Eur. J. Entomol. **110**(1): 39–45, 2013
http://www.eje.cz/pdfs/110/1/39
ISSN 1210-5759 (print), 1802-8829 (online)

Expression of cytochrome P450 CYP6B6 in the different developmental stages of the insect *Helicoverpa armigera* (Lepidoptera: Noctuidae)

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Key words. Lepidoptera, Noctuidae, *Helicoverpa armigera*, developmental stage, CYP6B6 expression

Abstract. In order to determine when during the development of *H. armigera* CYP6B6 is expressed, we measured the width of the head capsule, the length of the body and weight of the different life stages of the cotton bollworm reared under laboratory condition. The larvae took about 20 d to complete their development under these conditions, the pre-pupae about 4 d, pupae about 9 d, and the adults lived for about 9 d and the eggs took about 4 d to hatch. Knowing this it was possible to determine the pattern of cytochrome P450 *CYP6B6* expression at different stages in the life cycle of *H. armigera* using real-time quantitative polymerase chain reaction (RT-qPCR). The *CYP6B6* gene was expressed in all the developmental stages of the cotton bollworm. The results indicate that the level of expression was lower in the egg, pre-pupal, pupal and adult stages than in larvae. The level of expression of *CYP6B6* decreased from the 1st to the 3rd instar larva, with the lowest level recorded in the 3rd instar larvae and then increased reaching its highest value in 6th instar larvae. A similar trend in the expression of CYP6B6 was detected using immunohistochemistry. As the results show, more efficient control of the cotton bollworm can be achieved if insecticides are applied when the larvae of *H. armiger* are in the 3rd instar, because at that stage the eat little food and there is a low level of detoxifying enzymes in the larvae.

INTRODUCTION

Helicoverpa armigera (Lepidoptera: Noctuidae) is a major insect pest of a wide range of agricultural and commercial crops in many parts of the world, including China. The persistence of this pest in modern agriculture is mainly attributed to its resistance to commonly applied insecticides and ability to feed on a wide array of host plants (Tan et al., 1996; Forrester et al., 1993; Qiu et al., 2003). In insects, enzymatic detoxification of plant toxins and insecticides is a well documented, common and important mechanism of host plant adaptability and insecticide resistance (Forrester et al., 1993; Wu et al., 1997; Li et al., 2002; Yang et al., 2004; Chen et al., 2005). Among the insect detoxification systems, cytochrome P450 monooxygenases are the most prominent. The cytochrome P450s play many roles in insects, including synthesis of hormones and pheromones, fatty acid metabolism, and degradation of host plant toxins and insecticides (Feyereisen, 2005). An increase in insecticide detoxification by P450s and other enzymes is a common mechanism of insecticide resistance.

Inducibility is a general characteristic of cytochrome P450 (Harrison et al., 2001). Induction of cytochrome P450 genes by chemicals such as allelochemicals and some insecticides, is well documented (Feyereisen, 2005). There is a link between exogenous compounds acting as inducers and the induced enzymes metabolizing them in insect detoxification systems (Willoughby et al., 2006). Previous studies focused on the tissue distribution, developmental expression of P450 monoxygenases as a whole, their interactions with xenobiotic compounds and insecticide resistance (Qiu et al., 2000, 2001; Li et al., 2002). For example, the p-nitroanisole O-demethylase activity of P450s and the expression of *CYP6B6* mRNA induced by two plant secondary substances, 2-tridecanone and quercetin, have been investigated in the cotton bollworm, *H. armigera*. The O-demethylase activity is higher in the fat body than in the midgut of 6th instar larvae fed on artificial diets mixed with these allelochemicals. Similar results were obtained when induced by a combination of 2-tridecanone and quercetin at different concentrations (Liu et al., 2006). In *H. armigera* there are several mechanisms of resistance to pyrethroids (Ahmad et al., 1989; Gunning et al., 1991; McCaffery et al., 1997), but the characterization of individual cytochrome P450 in this insect is difficult due to the genetic diversity, broad substrate specificity and catalytic versatility of cytochrome P450s. However, molecular and immunology approaches have enabled investigators to study the expression profile of individual P450s in insects.

In the present study we determined the level of expression of *CYP6B6* and the distribution of CYP6B6 protein in the tissues of the different developmental stages of the cotton bollworm. This was done after determining the duration of each of its life stages. This study also provides evidence that it is best to apply pesticides to control the cotton bollworm when it is in the 3rd instar, because little food is ingested during this instar and the level of expression of detoxifying enzymes is low.

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MATERIAL AND METHODS

Insect

The laboratory strain of *H. armigera* came from China Agricultural University and was reared on an artificial diet at 28 ± 1°C, 70–80% relative humidity, under a photoperiod of 16L : 8D. Adults were kept under the same temperature and light conditions, and provided with a 10% honey solution. Composition of the artificial diet was: corn flour 300 g, soybeans powder 100 g, yeast extract powder 100 g, citric acid 2.5 g, vitamin C 10 g, sorbic acid 1.5 g, vitamin B 1.5 g, erythromycin 0.05 g, propionic acid 5 mL, vitamin E, water 2.5 L.

Reagents

The primary antibody was prepared by our research team, secondary antibody (goat anti-rabbit), diaminobenzidine (DAB) chromogenic kit were purchased from Zhangshan goldbridge in China, TRIzol reagent, real master mix SYBR green PCR kit from invitrogen in USA, DNase I, oligo(dT)18primer (50 mM), RNase M-MLV(RNase H-), RNase inhibitor, dNTP mixture (2.5 mM) from Takara Bio. Inc. in China, and all other reagents were of analytical quality and from Tianjin Fuyu Fine Chemical Co., Ltd.

Pattern of developmental of *H. armigera*

Daily measurements and observations were made on all stages from egg to adult, and the adults that emerged were transferred to a cage. During the larval period, the width of the head capsule of each larva (n = 180) was measured daily using a stereoscopic microscope (Nikon Instruments Inc. USA) and its body weight determined using an electronic balance (Mettler Toledo Inc. China). At the same time, the length of each larva was measured. The relationship between larval instars and developmental duration was analyzed using linear regression.

The relative expression of *CYP6B6* at different stages in the development

RNA isolation and cDNA synthesis

Total RNA from the different developmental stages of *H. armigera* was extracted using TRIzol according to the manufacture's instructions. The quality and concentration of RNA samples were determined using agarose gel electrophoresis and NanoDrop-1000 (Thermo Scientific Inc. USA). RNA was digested by DNase I in order to eliminate genomic DNA contamination. cDNA was synthesized by reverse transcription in 20 μL reaction system according to the manufacture's instructions. Three independent RNA preparations, representing three biological replicates, were used for the cDNA synthesis.

Real-time RT-PCR analysis

The levels of expression of the *CYP6B6* gene in the different developmental stages of *H. armigera* were quantified using a real-time quantitative polymerase chain reaction (RT-qPCR), a 7500 Real Time PCR System (Applied Biosystems, USA) and a real master mix SYBR Green PCR kit. The elongation β-actin was used as a reference gene to normalize the target gene expression levels recorded in the different samples. RT-PCR of each cDNA sample and template-free was performed in triplicate. All the primers sets used in this study were listed as follows. Specificity of the PCR amplification was checked by a melt curve analysis and by sequencing the PCR products. qTR-PCR was run in a 25 μL reaction system, which contained 12.5 μL of real master mix SYBR solution, 0.5 μL each of forward and reverse primers (10 μM), 1 μL of cDNA template and 10.5 μL of ddH_2O using the following cycling parameters: 95°C for 2 min, followed by 40 cycles of 95°C for 30 s, 62°C for 30 s and 72°C for 30 s. The relative expression levels of target genes were calculated using the comparative CT method. Primers of *CYP6B6* and β-actin (5'~3'): CYP6B6: F: TTCAAACTTATACCATGTCCACAATT, R: CCAATTGACGGAGCTCTAGAATCA; β-actin: F: ATCATCGACGCTCCCGGACA, R: TAGCTGCTTGACTCCGAGGGTG.

Presence and abundance of CYP6B6 protein in cross sections of the mid-gut of the different larval stages of *H. armigera*

The expression levels of CYP6B6 protein in the different larval atages were analyzed using an immunohistochemical method. The expression of CYP6B6 protein was determined in transverse sections of the body of 1st, 2nd and 3rd instar larvae, and transverse sections of the mid-gut of 4th, 5th and 6th instar larvae, after fixation, dehydration, embedding, sectioning, dewaxing, incubating with antibody and staining with DAB.

Statistical analysis

The relationship between larval instars and developmental durations was analyzed using linear regression. The number of instars was established graphically (Taylor, 1931) and confirmed using the confidence intervals of the measurements. The analysis was done using GraphPad Prism 4 software.

RESULTS

Larval period

Head capsule width, body length, body weight and duration of developmental of *H. armigera* are given in Table 1. The graphic method revealed six well defined frequency peaks at head capsule widths of 0.25, 0.41,

TABLE 1. Head capsule width, body length and body weight of *H. armigera*.

Developmental phase	Head capsule (mm) Mean ± SE	Length (mm) Mean ± SE	Weight (mg) Mean ± SE	Duration (d) Mean ± SE
1st larvae	0.25 ± 0.02	1.56 ± 0.24	1.1 ± 0.88	2.89 ± 0.65
2nd larvae	0.41 ± 0.02	3.88 ± 0.59	3.12 ± 1.76	3.23 ± 0.98
3rd larvae	0.63 ± 0.04	5.07 ± 0.93	8.33 ± 5.58	3.25 ± 0.98
4th larvae	1.06 ± 0.09	11.02 ± 2.57	13.58 ± 3.26	3.43 ± 0.91
5th larvae	1.6 ± 0.16	19.34 ± 3.6	19.32 ± 4.9	3.73 ± 0.69
6th larvae	2.53 ± 0.1	27.91 ± 3.9	28.59 ± 6.2	3.70 ± 0.50
prepupa			24.85 ± 2.28	3.89 ± 0.72
pupa			22.24 ± 3.34	8.92 ± 0.32
adult				9.21 ± 0.68
egg				3.80 ± 0.75

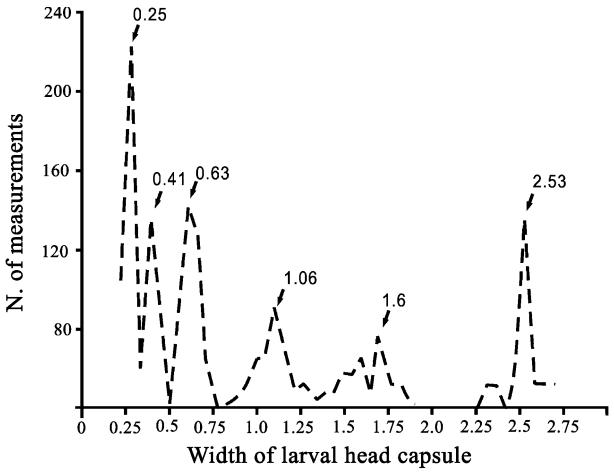

Fig. 1. Frequency distribution of head capsule widths of the different larval stages of *H. armigera* reared at 27 ± 1°C and 70–80% RH.

0.63, 1.06, 1.6 and 2.5 mm, indicating six larval instars (Fig. 1), in addition, the duration of development of 1st instar larvae was about 2.89 d and that of the other developmental stages about 3 d (Table 1). The period of time spent in each instar increased linearly was defined by the following relationship: $Y = 0.16X + 2.8$ ($R^2 = 0.93$), which indicates that the developmental time of each successive instar was 0.16 d longer than that of the previous instar. The total duration of development from the 1st to the 6th instar lasted about 20 d. We also observed that the dorsal surface of the larvae of *H. armigera* became more and more translucent and darker in colour and the crack in the middle of the head capsule increased in width in the latter instars (red arrows in Fig. 2). Similarly, the longitudinal line of the dorsum of the larvae became more and more translucent and darker in colour from the 1st instar larva to the prepupal stage (red arrows in Fig. 3) (Wu, 1977).

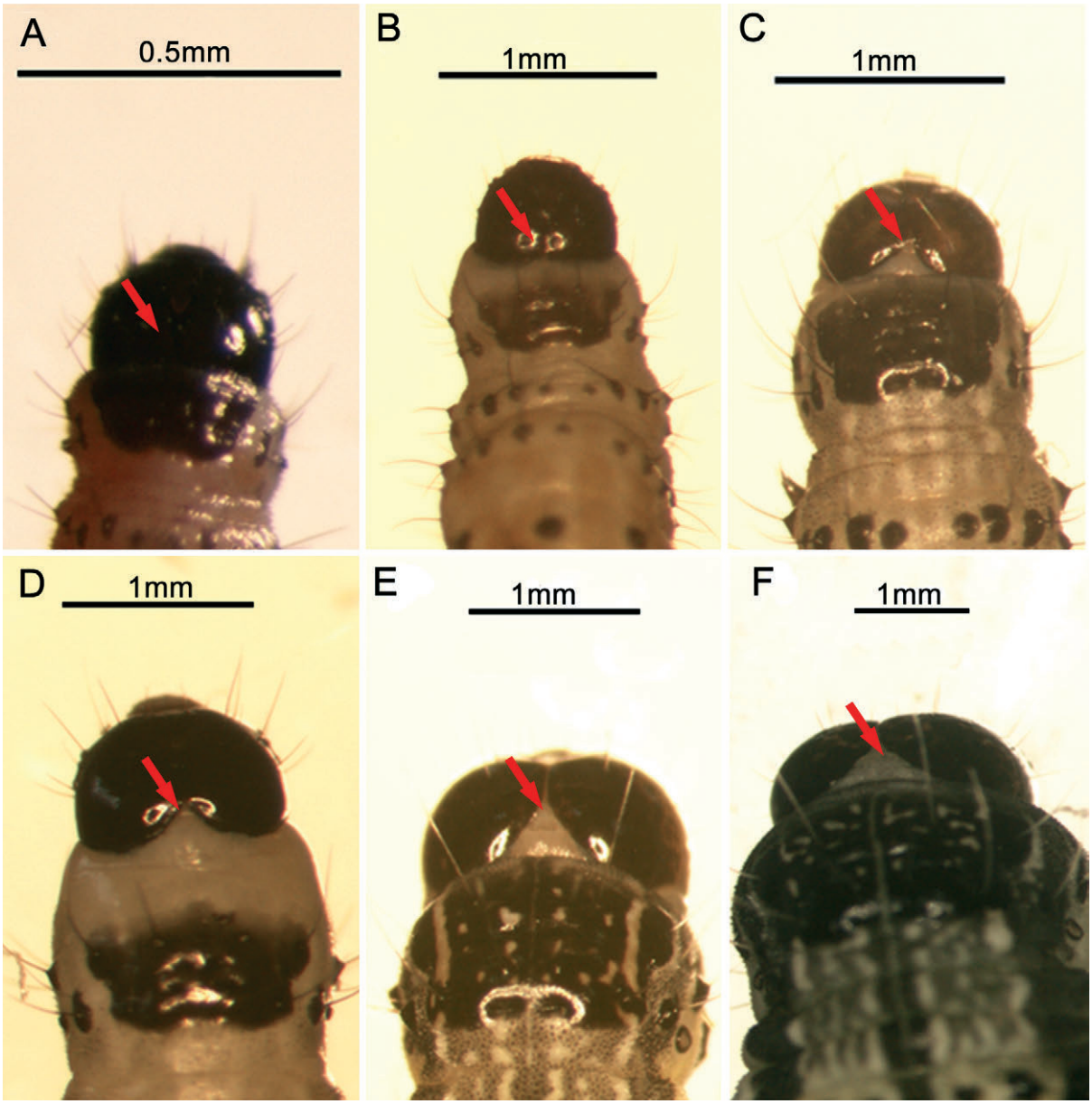

Fig. 2. Pictures illustrating the changes in head capsule width of *H. armigera* at different stages of development. For explanation of the red arrows see the corresponding text.

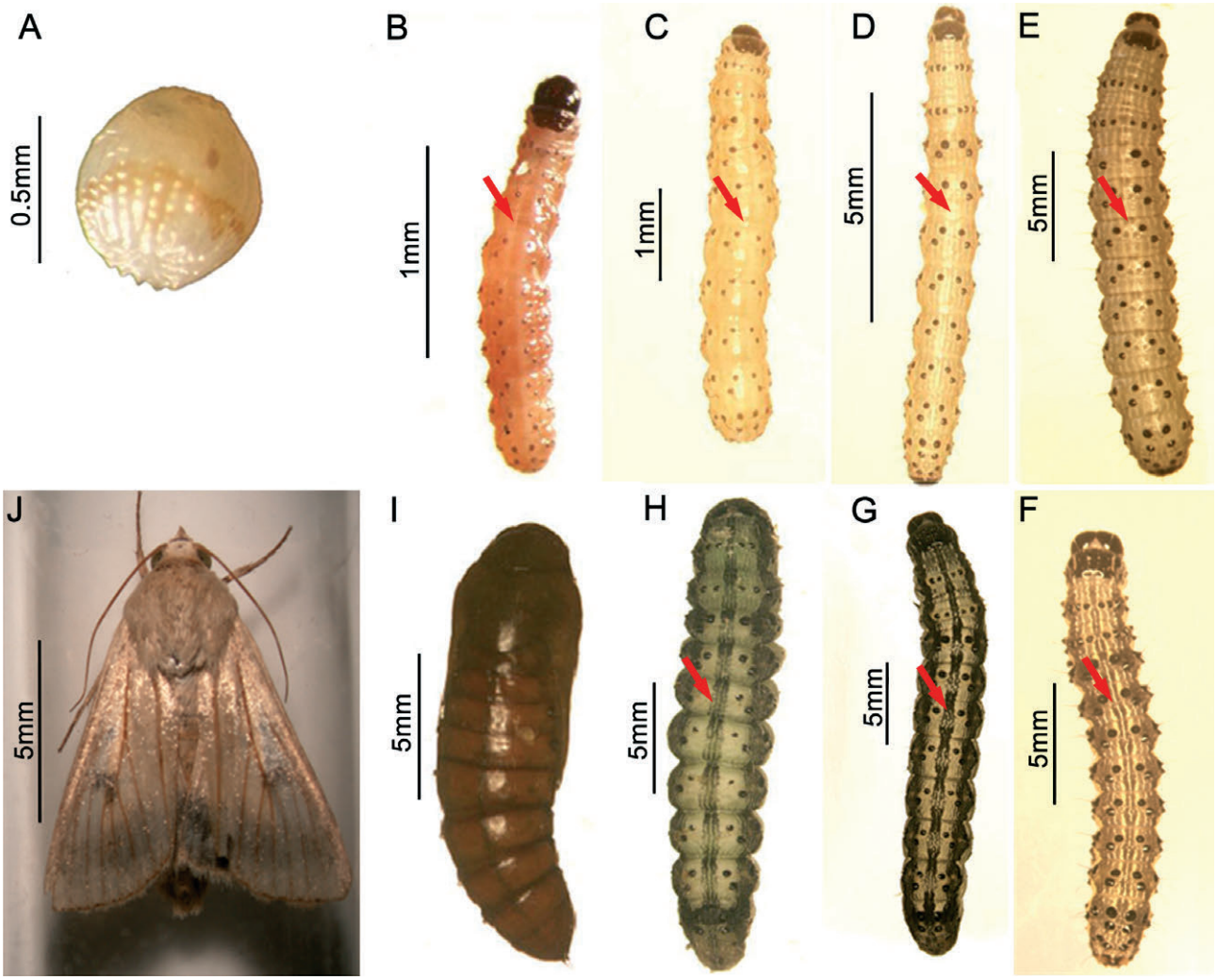

Fig. 3. The morphological features of *H. armigera* at different stages of development. A – egg; B–G – 1st to 6th instar active larvae; H – prepupa; I – pupa; J – adult. For explanation of the red arrows see the corresponding text.

The duration of prepupa, pupa, adult and egg

The duration of the prepupal period was 3.89 ± 0.72 d and the prepupa weighed 24.85 ± 2.28 mg. The duration of the pupal period was 8.92 ± 0.32 d and the pupa weighed 22.24 ± 3.34 mg. The adult lived for 9.21 ± 0.68 d and the eggs took 3.80 ± 0.75 d to hatch (Table 1).

Levels of expression of the *CYP6B6* gene in the different developmental stages

RNA was extracted from the different developmental stages of cotton bollworm using reverse transcription cDNA as a template, β-actin as a reference gene to calculate the relative level of expression of *CYP6B6* gene using $2^{-\Delta\Delta CT}$. The results show that *CYP6B6* was expressed in all the developmental stages of the cotton bollworm. From the 1st instar to the 3rd instar larvae there was a decrease in the level of expression of *CYP6B6*, reaching the lowest level in the 3rd instar larvae, and then increasing to a maximum in 6th instar larvae. The level of expression of *CYP6B6* was greater in the larval stages than in other developmental stages, in which the level was very low (Fig. 4).

Presence and abundance of CYP6B6 protein in cross sections of the mid-gut of larvae

The immunohistochemical reaction with the CYP6B6 antibody was mainly observed in larvae. CYP6B6 occurred mostly in the mid-gut and fat body (Fig. 5). The staining in the midgut became more intense as the larvae

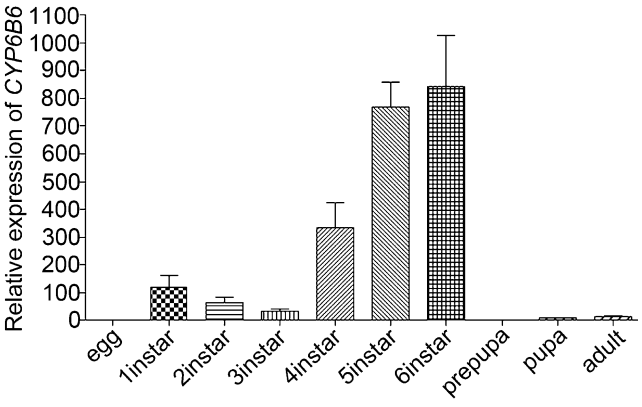

Fig. 4. The relative expression of *CYP6B6* in the different developmental stages of *H. armigera*.

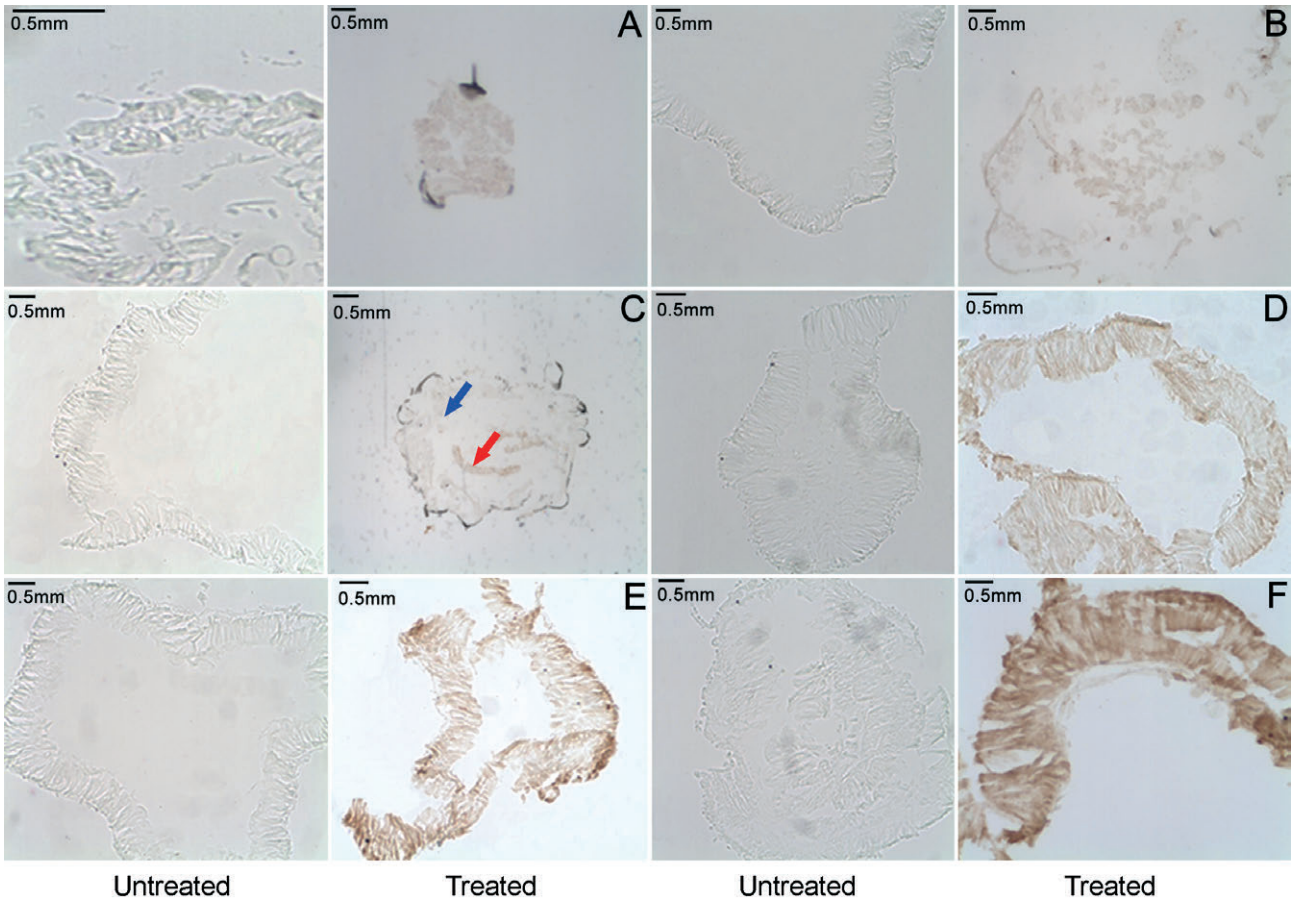

Fig. 5. Transverse sections of the mid-gut of larvae of *H. armigera* stained to show the presence and level of expression of cytochrome P450 CYP6B6. A–F – transverse sections through the mid-gut of 1st–6th instar larvae, respectively. Untreated group – not incubated with antibodies, treated group – incubated with antibodies. Red arrow indicates mid-gut and blue arrow fat body.

developed. From the 1st to 3rd instar larvae there was decrease in the amount of CYP6B6 protein reaching its lowest level in 3rd instar larvae, and then increasing to a maximum in 6th instar larvae, which is similar to the trend in *CYP6B6* mRNA expression.

DISCUSSION

The growth and development of cotton bollworm larvae is affected by a variety of factors, including temperature, humidity, photoperiod and host plant (Jiang et al., 1998; Hunter & McNeil, 1997; Liu et al., 2004). There is a big difference in the duration of development of larvae reared on an artificial diet or a host plant. The developmental time of immature stages ranged from 26.6 d to 35.1 d on six different host plants, cotton, tomato, hot pepper, tobacco and common bean (Liu et al., 2004). *H. armigera* larvae reared on different artificial foods take longer to complete their development. The lower amount of protein in artificial diets affects the growth and development of larvae and fecundity of adults (Wu & Li, 1993). In this study the larvae reared under laboratory conditions on an artificial diet took about 20 d to complete their development. The individual larval body lengths and weights recorded in each instar varied greatly whereas the head capsule widths did not (Table 1). Thus, the instar of individual larvae is more accurately identified using head capsule width than any of the other measurements.

Enzymatic and metabolic studies have long shown that insect cytochrome P450s play a major role in the detoxification of synthetic insecticides (Brattsten et al., 1986; Hodgson et al., 1995). The expression of cytochrome P450s is quite different in the different developmental stages or at different periods in the same developmental stage, and the enzyme activity and content are also different in the different developmental stages of *H. armigera*. Higher monooxygenase activity/content is usually associated with the mid-gut, fat body and malpighian tubules (Hodgson et al., 1983). In KQR and HDS strains of *H. armigera*, the content of cytochrome P450s and cytochrome b_5 increased from the 3rd to 6th instar larva, in which it was at the highest level and then decreased gradually with the lowest value recorded in the pupal stage. Similarly, the MROD (7-methoxyresorufin O-demethylation) activity of the cytochrome P450s increases from the 3rd to the 6th instar and is at its maximum in 6th instar larvae and then decreases with lowest activity occurring in pupae and moths (Qiu et al., 1999).

There is already evidence that the level of expression and degree of regulation of P450 enzymes is very variable even though individual insect P450s have not been

studied for very long (Scott et al., 1998). *CYP4D1* is expressed in all stages of development in *Drosophila* (Gandhi et al., 1992), while others, such as *CYP6Z1* is only expressed in the adults of *Anopheles gambiae* (Nikou et al., 2003) and *CYP6B2* only in the larvae of *Helicoverpa armigera* (Ranasinghe et al., 1997). Most of the insect P450s that have been studied are expressed in both sexes, although at least one, *CYP6L1*, is only expressed in male *Blattella germanica* (Wen & Scott, 2001) and *CYP312A1* in male *Drosophila melanogaste*r (Kasai & Tomita, 2003). Some P450s are found in only a few tissues, such as *CYP6L1* in the reproductive tissues of *Blattella germanica* (Wen & Scott, 2001), *CYP4C7* and *CYP15A1* in the corpora allata of *Diploptera punctata* (Sutherland et al., 1998; Helvig et al., 2004), *CYP4G15* in the central nervous system of *Drosophila melanogaste*r (Maîbèche-Coisine et al., 2000), while others are ubiquitous (*CYP6D1* in house flies) (Korytko & Scott, 1998). This study revealed that *CYP6B6* is expressed in all developmental stages and the levels of expression were higher in larvae than in other developmental stages. The levels of expression of *CYP6B6* in eggs, pre-pupae and pupae were very low, possibly because they derive little or no sustenance from the external environment. Adults were only fed 10% honey solution, in which there is little or no toxic secondary metabolites, which might account for the low level of *CYP6B6* recorded in adults.

The above results indicate that the artificial diet fed to the larvae not only affected their rate of development but also the level of expression of the *CYP6B6* gene. In addition there was a similar trend in the expression of CYP6B6 protein in the different larval stages with the lowest level recorded in 3rd instar and highest in 6th instar larvae. Therefore, in order to more effectively control cotton bollworm in the field it is important to determine when during its development CYP6B6 is expressed using the results of laboratory studies and predictions of its rate of development in the field.

ACKNOWLEDGEMENTS. This work was supported by the Science Foundation of Xinjiang Uygur Autonomous Region in China (2012211A018), Foundation of Student Research Training of Xinjiang University (XJU-SRT-Z11014) and the Natural Science Foundation of China (30960220).

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Received April 11, 2012; revised and accepted June 22, 2012