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Development and integrality of the regeneration leg in *Eupolyphaga sinensis*

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Abstract

Many arthropods are able to regenerate lost body parts after injury. Here, regeneration was studied in nymphs of *Eupolyphaga sinensis* (Walker) (Blattaria Polyphagidae) after amputating femur, tibia or tarsus of one hind leg. The development and integrality of regeneration leg was observed and the regeneration process was studied. The results showed that new leg formed inside the end of the stump. Development of the regeneration leg in the stump experienced four stages. At first epidermal cells of the stump edge formed scab and covered the wound. Then the underlying tissue became disorganized. Blastema grew rapidly and progressively differentiated from its proximal region to undergo morphogenesis, leading to the perfect replacement of the missing structures. Finally the regeneration leg developed well and detached to the old cuticle. Cell layers of blastema of the regeneration leg were epithelium, neurofibril, regeneration cells and tracheas. Only four segments were observed in the tarsus of new leg until adult stage when amputating the base of tarsus I. Tarsus II was the key position for the integrality of the regeneration leg when amputating different tarsus position. The regeneration cells were inside the end of the stump and at the beginning of the amputated segments.

Key words: *Eupolyphaga sinensis*, leg regeneration, development, integrality.

Introduction

Regeneration is a process of regrowth or repair the lost tissues or organs in organisms (Gui and Yi, 2002; Kumar *et al.*, 2007). The elegant and orderly process of regeneration not only provides clues to pattern formation mechanisms, but also offers the hope that the process might one day be manipulated in techniques to replace damaged human body parts (Marsh and Theisen, 1999). In insects, at least 36 genera of 11 orders were observed the phenomenon of limb regeneration (Bulliére and Bulliére, 1985; Beauchemin *et al.*, 1998; Tan *et al.*, 2004). Insects can regenerate lost appendages on head, chest or abdomen, such as antennae (Shaw and Bryant, 1974; French and Domican, 1982), legs (Bohn, 1974; Anderson and French, 1985; Bulliére and Bulliére, 1985; Mito *et al.*, 2002; Nakamura *et al.*, 2008) and cercus (Aiouaz, 1975; Švácha, 1995).

Nymphs of hemimetabolous insects such as cockroaches and crickets have three pairs of legs consisting of six segments, arranged along the proximodistal axis in the following order: coxa, trochanter, femur, tibia, tarsus, and pretarsus. After damage, nymphs of cockroaches and crickets possess functional legs with a remarkable capacity for regeneration, and the phenomenon of leg regeneration in cockroach nymphs is well studied (Bohn, 1970a; 1970b; 1971; French, 1976a; 1976b; Anderson and French, 1985). Tan *et al.* (2004) first studied the leg regeneration in *Eupolyphaga sinensis* (Walker) (Blattaria Polyphagidae) nymphs in 2004. At 30 ± 1 °C, the female *E. sinensis* moult 9-11 times before adult and need 240-320 days to grow to adult, the male *E. sinensis* moult 7-9 times before adult and need 160-240 days to grow to adult. The normal number of *E. sinensis* tarsal segment is five. The 4th instar nymph was often used for regeneration study because the body size was appropriate and the stadium was relatively short, which is about 20.0 ± 2.5 days (unpublished data). Tan *et al* (2004) reported that *E. sinensis* nymphs at all instars had regeneration ability and legs amputated at any site could regenerate. Besides, the regenerated legs could regenerate again when they were amputated. They also found that the regenerated legs had smaller size and lighter color compared with the normal ones, but the growth rate of the regenerated legs was faster (Tan *et al*, 2004). Tan *et al* (2007) reported that the duration of 4th instar increased with the number of amputated legs.

In the present study, we addressed three questions: (1) where did the regeneration leg developed when the leg was amputated at different segments, (2) how did the regeneration leg developed, (3) how was the integrality of the regeneration leg when the leg was amputated at different segments.

Materials and methods

Insect

Nymphs of *E. sinensis* were provided by Hubei Insect Resources Utilization and Sustainable Pest Management Key Laboratory (Huazhong Agricultural University), Wuhan City, Hubei Province, P. R. China. Nymphs were placed in plastic containers (7 × 4.5 cm at base and 8 cm height) with moist soil which water content was 15% (weight: weight). The rearing temperature was 30 ± 1 °C.

Regenerative position of nymph when amputated at different segments of one hind leg

In order to examine the regenerative position of nymph after amputating at different segment of leg, one hind leg of newly moulted 4th instar nymphs was amputated at femur base, tibia base or tarsus base respectively. The replicate number in each treatment was 10. The regenerate position was investigated a few days before the postoperative moult by dissecting residual coxa, femur or tibia of the hind leg.

Development of the regeneration leg

Newly moulted 4th instar nymphs were amputated at the femur base of one hind leg. Tissue section of the stumps was observed by microscope. The slicing was done at 8, 10, 12 d, respectively. The development of regeneration leg was observed by optical microscope. The regeneration cells were observed by transmission electron microscope.

The different segments of leg were cut and fixed for 24 h at 4 °C, phosphate-buffered 2.5% glutaraldehyde with a pH of 7.4. The different segments of leg were then rinsed twice in phosphate buffer at 10-min intervals and post-fixed for 3-4 h in 1% osmium tetroxide at room temperature, followed by two rinses in phosphate buffer and dehydration in a graded series of ethanol with 12 h stays at each concentration (30, 50, 70, 80, 90 and 100%), and embedded in araldite. Semi-thin sections for light microscopy were stained a few seconds with 1% methylene blue. Ultrathin sections for transmission electron microscopy (TEM), double-stained with uranyl acetate and lead citrate for a few minutes each, were examined in a Hitachi H-600 TEM microscope at 75 KV accelerating voltage.

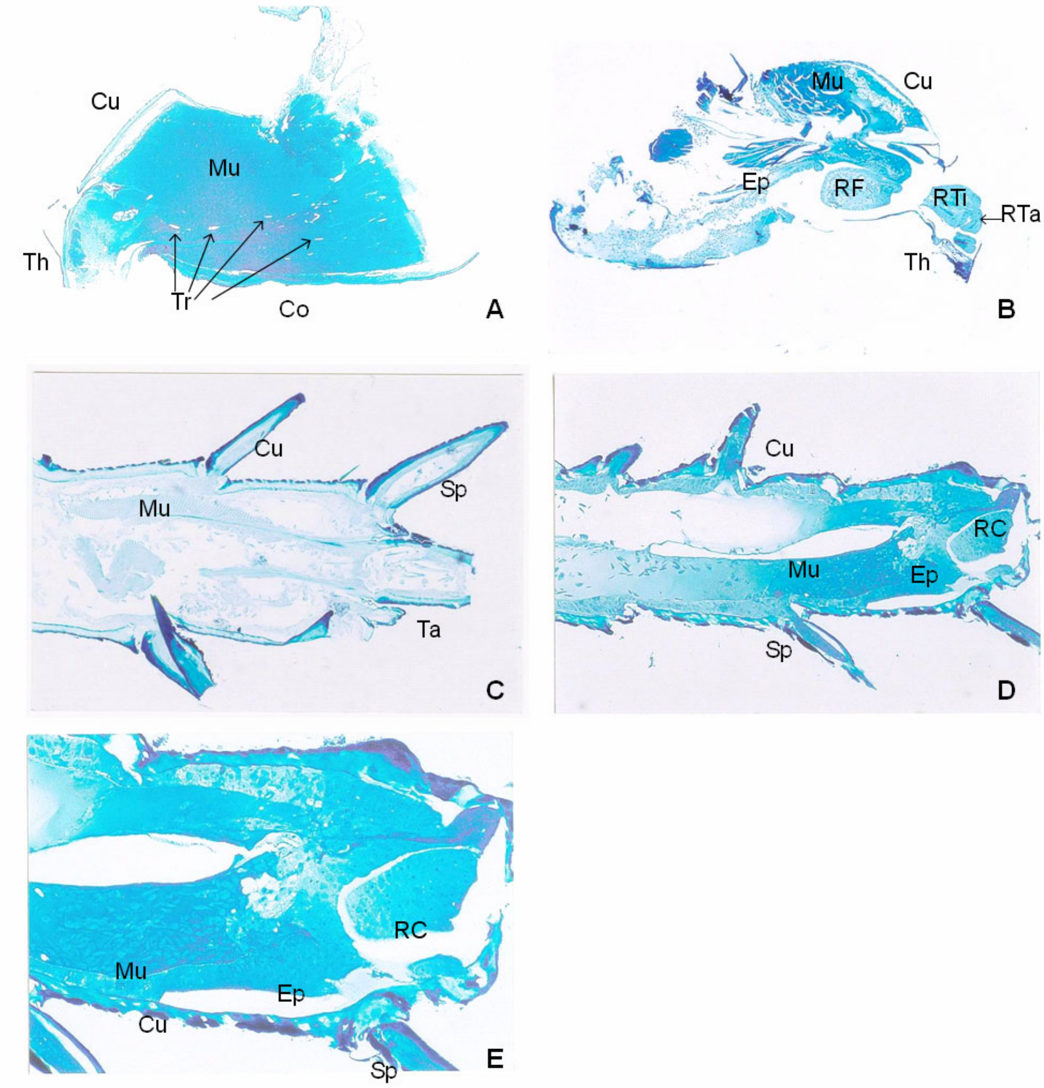


Figure 1. Longitudinal section of the regenerating appendages in 4th instar nymph of *E. sinensis*. (**A**) The coxa and trochanter of the intact hind leg (40×); (**B**) The regenerating leg develops inside the coxa and trochanter stump, 10 days after amputating at the femur base of the newly moulted 4th instar nymph (40×); (**C**) The tibia distal of the intact leg (40×); (**D**) The regenerating leg develops inside the tibia stump, 7 days after the tarsus of the newly moulted 4th instar nymph amputated (40×); (**E**) Enlarged portion of regeneration cells inside the tibia stump (100×). Co: Coxa; Ta: Tarsus; Th: Trochanter; Tr: Trachea; Cu: Cuticle; Ep: Epidermis; Fe: Femur; Mu: Muscle; RC: Regenerating Cell; RF: Regenerating Femur; RTa: Regenerating Tarsus; RTi: Regenerating Tibia.
(In colour at www.bulletinofinsectology.org)

Integrality of the regeneration leg when amputated at different segments of one hind leg

One hind leg of newly moulted 4th instar nymphs was amputated at the base of tarsus claws, tarsus V, tarsus IV, tarsus III, tarsus II, tarsus I, tibia or femur respectively. The replicate number in each treatment was 10. The segments of the regeneration leg were observed to the adult stage.

Results

Regeneration position of the amputated leg

The regeneration phenomenon was observed ten days after amputating at the base of the femur. At first, epidermal cells of the stump edge formed scab and covered the wound. Then the underlying tissue becomes disorganized. The femur, tibia and tarsus of new leg formed inside the end of the stump (figure 1B). Muscles were filled in the coxa and trochanter of the intact leg (figure 1A). The regeneration phenomenon was observed seven days after amputating at the base of the tarsus. Two bundles of muscles controlling the movement of the tarsus were observed in the tibia of the intact leg (figure 1C). However, the muscles in the tibia of the amputated leg were contracted and the regeneration cells were observed inside the end of the stump (figure 1D-E). A few days before the postoperative moult, the leg amputated at the base of femur, tibia or tarsus, was observed developing new leg at the end part of coxa, trochanter (figure 1B, figure 2A), femur (figure 2B) and tibia (figure 1D-E, figure 2C), respectively.

Development of the regeneration leg

One hind leg of new moulted nymph amputated at the base of the femur regenerated new leg 23 days after moulting. After 8 days (figure 3A), epidermal cells of the stump edge formed scab and covered the wound. The trochanter of the stump was filled with haemolymph. The underlying tissue becomes disorganized and muscles in the coxa were contracted. Blastema of the regeneration leg was connected with muscles. Then blastema grew rapidly and progressively differentiated from its proximal region to undergo morphogenesis, leading to the perfect replacement of the missing structures (figure 3B). 18 days later, incomplete regeneration leg occupied nearly one half of the stump coxa. The femur and tibia overlapped inside the end of the stump coxa, and half tarsus was in stump trochanter. Muscles in the femur and spines on the tibia were visible (figure 3C-D). After 22 days, the regeneration leg was developed well and detached to the old cuticle before moulting (figure 3E). Then the completely developed regeneration leg appeared after moulting (figure 3F).

Ultrastructure of the blastema of the regeneration leg

Cell layers of the blastema of the regeneration leg were epithelium, neurofibril, regeneration cells and tracheas.

There were many long irregularly ranked microvilli on the surface of the epithelium layer. The microvillus was emerged in the haemolymph. Primary lysosome, secondary lysosome and mitochondria were observed among the epithelial cells. Between the epithelial cells were intermediate junction complex and single desmosome (figure 4A-D-F-I).

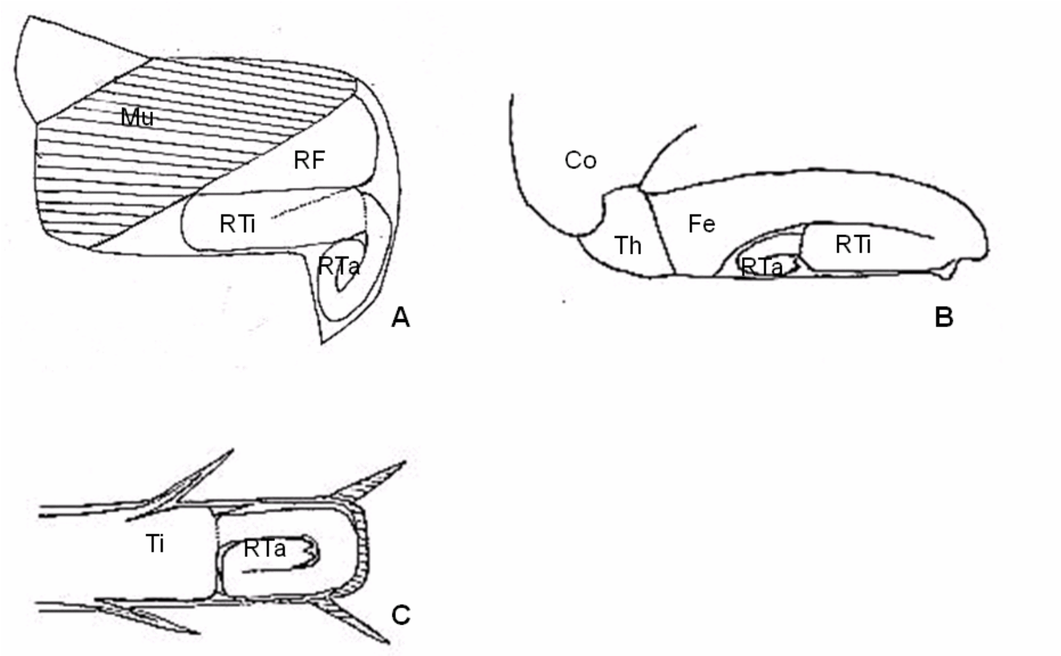


Figure 2. Schematic drawings of the regenerating leg after amputating the hind leg at different position of 4^{th} instar nymph of *E. sinensis* just before moulting to 5^{th} instar. (**A**) Schematic drawings of the regenerating leg after amputating the femur base; (**B**) Schematic drawings of the regenerating leg after amputating the tibia base; (**C**) Schematic drawings of the regenerating leg after amputating the tarsus base. Mu: Muscle; Co: Coxa; Th: Trochanter; Fe: Femur; Ti: Tibia; RF: Regenerating Femur; RTa: Regenerating Tarsus; RTi: Regenerating Tibia.

Neurofibril layer was composed of peripheral glial cells, inner glial cells and axon. There were two ring cell layers in perineurium, in which glycogen granules and lipid granules were rich. One or two axons were found in the neurofibril. There were many microtubules inside the axon (figure 4D-E).

Regeneration cells layer: much interspaces of the regeneration cell were filled with nucleus. Nucleoli were gathered first, then distributed uniformly, and dispersed abnormally at last (figure 4A-B-C). There were lipid granules, mitochondria, and lysosome in the regeneration cells. Many lipid droplet were among regeneration cells and junction points between regeneration cells and muscles in coxa (figure 4H).

Nucleus in trachea cell was large. The large numbers of thick or thin tracheoles showed that the trachea cells were in division and proliferation phase.

Integrality of the regeneration leg when amputated at different position

The leg regeneration was observed when the leg was amputated at the base of tarsal claws, tarsus V, tarsus IV, tarsus III, tarsus II, tarsus I, tibia or femur respectively. Complete leg was formed when amputated at the base of tarsal claws, tarsal V, tarsal IV, tarsal III or tarsal II, whose tarsus of new leg was five segments (figure 5A-B-C-D). However, the regeneration leg was incomplete in the tarsus. The leg was observed to the

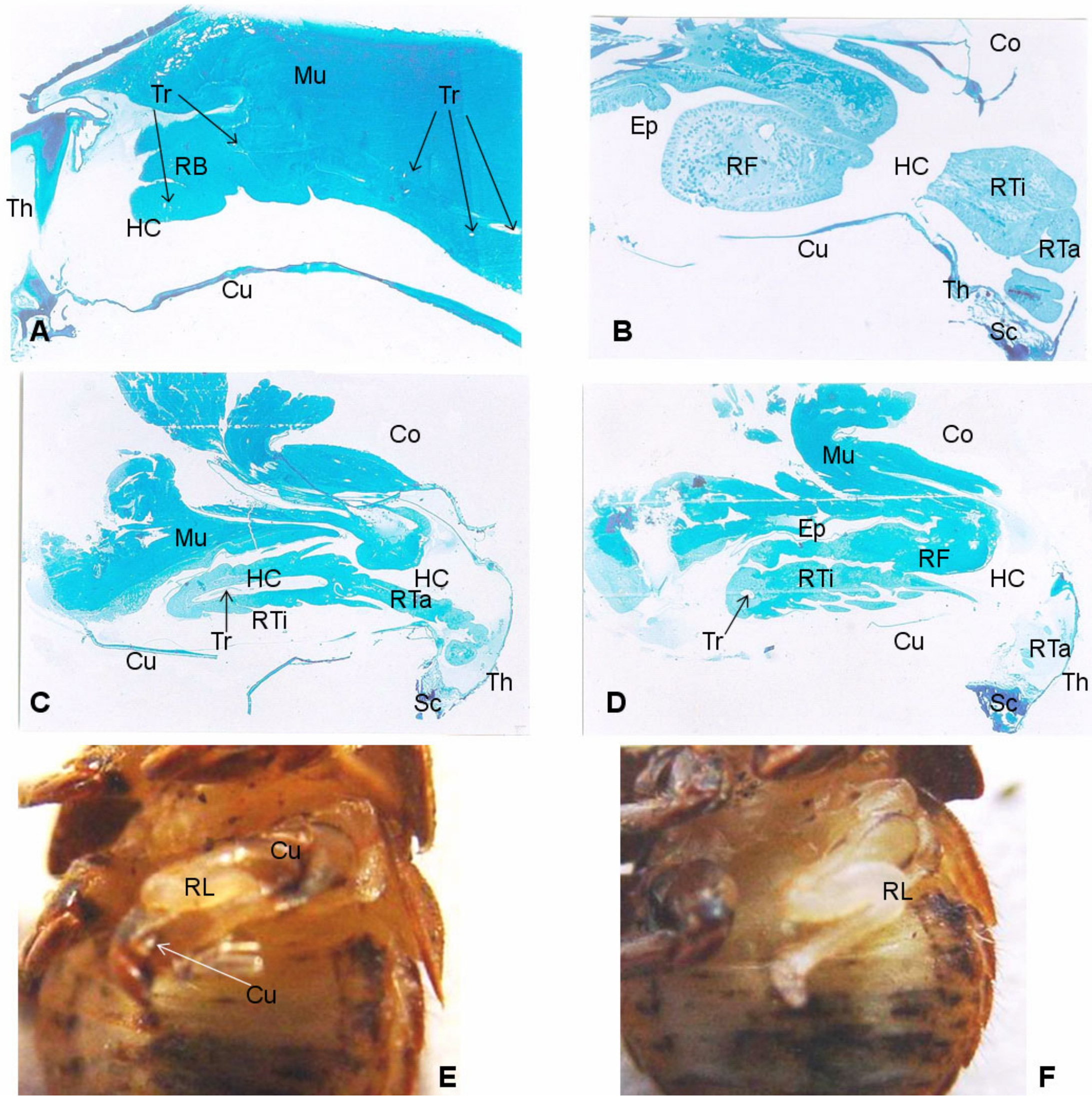
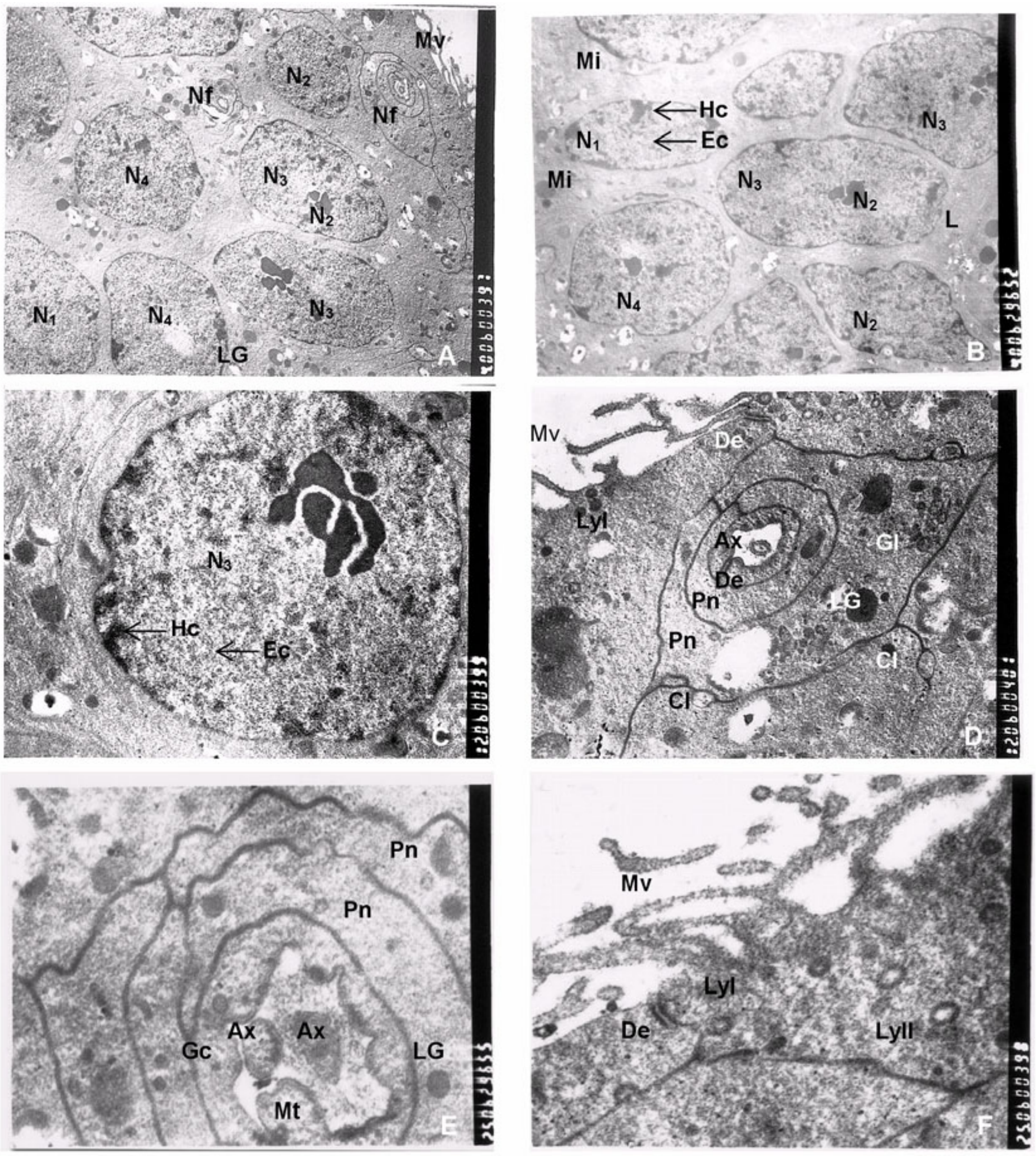


Figure 3. Development of the regenerate leg in 4th instar nymph of *E. sinensis* when amputated at the base of the femur and metathoracic of 4th instar nymph amputated at the base of the femur of one hind leg. (**A**) Terminal of the stump leg, 8 days after amputated (100×); (**B**) Terminal of the stump leg, 10 days after amputated (100×); (**C**) Upside of the terminal of the stump leg, 18 days after amputated (40×); (**D**) Underside of the terminal of the stump leg, 18 days after amputated (40×); (**E**) The regeneration leg dissected medial from residual coxa, 22 days after amputated; (**F**) Newly moulted regeneration leg. Co: Coxa; Cu: Cuticle; Ep: Epidermis; Mu: Muscle; RF: Regenerating femur; RTa: Regenerating tarsus; RTi: Regenerating tibia; Sp: Spine; Tr: Trachea; Th: Trochanter; HC: Hemolymph cavity; RB: Regenerating leg blastema; RL: Regeneration leg.
(In colour at www.bulletinofinsectology.org)

adult stage, only four segments were seen in the tarsus of new leg when amputated at the base of tarsus I (figure 5E). Tarsus II was the key position for the integrality of the regeneration leg when amputated at different position.



(continued)

Figure 4. The ultrastructure of the blastema of the regeneration leg, 8 days after amputating at the the femur base of the newly moulted 4th instar nymph of *E. sinensis*. (**A**) The periphery regenerative cell structure in regenerating leg blastema, showing microvilli, epithelial cell, neurofibril and regenerative cell group (4,000×); (**B**) The regenerative cells group in regenerating leg blastema (4,000×); (**C**) The regenerative cell in regenerating leg blastema (12,000×); (**D**) Microvilli, epithelial cell and neurofibril in regenerating leg blastema (12,000×); (**E**) The neurofibril in regenerating leg blastema (25,000×); (**F**) The microvilli and epithelial cell in regenerating leg blastema (25,000×); (**G**) The trachea in regenerating leg blastema (3,000×); (**H**) Connection department between regenerating leg blastema and the coxa muscle cell (3,000×); (**I**) The microvilli and the structure between epithelial cell connection department in regenerating leg blastema (25,000×). Ax: Axon; CI: Cell membrane are intercalated with one another; De: Desmosome; GC: Glial Cell; Gl: Glycogen granule; IJ: Intermediate junction; L: Lipid droplet; LG: Lipid granule; LyI: Primary Lysosome; LyII: Secondary lysosome; Mi: Mitochondria; Mt: Microtubule; Mv: Microvilli; Nf: Neurofibril; Pn: Perineurium; TC: Trachea cell; TJ: Tight Junction; Tr: Trachea; N_0: intact nucleus; N_1: nucleus in interphase of mitosis; N_2: nucleus in prophase of mitosis; N_3: nucleus in metaphase of mitosis; N_4: nucleus in anaphase of mitosis; Hc: Heterochromatin; Ec: Euchromatin.

(Figure 4 continued)

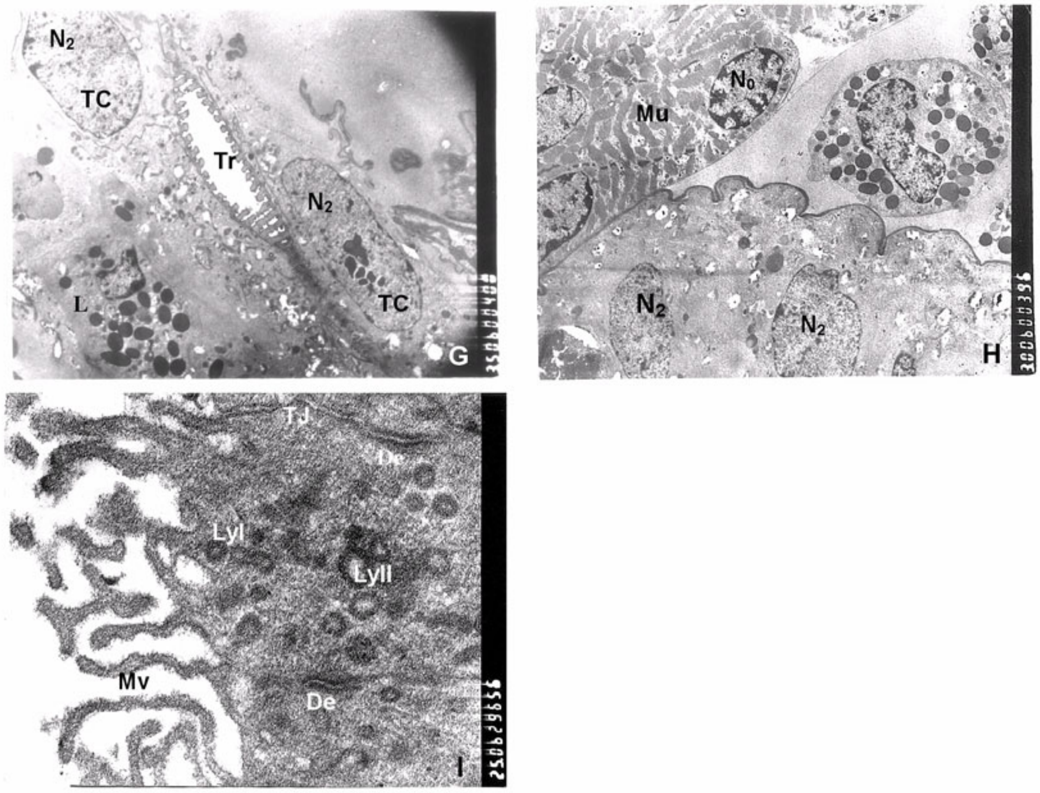


Figure 4. The ultrastructure of the blastema of the regeneration leg, 8 days after amputating at the the femur base of the newly moulted 4th instar nymph of *E. sinensis*. (**A**) The periphery regenerative cell structure in regenerating leg blastema, showing microvilli, epithelial cell, neurofibril and regenerative cell group (4,000×); (**B**) The regenerative cells group in regenerating leg blastema (4,000×); (**C**) The regenerative cell in regenerating leg blastema (12,000×); (**D**) Microvilli, epithelial cell and neurofibril in regenerating leg blastema (12,000×); (**E**) The neurofibril in regenerating leg blastema (25,000×); (**F**) The microvilli and epithelial cell in regenerating leg blastema (25,000×); (**G**) The trachea in regenerating leg blastema (3,000×); (**H**) Connection department between regenerating leg blastema and the coxa muscle cell (3,000×); (**I**) The microvilli and the structure between epithelial cell connection department in regenerating leg blastema (25,000×). Ax: Axon; CI: Cell membrane are intercalated with one another; De: Desmosome; GC: Glial Cell; Gl: Glycogen granule; IJ: Intermediate junction; L: Lipid droplet; LG: Lipid granule; LyI: Primary Lysosome; LyII: Secondary lysosome; Mi: Mitochondria; Mt: Microtubule; Mv: Microvilli; Nf: Neurofibril; Pn: Perineurium; TC: Trachea cell; TJ: Tight Junction; Tr: Trachea; N_0: intact nucleus; N_1: nucleus in interphase of mitosis; N_2: nucleus in prophase of mitosis; N_3: nucleus in metaphase of mitosis; N_4: nucleus in anaphase of mitosis; Hc: Heterochromatin; Ec: Euchromatin.

Position of the regenerative cells in the stump when the leg was amputated at different site of tarsus

The leg was amputated at the base of tarsus V, tarsus IV, tarsus III, tarsus II, and tarsus I, respectively. The regeneration cells were inside the end of the stump and at the beginning of the amputated segments (figure 6).

Discussion

Nymphs of *E. sinensis* can regenerate the leg when amputated at different segments of the leg. Renewable cells appeared inside the end of the leg stump a few days after amputation. However, the regenerated leg was a little smaller than the normal size. This was similar to *Leucophaea maderae* F. described by Bohn (1970b). Amputation of four segments of the tarsus resulted in shortened leg segments. Complete new tarsus (five segments) was formed when the leg had been amputated less than four segments. If four segments of the tarsus were lost, new tarsus regenerated only four segments. Tarsus II was the key position for the integrality of the regeneration leg when amputated at different position. Lüdke and Lakes-Harlan (2008) reported the similar phenomenon in *Schistocerca gregaria* (Forskal).

Stocum (1984) reported that the regeneration blastema possesses a memory of its position along the appendage. In our study, the cells in the regeneration blastema have a variety of structures and their related functions may also guarantee the normal development of regeneration leg.

Villiform structure on the microvillus named sugar-coat was the element of glycoprotein for the cell membrane and dissociative sugar chain of glycolipid. These microvilli increased the absorbing and exchanging ability

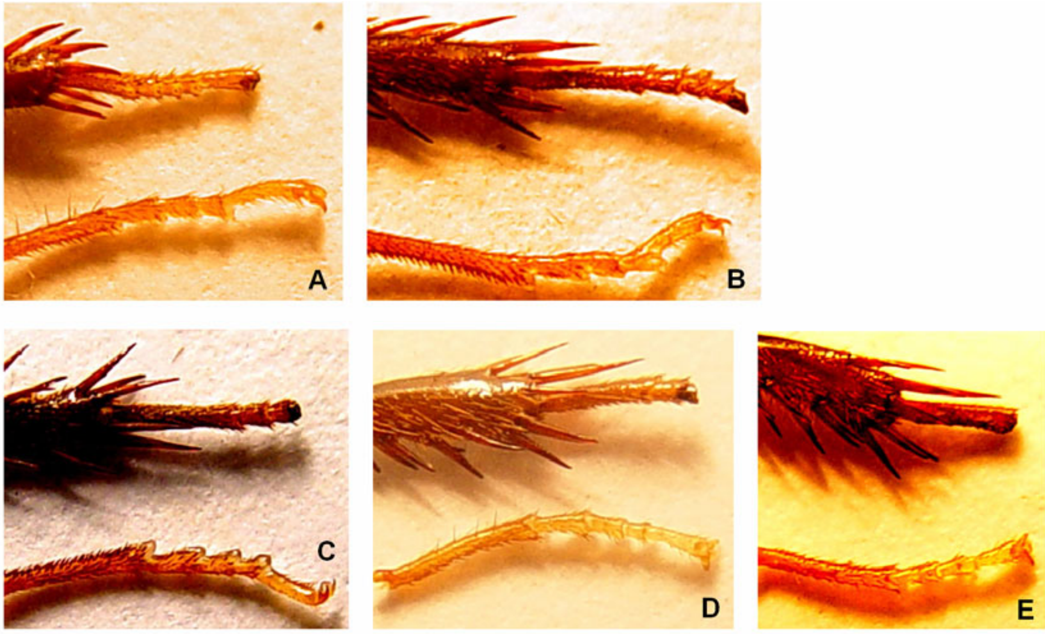


Figure 5. The number of tarsal segments for regeneration leg after amputating at the different position of tarsus in 4th instar nymph of *E. sinensis*. The upper ones are amputated legs of distal tibiae and remaining tarsi of 4th instar nymphs and lower ones are regeneration legs of tarsi of 5th instar nymphs. (**A**) Regeneration leg has five segments after amputating at the base of tarsal claws; (**B**) Regeneration leg has five segments after amputating at the base of tarsus V; (**C**) Regeneration leg has five segments after amputating at the base of tarsus IV; (**D**) Regeneration leg has five segments after amputating at the base of tarsus III; (**E**) Regeneration leg has four segments after amputating at the base of tarsus II.
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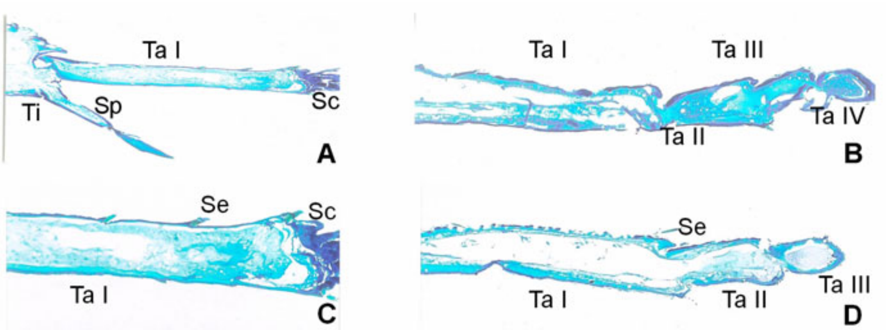

Figure 6. The regeneration cells in the stump when the leg was amputated at different site of tarsus 7 days later in 4th instar nymph of *E. sinensis*. (**A**) The regeneration cells appeared in the tarsus I stump after the tarsus II base amputated (40×); (**B**) The regeneration cells appeared in the tarsus IV stump after the tarsus V base amputated (100×); (**C**) Enlarged portion of regeneration cells from figure 6A (100×); (**D**) The regeneration cell appeared in the tarsus III stump after the tarsus IV base amputated (40×). Sc: Scab; Se: Seta; Sp: Spine; Ta V: Tarsus V; Ta IV: Tarsus IV; Ta III: Tarsus III; Ta II: Tarsus II; Ti: Tibia.
(In colour at www.bulletinofinsectology.org)

of epithelial cells, which helped the blastema of the regeneration leg grow. Many lysosomes were found in the blastema of the regeneration leg. All these were helpful to the leg regeneration of the nymph.

Tight junctions among epithelial cells closed the cell space at the top, which formed a barrier preventing macromolecules outside the cells from entering into the cells and preventing cell fluids from spilling outside. Intermediate junctions plays adhesive role. They can keep cell shape and hand on cell contraction. Desmosome, a strong cell connection structure, is rich in the organization vulnerable to mechanical stretch (Gao, 2006). These special structures were ubiquitous in the blastema of the regeneration leg.

In newt, nerve tissues were relative to the limb regeneration. The stump can regenerate when the nerve existed, but when the nerve was cut off prior to amputation operation, the stump can not regenerate (Fan and Bai, 2002). In blastema of the regeneration leg of *E. sinensis* nymphs, many nerves and neurofibrils were found. This suggests that nerve and neurofibrils were important to limb regeneration for organism.

Acknowledgements

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L.-F. Tan and Y. Zhao contributed equally to this work.

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