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ABSTRACT

The southern cattle fever tick, *Rhipicephalus microplus*, is a livestock pest worldwide in tropical and subtropical climates, including South Texas, and can vector *Babesia* spp., the causal agents of bovine babesiosis. Artificial rearing methods for *R. microplus* are needed, especially for rearing specialist tick parasitoids that are under evaluation for classical biological control. In this study, we tested the efficiency of artificial feeding of *R. microplus* larvae, nymphs, and adults on a siliconized substrate (goldbeater's membrane, lens paper, or Hemotek), or on non-siliconized goldbeater's membrane or Hemotek. Other variables tested were a warm water bath, incubator, positioning blood above or below ticks, using various attractants to stimulate attachment to membrane, incubating with or without 5% CO_2, changing static blood once a day versus peristaltic pumping of blood, and using heparinized versus defibrinated blood. Peristaltic pumping of blood across the membrane inside the incubator significantly increased larval attachment. We found that up to 25% percent of these life stages would attach to the siliconized goldbeater's membrane and feed, although none molted or completed their entire life cycle. A red color observable in the "fed" ticks' legs seemed to indicate that bovine hemoglobin had penetrated the gut and entered the hemolymph of the ticks. We were successful rearing unfed nymphs to the engorged stage, which is the pre-requisite for rearing *Ixodiphagus* tick parasitoids. Suggestions for future experimentation for rearing *R. microplus* on artificial membranes are discussed.

Additional index words: Livestock entomology, artificial rearing of ticks

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The southern cattle fever tick (SCFT), *Rhipicephalus* (=*Boophilus*) *microplus* Canestrini (Acari: Ixodidae) has a wide geographic distribution, spanning tropical and subtropical regions between parallels 32°N latitude and 35°S latitude (Goolsby et al. 2016a). It causes huge economic losses to milk and meat production by transmitting *Babesia* spp. that cause bovine babesiosis (Pérez de León et al., 2012; Grisi et al. 2014). Southern cattle fever tick and bovine babesiosis are estimated to have caused losses close to $3 billion annually to the U.S. livestock industry in today's currency before they were eradicated from the U.S. (Graham and Hourrigan 1977; Anderson et al. 2010; USDA Texas Cattle Fever). An eradication program based on continuous surveillance by mounted inspectors and the use of acaricides has been implemented in the U.S. along the Texas-Mexico border to manage any periodic outbreaks. However, novel strategies are

needed for the continued detection, suppression and eradication of SCFT in the permanent quarantine zone (PQZ) due to the emerging role of 1) exotic nilgai antelope, *Boselaphus tragocamelus* (Pallas), and white-tailed deer, *Odocoileus virginianus* (Zimmerman), as alternate SCFT hosts; 2) growing evidence of acaricide resistance; and 3) the invasion of pathogenic landscape-forming weeds such as the giant reed, *Arundo donax*, and Guineagrass, *Megathyrsus maximus*, that contribute to suitable habitat for the SCFT (Perez de León et al. 2012; Busch et al. 2014; Esteve-Gassent et al. 2016; Foley et al. 2017). Many of the research strategies being investigated involve *in vivo* rearing of *R. microplus* on live animals, which can be both costly and requires careful consideration of animal care and use guidelines. Artificial rearing of *R. microplus* would not completely replace *in vivo* rearing and testing but would provide new options for researchers.

One of the research strategies that would particularly benefit from an artificial rearing system is evaluation of classical biological control with specialist parasitoid insects (Goolsby et al. 2016b). An artificial rearing system would allow for small scale rearing of *R. microplus* and candidate parasitoids. Parasitoids, such as *Ixodiphagus hookeri* (Hymenoptera: Encyrtidae), are known to parasitize unfed tick nymphs and emerge from fully engorged nymphs (Wood 1911; Collatz et al. 2011). Therefore, even artificial rearing of selected tick life stages would be useful for evaluation of biological control insects.

Artificial membrane feeding of ticks has been achieved by a number of investigators but still remains technically challenging for some tick species including *R. microplus*. The utility of artificial membrane rearing of ticks for many aspects of tick research has been demonstrated by studies on acaricides (Kröber and Guerin 2007), pathogen transmission (Waladde et al. 1996; Oliver et al. 2016; Koci et al. 2018; Hart et al. 2018; Vimonish et al. 2020; Korner et al. 2020), and vaccine development (Lew-Tabor et al. 2014; Trentleman et al. 2019) as well as tick biology (Bullard et al. 2016). Methods of artificially feeding ticks with capillary tubes and membranes have been reviewed by Bonnet and Liu (2012) and Romano et al. (2018).

Our aim in developing an artificial feeding protocol for *R. microplus* was to be able to provide engorged nymphs for rearing of the tick parasitoid *Ixodiphagus* sp. (Hymenoptera: Encyrtidae) *in vitro*. Cattle fever tick parasitoids from the native range in Asia could potentially be used as biological control agents to limit the spread of *R. microplus*, especially on wildlife host species such as nilgai and white-tailed deer in Texas and along the transboundary region with Mexico (Goolsby et al. 2016b).

MATERIALS AND METHODS

Engorged cattle fever ticks, *R. microplus* adult females were collected from cattle that had been previously infested with larvae. The females were stored in glass vials at 27°C and 92%RH. Larvae that hatched from eggs deposited by females in these vials were used in the experiments. All tests were done with *R. microplus* except for one test done with *Rhipicephalus annulatus* Say collected from cattle.

Blood from calves at the USDA ARS Cattle Fever Tick Laboratory, Edinburg, Texas, was collected directly into 10 ml sodium heparin-coated Vacutainer (Becton Dickinson) tubes. To prepare defibrinated blood, the blood was collected directly into a sterile Erlenmeyer flask containing sterile glass beads. The blood was defibrinated by continuous swirling for 20 minutes and then decanted into a sterile glass bottle. Solid glucose was added to achieve a concentration of 2mg/ml. Blood was stored at 4°C. Immediately before use the blood received 0.1M disodium ATP (Sigma A6419) in 0.9% NaCl to produce a concentration of 0.001M disodium ATP and 10 mg/ml gentamicin (Sigma G1272) to produce a concentration of 5µg/ml gentamicin in the blood. We adhered to protocols for the care and use of animals as required by the presiding USDA-ARS Institutional Animal Care and Use Committee (IACUC). The facilities are fully accredited by the American Association of Laboratory Animal Care.

Collection of nilgai hide volatiles was done using a push-pull based vacuum air delivery system (Sigma Scientific LCC, Gainesville, FL) in 4L glass domes. Volatiles from ~250g of nilgai hide were collected onto a SuperQ based filter for 4 hours at a push flow rate of 1.5l/min and a pull flow rate of 1.2l/min. Before collection, the hide previously stored in -80°C was thawed to room temperature and cut into samples of ~250g and weighed. After the collection, the filter was eluted with 150 µl of dichloromethane and stored at -80°C until further use (for detailed methodology of volatiles extraction see Kariyat et al. 2012 and 2013). Volatiles were collected 8 times from a single hide, but from different sections of the hide. A small volume (20-50µl) of volatiles (in dichloromethane) was pipetted onto the tick side (siliconized side) of the membrane, and the solvent was allowed to evaporate before adding ticks.

The methods for preparing feeding units followed on from those of Kröber and Guerin (2007) and Oliver et al. (2015). Lens paper, goldbeater's membrane (www.talasonline.com), Hemotek membrane, and nylon mesh (38µ openings) were coated on one side with Ecoflex 00-10 (mixed according to the manufacturer's directions; Smooth-On.com) and scraped smooth with a hard-plastic blade to leave a very thin layer of silicone. Siliconized membranes were left to cure overnight. Feeding units were 4 cm long sections of Plexiglas tubing (32 mm O.D., 25.5 mm I.D.). The cylinder was glued to the siliconized side of a membrane with GE 2+ clear silicone caulk and left overnight. Excess membrane was trimmed from the cylinder.

For tests with the ticks positioned on top of the membrane above the blood, the above described single cylinders were used. In some tests, cow hair, *R. microplus* frass, or volatiles collected from nilgai hide were added to the siliconized side of the membrane before tick larvae were enclosed by sealing the open end of the cylinder with Parafilm. Approximately 3 ml of bovine blood that was warmed to 37°C was put in the well of a 6-well tissue culture plate. A snugly fitting acrylic ring was placed around the cylinder and adjusted so that the membrane end of the cylinder was

slightly immersed in the blood. The plate was then kept either on the bench at 25°C, in a humidified aquarium at 27°C, in a 37°C water bath, or in a 37°C incubator (Eppendorf Galaxy 48R). For all tests, blood was changed about every 24hrs, and the blood side of the membrane was rinsed with sterile 0.9% NaCl. In tests in which CO_2 was used, the incubator was connected to a CO_2 tank and regulated to 5% CO_2.

For tests with ticks positioned below the blood, a second cylinder was glued to the other side of the membrane thus creating a double cylinder (Fig 1). The ticks were contained by a square of nylon mesh held inside the cylinder with a rubber stopper that had a 1 cm hole bored in the center. About 3ml of blood was added to the non-siliconized side and this upper cylinder was sealed with Parafilm.

For tests involving peristaltic pumping of blood, a 1 mm diameter hole was drilled in the upper cylinder (before gluing to the membrane) about 4 mm from the end. This was reamed out slightly to fit a 19 mm long piece of 18-gauge stainless steel cannula. This was glued in place with superglue. On the opposite side of the cylinder a 4.8 mm hole was drilled and reamed slightly larger. It was centered about 7 mm from the end of the cylinder. A 13 mm piece of a 1 ml disposable polyethylene pipet was cut. The end was wrapped in a single layer of Parafilm and inserted into the hole. Silicone adhesive was smeared around the outside of the slightly protuberant end of the pipet piece on the inside of the cylinder for a better seal. A 20 cm piece of 4.76 mm ID silicone tubing was attached to the pipet piece. Inside the CO_2 incubator, a 280 mm piece of 12.7 mm wooden dowel was attached to two horizontal shelf supports to make a vertical mast to support the feeding unit at a suitable height. A 76 mm piece of adhesive-backed Velcro was attached lengthwise to the dowel just above the midpoint of the dowel. A small square of adhesive-backed Velcro was attached to the side of a 30 ml Dixie condiment cup to attach the cup to the dowel. The double-cylinder feeding unit was placed in the condiment cup. A MiniStar peristaltic pump (World Precision Instruments) was set up in the CO_2 incubator. The 1 mm ID tubing was used to pump blood from a 10 ml vial of heparinized or defibrinated cow's blood to the upper cylinder through the 18-gauge cannula. The blood flowed across the membrane and returned to the vial through the pipet piece and 4.76 mm ID silicone tubing.

Statistical analyses:

We used binomial logit regression to examine whether the three membrane types affected the proportion of *R. microplus* larvae attachment. Similarly, we ran a sep-arate binomial logit regression to examine whether adding adult *R. microplus* affected the proportion of nymphs attached to siliconized goldbeater's membrane. The analyses were carried out using the statistical software JMP (SAS 9.0, Carey, NC) and plots were made using the graphing software Graphpad Prism (LaJolla, CA).
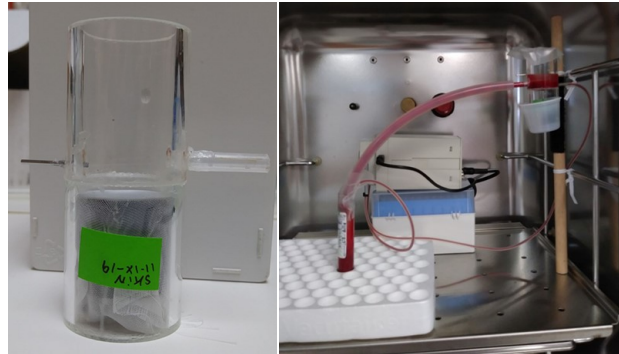

Fig. 1. Set up of artificial rearing system for larval and nymphal cattle fever ticks, *Rhipicephalus microplus*. (left) Double cylinder with inlet/outlet and with stopper with mesh in tick chamber and (right) Peristaltic pumping of blood over artificial membrane in double cylinder in incubator. Cattle fever ticks, *R. microplus*, are in lower cylinder.

RESULTS

Larvae. In tests using siliconized lens paper, single cylinders, with ticks positioned above blood, we found that larvae could not attach to the membrane whether it was in the 20-40μ thick range (3 replicates; >60 larvae total) or thicker (not measured; 6 replicates; >60 larvae total). These tests included those done on the benchtop at 25°C, in an aquarium at 27°C, and in a water bath at 37.5°C. No attachment of larvae to siliconized nylon mesh (50-90μ thick) occurred using these methods (ticks above blood; 2 replicates; >40 larvae total).

We found some larval attachment to siliconized goldbeater's membranes (Fig 2, Appendix 1). The mean proportion of *R. microplus* larvae attached to siliconized goldbeater's membranes is shown under different conditions: Membrane 1 - larvae above blood, test done in a water bath, and blood static except for being manually changed 1X/day; Membrane 2 - larvae below blood, test done in an incubator, and blood static except for being manually changed 1X/day; Membrane 3 - larvae below blood, test done in an incubator, blood recirculated by peristaltic pumping and manually changed 1X/day (Fig 3). Proportions of larval attachment for membranes 1 and 3 were not significantly different from each other, but both were significantly different from membrane 2 (Binomial

logistic regression; Chi-square =54.79; P =0.00).

Fig. 2. *Rhipicephalus microplus* larva attached to siliconized goldbeater's membrane as seen from below.
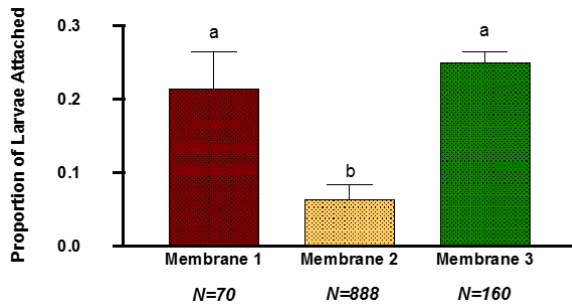

Figure 3. Proportion of *Rhipicephalus microplus* larvae attached to siliconized goldbeater's membranes under different conditions: Membrane 1 - larvae above blood, test done in a water bath, and blood static except for being manually changed 1X/day; Membrane 2 - larvae below blood, test done in an incubator, and blood static except for being manually changed 1X/day; Membrane 3 - larvae below blood, test done in an incubator, blood recirculated by peristaltic pumping and manually changed 1X/day. Different letters on bars show statistical significance at a P of <0.05

Three non-siliconized membranes in single cylinders with *R. annulatus* larvae above blood were tested in the incubator. About 5% of larvae attached to the goldbeater's membrane which was 10-20μ thick. The Hemotek membrane positioned with knobs up had about 6% of larvae attached while the Hemotek membrane positioned with knobs down had no larvae attached. The Hemotek membranes were 30μ thick. When these same three types of non-siliconized membranes were tested in double cylinders with *R. microplus* larvae below the blood, the membranes became too hydrated and trapped the larvae with no attachment noted.

Twelve tests using nilgai hide volatiles added to

siliconized goldbeater's membranes were conducted in double cylinders within the incubator. We evaluated 0, 20, 30, 40, or 50 μl of nilgai hide volatiles on the membrane. Larval attachment was highly variable and did not significantly correspond to quantities of nilgai hide volatiles. A peristaltic pump was used to circulate blood over the membrane (see Fig 1), and this resulted in about 25% attachment of larvae when using either heparinized (2 replicates; total of 40 attached larvae out of >160) or defibrinated (1 replicate; total of 20 attached larvae out of >80) blood. The blood in both cases remained bright red compared to static blood under the same conditions.

Nymphs and Adults. The addition of adult *R. microplus* to the cylinder resulted in significantly higher attachment of nymphs to the siliconized goldbeater's membrane (Binomial logistic regression; Chi-square =8.96; P =0.003) (Fig 4, Appendix 2). We observed pulsing of blood in the gut lumen of a tick which demonstrated active feeding and engorgement by nymphs (Fig 5). The addition of 5% CO_2 did not significantly increase the attachment of nymphs to siliconized goldbeater's membranes. The mean percentages of attachment in the presence and absence of 5% CO_2 were 6.8% and 4%, respectively. Twenty-seven percent of adult *R. microplus* (2 out of 7 ♀ and 2 out of 8 ♂) were able to attach to a siliconized (on non-knobby side) Hemotek membrane. In all of the tests, although some larvae, nymphs, and adults appeared to be somewhat engorged (darker color; plumper), all of the ticks died within a few days after attachment.
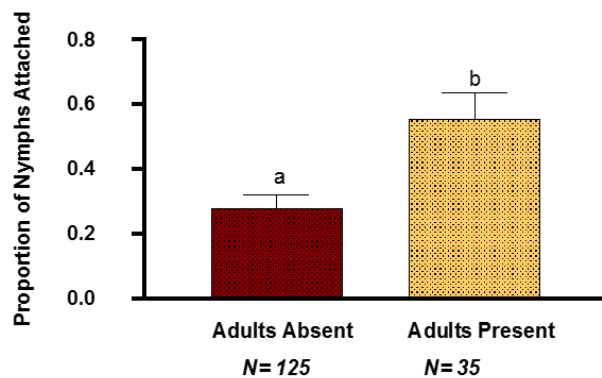

Figure 4. Proportion of *Rhipicephalus microplus* nymphs attached to siliconized goldbeater's membrane in the presence and absence of adults. Different letters on bars show statistical significance at a P of <0.05.

[▶] **Fig. 5.** Video shows the pulsing of blood in the gut lumen of a *Rhipicephalus microplus* nymph feeding on a siliconized goldbeater's membrane.

DISCUSSION

To develop an artificial rearing system for *R. microplus* we tested a number of different conditions associated with artificial membranes, blood conditions, peristaltic pumping and elevating CO^2 levels. Other *Rhipicephalus* spp. have been fed through artificial membranes. The short hypostomes of *Rhipicephalus* larvae (approximately 70-90μ; Estrada-Peña et al. 2012; Abdel-Shafy et al. 2013) set the requirement that artificial membranes be less than 40μ thick. Overall, the attachment of larvae and nymphs in our experiments was low compared to the 50 to >70% larval attachment of *R. microplus* reported by Kuhnert (1996). Trentleman et al. (2017) found an engorgement rate of 13.6% of *R. australis* larvae on goldbeater's membranes from which the liquid silicone had been scraped and 29.4% to 71% on membranes where the silicone was applied with a gloss paint roller. They attribute the variability in engorgement between membrane types to the irregular surface made by the paint roller causing many spots to be thinner than average. They ascribe the variability between individual rolled membranes to differences in viability between batches of larvae. Trentleman et al. (2019) reported that an average of 40% of *R. australis* larvae attached to rolled membranes and fed on bovine serum. Waladde et al. (1995) achieved an engorgement of 91% of *R. appendiculatus* nymphs. Krull et al. (2017) reported that *Dermacentor reticulatus* Fabricius larvae and nymphs, which have hypostome lengths of 72μ and 125μ, respectively, were unable to feed on siliconized lens paper (50μ thick) or siliconized goldbeater's membranes (20μ to 50μ) in significant numbers even though the membranes were thin enough to be penetrated by the hypostomes. In our tests siliconized lens paper proved to be unsuitable for *R. microplus* larvae to feed through even when the thickness was 20-40μ. Membrane factors other than thickness may influence tick attachment.

Most methods for rearing ticks on artificial membranes use some type of attractant such as host hair, tick frass, or a chemical extract of host hair or skin (reviewed by Kuhnert 1996, Bonnet and Liu 2012, and Romano et al. 2018). These attractants may induce attachment by the tick and successful feeding and development on an artificial membrane. In most tests we used volatiles collected by vacuum from air passing over nilgai hide to encourage attachment. However, there was no clear effect of any of these attractants on attachment in our experiments. Questing larval *R. microplus* crawl up on to plants and attach to host animals as they brush up against them. Volatiles such as CO_2 from host animals should be important to larvae in their questing behavior and stimulate attachment to the host. *In vitro* feeding was improved with 5% CO_2 in the three-host ticks *Amblyomma variegatum* (Voight et al. 2009) and *Dermacentor reticulatus* (Krull et al. 2017). However, we found no stimulating effect of 5% CO_2 on *R. microplus* nymphal attachment. It may be that CO_2 is only important for larval attachment in a one-host tick. Attractants may not be as important for *R. microplus* nymphs since this is a one host species, and therefore nymphs do not need to seek out hosts.

Blood quality, flow rate and temperature are important parameters in an artificial rearing system for ticks. De la Vega et al. (2004), Boehme et al. (2018), and Vimonish et al. (2020) describe *in vitro* tick feeding systems that incorporated a peristaltic pump in their rearing systems. In our tests, peristaltic pumping of blood across the membrane maintained the bright red color of the blood compared to blood that was static and significantly increased *R. microplus* larval attachment in tests done in the incubator. Changing the blood at least twice a day as well as peristaltic pumping may be beneficial and increase the length of time larvae and nymphs remain attached. Although our limited comparison showed equal attachment rates of larvae to the membranes, we believe that peristaltic pumping will be important for rearing of *R. microplus*, especially to the fully engorged adult stage. Additional tests are recommended that vary blood temperature and flow rates which may better mimic *in vivo* conditions.

Mortality of the ticks in our experiments was high in both the ticks that did not appear to feed and those that apparently fed, i.e. looked darker and plumper. The legs of the fed ticks were often observed to have an unusual red coloration that may indicate that bovine hemoglobin had penetrated the gut and entered the hemolymph of the ticks. This may have been due to a fungal or bacterial infection. Incorporating a fungicide and changing the blood more often might correct this problem. Agbede and Kemp (1986) reported that some of the *R. microplus* adults that fed on cattle vaccinated with whole tick extract had leakage of bovine erythrocytes or hemoglobin into the hemolymph due to gut damage. The damage observed by Agbede and Kemp (1986) could be similar to the symptoms observed in our tests. Further evaluation of the potential effect of bacterial and fungal infections on rearing success should be investigated.

To conclude, our work shows the feasibility of feeding *R. microplus* on artificial membranes.

Continued development of these methods should allow the rearing of *Ixodiphagus* sp. in *R. microplus* nymphs *in vitro* for biological control. Further development of a standardized artificial rearing system for *R. microplus* could result in substantial cost savings and accelerate the testing of novel methods for control of this important worldwide pest of wildlife and livestock.

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