



## *Haemaphysalis longicornis*: the life-cycle on dogs and cattle, with confirmation of its vector status for *Theileria orientalis* in Australia

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### ARTICLE INFO

#### Keywords:

*Theileria orientalis*  
Ikeda  
Theileriosis  
Transmission  
Ticks  
*Haemaphysalis*

### ABSTRACT

The intracellular protozoal parasite *Theileria orientalis* ikeda has rapidly spread across South-eastern Australia since 2006, causing deaths and production losses in cattle. The 3-host “bush tick” *Haemaphysalis longicornis* (Neumann) appears the principal biological vector in the endemic regions. To generate sufficient numbers of ticks to produce stabilate for infection to confirm vector competency and for acaricide trials, the optimal conditions and stage-specific intervals for the generational life-cycle of *H. longicornis* was defined on two dogs and two steers. To determine whether *H. longicornis* was a definitive host for *Theileria orientalis*, nymphal stages were fed on a steer infected with *T. orientalis* and moulted adults were permitted to feed for 3 days on an uninfected calf prior to harvest. Subsequent detection of infection after inoculation of four naïve calves with stabilate produced from ground-up adult ticks or dissected salivary glands confirmed *H. longicornis* as one final (definitive) host for *T. orientalis* in Australia.

### 1. Introduction

The 3-host Ixodid tick *Haemaphysalis longicornis* (Neumann) has been reported from countries with temperate climates including Australia, New Zealand, Fiji, New Caledonia, China, former USSR, Korea and Japan (Hoogstraal et al., 1968; Roberts, 1963, 1970; James et al., 1984) and several Pacific Islands, including Hawaii, as well as emergence noted in the USA (Chomel, 2011). This species is distinct from *H. bispinosa* (Neumann), which has a tropical distribution that does not overlap with *H. longicornis* (Hoogstraal et al., 1968). The tick has a wide host range across livestock and wildlife (Cane, 2014), with cattle being the principal host. *H. longicornis* is appreciated as an important vector of protozoal parasites in East Asian countries, including *Babesia ovata* and *B. gibsoni*, as well as parasites described as *Theileria sergenti* and *T. mutans* (Galay et al., 2012; Hoogstraal et al., 1968). In a recent study from North Korea, *H. longicornis* collected from goats were positive for *Anaplasma bovis*, *A. phagocytophilum*, *Bartonella grahamii*, *B. henselae* and *Borrelia* spp. based on 16S ribosomal RNA and ITS species-specific nested PCR (Kang et al., 2016). Populations of *H. longicornis* in livestock in Australia and New Zealand are parthenogenetic with small numbers of sterile males recovered (Hoogstraal et al., 1968; Roberts, 1970).

While *Rhipicephalus (microplus) australis* is the primary tick vector of *Babesia bovis* and *B. bigemina* in Australia, and *H. humerosa* and *H. bancrofti* vectored the “benign” *T. buffeli*, (Riek, 1982; Stewart et al., 1987), *H. longicornis* has been implicated as the definitive host for the more pathogenic *T. orientalis* ikeda in Australia (Hammer et al., 2015; Yano et al., 1988). In related studies, mechanical transfer of bovine blood infested with *T. orientalis* ikeda resulted in persistent infection (as determined by PCR) but did not elicit clinical disease or detectable parasitaemia (Hammer et al., 2016; Gibson, 2017). In splenectomised calves, marked parasitaemias and PCV reductions were elicited after similar mechanical transfer, but infection was also self-limiting (Tick Fever Centre data). This indicated that sexual reproduction in the definitive tick host could be essential to retain virulence in the parasite, but confirmation of the vector was required to instigate vaccination or tick control.

Consequently, to confirm the transmission of *T. orientalis* ikeda by *H. longicornis*, and to examine both the early pathogenesis of infection and the generation of immunity to *T. o. ikeda* in Australian cattle, an infective tick stabilate was needed. To obtain large numbers of larval ticks for infection with genotypic isolates of *T. orientalis* and to generate ticks for acaricide trials, this study reports the life cycle requirements for a generation of *H. longicornis* raised on dogs and cattle, the successful

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production of infective stabilate, and confirmation of the definitive host status of *H. longicornis* for transmission of *T. orientalis* ikeda in Australia.

## 2. Methods

### 2.1. Method for feeding ticks on dogs

#### 2.1.1. Source and storage of ticks prior to infestation

Engorged females were originally sourced from Stroud NSW. The ticks were maintained in two incubators to examine several temperatures and humidities for development; either 25 °C ( $\pm 1$  °C) and 85–95% relative humidity or 27 °C ( $\pm 1$  °C) and 70–80% relative humidity. Hatching and subsequent development throughout all stages of the life cycle were monitored and recorded. Ticks that showed the best development were chosen for infestation.

#### 2.1.2. Animals and facilities

Dogs of quiet temperament with light, short coats were used for all stages of infestation. Two dogs were maintained in a specially designed pen for a maximum period of 12 days at a time. The pen has a raised floor system made of an open weave metal with a plastic coating to allow engorged ticks to fall through into the water catchment below. The facility had automated lighting set to give the dogs 12 h of light a day. Dogs were provided with ad lib water and fed twice daily as per their normal feeding regime. In addition, dogs were socialised twice daily for a minimum 15 min.. The Elanco AEC committee covered animal ethics (Approval number ELA1700007).

#### 2.1.3. Infestation and collection of ticks

On day 0, a maximum of 20 000 larval ticks per dog were deposited evenly along the backline of the dog from the head to the tail. Immediately after infestation the dogs were kept idle to avoid dislodging the ticks. The water catchment of the pen was kept dry for the first 24 h to allow some time for any ticks that became dislodged in this initial period to re-attach. The dogs were monitored at least twice daily to allow socialisation, cleaning and tick collections.

A 250–300  $\mu\text{m}$  sieve (maximum) was placed beneath the outlet from the pen to collect ticks and debris that had collected in the water catchment below. Collections were performed at least twice daily from day 2 to 12, with up to three collections performed on days of peak drop. The days on which the start, peak and end of drop occurred were recorded. Collected ticks were rinsed through sieves of decreasing size to separate ticks from debris. Once cleaned, ticks collected were placed in large ventilated containers with paper towelling at the bottom, allowed to dry out under a heat lamp, before equal numbers were placed in one of the two incubators to moult, harden and continue development at either 25 °C or 27 °C.

Once the feeding period was complete, a visual and tactile check was completed on the dogs to locate any remaining attached ticks, ensuring to check inside ears, toes and pads. If found, remaining ticks were detached mechanically using a pincer or a flea comb. Ticks removed mechanically were assessed, and if considered intact and sufficiently developed they were added to the collected ticks; if not, they were discarded. To help ensure they were tick free, the dogs were washed and kept isolated from other dogs for a minimum of 24 h prior to returning to the dog facility.

### 2.2. Method for feeding ticks on naïve and infected cattle

#### 2.2.1. Source and storage of ticks prior to infestation

Ticks were maintained in incubators at 27 °C and 85% relative humidity.

#### 2.2.2. Animals and facilities

Splenectomised bovine calves, aged approximately 5–9 months, were used for tick application at the Tick Fever Centre, Wacol,

Brisbane. These calves had been housed in insect proof sheds on mesh flooring in the 3–4 months since splenectomy, and had been tested negative for *Theileria orientalis*, based on weekly blood smear examination since splenectomy. The calves were moved to separate single insect proof moated isolation pens prior to tick infestation. All studies were conducted under Queensland Department of Agriculture and Fisheries Staff Access Animal Ethics Committee Approval SA 2017/05/604.

Infectivity trials were conducted at the University of Sydney Moffitt's Farm, Camden Campus, using Holstein steers aged 6–8 months. Studies were conducted in accordance with USyd Animal Ethics protocol 2018-1328.

#### 2.2.3. Infection of calves with *Theileria orientalis*

Initially, nymphal ticks were allowed to feed on an infected steer (by positive blood smear) before being tested by PCR after moulting to unfed adults. These ticks tested negative for *T. orientalis* by PCR. Therefore, around 100 newly moulted and infected adult *H. longicornis* were allowed to feed for 3d on an uninfected calf to allow for sporozoite development and maturation, before being harvested for preparation of stabilate. This follows a similar methodology which allows maturation for sporozoites of *T. parva*, except feeding is conducted on rabbits (Kimbita and Silayo, 1997), due to the much higher virulence of *T. parva*.

#### 2.2.4. Infestation and collection of ticks

Infestation techniques were based on the methods of the International Livestock Research Institute, Nairobi, Kenya (ILRI; Naftaly Githaka, pers. comm.) and USDA Animal Disease Research Unit, Pullman WA (Glen Scoles, pers. comm.). An area on the back of the calf sufficient to allow attachment of the patch was clipped using #10 clipper blades. Natural calico (purchased from Spotlight) patches were sewn with corner pleats to elevate the patch off the skin of the calf, and the edges overlapped to prevent excess fraying. The patch size varied between applications, but a piece of calico approximately 42 cms long and 37 cms wide, when pleated and glued into place on the back of the calf (Kamar® Heatmount Detector liquid adhesive) with a 30–40 mm contact area all around the edge of the patch, resulted in a feeding area under the patch of approximately 25–30 cm  $\times$  20–25 cm. The calves were not tethered in any way (by either halter or stanchion) and were able to move freely in the pens (and lick the patches!). The patch was applied the day before tick infestation and remained intact for the period of feeding.

The ticks were applied by two methods. For larvae, nymphs and adults, a small incision was made in the centre of the patch, and the tube inserted through the incision. The lid was removed through the patch, and ticks could be released onto the calf. This was assisted using a small art brush to remove the ticks from the tube. For adults, where application numbers were smaller and the ticks larger, the tubes were also emptied into a funnel with the end poked down through the incision, and the ticks brushed down the funnel onto the calf. The incision was closed with a mattress suture using an ordinary sewing needle and thread. A piece of sticking plaster was applied over the incision, but the plaster used did not always stick well.

Ticks which landed outside the patch during the application process (especially larvae and nymphs) were collected using sticky tape. A bucket with sufficient ethanol to just cover the base was used to throw empty tubes, used sticky tape and used brushes at the end of the procedure, so that any residual live ticks were killed.

Once engorged and unattached, larvae and nymphs were collected with suction applied via a small vacuum pump, using rubber tubing inserted through an incision in the patch, and attached to a small 500 ml Schott bottle as the collection vessel. Engorged adults, which had detached after feeding, were collected by hand picking. Attached semi-fed adults, collected on Day 3 after application, were picked off using a commercial tick removal tool available at pet stores (Tick Twister®).

The ticks were transported to the Biosecurity Sciences Laboratory, Coopers Plains, Brisbane and processed prior to incubation according to life stage.

Engorged larvae were passed through a coarse sieve into a kitchen strainer to separate them from the detritus collected in the vacuum collection process. Larvae were then weighed into ventilated 20 ml polystyrene tubes in 0.5 g (approx. 1100 larvae) and 1.0 g (approx. 2200 larvae) allocations.

Engorged nymphs were sieved in the same manner and tubed in 0.5 g lots. Counts made of four repeated 0.5 g aliquots indicated around 110 engorged nymphs with good consistency.

Engorged females were rinsed clean and dried thoroughly with tissue, then stuck to the lid of a takeaway food container with double-sided adhesive tape (Scotch 3 M 969 adhesive transfer tape) and given two weeks incubation period to lay eggs. The eggs were then weighed into ventilated 20 ml polystyrene tubes in 0.5 g and 1.0 g allocations.

All tick life stages were incubated at 27 °C and 85% relative humidity, conditions optimised for maintaining cultures of *Rhipicephalus (microplus) australis*.

### 2.3. Tick development observations

Once the feeding period was complete, collected ticks were weighed and stored in ventilated syringes or vials in the incubators. The number of ticks was calculated from the weights (Zheng et al., 2011), which were validated in this experiment through an average of three standardised counts of samples at each life stage. Ticks were checked daily to monitor hatching and moulting. Start, peak and end of hatch or moult was recorded as well as percentage of ticks which did not develop. Ticks were allowed a minimum of 5 days post hatch or moult to allow sufficient hardening of their exoskeleton prior to their next infestation. A poor yield of engorged nymphs was noted on cattle when unfed nymphs were applied at Day 15 post collection of engorged larvae. While it is not certain that too early application of nymphs after the moult was a contributing factor, it is considered prudent to wait for a longer period after the moult is largely complete.

### 2.4. Production of theilerial stabilate(s)

#### 2.4.1. Ground-up tick supernatant fluid (GUTS)

Ground-up tick supernatant fluid (GUTS) containing *T.orientalis* ikeda was produced according to the method described for *T.parva* in the OIE Terrestrial Handbook (2008, p799-80). Briefly, batches of 1000–1500 *H.longicornis* adult ticks, infected as nymphs, were collected after 3 days of feeding. Ticks were disinfected in 70% ethanol and rinsed in distilled water before being partially “cut” through the caudal half of the abdomen and placed into a sterile glass homogeniser flask containing around 25 ml of cold Hanks BSS (HBSS) with 10% bovine serum albumin (BSA; Australian sourced to avoid BSE biosecurity issues). The incisions allowed for easier grinding. Ticks were then ground using a tight fitting glass homogeniser in an ice bath for 2 min before centrifugation at 50 g for 5 min at 4 °C and the supernatant harvested. An equal volume of cold 15% glycerol in HBBS-BSA was added dropwise while stirring on a magnetic stirrer, giving a final equivalent of 20 ticks ml<sup>-1</sup>. The stabilate was aliquoted in 1.5 ml volumes into cryovials

**Table 1**

Oviposition by *Haemaphysalis longicornis* at constant temperatures and relative humidity within the laboratory.

Temperature and Relative Humidity	Preoviposition Period		Oviposition Period		Mean number of eggs deposited per mg body weight
	Average	Range	Average	Range	
25 °C ( ± 1) 85–95% RH (5)	6	4–7	19	18–20	11.8
27 °C ( ± 1) 70–80% RH (5)	5	4–6	14	9–18	9.6

Intervals are in days. The number of engorged females are in parentheses.

(Nunc) before being gradually frozen, initially over 30–45 min in the vapour phase of liquid nitrogen, then stored in liquid nitrogen until use.

#### 2.4.2. Salivary gland stabilate

Tick salivary glands were dissected from around 1500 disinfected, washed ticks (view Patton et al., 2012) and progressively accumulated into 25 ml cold HBBS-BSA in a glass homogenising flask. They were subsequently ground and processed as for GUTS above, frozen and stored in HBSS-BSA with 7.5% glycerol (final concentration) as 1.5 ml aliquots (20 ticks ml<sup>-1</sup>) in liquid nitrogen.

#### 2.4.3. Infection and monitoring of calves

To test the ability of infected *H.longicornis* to transmit *T.orientalis* ikeda prior to committing large numbers for the production of stabilate, around 100 unfed, adult ticks, which had been infected with *T.orientalis* ikeda as nymphs, were placed under a backline patch onto an uninfected, splenectomised calf and removed 3d later. This followed a similar rationale and feeding times on rabbits, enabling maturation of *T.parva* sporozoites in *Rhipicephalus appendiculatus* (Kimbata and Silayo, 1997). Subsequently, 5d after tick application, the regional prefemoral lymph node was biopsied using a 19 g needle into PBS-EDTA to search for schizonts. A total of four impression smears were made and stained with Giemsa. Blood was collected twice-weekly with smears examined for the presence of piroplasms and the haematocrit determined. This calf became positive for *T.orientalis* after 13 days and samples of the 3day-fed ticks were also positive for *T.orientalis* ikeda by PCR. Therefore, for stabilate production, another batch of around 3000 infected ticks were allowed to feed for 3d on another uninfected calf before being removed and made into stabilate.

For infection, tick stabilate was thawed in water at room temperature immediately prior to inoculation. A volume of 1.5 ml GUTS was inoculated subcutaneously (SC) into the right neck of each of 2 calves and a similar volume of the salivary gland stabilate was administered to an additional 2 calves. Both volumes were the equivalent of around 30 ticks. Calves were monitored daily, with 5 ml blood collected twice weekly into EDTA vacutainers (Vacurette, Griener Bio-one) from d14 post-inoculation. The study was terminated at d28 when all calves tested positive by PCR.

#### 2.4.4. PCR diagnostic monitoring of ticks and calves

For all diagnostic PCR, DNA extraction was performed using the KingFisher® MO BIO PowerMag® Microbiome robotic program before PCR was conducted using the Ausdiagnostics kit (AusDiagnostics Pty Ltd: Easy-Plex™ 96 System. NSW), according to the manufacturer's instructions (Perera et al., 2015). The assay is based on genotypic variation in the major piroplasm surface protein (MSPS) gene and can detect 0.25 parasites ml<sup>-1</sup> of blood (Perera et al., 2015). Intensity of infection was determined from the Cycle take-off (Ct) value: negative, > 25; positive, 15–25; strongly positive, < 15.

## 3. Results

### 3.1. Lifecycle timelines on dogs and within the laboratory

Oviposition occurred after a relatively consistent pre-oviposition

**Table 2**  
Egg hatchability at constant temperatures and relative humidity for *Haemaphysalis longicornis* within the laboratory.

Temperature and Relative Humidity	N	Hatch Ratio (%)	Period (days)	
			Average	Range
25 °C ( ± 1) 85–95% RH	150	97	25	17–30
27 °C ( ± 1) 70–80% RH	150	65	20	15–23

period of 4–7 days (Table 1). The pre-oviposition and oviposition periods were slightly shorter at 27 °C and 70–80% relative humidity but resulted in a reduced number of eggs per mg of bodyweight being deposited by the females (Table 1). Egg hatchability was notably higher (97%) at 25 °C and 85–95% relative humidity than at 27 °C (65%). However, hatching at 25 °C took longer (25 days) than at 27 °C (20 days) (Table 2). Larval and nymphal moulting occurred over a shorter period and lower ratio at 27 °C and 70–80% relative humidity when compared to those stored at 25 °C and 85–95% relative humidity (Table 3).

Larval feeding was completed within 3–5 days post infestation (pi) with the highest number of ticks dropping at 4 days pi (Table 4). Nymphal feeding occurred within 4–7 days pi with the highest number of ticks dropping off 6 days pi (Table 4). Adults commenced detaching after 6 days pi with the highest number of ticks falling off on day 10 pi. Since dogs were only kept in the containment facility for a maximum of 12 days, the full length of adult feeding could not be monitored past 11 days pi. Ticks remaining attached at day 11 were removed and end of adult feeding was recorded as Day 11 + (Table 4). The total life cycle was around 111 days when compared with other international studies (Table 5).

When maintained at 25 °C and 85–95% relative humidity, hatching larvae had the highest attrition of non-feeding life stages at 3% versus 0% for moulting larvae and nymphs (Fig. 1). Attrition during feeding was highest for engorged larvae at 46% followed by engorged nymphs at 28% and engorged adults at 15% (Fig. 1).

### 3.2. Lifecycle timelines on cattle and within the laboratory

Oviposition occurred after a pre-oviposition period of 4 days and lasted for a period of 13 days (Table 5). Oviposition to hatching period was approximately 28 days with larvae resting for a period of 21 days before being infested onto cattle (Table 5). Larval feeding was completed over a period of 4–6 days with moulting occurring over a period of 2–11 days post collection (Pc) (Table 5). Nymphs took 4–9 days to harden before feeding for a period of 3–7 days. The nymphs started moulting 9 days Pc with moulting complete at 15 days Pc. Adult ticks were mostly all engorged and detached by Day 6 of feeding on cattle and laid approximately 200 mg of eggs per female (Table 5).

### 3.3. Development of theileriosis after tick application or stabilate inoculation

Following application of unfed adult ticks (infected with *T.orientalis* ikeda as nymphs) onto an uninfected, splenectomised calf, the host

**Table 3**  
Moulting periods of engorged larvae and nymphs at constant temperatures and relative humidity for *Haemaphysalis longicornis* within the laboratory.

Temperature and Relative Humidity	Larval Moulting				Nymphal Moulting			
	N	Ratio %	Period (days)		N	Ratio %	Period (days)	
			Average	Range			Average	Range
25 °C ( ± 1) 85–95% RH	150	100	13.5	8–16	150	100	13	9–16
27 °C ( ± 1) 70–80% RH	150	65	11.5	6–18	150	88	11	8–12

**Table 4**  
Day post infestation (day 0) when engorged ticks dropped off dogs.

Life Stage	Days post infestation		
	Start	Peak	End
Larvae	3	4	5
Nymphs	5	6	7
Adults	6	10	11+

developed a detectable parasitaemia 13 days later, confirmed by PCR (Ausdiagnostics) as *T.orientalis* ikeda genotype. Although biopsies of the prefemoral nodes were negative when taken 5d after application, piroplasms were detected in blood smears from day 13 post-application.

The inoculation of GUTS or salivary glands produced parasitosis detectable by PCR, in all 4 calves within 26 days. Three calves became positive for *T.orientalis* ikeda on day 22 (both salivary glands and 1 GUTS) and day 26 (GUTS). Clinical disease was avoided as the study was terminated at this point as a permit to use buparvaquone for treatment had not been issued. Throughout the same 26-day period, blood smears were negative for piroplasms and the haematocrit remained unchanged.

## 4. Discussion

Since previous reports did not detail any colony conditions for *H.longicornis*, (Riek, 1982; Stewart et al., 1987), the current study demonstrates the successful cultivation of *H.longicornis* on dogs and cattle in Australia where the average generation cycle of the tick was approximately 111 days. This is consistent with similar research on the life cycle from Japan (Hoogstraal et al., 1968) but is notably shorter than the 135.8 days reported from China (Lui and Jaing, 1998) (see Table 5). However, a field study in China demonstrated that generational cycle could vary greatly depending on the time of year in which each stage of the life cycle occurred (Lui and Jaing, 1998). This could explain the notable differences between some of the stages in each of the studies and contrasts the annual seasonality in the field that is substantially accelerated under the optimal, climate-controlled conditions used in this study. The lengthening of the life cycle to around a year in the field is also noted in Australia and New Zealand, with early summer noted for the appearance of adult stages of the parasite (Heath, 1998; Wagland et al., 1979). In the cattle studies here, temperature in the pens at the time of feeding on cattle was not noted; conditions of incubation of the laboratory stages were noted; but in general, the effect of season and temperature was not compared.

The larval and nymphal feeding periods on dogs of the current study were similar to results from Japan and China (Hoogstraal et al., 1968; Lui and Jaing, 1998) (Table 5). The average feeding periods for larvae only differed by 0.7 days with an average feeding period of 4, 4.5 and 3.8 respectively (Table 5). Similarly, average nymphal feeding periods exhibited a difference of only 0.6 days with average feeding periods of 6, 6 and 5.4 respectively. Adult feeding periods displayed the greatest difference between the 3 studies, with a difference of 6 days occurring between the averages reported by Japanese (15 days) and Chinese researchers (9.4 days) (Table 5). The average of 10 days for the adult feeding period in this study is consistent with several previous reports

**Table 5**  
Comparison of life-cycle timelines for *Haemaphysalis longicornis* in Australia, Japan and China.

Life cycle stage	Australia: Current study			Japan**		China***
	Range (Days)	Dogs	Average	Range (days)	Average	Average
		Range (days)				
Oviposition to hatching	28	17–30	25	24–38	27.5	38.5
Resting (Larvae)	21	3–5	4	3–5	4	5.3
Feeding (larvae)	4–6	3–5	4	4–5	4.5	3.8
Larval moult	2–11	8–16	13.5	14–17	15.5	13.9
Hardening (nymph)	4–9	4–6	5	2–3	2.5	7.2
Feeding (nymph)	3–7	5–7	6	5–7	6	5.4
Nymphal moult	9–15	9–16	13	12–16	14	16.9
Resting (adults)		6–8	7	4–6	5	7.6
Feeding (adults)	3–6	6–11 +	10	11–19	15	9.4
Preoviposition Period	4	4–7	6	3–7	5	7.8
Oviposition period	13	18–20	19	11–27	19	20
Numbers of eggs*	200		11.8		2024	
% hatch rate			97		94	
Generational cycle	97–128	83–131 +	111		118	135.8

\* Number of eggs deposited per mg of body weight.  
 \*\* Kitaoka (1961), cited by Hoogstraal et al. (1968).  
 \*\*\* Lui and Jaing (1998).

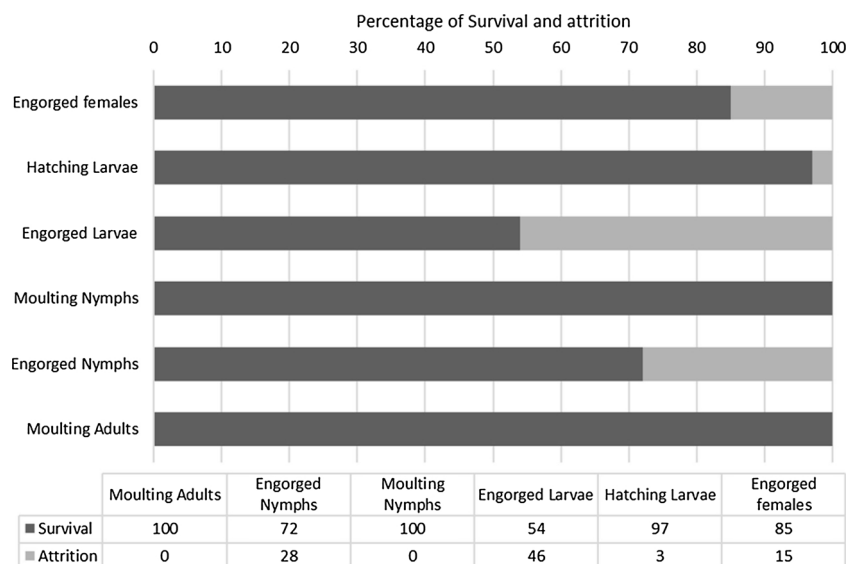
(Kang, 1980; Lui and Jaing, 1998). In this study, stabilates containing either *T.orientalis* ikeda or *T.orientalis* buffeli- infected erythrocytes (iRBCs) were inoculated intravenously into two splenectomised calves retained in small pens. In both cases, although the parasitaemias reached levels up to approximately 31% and 6% iRBCs respectively, and haematocrit reductions of approximately 45% and 38% respectively were recorded (from 33% to a minimum of 18% PCV; and from 32% to 20%), the calves did not show any change in demeanour or appetite, and the infection was self-limiting.

In the current study, the larval feeding period was slightly shorter on dogs than on cattle. This is consistent with previous research of feeding periods on cattle (Wagland et al., 1979). However, nymphal and adult feeding periods were longer on dogs when compared to cattle. This difference could be the result of a number of factors including host immune response, differences in host anatomy affecting feeding or environmental differences between the facilities in which each trial was run. Resistance to *H.longicornis* in cattle has been previously demonstrated by Sutherst et al (1979), which resulted in greater losses of all life stages of ticks feeding on previously exposed host

animals when compared to the ticks feeding on naïve hosts.

During collections of each stage of the life cycle from dogs, it was noticed that collections were greatest in the morning. This could suggest that most feeding by *H.longicornis* occurs nocturnally or in response to the host periods of inactivity. A previous study in Queensland, Australia, suggested that the seasonality of *H.longicornis* could be the result of a photoperiod-induced diapause (Sutherst and Bourne, 1991), as a greater number of adult ticks engorged when exposed to 16 h of daylight in comparison to 10 h of daylight before infestation (Sutherst and Bourne, 1991). The results of both studies imply that light stimuli could impact the feeding behaviour of *H.longicornis*, but more research is needed for any confirmation. In the cattle studies here, it is not possible to make any comment on the effect of collection time, because of patches and infrequent collections.

Considering the sequential stages of the life cycle, larval and nymphal moulting times were slightly shorter than the Japanese research (Hoogstraal et al., 1968) while the nymphal moult was notably shorter than results from China (Lui and Jaing, 1998). The average period between oviposition and hatching (25 days) was also substantially



**Fig. 1.** Survival and attrition of various life stages of *Haemaphysalis longicornis* fed on dogs and maintained in 25 °C ( ± 1) and 85–95% RH incubator.

longer in both Japan and China (27.5–39 days) (Hoogstraal et al., 1968; Lui and Jaing, 1998).

The pre-oviposition, oviposition and oviposition to hatching periods in the current study are analogous to those found at similar temperatures and humidity in other studies (Heath, 1979; Kang, 1980; Table 5). The number of eggs (11.8) per mg of engorged female bodyweight is equivalent to that found in previous research (Yano et al., 1987; 1988). The temperature threshold for normal development of *H. longicornis* varies between 12–19 °C (Sutherst and Bourne, 1991; Yano et al., 1987), under which development is either temporarily halted or does not occur. The minimum temperature for the development of *H. longicornis* also differs in the literature. This could be a result of geographically-isolated strains of *H. longicornis* adapting to the local climatic conditions where each study was conducted. While the temperature threshold was not examined in the current study, development occurred more favourably at 25 °C, which is consistent with several other reports (Heath, 1979, 1981; Kang, 1980; Lui and Jaing, 1998; Yano et al., 1987). The correlation between humidity and rate of moult or hatch found in this study was also consistent with published research (Heath, 1979, 1981; Kang, 1980; Yano et al., 1987).

The attrition during feeding periods on dogs seen in the current study is likely due to the method of collection rather than being representative of natural attrition rates (Fig. 1). A similar model used for breeding *Rhipicephalus sanguineus* has shown significant decrease in attrition rates over time (DM, unpublished data). This suggests that the ticks may better adapt to the model over time or that technicians are better able to refine collecting techniques with increased experience working with the species or the population sizes of each life cycle stage (Elanco Animal Health, in-house data). Zheng et al. (2011) examined development of *H. longicornis* and noted that survival of various life stages varied with seasonality, emphasising that attrition rates are greatly affected by climatic conditions when these are not controlled. Physical trauma from cattle licking the patches and ticks getting stuck in the glue at the edge of the feeding area may also contribute to reduction in recovery of ticks harvested from cattle.

Once the optimal life cycle requirements for *H. longicornis* were established, GUTS or salivary gland stabilates were produced from adult ticks following methodology for *T. parva* (Kimbata and Silayo, 1997; OIE Terrestrial Manual, 2008). It was noted that levels of infection were poorly positive by PCR in ticks and salivary glands from infected, unfed adults, but improved markedly after 3 or 5 days of feeding. Therefore, feeding of infected adults for 3–5 days was considered necessary to enable *T. orientalis* sporozoites to multiply and mature, as is required for *T. parva* (Kimbata and Silayo, 1997), but feeding could occur on uninfected cattle, due to the lower virulence of *T. orientalis*. Subsequently, the successful production of parasitosis from both GUTS and salivary gland stabilates within 26 days on calves, confirmed the role of *H. longicornis* as a final host and vector for *T. orientalis* ikeda. While this status has been previously confirmed in Japan (Kamio et al., 1989) and highly suggested in New Zealand (Watts et al., 2016), its role required confirmation by the use of stabilate (Hammer et al., 2015), due to the induction of parasitosis following inoculation of blood containing piroplasms (Hammer et al., 2016; Gibson, 2017).

## 5. Conclusions

The 3-host tick, *Haemaphysalis longicornis*, was successfully raised on dogs and cattle with a generational life cycle of around 110 days. With this information, *H. longicornis* nymphs were successfully fed on parasitised cattle and infected with *Theileria orientalis* ikeda. Following moulting, adult infected ticks required an additional 3–5 days of feeding to mature Theilerial sporozoites. Subsequently, stabilates made from ground-up ticks (GUTS) or dissected salivary glands initiated detectable Theilerial parasitism in naïve calves within 26 days, confirming the competence of *H. longicornis* to transmit *T. orientalis* ikeda in Australia.

## Declaration of Competing Interest

The authors declare that they have no competing interests

## Acknowledgements

The research in this paper was supported by Meat & Livestock Australia (P.PSH.0832) and partly through the Dugdale-Peele bequest at the Sydney School of Veterinary Science for the study of control of tick-borne conditions in dogs. Staff at Biosecurity Sciences Laboratory, Coopers Plains QLD assisted with processing and incubation of ticks in the laboratory. The excellent technical assistance of Ms Maira Meggiolaro with PCR was much appreciated.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.vpoa.2019.100022>.

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