvolupament i augmenta el nombre de mudes de larves desenvolupades, i pot estar relacionat com un mecanisme de defensa contra la toxina.