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The development of oocytes in the ovary of a parthenogenetic tick, *Haemaphysalis longicornis*

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ABSTRACT

Haemaphysalis longicornis is an important vector of various pathogens in domestic animals and humans. The tick is a unique species with bisexual and parthenogenetic races. Although mating induces oocyte development, it is possible in the parthenogenetic race to complete oogenesis without copulation. Here we examined the developmental process of oocytes from unfed to the oviposition period in parthenogenetic *H. longicornis*. We classified the developmental stages of oocytes into five stages: stage I, germinal vesicle occupies more than half of the cytoplasm; stage II, germinal vesicle occupies less than half of the cytoplasm; stage III, germinal vesicle migrates from the center in the oocyte to the vicinity of the pedicel cells; stage IV, the cytoplasm is filled with yolk granules of various sizes; stage V, the cytoplasm is occupied by large yolk granules. Oocytes at the unfed period were undeveloped and classified as stage I. Stage I and II oocytes were observed at the rapid feeding period, indicating that oocyte development began after the initiation of blood feeding. All developmental stages of oocytes were observed at the pre-oviposition period. At 10 days after the beginning of the oviposition period, the ratios of stage I and II oocytes were higher than those of the previous period, suggesting that the ovarian development and activity may be continuing. Based on these findings, we propose classification criteria for the oocyte development in the parthenogenetic *H. longicornis*. The criteria will be useful for understanding the mechanisms of tick reproduction and transovarial transmission of pathogens.

1. Introduction

Ticks are hematophagous ectoparasites, acting as vectors of various pathogens for vertebrates. They are associated with significant economic losses in livestock animals and also cause great suffering to humans worldwide. The hard tick *Haemaphysalis longicornis*, the most dominant tick species in Japan, is distributed in Australia, New Zealand, New Caledonia, the Fiji Islands, Korea, China and Russia [1]. *H. longicornis* is characterized by having both parthenogenetic and bisexual races. The parthenogenetic race of *H. longicornis* is distributed widely across Japan, and the bisexual race is distributed only on Kyushu (the most southwesterly of Japan's four main islands) and at scattered sites across southern Japan [2]. *H. longicornis* is known as a vector of *Theileria orientalis* and *Babesia ovata*, which cause piroplasmosis in cattle, *B. gibsoni* causing babesiosis in dogs, and *Rickettsia japonica* causing Japanese spotted fever in humans [3–5]. In addition, severe fever with thrombocytopenia syndrome (SFTS) was first reported in China, and the first patient in Japan was reported in 2013 [6]. The SFTS virus gene has been detected in several tick species, including *H. longicornis*. The significance of this tick species on the public health problem has become increasingly evident because the SFTS virus is widely distributed in Japan and has an approximately 30% fatality rate in humans.

In *H. longicornis*, some pathogens are transmitted transovarially [7–11]. Observation of *Babesia major* in *H. punctata* oocytes led to hypothesize that the parasites invade the developing oocytes, especially

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immature oocytes [12,13]. Recently, tick-*Babesia* experimental model revealed the time course of *B. ovata* migration in the internal organs including the ovary [14]. It was also reported that *B. ovata* DNA exists in eggs laid a few days after the beginning of oviposition in *H. longicornis* [15]. However, how and when the *Babesia* parasites invade into the oocytes of *H. longicornis* remains unclear.

The oocytes on female hard ticks begin to develop asynchronously after blood feeding. The oocyte maturation process of *Amblyomma cajennense*, parthenogenetic *A. rotundatum*, and bisexual *H. longicornis* were observed and the developmental stages of oocytes were defined [16–18]. Although parthenogenetic *H. longicornis* have also been examined with regard to the development of the ovaries [19,20], there was no description about the classification of the developmental stages of oocytes.

Simplicity of maintenance of parthenogenetic *H. longicornis* in laboratory has made it an excellent model for studies on biology and physiology of hard ticks [14]. Therefore, it is necessary to establish the classification criteria for oocytes of parthenogenetic *H. longicornis* toward understanding the mechanisms underlying the transovarial transmission of pathogens. In the present study, we examined oocyte maturation in parthenogenetic *H. longicornis* and classified the oocytes into developmental stages.

2. Materials and methods

2.1. Ticks and animals

The parthenogenetic tick *H. longicornis* (Okayama strain) was maintained at National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, feeding on the ears of Japanese white rabbits (Japan SLC, Shizuoka, Japan) by the cotton bag method as described [21]. The rabbits were cared for in accordance with the guidelines approved by the Animal Care and Use Committee of Obihiro University of Agriculture and Veterinary Medicine (approval no. 28–34). They were maintained in a temperature- and humidity-regulated room (at 25 °C and 40%, respectively) under controlled lighting (lights on from 6:00 to 19:00), with free access to tap water and commercial regular chow (CR-3; CLEA Japan, Tokyo) throughout the experiments.

2.2. Histological observation of the ovaries

Ovaries were collected from female *H. longicornis* at the unfed, the slow feeding (3 days after attachment), the rapid feeding (4–6 days after attachment) and the engorgement (5–7 days after attachment) periods. Engorged females were transferred into individual vials (AS ONE, Osaka, Japan) that were placed in a chamber and then kept at 25 °C with saturated humidity in continuous darkness.

Ovaries were also collected at 4 days (pre-oviposition), 7 days (the beginning of oviposition) and 17 days (10 days after the beginning of oviposition) after engorgement. A total of 161 ovaries were used in our study. The morphology of each ovary immersed in phosphate-buffered saline (PBS) was observed and photographed using a stereomicroscope (SZX16, Olympus, Tokyo) coupled with a digital camera (DP21; Olympus). Following the morphological observation, ovaries were fixed with 4% paraformaldehyde-2.5% glutaraldehyde in PBS for 24 h at 4 °C. They were pre-embedded in 3% agarose (Sigma, St. Louis, MO, USA) in PBS and then embedded in paraffin (Sakura Finetek Japan, Tokyo). The paraffin sections were cut at 5-μm thick using a microtome (REM-700, Yamato Kohki Industrial, Saitama, Japan). They were stained with hematoxylin (Muto Pure Chemicals, Tokyo) and eosin (Muto Pure Chemicals) following routine histological procedures. The stained sections were observed and photographed using a light microscope (BX53, Olympus) coupled with a digital camera (DP73, Olympus).

2.3. Classification of oocytes

We classified the developmental stages of the oocytes in parthenogenetic *H. longicornis* into five stages as shown in other reports; *A. brasiliense* [22], *A. cajennense* [16], parthenogenetic *A. rotundatum* [17], *A. triste* [23], *A. varium* [24], bisexual *H. longicornis* [18], *Rhipicephalus* (*Boophilus*) *annulatus* [25], *R. (Bo.) microplus* [26] and *R. sanguineus* [27,28]. Four histological sections were selected randomly in each sampling period to calculate the ratio of each developmental stage to all oocytes observed in each section. The area per oocyte on a section at each sampling time point was measured using the BZ-H3AE Analysis application (Keyence, Osaka, Japan).

3. Results

3.1. Classification of oocytes

Based on area size, shape, location of germinal vesicle, cytoplasm appearance, and presence of chorion, oocytes of parthenogenetic *H. longicornis* were classified into 5 stages as follows:

3.1.1. Stage I

The stage I oocytes are small (3.60×10–$1.86 \times 10^3\,\mu m^2$; median $814\,\mu m^2$) and have round to elliptical shapes (Table 1, Fig. 1A). The germinal vesicle is located in the center of the oocytes and occupies more than half of the cytoplasm. The cytoplasm is homogeneous and basophilic and does not possess yolk granules. These oocytes are attached to the ovarian wall by pedicel cells.

3.1.2. Stage II

The area of stage II oocytes is approximately four times larger (1.91×10^3–$5.73 \times 10^3\,\mu m^2$; median, $2945\,\mu m^2$) than that of stage I (Table 1). The oocytes have an elliptical shape and attached to the ovarian wall, where are supported by pedicel cells (Fig. 1B). Germinal vesicle occupies less than half of the cytoplasm. The cytoplasm is eosinophilic (acidophilic), indicating that the stage II oocytes begin to the yolk granulation.

3.1.3. Stage III

The area of stage III oocytes is 5.96×10^3–$1.81 \times 10^4\,\mu m^2$ (median $10{,}386\,\mu m^2$) (Table 1) and is supported by pedicel cells. In these oocytes, the germinal vesicle has migrated from the center of the oocytes to the vicinity of the pedicel cells. Progressed yolk granulation is observed in the cytoplasm of stage III oocytes (Fig. 1C). Choriogenesis appears to begin at this stage.

Table 1
Characteristics of the oocytes at each developmental stage in *H. longicornis*.

Oocyte developmental stage	Area per oocyte (μm^2)	Location of germinal vesicle	Yolk granules	HE staining properties
Stage I	3.60×10^1–1.86×10^3	Center of cytoplasm	Not present	Basophilic
Stage II	1.91×10^3–5.73×10^3	Center of cytoplasm	Not present	Eosinophilic
Stage III	5.96×10^3–1.81×10^4	Close to chorion	Not present, or small	Eosinophilic
Stage IV	1.92×10^4–4.48×10^4	Close to chorion	Various sizes	Eosinophilic
Stage V	4.51×10^4–1.69×10^5	Close to chorion	Large and polygonal shapes	Eosinophilic

HE, hematoxylin and eosin.