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ORIGINAL ARTICLE

Effects of 20-hydroxyecdysone on the development and morphology of the red flour beetle, *Tribolium castaneum* (Coleoptera: Tenebrionidae)

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Key words. Coleoptera, Tenebrionidae, *Tribolium castaneum*, 20-hydroxyecdysone, wing growth, α-amylase, prothetelic

Abstract. The red flour beetle, *Tribolium castaneum*, is a pest of stored products. It is also regarded as a model species for studying development, genetics, biology, physiology and biochemistry. Recently, it has become a model for use in RNA interference experiments. 20-hydroxyecdysone (20E) is involved in insect metamorphosis and its role in organ development in *T. castaneum* are based on hormonal treatment in conjunction with RNAi. However, information on the biological, morphological and physiological effects of 20E on *T. castaneum* is still limited. This study reveals the responses of *T. castaneum* larvae to injections with various doses of 20E (100, 200, 300, 400 and 500 ng/insect). The results show that larvae injected with 20E reached the prepupal, pupal and adult stages earlier than the control group. Different degrees of morphological change were observed in nine traits, including the appearance of pupal prothetelic organs in the larvae. Moreover, an injection of a high dose of 20E reduced the body weights of the resulting insects at each stage, as well as the length and width of elytra. The enzymatic activity of α-amylase in the resulting adults also decreased significantly. This indicates that injection of 20E caused precocious metamorphosis in *T. castaneum* by inducing changes in morphology and α-amylase activity, and the optimal concentrations that induce such phenomena were in the range of 100–200 ng/insect. Further investigations are needed to examine the roles of 20E in the regulation of α-amylase in *T. castaneum*.

INTRODUCTION

The red flour beetle *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) is an important pest of stored products, especially in tropical areas. However, it has also been used as a model species for development and genetic studies (Kingler, 2004). Recently, its genome was completely sequenced, resulting in *T. castaneum* becoming an important model for RNA interference experiments for exploring the functions of genes. There are numerous reports of the high efficiency of RNAi in *T. castaneum*, and it is thus regarded as a valuable system for studying any basic problem in insect biology and physiology.

The steroid hormone 20-hydroxyecdysone (20E) is a major developmental hormone in insects and is involved in various developmental transitions, including ecdysis and metamorphosis. This hormone functions by activating target genes in a stage-specific and tissue-specific manner. In the last larval instar, high levels of ecdysone initiate the transition to pupal development (Smagghe, 2009). There are many reports of using RNAi in conjunction with treatments of 20E or juvenile hormone to reveal the relationship between a gene of interest and its hormonal regulation. One such study reports that 20E regulates ovarian growth, oocyte maturation and the development of *T. castaneum* (Parthasarathy et al., 2010). Mid gut remodeling in *T. castaneum* is also regulated by 20E according to an RNAi experiment with the Broad gene (*TcBr*) (Parthasarathy et al., 2008).

There are many reports on the involvement of RNAi genes in the biosynthesis and action of 20E, but there is no information on the biological and morphological effects of 20E on *T. castaneum*. Accordingly, we examined the effects on development and morphology of various doses of 20E. The goal is to determine the proper doses for experiments examining the molecular bases of the effects of 20E on various biological events, such as growth, wing development and wing expansion. This study examines the effects of the injection of 20E during the larval period of *T. castaneum*. We report the effects of injecting various concentrations of 20E on the percentage of insects entering the prepupal, pupal and adult stages. Morphological changes were also recorded. There are reports that 20E and an insulin-like growth factor are the key extrinsic regulators of the growth and differentiation of the wing imaginal disc in

Lepidoptera and Diptera (Tobler & Nijhout, 2010). In addition, the length and width of adult elytra of *T. castaneum* were measured after injecting larvae with 20E.

Alpha-amylase (α-1,4-glucan-4-glucohydrolases; EC. 3.2.1.1) is a crucial enzyme for starch digestion in insects that feed on starch or grain powder, including the red flour beetle. This enzyme converts complex carbohydrates into oligosaccharides, which are hydrolyzed to glucose by α-glucosidases (Terra et al., 1996). The changes in α-amylase activity are related to the feeding behaviour and development in *T. castaneum* (Tatun et al., 2014). In addition, changes in α-amylase activity are associated with different types of plant diets in *Helicoverpa armigera* (Kotkar et al., 2009). Accordingly, we examined the effects of 20E on changes in α-amylase activity in order to determine whether the enzyme is hormonally regulated and reveal how it responds.

MATERIAL AND METHODS

Insect cultures

Stock cultures of *T. castaneum* were obtained from the Postharvest and Processing Product Research and Development Office, Department of Agriculture, Thailand. The insects were maintained on wheat flour mixed with 5% yeast and kept at 30 ± 1°C and 70 ± 5% relative humidity. Mature larvae (22 days old) were used in this study.

Hormone injection

A stock solution of 20E (Sigma, St. Louis, MO, USA) was dissolved in absolute ethanol at a concentration of 1 mg/ml and then diluted with distilled water to obtain concentrations of 100, 200, 300, 400 and 500 ng/100 nl. The larvae (n = 75) were anesthetized with diethyl ether in an air-tight chamber for 5 min and then moved onto a glass slide, where they were held in place with sticky tape. Next, 100, 200, 300, 400 or 500 ng of 20E (approximately 100 nl) were injected into the lateral side of the second abdominal segment of each larva using an aspirator tube that was tightly fitted with a 3.5-in glass capillary tube (Drummond, PA, USA) and pulled by a needle puller (Model P-2000, Sutter Instrument Co., CA, USA). Control larvae were injected with sterilized deionized water. After injection, larvae were kept at room temperature to recover from anesthesia and then kept individually in 24-well plastic plates containing wheat flour mixed with 5% (w/w) yeast. Every 2 days, until 14 days post-injection, we recorded the number of larvae that changed to the prepupal stage and exhibited a C-shape (crooked posture) and ceased feeding, as well as those that reached the pupal and adult stages. In addition, the body weights of the larvae, prepupae, pupae and adults were measured after injection with 500 ng of 20E during the larval stage. Three replicates were performed per experiment.

Morphological observations

The insects injected with 20E were digitally photographed under an Olympus SZ51 stereomicroscope (Olympus Corporation, Tokyo, Japan). The length and width of the elytra were measured using cellSens Imaging software version 1.8 (Olympus Corporation, Tokyo, Japan). Adobe Photoshop CS3 (Adobe Systems Incorporated, San Jose, CA) was used to document the insect images.

Measurement of α-amylase activity

To measure α-amylase activity, larvae (2 days post-injection), pupae (3 day-old) and adults (7 day-old) (n = 5) were homogenized in 500 µL of 20 mM sodium acetate buffer at pH 5.5 containing 10 mM NaCl and 20 mM $CaCl_2$. The resulting homogenates were then filtered through cheesecloth and centrifuged at 10,000 xg for 15 min at 4°C. The supernatant was transferred to a new tube and kept at –20°C until used as an enzyme extract. The amount of protein in each sample was measured prior to the α-amylase assay using a protein dye-binding method (Bio-Rad, Hercules, CA, USA). The activity was determined by incubating 15 µL of the insect extract with 100 µL of starch solution (0.2% starch (Sigma, St. Louis, MO, USA) in 20 mM sodium phosphate buffer, pH 6.0) at 37°C for 10 min. After incubation, 20 µL of 1 M HCl was added to the reaction tube to stop the enzymatic reaction, followed by the addition of 100 µL of iodine solution (0.5% I_2 and 5% KI). The 250-µL reaction mixture was then transferred to a 1.5-mL plastic cuvette, and the quantity of remaining starch was determined spectrophotometrically at 580 nm (Thermo Scientific Biomate3s, Thermo Fischer Scientific Inc., MA, USA). The α-amylase activity is presented in terms of µg of hydrolyzed starch/µg of protein/min. Three replicates were performed per experiment.

Statistical analysis

One-way Analysis of variance (ANOVA) and a least-significance-difference (LSD) multiple-range test were used for all sta-

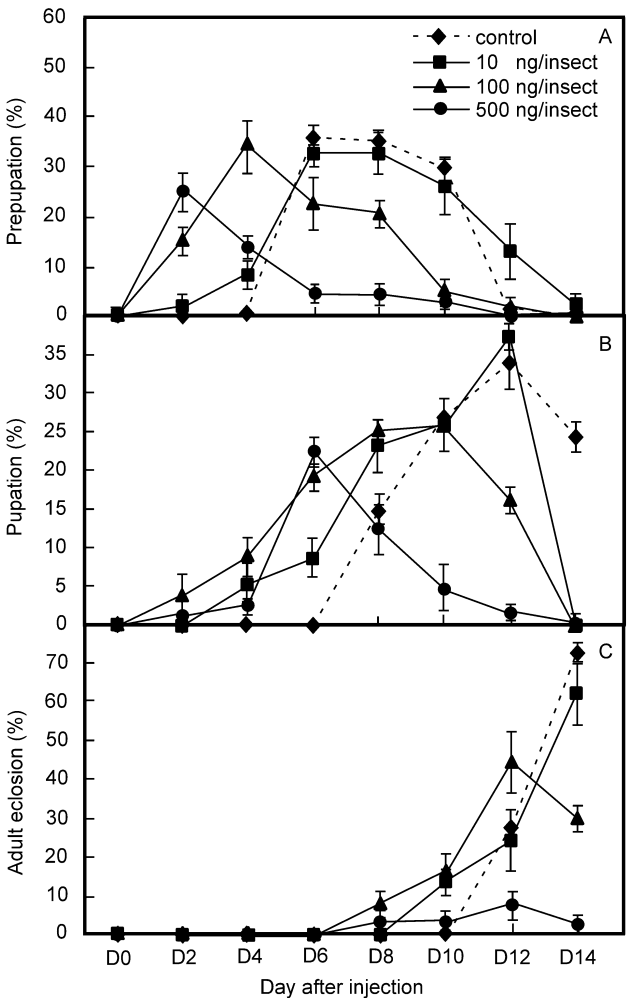

Fig. 1. Percentage of insects entering prepupal (A), pupal (B) and adult stages (C) after being injected with 10, 100 and 500 ng of 20-hydroxyecdysone (20E) in the larval stage (n = 75). Numbers of prepupae, pupae and adults were recorded every 2 days until adult emergence. The experiment was performed in triplicate and each error bar indicates the SD of the mean.

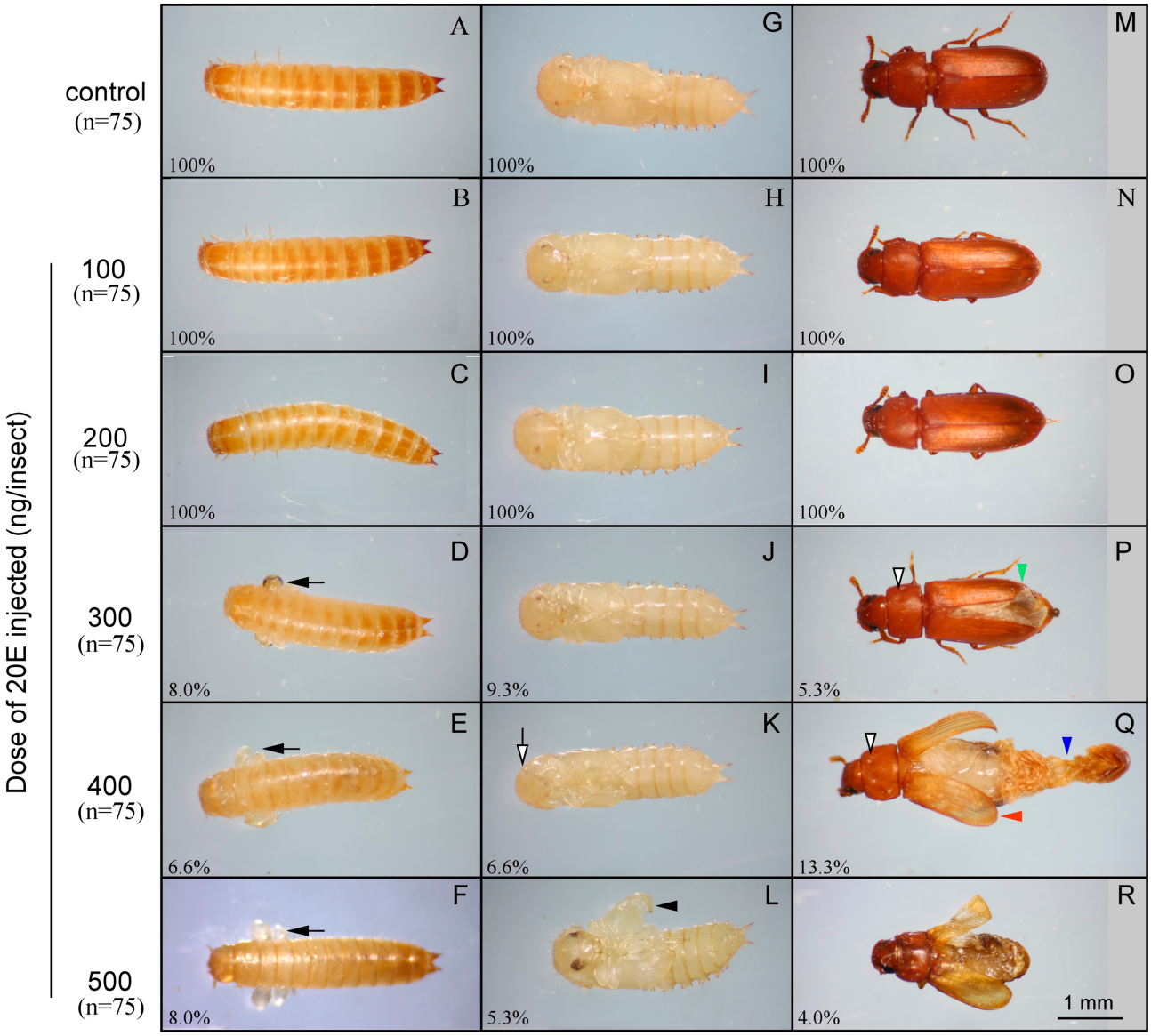

Fig. 2. Morphological changes in the larvae, pupae and adults of *T. castaneum* after injection of 20-hydroxyecdysone (20E) during the larval stage (n = 75). Control insects were injected with sterile distilled water (A, G, H). Larvae were injected with 100, 200, 300, 400 and 500 ng 20E, which resulted in larvae (B–F), pupae (H–L) and adults (N–R). White arrow and black arrowhead indicate detached wing and antennae of 20E treated pupae. Red arrowhead indicates split and short elytra. White arrowhead indicates small depression on dorsum of thorax. Blue arrowhead indicates pupal cuticle attached to the posterior end of abdomen. Percentage of occurrence of each trait is shown in the bottom left corners of each of A to R.

tistical analyses (SPSS program version 11.5). The significance level was set to 0.05 ($P < 0.05$).

RESULTS

Effects of 20E on the postembryonic development of *T. castaneum*

At day 2 post-injection, 2.17%, 15.79% and 48.57% of the larvae injected with 10, 100 and 500 ng of 20E, respectively, changed to the prepupal stage (larvae with a crooked posture, inactive, and non-feeding). The prepupal stage was not achieved in the control group until 4-days post injection (Fig. 1A). The highest number of larvae entering the prepupal stage at day 4 post-injection was recorded for the larvae injected with 100 ng of 20E. In the control group and insects injected with 10 ng of 20E, the highest number of larvae entering the prepupal stage was recorded at day 6 post-injection (35.29 and 32.61%, respectively).

The percentage pupating after injection with 20E revealed that larvae injected with 100 and 500 ng of 20E started to pupate at day 2 post-injection (9.09%) (Fig. 1B). Pupation in the control group occurred on day 8 (14.63%), whereas for those injected with 10 and 100 ng of 20E it occurred on day 4 (5%) and day 2 (3.9%) post-injection, respectively. The highest percentage pupation of larvae injected with 100 and 500 ng of 20E was recorded on day 10 and day 6, respectively. The highest percentage of larvae injected with 10 ng of 20E and the control group occurred on day 12.

Adult eclosion was recorded on day 8 when the larvae were injected with 100 and 500 ng of 20E (Fig. 1C). The larvae injected with 10 ng of 20E changed to the adult stage

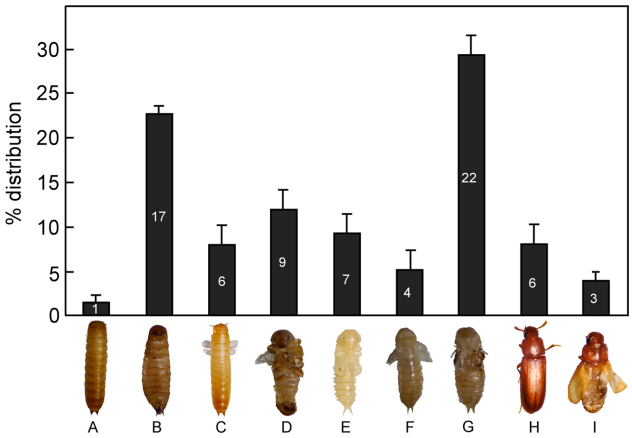

Fig. 3. Columns indicate the percentage occurrence of different characteristics resulting from injecting the larvae with 500 ng 20-hydroxyecdysone (20E) (n = 75) and in the control group injected with sterile-deionized water. (A) normal looking larvae (B) larvae with flattened abdomens, (C) larvae with projecting small wing pads on meso- and meta-thorax, (D) incomplete pupae with larval cuticle at posterior end, (E) normal pupae, (F) pupae with unfolded wings, (G) pupae with unfold legs and detached antennae, (H) normal adult, (I) incomplete adult. The numbers in the columns are the number of insects.

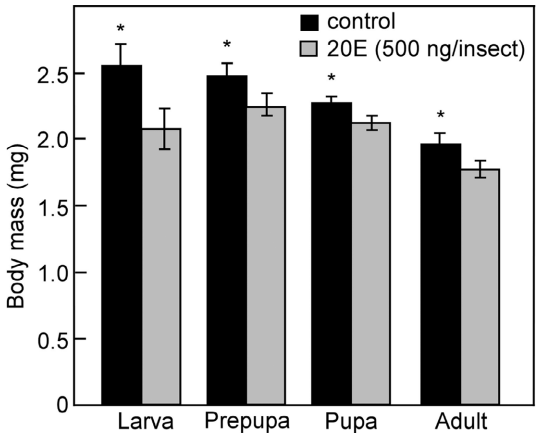

Fig. 4. Changes in the body weight of *T. castaneum* resulting from the injection with 500 ng 20-hydroxyecdysone (20E) during larval stage (n = 75). The error bars indicate SDs and asterisks indicate significant difference between control and groups injected with 20E ($P < 0.05$).

on day 10, whereas adult eclosion in the control group was first recorded on day 12 and the highest percentage on day 14. There were lower percentages of eclosion in the groups treated with 100 and 500 ng than in the control group and those that received lower doses of 20E. This occurred because incomplete larvae and pupae developed, which failed to develop into adults. The percentage of adult eclosion in the control group was about 75%.

Effects of 20E on the morphology of *T. castaneum*

When the last-instar larvae were injected with various doses of 20E, morphological abnormalities were observed at each developmental stage (Fig. 2). In the control group, the injection of sterile water had no effect and resulted in the formation of normal pupae that subsequently moulted into normal adults (Fig. 2A, G, M). The larval body lengths of larvae injected with 100, 200, 300, 400 and 500 ng of 20E were shorter than those of the control group (Fig. 2B–C). Interestingly, larvae injected with 300, 400 and 500 ng of 20E exhibited 2 pairs of small wing pads that projected out of the larval cuticle on the second and third segments of the thorax (Fig. 2D–F; black arrow). In addition, other larval organs were also observed in those larvae, including antennae, cuticle and eyes, and they died within a few days.

The control larvae developed normally into pupae on day 12, as shown in Fig. 1B. The pupae developed from larvae injected with low doses of 20E (100 and 200 ng of 20E) formed pupae that looked normal, while those that developed after high doses of 20E (300, 400 and 500 ng of 20E) exhibited small wings and more rounded abdomens (Fig. 2J, K, L). The overall body sizes of these pupae were not different from those of the water-injected control pupae, but the wing lengths were substantially shorter. At doses of 400 and 500 ng of 20E, the wings and antennae of the resulting pupae were not attached to the body (Fig. 2K, L; white arrow and black arrow).

The injection of 100 and 200 ng of 20E had no apparent effect on adult morphology (Fig. 2M–O). After injecting 300 ng of 20E, adults exhibited split and short elytra (Fig. 2P; green arrowhead), and there were small depressions on the dorsum of the thorax (Fig. 2P, white arrowhead). The injection of 400 ng of 20E had more severe effects on the adults, resulting in split elytra (Fig. 2Q, red arrowhead), an uncovered abdomen and the pupal cuticle attached to the posterior end of the beetle (Fig. 2Q, blue arrowhead).

The adults that emerged after injection with 500 ng of 20E had a flattened body, split elytra, and a small depression on the dorsum of the thorax (Fig. 2R). When larvae were injected with 400 or 500 ng of 20E, the resulting adults survived for only about 5 days after eclosion. In addition, there were various morphological abnormalities after injection with 500 ng of 20E, such as a flattened abdomen (17%) (Fig. 3B) and larvae with small, projecting wing pads on the thorax (6%) (Fig. 3C). About 9% of the pupae were incomplete and entrapped within the larval cuticle (Fig. 3D). About 7% of the pupae were normal (Fig. 3E), 4% had unfolded wings (Fig. 3F) and 22% had unfolded legs and detached antennae (Fig. 3G). After the larvae injected with 20E developed into adults, about 6% appeared normal, and about 3% were incomplete adults with small bodies, unfolded wings, a flattened abdomen and a bump on the thorax (Fig. 3I). We next determined body weight after injection with 500 ng of 20E (Fig. 4). The results showed that the injection decreased the body weight of the larvae, prepupae, pupae and adults ($P < 0.05$).

Effects of 20E on elytral width and length in *T. castaneum*

Since the adults that emerged from larvae injected with 20E obviously had small elytra, we determined the effect of different doses of 20E on the length and width of elytra (Fig. 5). At 100 and 200 ng of 20E, the elytral lengths were 2.76 and 2.5, respectively, which are not much different from the length of 2.66 mm of the control adults (Fig. 5A).

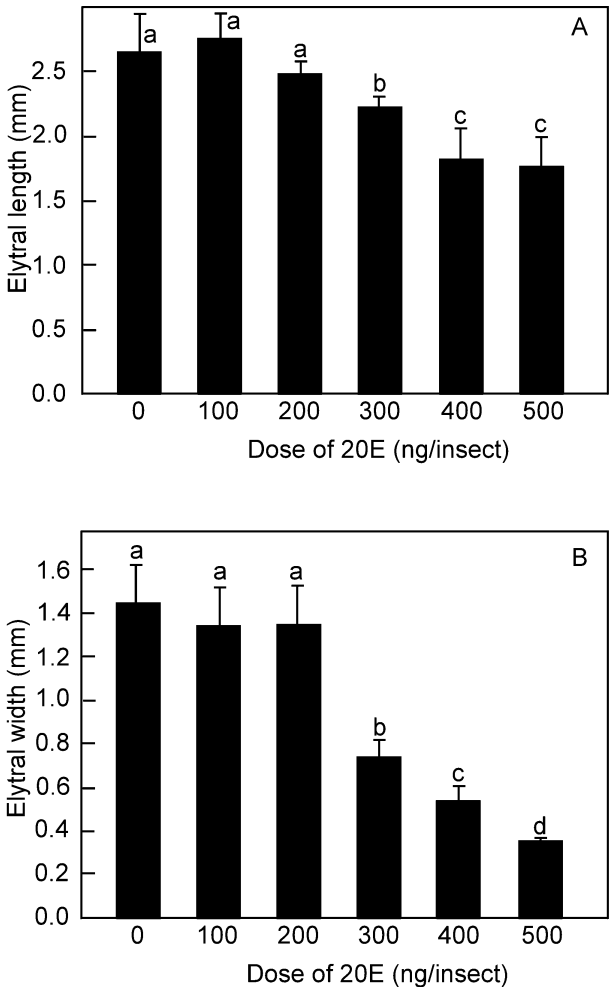

Fig. 5. The length (A) and width (B) of elytra of *T. castaneum* adults that as larvae were injected with either 100, 200, 300, 400 or 500 ng 20-hydroxyecdysone (20E). The error bars indicate SDs and values labelled with different letters differ significantly (P < 0.05).

At 300, 400 and 500 ng of 20E, however, the adults had shorter elytra of 2.23, 1.83 and 1.77 mm, respectively. There was not much difference in the elytral widths between control adults and those treated with 100 and 200 ng of 20E (1.45, 1.35, and 1.34 mm, respectively) (Fig. 5B). However, the elytra were significantly narrower in adults that emerged from larvae injected with 300, 400 and 500 ng of 20E. These results demonstrate that injection with 300–500 ng of 20E significantly reduced the length and width of elytra in adult *T. castaneum*.

Effect of 20E on the activity of the enzyme α-amylase

The activity of α-amylase was measured in larvae, pupae and adults that were injected with various doses of 20E as larvae (Fig. 6). The activity of α-amylase in 20E-treated larvae (2 days post-injection) was not different from that of the control larvae (Fig. 6A). Similarly, the α-amylase activity in the pupae (3 day-old) was at the same level as in the control pupae (Fig. 6B). In contrast, the α-amylase activity decreased significantly after injection with 200, 300 or 400 ng of 20E. Particularly, the group injected with 500 ng of 20E had the lower α-amylase activity compared to that

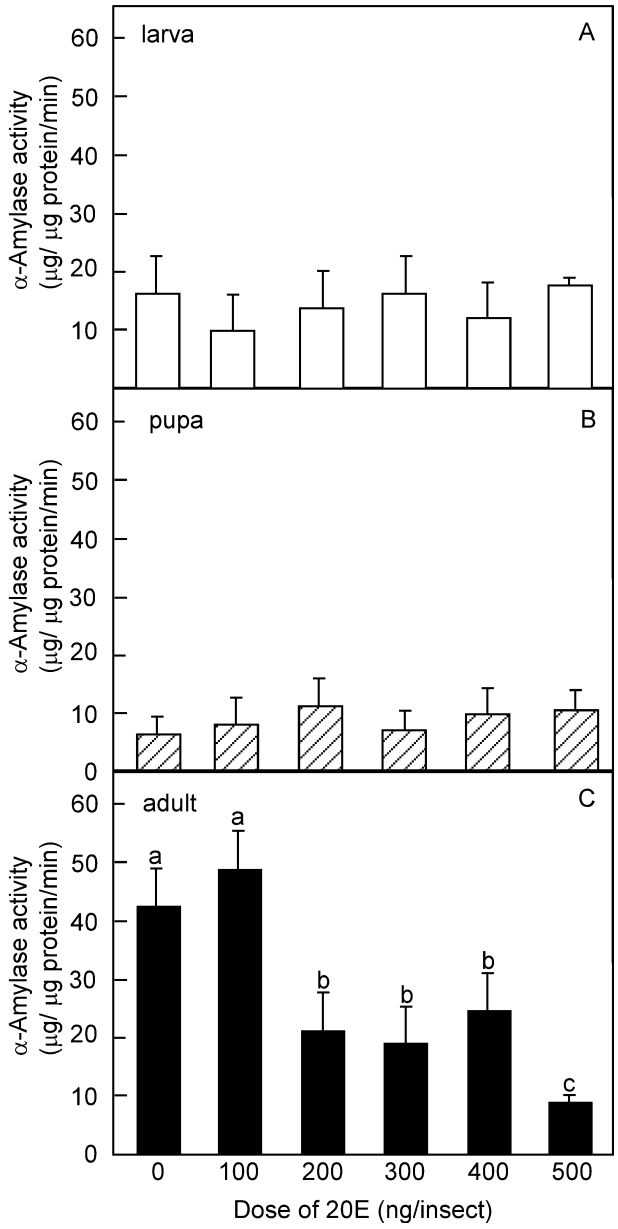

Fig. 6. The enzymatic activity of α-amylase recorded in *T. castaneum* larvae (A), pupae (B) and adults (C) that were injected with 20-hydroxyecdysone (20E) as larvae. The error bars indicate SDs and values labelled with different letters differ significantly (P < 0.05).

recorded after injection of lower doses (Fig. 6C). However, the enzyme activity in larvae injected with 100 ng of 20E was not different from that of the control group.

DISCUSSION

The active form of the ecdysteroid, 20E, coordinates the differentiation of adult structures during insect moulting and metamorphosis (Dubrovsky, 2005). Alterations in the levels of 20E could affect the timing of moulting in different developmental stages in insects, as well as the percentages pupating and emerging as adults (Gelman et al., 2002; Noriega et al., 2002). In the present study, we injected various doses of 20E into 22-day-old larvae, which were in the last instar. Injection of 20E resulted the larvae of *T.*

castaneum entering the prepupal and pupal stages 2 days and 6 days earlier, respectively, than the control group. In the groups injected with 20E, adult eclosion occurred 4 days earlier than in the control group, indicating that 20E caused precocious metamorphosis in *T. castaneum*. This result is consistent with a study on *Drosophila,* in which ecdysone controls developmental transitions and regulates the duration of the growth period (Herboso et al., 2015). Parthasarathy et al. (2008) report that ecdysteroid levels in *T. castaneum* are low during the final instar of the larval stage (ca. 50–100 pg/insect), begin to increase at the beginning of the prepupal stage (ca. 300–600 pg/insect) and reached maximum levels prior to pupation (ca. 500–3500 pg/insect). The ecdysteroid titers were low at the beginning of the pupal stage and reached the lowest levels prior to adult eclosion. Exogenous 20E accelerated the larval-pupal transformation, as shown in Fig. 1. This is consistent with a study by Nijhout et al. (1974), in which a small rise in 20E levels during the final instar of the larval stage terminated larval feeding and initiated premetamorphic behaviour as well as a commitment to pupate. The *T. castaneum* larvae showed changes in their behaviour, such as, cessation of feeding, changes in the way they moved and the adoption of a crooked posture at day 2 post-injection. These changes were dose-dependent, indicating that the increases in ecdysteroid in larval haemolymph probably commits *T. castaneum* larvae to pupating. This is consistent with studies on other insects, including the tenebrionid beetles *Tenebrio molitor*, *Tribolium freemani* and *Zophobas atratus* (Connat et al., 1984; Hirashima et al., 1995; Aribi et al., 1997).

Morphological changes in various traits in *T. castaneum* in response to the injection of 20E were observed. The most apparent morphological changes were the projecting pairs of wing pads on the meso- and meta-thoraces of larvae treated with 300, 400 and 500 ng of 20E. In addition, the postures of wings and antennae in the pupal stage were different from those of the control pupae. The occurrence of these prothetelic morphological changes has been reported previously. Prothetelic larvae with the early development of imaginal characteristics result from an improper balance of hormones during development, and prothetely is reported in 22 species belonging to 10 families of Coleoptera (Wigglesworth, 1954).

This study showed that the injection of 20E (300, 400 and 500 ng/insect) into mature larvae of *T. castaneum* caused the formation of prothetelic larvae, which usually also have some other organs more characteristic of pupae, such as small wing pads. The injection of a high concentration of 20E may cause hyperecdysonic conditions in larvae and subsequently lead to the appearance of pupal organs. In a similar way, a study on *T. molitor* reveals that the injection of 20E (10 µg/insect) into the last larval instar induces the development of prothetelic larvae and larval-pupal intermediates (Quenedey & Quennedey, 1990). Moreover, prothetelic larvae of *T. molitor* also have small projecting wing pads called wing anlagen and do not moult to the adult stage (Quenedey & Quennedey, 1993).

The effects of exogenous 20E are reported in many species of insects and affect various aspects, including development and survival. However, each species of insect varies in its susceptibility to injection with 20E. The present study demonstrated that the body weight of all the developmental stages (larval, prepupal, pupal and adult) of *T. castaneum* was decreased by injections with 20E (Fig. 4). High concentrations affected the growth of adult wings of *T. castaneum* in that the elytra are shorter and narrower (Fig. 5).

Just before pupal ecdysis in *T. molitor*, the proliferation of anlagen wing cells decreased (embryonic tissues that are capable of forming adult structures). Furthermore, the mitotic index dropped suddenly, and 40% of the anlagen cells started to degenerate. This is correlated with an increase in ecdysteroid levels in the haemolymph of *T. molitor* (Quennedey & Quennedey, 1990). This suggests that the injection of high doses of 20E into *T. castaneum* larvae interfered with the process of wing development by initiating the termination of cell proliferation in the imaginal wing disk. Similar effects of 20E are reported in *Plutella xylostella*, in which high concentrations result in the reduction of larval body weight and average pupal body weight (Sun et al., 2015). A study on *Drosophila melanogaster* reveals that ecdysone accelerate metamorphic development and reduce body size (Ono, 2014). Furthermore, 20E slowly reduces food consumption and then induces starvation, resulting in fat body lipolysis (Wang et al., 2010). Thus, 20E may cause a reduction in insect body mass by decreasing food consumption.

There are many experiments that indicate that ecdysone is required to promote wing growth in insects. For example, reducing ecdysone levels impairs the growth of wings and ovaries in *Blattella germanica* and *Drosophila* (Herboso et al., 2015). Animal size and nutritional status are monitored by the larval fat body by integrating 20E signaling with the insulin signaling pathway, which indicates that treatment with 20E may interfere with the signaling pathway and lead to abnormal biosynthesis or metabolism (Nichole et al., 2010). Several studies show that ecdysone positively regulates organ growth in other species (Sun et al., 2015). However, ecdysone has different roles in Lepidoptera, in which a low level of ecdysone is required for the growth of imaginal discs (Nijhout et al., 2007), whereas high concentrations of ecdysone lead to the termination of cell growth and proliferation (Champlin & Truman, 1998). Furthermore, ecdysone appears to negatively regulate the growth of many tissues including fat body in *Drosophila* and enhances the growth of wing imaginal discs in fruit flies and Lepidoptera (Nijhout et al., 2014). Taken together, the results indicate that the level of ecdysone is important in determining the nature of the response in insects.

In many insects, the release of digestive enzymes is generally known to be regulated by hormones, including FMRF amides, allatostatins, allatotropins and sulphakinins (Lehane et al., 1995; Terra et al., 1996). Recently, studies on *Periplaneta americana* revealed that the adipokinetic hormone (AKH) stimulates the activity of the digestive

enzyme α-amylase in the mid gut of insects (Bodláková et al., 2017). This indicates that α-amylase is one of the digestive enzymes that are regulated by hormones. In the red crayfish (*Procambarus clarkii*) the gene expression of *amylase* (*Pc-Amy*) is significantly down-regulated after injection with 20E (Pen et al., 2015). Furthermore, RNA interference with the *ecdysone receptor* gene indicates that *Pc-Amy* is one of the ecdysteroid-responsive genes.

The present study revealed that injection with 20E (200–500 ng/insect) decreased the activity of α-amylase in the resulting adults of *T. castaneum* (Fig. 6), indicating that 20E may play a role in the regulation of α-amylase during the adult stage. Accordingly, based on the percentage pupation and eclosion, morphological changes and changes in the activity of α-amylase, the optimal concentration to induce larval-pupal-adult transformations in *T. castaneum* is 100–200 ng of 20E. Higher doses induce pupation and adult emergence, but caused deformities in the pupae and adults and a reduction in α-amylase activity. It is reported that changes in *amylase* gene expression and α-amylase activity in *T. castaneum* (*Tc-Amy*) correlate well with developmental profile, with a high expression of amylase in the larval stage, a very low level in pupae and the highest activity in adults, with in the latter it is about 2-fold higher than that in larval stage (Tatun et al., 2014). Thus, further investigations are needed to reveal the relationship between *Tc-Amy* and ecdysteroid in the red flour beetle.

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DECLARATION OF INTEREST. The authors report no conflicts of interest.

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