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Purification of vitellin and dynamics of vitellogenesis in the parthenogenetic tick *Haemaphysalis longicornis* (Acari: Ixodidae)

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Abstract Vitellin (Vt) was purified from eggs of parthenogenetic bush tick *Haemaphysalis longicornis* by gel filtration and ion exchange chromatography. Our results revealed that only one single Vt existed in parthenogenetic bush tick, and the purified Vt was proved to be a hemoglycolipoprotein consisting of nine polypeptides with molecular weights of 203, 147, 126, 82, 74, 70, 61, 47 and 31 kDa, respectively. Polyclonal antibody and monoclonal antibody against Vt were produced using the purified Vt. The change in vitellogenin (Vg) and Vt levels over time of the parthenogenetic *H. longicornis* was established, and the Vg content in haemolymph and Vt in ovary at different feeding or engorgement statuses was also determined using a double antibody sandwich enzyme-linked immunosorbent assay. The Vg level in haemolymph was distinctly increased on the day of engorgement (1.785 mg/mL) and continued to increase until 2nd day post-engorgement (5.611 mg/mL). There was a slight decrease in Vg level after 4 days of engorgement, and a second peak was observed on day 2 post-oviposition (10.774 mg/mL). Subsequently, Vg content continuously decreased and reached a low level on the 10th day post-oviposition. The Vt content in ovary continuously increased once the female reached its critical weight (0.024 mg per female), and reached the maximum level on day 2 post-oviposition (1.942 mg per female). Afterwards, Vt content rapidly decreased.

Keywords *Haemaphysalis longicornis* · Parthenogenesis · Vitellogenesis · Vitellin · Haemolymph · Ovary · ELISA

Introduction

Ticks are well known vectors for a great variety of infectious pathogens, including viruses, rickettsia, bacteria, spirochetes protozoa and nematoda, all of which can cause severe

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damage to both animals and humans as well as significant economic losses (Deng and Jiang 1991; Sanches et al. 2012). Therefore, ticks have attracted much scientific and public attention due to their medical and veterinary importance (Jongejan and Uilenberg 2004).

Although most described tick species reproduce bisexually, a few species are able to reproduce by parthenogenesis (Oliver 1974, 1977). Parthenogenesis in ticks has been recognized in several genera, including *Amblyomma, Hyalomma* and *Haemaphysalis*, with certain species of ticks lacking males altogether (Aragão 1912; Pervomaisky 1949; Zhmaeva 1950; Kiszewski et al. 2001).

The bush tick *Haemaphysalis longicornis* Neumann is widely distributed in Australia, New Zealand, Korea, Japan (Tenquisf and Charleston 2001) and 17 provinces of China (Deng and Jiang 1991). Moreover, it can transmit a large variety of pathogens, including *Theileria* (Li et al. 2009), *Babesia* (Guan et al. 2010) and *Rickettsia* (Zou et al. 2011). Most interestingly, *H. longicornis* consists of a diploid bisexual population, a triploid obligatory parthenogenetic population and an aneuploid population, which are capable of parthenogenetic and bisexual reproduction (limited in Cheju Do) (Oliver 1977; Chen et al. 2012).

Reproductive success in ticks highly relies on the vitellogenesis and ovarian development (Boldbaatar et al. 2010). However, most studies have been conducted on bisexual ticks rather than parthenogenetic species (Sonenshine 1991; James and Oliver 1996; Denardi et al. 2004; Friesen and Kaufman 2004; Saito et al. 2005; Sanches et al. 2010). We have previously described several characteristics of the parthenogenetic population of *H. longicornis*, including some important micro-structures and synchronized life-cycle features (Chen et al. 2012). However, only limit research on vitellin (Vt) or vitellogenesis in the parthenogenetic population has been performed. In this study, Vt was purified from eggs of parthenogenetic *H. longicornis*, and the change in Vt levels over time was also investigated, providing basic background for further studies.

Materials and methods

Sample collection and tick rearing

Nymphs of parthenogenetic *H. longicornis* were collected from vegetation by flag dragging in Cangxi County (31°37′–32°10′N, 105°43′–106°28′E) of Sichuan Province, Southwest China, and reared in our laboratory for two generations. The emerged adults were fed on rabbits and removed once a suitable feeding status or engorgement stage was achieved. Unfed and engorged ticks were kept in cotton-plugged glass tubes in an incubator (27 ± 1 °C, 90 % RH and 12-h photoperiod).

Collection of eggs and preparation of crude egg extract

Eggs were collected and weighed daily from ovipositing females. Briefly, 1 g of eggs was homogenized in 2 mL distilled water containing protease inhibitor (PMSF) using a glass-Teflon homogenizer, and the homogenate was centrifuged at 9,000 rpm for 15 min at 4 °C. Subsequently, crude egg extract (supernatant) was obtained by discarding the fat layer and then frozen at −20 °C prior to further purification.

Purification of Vt from crude egg extract

Crude egg extract (1 mL) was applied onto a Sepharose CL-4B gel filtration column (1.0 × 90 cm) and eluted with water at 4 °C, followed by the collection of 5-mL fractions.

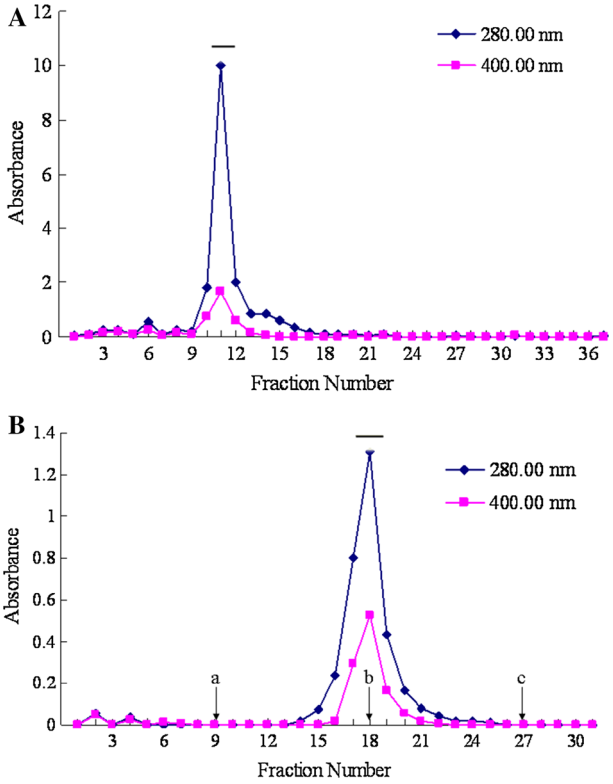

Fig. 1 Elution profile of Vt from crude egg extract on chromatography. **a** Chromatogram on Sepharose CL-4B; **b** chromatogram on DEAE-cellulose (52); *a* 0.02 M Tris–HCl buffer (pH 8.0) + 0.1 M NaCl; *b* buffer + 0.2 M NaCl; *c* buffer + 0.3 M NaCl. The fractions indicated with bars were pooled as Vt

Proteins and heme proteins in the eluate were photometrically monitored at wavelengths of 280 and 400 nm, respectively (Chinzei et al. 1983). Fractions containing Vt (the largest peak, Fig. 1a) were pooled and dialyzed against dialysis buffer (0.02 M Tris–HCl, pH 8.0) at 4 °C for 12 h. The dialyzed Vt was applied onto a diethyl-amino-ethyl (DEAE)-cellulose column (1.6 × 30 cm), which was previously equilibrated with same buffer. Vt purification was conducted using a stepwise gradient elution (0.0–0.8 M NaCl). Similar to gel filtration, the eluate of Vt was collected and monitored at 280 and 400 nm. The Vt fractions were pooled and dialyzed against water at 4 °C for 24 h and then stored at −20 °C. The Vt concentration was determined using the Bradford assay (Bradford 1976).

Polyacrylamide gel electrophoresis (PAGE)

Purity of Vt was examined by native-PAGE and sodium dodecyl sulfate (SDS)-PAGE. Native PAGE was carried out on 7.5 % separating gels (1.5 M Tris–HCl buffer, pH 8.8) with 4 % stacking gels (0.5 M Tris–HCl buffer, pH 6.8). Samples (5–20 μg protein) were dissolved in 0.1 M Tris–HCl buffer (pH 6.8) containing 10 % glycerol and bromophenol blue. Electrophoresis was performed in 0.05 M Tris–glycine buffer (pH 8.3) at a constant voltage (120 V) for 4–5 h. SDS-PAGE was carried out on 7.5 % separating gels with 4 %

stacking gels containing 0.1 % SDS. Samples were dissolved in 0.1 M Tris–HCl (pH 6.8) containing 2 % SDS, 5 % 2-mercaptoethanol, 10 % glycerol and 0.05 % bromophenol blue, and then boiled for 2 min. Electrophoresis was carried out in 0.05 M Tris–glycine buffer (pH 8.3) containing with 0.1 % SDS at a constant voltage (120 V) until the tracking dye reached to the end of gel.

After electrophoresis, both native-PAGE and SDS-PAGE gels were stained with Coomassie blue, whereas the native-PAGE gel was further stained with periodic acid-Schiff (PAS) for carbohydrates or Sudan black B for lipids.

Antibody preparation and immunological tests

Polyclonal antibodies (PcAb) were prepared according to James and Oliver (1997). The purified Vt (500 μg) was emulsified in Freund's complete adjuvant and then subcutaneously injected into rabbits. Two booster injections of Vt were given with the same procedure but using Freund's incomplete adjuvant. Serum was collected after 4–6 weeks, when high antibody titer was observed against Vt. IgG fractions were isolated from antisera using caprylic acid ammonium sulfate precipitation (Li et al. 2008).

Monoclonal antibody (McAb) was produced in BALB/c mice, which were subcutaneously injected with 50 μg Vt antigens emulsified in Freund's complete adjuvant. After that, booster injection was performed twice using the same antigen emulsified in Freund's incomplete adjuvant at intervals of 14 days. Splenocytes from immunized mice were fused to myeloma cells, SP2/0, using polyethylene glycol. Hybridomas were selected in HAT medium, and the hybridoma culture supernatants were screened by enzyme-linked immunosorbent assay (ELISA) in 96-microtiter plates and Western blot.

ELISA was performed according to Li et al. (2008). The positive hybridoma cells were cloned three times. The immunoglobulin of ascites was purified with the method of caprylic acid ammonium sulfate precipitation, while titers and specificity of McAbs were analyzed as previously described (Li et al. 2008). The immunoglobulin isotype was determined using Mouse Monoclonal Antibody Isotyping Reagents.

For Western blot analysis, 10 μg Vt antigens were separated on a 7.5 % SDS-PAGE gel under the reduced condition, and then the gel was electro-transferred onto polyvinilidene difluoride (PVDF) membranes at 4 °C for 1 h. The PVDF membranes were incaubated with McAb and then reacted with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG at room temperature for 1.5 h. Reactive bands were revealed using DAB reagent (3, 3′-diaminobenzidine).

Collection of haemolymph and ovary samples

Haemolymph and ovary of female ticks at different feeding durations or different periods after engorgement were prepared as follows.

Briefly, haemolymph was collected using a calibrated glass micropipette, which could measure the sample volume based on the length of the fluid column, and then samples were diluted in 0.01 M Tris–HCl (pH 8.0) containing 0.02 M Ethylene glycol bis (2-aminoethyl) tetraacetic acid (EGTA) and a small amount of phenylmethanesulfonyl fluoride (PMSF).

Ovaries were dissected and homogenized in 100 μL of 0.01 M Tris–HCl (pH 8.0). The ovarian homogenates were centrifuged at 9,000 g for 10 min. Supernatant was collected into a clean Eppendorf tube and stored at −20 °C until analysis.

Determination of vitellogenin (Vg) content

Vg content was determined using double antibody sandwich ELISA (DAS-ELISA). Briefly, rabbit anti-Vt polyclonal IgG (21.0 μg/mL) was used as capture antibody to coat microtiter plates at 4 °C overnight. After washing three times with phosphate-buffered saline containing 0.05 % (v/v) Tween-20 (PBST), wells were blocked with 10 % fetal bovine serum (FBS) in PBST at 37 °C for 2 h. Subsequently, the plates were washed three times with PBST, and then incubated with 100 μL test samples at 37 °C for 45 min. After six washes with PBST, the anti-Vt McAb was added into each well at 37 °C for 45 min. Wells were washed again, and then the plates were incubated with HRP-conjugated goat anti-mouse IgG at 37 °C for 30 min. After three washes with PBS, chromogen and substrate were added (10 μg 3,3′,5,5′-Tetramethylbenzidine, 5 μL H_2O_2 in 0.1 M citrate–phosphate buffer, pH 5.0) and incubated at 37 °C for 15 min. The reaction was terminated with 2 M H_2SO_4. Finally, optical density (OD) was determined at 450 nm. Meanwhile, a serial dilution of the purified Vt (from 20.48 μg/mL to 1.25 ng/mL) was used to generate a standard curve in order to quantitatively evaluate test samples.

Chemicals

All chemicals were purchased from Sigma Chemical (USA), except for DEAE-cellulose (Whatman Chemical, USA).

Results

Purification of Vt

Vt was purified from crude egg extract by gel filtration (Sepharose CL-4B) and ion exchange chromatography on DEAE-cellulose. The soluble fractions of crude egg extract were eluted from Sepharose gel in several peaks, and the first peak was the only peak containing a heme protein, which was proved by its absorbance at 280 and 400 nm (Fig. 1a). Heme proteins (fractions from the first peak) were pooled and subsequently loaded onto a DEAE-cellulose ion exchange column. The pooled sample was eluted as one peak from column with 0.2 M NaCl, and it was designated as Vt (Fig. 1b).

Purity of Vt was assessed under non-reducing and non-denaturing conditions (native-PAGE) (Fig. 2a). Positively stained Vt revealed the presence of carbohydrates and lipids (Fig. 2b, c), indicating that it was a hemoglycolipoprotein.

Polypeptides of Vt

Purified Vt was tested under reducing conditions (SDS-PAGE) (Fig. 3), and nine polypeptide bands of Vt were detected. The molecular weights of these polypeptides were 203, 147, 126, 82, 74, 70, 61, 47 and 31 kDa, respectively.

Production of McAb against Vt

In the present study, we generated seven hybridoma cell lines secreting McAb against Vt by fusing myeloma cells (SP2/0) with splenocytes from BALB/c mouse immunized with

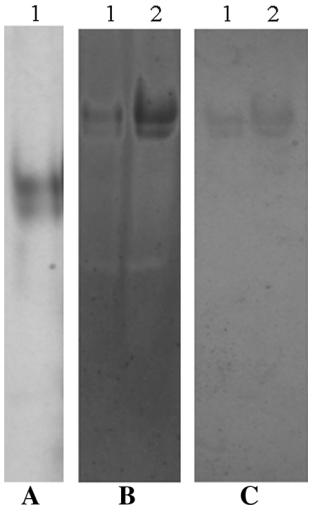

Fig. 2 Native-PAGE of Vt stained with Coomassie blue, periodic acid-Schiff or Sudan black B. **a** Stained with Coomassie blue; **b** stained with periodic acid-Schiff; **c** stained with Sudan black B; *Lane1* purified Vt; *Lane2* crude extract of egg

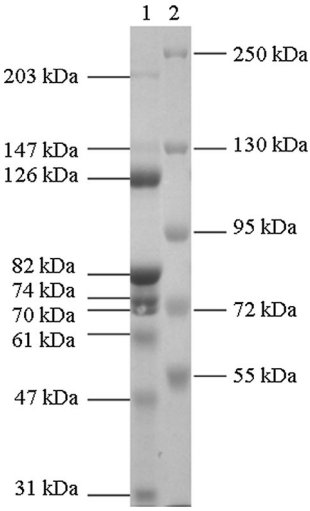

Fig. 3 Polypeptides of Vt separated by 7.5 % SDS-PAGE (stained with Coomassie blue). *Lane 1* Polypeptides of Vt; *Lane 2* molecular mass standards

Vt antigen. The hybridoma cell line that produced IgG isotype with the highest specificity and titer was named as 4G11.

The IgG isotype of 4G11 was identified, and data showed that 4G11 was of the isotype IgG2b. The IgG of 4G11 was purified, and SDS-PAGE revealed that molecular weights of its heavy chain and light chain were 55.8 and 20.9 kDa, respectively (Fig. 4).

Western blot analysis showed that purified 4G11 had specific immunological reaction with only two polypeptides (82, 61 kDa) of Vt (Fig. 5).

Vg dynamics of the haemolymph at different feeding or engorgement statuses

Vg level in haemolymph of the parthenogenetic *H. longicornis* obviously varied at different feeding statuses or after different periods post-engorgement. Vg content in

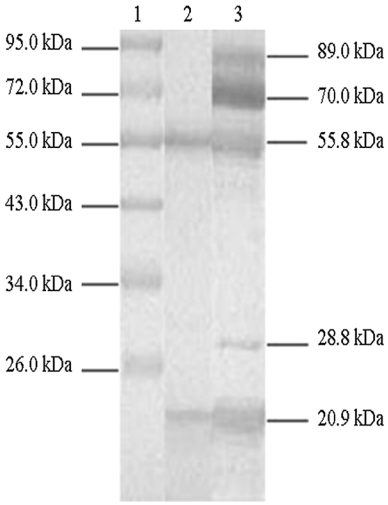

Fig. 4 SDS-PAGE of IgG from the hybridoma cell line (named as 4G11) before and after purification. *Lane 1* Molecular mass standards; *Lane 2* IgG of 4G11 purified by caprylic acid ammonium sulfate precipitation; *Lane 3* BALB/c mouse ascites of 4G11 before purification

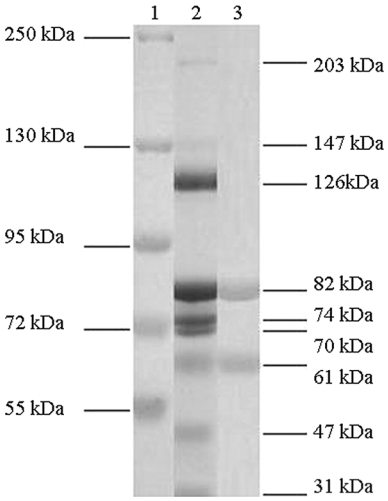

Fig. 5 Western blot of the purified 4G11 and the polypeptides of Vt. *Lane 1* Molecular mass standards; *Lane 2* polypeptides of Vt; *Lane 3* polypeptides (82, 61 kDa) of Vt showed specific immunological reaction with Purified 4G11

haemolymph was extremely low at unfed stage and at early stage of attachment (0.112, 0.137 mg/mL). However, it distinctly increased at the engorgement stage (1.785 mg/mL) and then reached a relatively high level on 2nd day post-engorgement (5.611 mg/mL). Shortly afterwards, the Vg content exhibited a gradually decrease from 4th day post-engorgement to the beginning of oviposition (3.559 mg/mL), but increased again and rapidly reached its maximum level on 2nd day post-oviposition (10.774 mg/mL). Subsequently, the Vg content continued to decrease and reached a low level on 10th day post-oviposition (0.107 mg/mL) (Table 1; Fig. 6).

Vt dynamics of the ovary at different feeding or engorgement statuses

The content of Vt in ovary was very low at the early stage of attachment, leading to difficulty in detection until the day of engorgement (0.024 mg/ovary). After engorgement,

Table 1 Levels of Vg in haemolymph and Vt in ovary at different feeding or engorgement statuses

Stages	Vg in haemolymph (mg/mL)		Vt in ovary (mg/ovary)	
	Mean ± SEM	Tested ticks (n)	Mean ± SEM	Tested ovaries (n)
U	0.112 ± 0.056	30	0.000 ± 0.000	30
F	0.137 ± 0.047	20	0.000 ± 0.000	12
E0	1.785 ± 0.427	6	0.024 ± 0.008	12
E2	5.611 ± 1.343	6	0.049 ± 0.011	6
E4	4.499 ± 0.755	6	0.355 ± 0.110	6
O0	3.559 ± 1.070	6	1.115 ± 0.179	6
O2	10.774 ± 2.043	6	1.942 ± 0.215	6
O4	5.161 ± 1.101	6	0.364 ± 0.072	6
O6	3.019 ± 0.696	10	0.340 ± 0.089	8
O8	1.748 ± 0.554	12	0.065 ± 0.029	12
O10	0.107 ± 0.045	15	0.024 ± 0.003	15

The different feeding or engorgement statuses are represented by the following capital letters or the combination of the letters and numbers (in this and in subsequent tables and figures)

U: Unfed stage; F: The 3rd day after feeding; E0: Day of engorgement; E2, E4: The 2nd and 4th day after engorgement; O0: Day of oviposition; O2, O4, O6, O8, O10: The 2nd, 4th, 6th, 8th, 10th day after oviposition

ovarian Vt level was obviously increased, exhibiting a 46-fold increase on the day of oviposition (1.115 mg/ovary), and it reached the maximum level on 2nd day post-oviposition (1.942 mg/ovary). Afterwards, the Vt content began to decline rapidly (0.364 mg/ovary) and reached a lower level on 10th day post-oviposition (0.024 mg/ovary) (Table 1; Fig. 6).

Discussion

Vt from eggs of the parthenogenetic tick, *H. longicornis* was purified and partially characterized in the current study. The property of Vt is similar to that reported in bisexual population of *H. longicornis* (Yang et al. 2004). The Vt here also contains heme, lipid and carbohydrate components. Derived from the digestion of host hemoglobin, the heme component is the characteristic of Vt found in ticks (Diehl et al. 1982). In general, Vts from ticks are similar to insect Vts in terms of carbohydrate and lipid composition (James and Oliver 1997).

Previous studies have shown that many bisexual ixodid ticks in general have two Vts, such as *Rhipicephalus appendiculatus* and *Dermacentor variabilis* (Dhadialla 1986; Rosell and Coons 1991). However, the argasid tick, *Ornithodoros moubata*, possesses only one single Vt (Chinzei et al. 1983), and the prostriate tick, *Ixodes scapularis*, which is thought to be phylogenetically intermediate between argasids and metastriates, has only one single Vt (James and Oliver 1997). Similarly, we showed that only one single Vt existed in bisexual population of *H. longicornis*. Whether there is a relationship between the number of Vts and the species of ticks remains unknown.

Vt of the parthenogenetic *H. longicornis* consisted of nine polypeptides with molecular weights ranging from 31 to 203 kDa, which was inconsistent with other bisexual tick

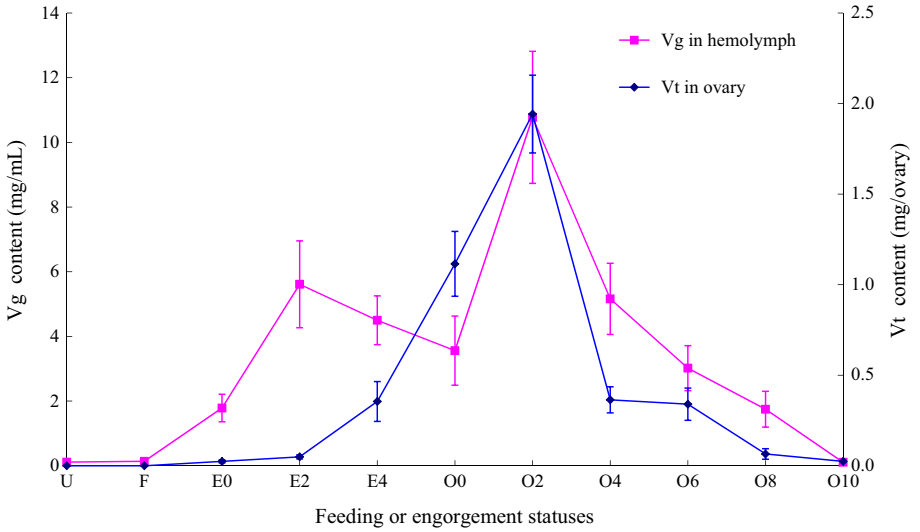

Fig. 6 Dynamics of Vg or Vt content in haemolymph or ovary at different feeding or engorgement statuses. The different feeding or engorgement statuses are represented by the following *capital letters* or the combination of the *letters* and *numbers*. U: Unfed stage; F: The 3rd day after feeding; E0: Day of engorgement; E2, E4: The 2nd and 4th day after engorgement; O0: Day of oviposition; O2, O4, O6, O8, O10: The 2nd, 4th, 6th, 8th, 10th day after oviposition

species as previously reported. Vt of the bisexual *H. longicornis* is composed of eight subunits, and their molecular weights are 112, 103, 80, 78, 71, 68, 62 and 52 kDa, respectively (Yang et al. 2004). Whereas both *D. variabilis* and *R. appendiculatus* have two Vts with polypeptides, of which the molecular weights range from 35 to 135 kDa and from 43 to 160 kDa, respectively (Rosell and Coons 1991; Dhadialla 1986). *O. moubata* has only one Vt with six polypeptides, and the molecular weight of its subunits ranges from 52 to 112 kDa (Chinzei et al. 1983). The Vt in *I. scapularis* contains seven subunits with molecular weights ranging from 35 to 154 kDa (James and Oliver 1997).

In insects, Vgs are large (200–700 kDa) homologous phosphoglycolipoproteins with monmers consisting of one to four subunits. The Vg monomers of most insects are composed of one large (>150 KDa) and one small (<65 KDa) subunit (Kunkel and Nordin 1985; Raikhel and Dhadialla 1992; Valle 1993; Sappington and Raikhel 1998). However, in ticks, no such similar rule was observed in terms of the molecular weight and subunit number of Vt or Vg.

Since the first full-length Vg cDNA sequence for tick was reported from *D. variabilis*, multiple Vg genes have been isolated from ticks, with full-length sequence available from *D. variabilis, H. longicornis,* and *O. moubata*, and partial sequence available from *R. microplus* and *I. scapularis* (Thompson et al. 2007; Boldbaatar et al. 2010; Khalil et al. 2011). Notably, three potential Vg sequences have been characterized from parthenogenetic *H. longicornis* (HlVg1–3) (Boldbaatar et al. 2010), but further analyses cast doubt on HlVg2 and HlVg3 actually being Vg sequences due to their similarity to the common tick storage protein CP (Khalil et al. 2011). In the current study, Vt subunit sizes were compared with the possible products from the predicted RXXR cleavage sites of the three previously described HlVgs. The 61 KDa Vt subunit resolved on the SDS-PAGE was presumably consistent with the expected cleavage product of 60.8 KDa found in HlVg1,

from the first cleavage site (RGTR, aa 253–256) to the second cleavage site (RAIR, aa 779–782), whereas no other Vt subunits were found consistent with the expected products obtained from the suggested cleavage sites of HlVg2 and HlVg3. Undoubtedly, those results strengthened the idea that HlVg1 is the precursor to Vt in *H. longicornis*. However, further analysis remains necessary to explore their relationship.

In arthropods, Vg is transported from its original site by haemolymph to ovary and specifically accumulates in oocytes, and Vg becomes Vt with further processing (Raikhel and Dhadialla 1992; Friesen and Kaufman 2004). In ticks, it has been widely recognized that Vg is produced from fat body and gut and regulated by ecdysteroids (Horigane et al. 2010; Khalil et al. 2011).

The Vt makes up 80 % of the total protein present in eggs (Chinzei et al. 1983), which can be used as a source of nutrient during embryonic development. In ticks, reproductive success is closely related to the process of Vg transportation and uptaking (Boldbaatar et al. 2010). Vgs and Vts are structurally, biochemically and immunologically similar in majority of insects and ticks (Kunkel and Nordin 1985; Kaufman 2004). For the first time, PcAb and McAb against Vt of parthenogenetic population of *H. longicornis* was produced. In addition, a series of identifications were performed on the purified McAb, providing an important tool for further elucidating vitellogenesis and its regulatory mechanism in the parthenogenetic *H. longicornis*.

The Vg or Vt content of the haemolymph and ovary in the parthenogenetic *H. longicornis* was determined using DAS-ELISA. The Vg content of haemolymph was extremely low at the unfed stage and the early stage of attachment, while it was distinctly increased after engorgement. This indicated that feeding initiated Vg production, and Vg was synthesized at an accelerating rate and rapidly released into haemolymph after engorgement.

There was a correlation between Vg content in haemolymph and Vt content in ovaries of the parthenogenetic *H. longicornis*. The Vg content was distinctly increased in haemolymph on the day of engorgement, when Vt began to appear in ovary with a very low content. Then Vg content in haemolymph reached a relatively high level on 2nd day post-engorgement. Shortly afterwards, Vg content was gradually decreased from 4th day post-engorgement to the beginning of oviposition. In contrast, the ovarian Vt level was continuously increased, and it reached a maximum level on 2nd day post-oviposition. Therefore, the decrease of Vg content in haemolymph at 4 days after engorgement was attributed to an increasing rate of Vg uptake by oocytes.

When ovipositing began, Vg content in haemolymph was increased again and reached its highest level on 2nd day post-oviposition, and this increase might be due to a decrease of Vg uptake into ovaries after oviposition. The reduced Vg uptake by ovaries could induce an accumulation of a high content of Vg in haemolymph, resulting in termination of Vg production. Subsequently, Vg content in haemolymph and Vt content in ovaries were continuously decreased and reached a low level on 10th day post-oviposition.

Even though the vitellogenesis and ovarian development of the parthenogenetic and bisexual populations of *H. longicornis* were similar to each other, some important differences also existed. Early studies on bisexual population showed that Vt is detected in ovary at 2 days after engorgement (Yang et al. 2004). The current work revealed that Vt appeared in ovary of the parthenogenetic population 2 days earlier than that of bisexual population. This could be regarded as an adaptative strategy due to the absence of copulation and lack of male factor transfer.

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Conflict of interest All the authors declare no conflict of interest.

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