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Rationale for Classical Biological Control of Cattle Fever Ticks and Proposed Methods for Field Collection of Natural Enemies

John A. Goolsby[1*], Dennis T. Mays[2], Greta L. Schuster[2], Javid Kashefi[3], L. Smith[3], D. Amalin[4], M. Cruz-Flores[4], A. Racelis[5], and A.A Pérez de León[6]

[1]*U. S. Department of Agriculture, Agricultural Research Service, Cattle Fever Tick Research Laboratory, Moore Air Base Bld. #6419, Edinburg, TX 78539*

[2] *Texas A&M University - Kingsville, Dept. of Agriculture, Agribusiness and Environmental Sciences, Kingsville, TX*

[3] *USDA-ARS, European Biological Control Laboratory, Thessaloniki, Greece and Montpellier, France*

[4] *De La Salle University, Dept. of Biology, Manila, Philippines*

[5]*University of Texas – Rio Grande Valley, Dept. of Biology, Edinburg, TX*

[6] *USDA-ARS, Knipling-Bushland U.S. Livestock Insects Research Laboratory, Veterinary Pest Genomics Center, Kerrville, TX 78028*

*Corresponding author, email: john.goolsby@ars.usda.gov

ABSTRACT

Classical biological control using specialist parasitoids, predators and/or nematodes from the native ranges of cattle fever ticks *Rhipicephalus microplus* and *Rhipicephalus annulatus* could complement existing control strategies for this livestock pest in the transboundary region between Mexico and Texas. Methods for field collection of cattle fever tick natural enemies are discussed, including exposure of infested cattle to collect insects that are parasitic on the nymphs and adults, time lapse photography to observe predators of questing larvae, and soil assays to detect entomopathogenic nematodes.

Additional Index Words: livestock entomology, transboundary disease vectors, pathogenic landscapes, *Ixodiphagous* spp.

Cattle fever tick(s) (CFT) *Rhipicephalus (=Boophilus) microplus* (Canestrini) and *Rhipicephalus* (=*Boophilus*) *annulatus* (Say) are native to south Asia and Mediterranean Europe, respectively, and common livestock pests in tropical, subtropical, and warm temperate areas across the world (Wharton 1974). Because cattle fever ticks are vectors of bovine babesiosis and anaplasmosis, they present an important obstacle to livestock production (Pérez de León et al. 2014). Graham and Hourrigan (1977) estimated that cattle fever ticks and bovine babesiosis caused losses to the US livestock industry close to $3 billion annually in today's currency before they were eradicated from the US. An aggressive eradication program based on the use of acaricides has been implemented in the US to manage periodic outbreaks, but due to growing evidence of acaricide resistance, the emerging role of white-tailed deer and exotic nilgai as tick hosts, and invasion of giant reed and other exotic plant species, novel strategies need to be examined and implemented for the continued suppression and eradication of CFT from this area (Ghosh et al. 2005, Perez de León et al. 2010, 2012, Racelis et al. 2012). Alternative strategies that can have an impact on CFT off the host animal are required for sustainable CFT eradication and control in the U.S. and worldwide, respectively. Biological control using free-living, host specific organisms from the native ranges of CFT warrants exploration as a management approach to achieve that goal. Further, the native range of these two species has never been explored for biological control agents. Direct biological control of

CFT could potentially be integrated into the U.S. Dept. of Agriculture, Animal and Plant Health Inspection Service, Veterinary Services, Cattle Fever Tick Eradication Program (CFTEP) management strategy to increase levels of mortality in the CFT Permanent Quarantine Zone (PQZ) between Del Rio and Brownsville, TX and especially across the Rio Grande River in Mexico where CFT have not been eradicated. Biological control is not a stand-alone strategy but could complement existing strategies such as acaricides and vaccines (Pérez de Léon et al. 2010, 2012). It may be the only method for control of CFT on wild hosts such as whitetailed deer and exotic nilgai, which have become important vectors of the tick in the transboundary region between Texas and northeastern Mexico (Moczygemba et al. 2012).

To date, only cursory efforts have been made to search for classical biological control agents in the native range of CFT. Biological control relies on the discovery and utilization of highly specific natural enemies from a pest's native range (Goolsby et al. 2006). The native range of *R. microplus* extends across Southeast Asia from Indonesia to India (Wharton 1974) (Fig. 1). In India, *R. microplus* has numerous wild hosts including Sambar deer, barking deer, and nilgai (Ghosh et al. 2007). This indicates a long evolutionary history with its bovid and cervid mammal host complex, and India is thus an important region to search for potential biological control agents. Closely-related *Rhipicephalus australensis* is found in Australia and it intergrades with *R. microplus* in southeast Asia (Chevillon et al. 2013). *Rhipicephalus annulatus* is native to Mediterranean Europe, northern Africa, and southwestern Eurasia and invasive in northern Mexico (Estrada-Peña et al. 2006). To date, no surveys for classical biological control agents have been conducted in the native range of *R. annulatus*. Other survey locations may come from the origins of domesticated cattle which are one of the primary hosts of CFT. Domesticated cattle species *Bos taurus* and *B. indicus* were domesticated from *Bos primus* independently in the Fertile Crescent and the Indus Valley (Ajmone-Marsam et al. 2010). In summary, large areas of the native ranges of CFT are yet to be fully explored for biological control agents.

Rationale for Classical Biological Control of CFT

Biological control has been extensively employed for both insect crop pests and rangeland weeds, but there have been relatively few efforts to control livestock pests. Old World dung-burying scarab beetles were introduced into Australia and successfully reduced populations of blood-feeding bush flies (*Musca vetustissima*) and buffalo flies (*Haematobia irritans*) which breed in high numbers from bovine dung pads (Waterhouse 1974). Control of filth flies, including *Musca domestica,* is improved in feedlots

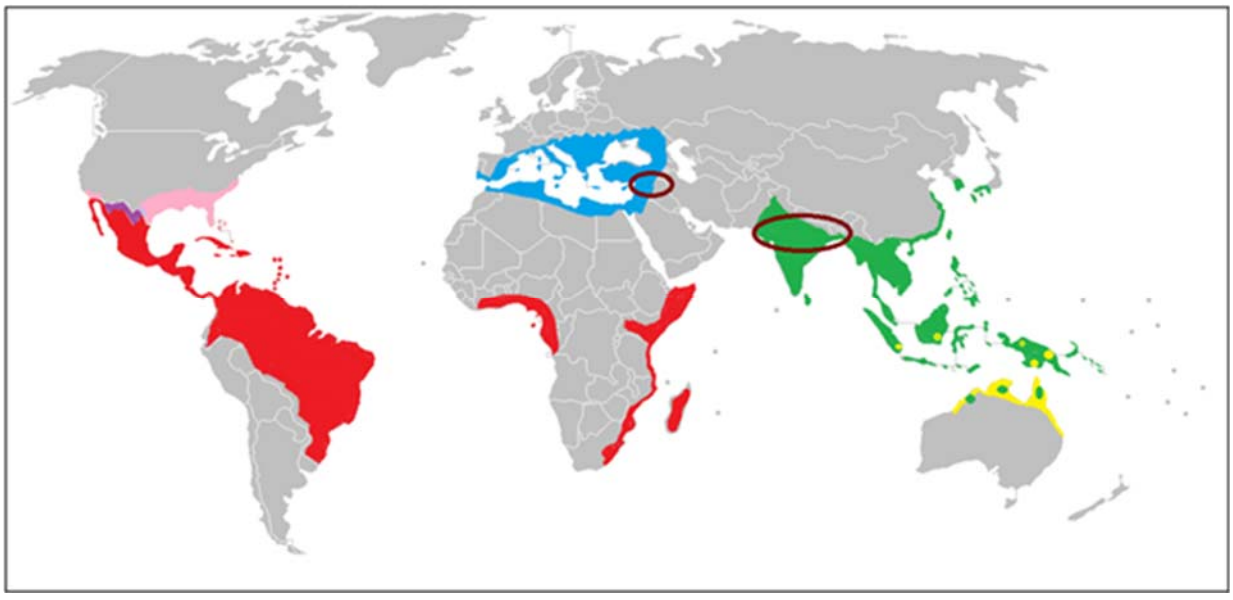

Fig. 1. Native and introduced ranges of cattle fever ticks (*Rhipicephalus* spp.) Red = introduced range of *R. microplus*, Pink = *R. microplus* eradicated, Blue = native range of *R. annulatus*, Purple = introduced range, Green = native range of *R. microplus*, Yellow = native range of *R. australensis*. Brown ovals indicate areas of domestication for cattle *Bos taurus* (Fertile Crescent) and *Bos indicus* (India).

and poultry houses by using periodic releases of *Muscidafurax* and *Spalangia* parasitic wasps (e.g., Geden et al. 1992, Petersen et al. 1992, Geden and Hogsette 2006, Kaufman et al. 2012, Machtinger et al. 2015).

Biological control of ticks has focused mainly on the use of *Ixodiphagous* (=*Hunterellus*) spp. (Hymenoptera: Encyrtidae) (Ostfeld et al. 2006). There are seven recognized species: *Ixodiphagus texanus* Howard, *Ixodiphagus hookeri* (Howard), *Ixodiphagus mysorensis* Mani, *Ixodiphagus hirtus* Nikolskaya, *Ixodiphagus theilerae* (Fielder), *Ixodiphagus biroi* Erdos, and *Ixodiphagus sagarensis* (Geevarghese). These wasps have been found parasitizing ticks belonging to the genera *Ornithodoros*, *Amblyomma*, *Dermacentor*, *Hyalomma*, *Haemaphysalis*, *Ixodes*, and *Rhipicephalus*. These parasitic encyrtid wasps oviposit into nymphs, emerge from engorged adults, and have 1-2 generations per year. Hu et al. (1998) reviewed the history of these biological control programs, including the intensive releases of *Ixodiphagous* parasitoids in Russia and the northeastern USA in the 1920s and 50s, respectively. Knipling and Steelman (2000) developed a conceptual model for area-wide biological control of *Ixodes scapularis*, the vector of Lyme disease using augmentative releases of *I. hookeri*. Lopes et al. (2012) reported parasitism of *Rhipicephalus sanguineus* and *Amblyomma* spp. by *I. hookeri* and *I. texanus* in several geographic regions of Brazil with distinct climates. Mwangi et al. (1997) conducted small-scale inoculative releases of *I. hookeri* (Hymenoptera: Encyrtidae) and reported more than 49% parasitism of *Amblyomma variegatum* nymphs on cattle in Kenya. Collatz et al. (2010) reported unique host finding and oviposition behaviors of *I. hookeri* when collected and exposed on their original co-evolved tick host. They further speculated that even though this parasitoid may have a global distribution, it may be specialized to attack its native hosts and has biological traits, such as a lengthy diapause, to adapt to local climates. This may explain the apparent lack of success from introducing *Ixodiphagous* spp. against novel hosts in different climates. Following this logic, *I. mysorensis* originally collected from Mysore, Karnataka, India may be best adapted to *R. microplus* since these two species are native to the same region and this area of central India is also climatically similar to the subtropical areas of south Texas and northern Mexico where CFT are invasive. Similarly, *Ixodiphagous* spp. collected from *R. annulatus* in Europe may be better adapted to the colder, northern part of the PQZ between Laredo and Del Rio, TX, where this CFT species is invasive.

Other types of parasitoids also hold promise; Samish and Rehacek (1999) report that *Megaselia rufipes* and *Megaselia scalaris* flies (Phoridae) infested *Amblyomma*, *Anocentor*, *Rhipicephalus* (=*Boophilus*), and *Ixodes* ticks in laboratory colonies, with *Megaselia* larvae emerging from the ticks and infesting their eggs. In Zimbabwe, Phoridae flies were found infesting *Rhipicephalus* (= *Boophilus*) *decoloratus* and *R. microplus* female ticks, which produced half as many eggs as the fly-free control group (Mwangi et al. 1997). Andreotti et al. (2003) identified a phorid fly parasitoid of *R. microplus* in Brazil. Little is known about the field biology of these flies, and their impacts may have gone unrecognized.

Specialized arthropod tick predators may also be useful as classical biological control agents. Specialized predators of CFT nymphs and egg masses may have gone unnoticed in the native range. In the introduced range, insect predators are known to have significant impacts on ticks. In a review of the literature records of predation on ticks, Samish & Alekseev (2001) documented that ground dwelling predators (e.g., ants, beetles and spiders) are the major natural enemies of ticks. Ants are also known to be important predators of ticks in TX. Fleetwood et al. (1984) documented reduced populations of Lone Star ticks, *Amblyomma americanum*, in pastures with abundant red imported fire ants, *Solenopsis invicta.* Fire ant predation is generally believed to reduce the incidence of tick vectored pathogens of livestock. In Louisiana, fire ant predation of *Ixodes* ticks was associated with a reduced incidence of anaplasmosis in cattle (Jemal and Hugh-Jones 1993.)

Nematodes have also been investigated as biological control agents for ticks (Samish and Glazer 1991; Samish and Rehacek 1999; Samish et al. 2004). Most of the research involved use of commercially available entomopathogenic nematodes of the families Heterorhabditidae and Steinernematidae. The only free-living stage of the nematode, the third/infective juvenile, actively locates and enters the host via natural openings, and then releases symbiotic bacteria that kill the host within 24–72 h. The nematodes then multiply within the host cadaver, and by 6–18 days post infection, thousands of invective juveniles are released into the environment. However, currently available entomopathogenic nematodes such as *Steinernama carpocapse*, can infect and kill CFT, but do not reproduce (Samish et al. 2004, Molina-Ochoa et al. 2009). The most common natural habitat of these nematodes is moist ground. Nematodes collected from *Rhipicephalus* spp. in the native range may be better adapted than commercially available nematodes. Additionally, nematodes may persist in locations where white-tailed deer groom and bed down for the night and infective juvenile nematodes are most likely to find new CFT hosts.

A considerable amount of lab and field re-

search has been conducted to investigate the potential of entomopathogens such as *Beauveria, Isaria* and *Metarhizium* and is reviewed by Samish et al. (2004). Commercial products containing these pathogens are available and have been demonstrated to be effective as biopesticides, especially in tropical climates with high humidity. The methods described in this paper are not specifically designed or intended to search for novel microbial pathogens of CFT. Our focus is on macro-organisms that have the potential to be free-living classical biological control agents of CFT.

Methods to discover and evaluate natural enemies of CFT are needed to investigate the potential for classical biological control. These methods must be able to detect parasitism and predation on all life stages (eggs, larvae, nymphs, adults) of CFT, both on and off the host animal. The purpose of this paper is to identify the challenges to discovering candidate biological control agents including nematodes, parasitoids, and specialized predators and develop methods to be used in foreign exploration. *Ixodiphagous* parasitoids of *Rhipicephalus* and *Ixodes* spp. and other species are known to oviposit into nymphs attached to the host animal and then begin development after the fully engorged females fall from the host. Our methods using CFT infested calves are designed to detect parasitism of CFT nymphs and adults associated with domesticated cattle, however it is possible that the parasitoids are attracted to specialized cues found only on the original mammal hosts, such as nilgai antelope. Methods for detection of parasitoids and predators of CFT eggs and nymphs rely on time lapse photography to observe and identify these natural enemies. It is possible that specialized parasitoids or predators attack this life stage and could be investigated as biological control agents. Entomopathogenic nematodes that attack and reproduce in CFT may also be useful as biological control agents. We propose that nematodes may exist in the native range of CFT, perhaps living in animal wallows or bedding places where their large mammal hosts frequent. Based on these hypotheses, we propose to use these methods to search for potential biological control agents of CFT.

MATERIALS AND METHODS

These studies were conducted at the USDA-ARS Knipling-Bushland U.S. Livestock Insect Research Laboratory, Cattle Fever Tick Research Laboratory (CFTRL) at Moore Airbase in Edinburg, Texas (28° 23’ 56.33” N; 98° 20’ 38.21” W). The animals used in the study had not received any acaricide treatments and were cared for using the guidelines established by the Institutional Animal Care and Use Committee (IACUC). The pen and adjacent 12 ha. pasture has no history of acaricide use.

CFT Infested Cattle Exposure Technique. On 5 May 2015, two eight month old Black Angus heifers (*Bos tarus*) were placed in a pen and tagged with two different ear tags to distinguish between the two (Fig. 2F). On the same day as they were penned, a vial of 500 *R. annulatus* questing larvae and 500 questing *R. microplus* larvae were released to the heifers (Fig. 2A). Each vial was glued to the top of their shoulder using Livestock Identification Tag Cement (Nasco; Fort Atkinson, WI.) (Fig. 2B, D, E). Eight days after infestation the heifers were moved into a working chute to scratch for ticks and take pictures of their development (Fig. 3). Starting on 19 May 2015 the two heifers were moved into the chute once a day until the end of the test to take pictures of the development of the ticks. The daily developmental observation of the ticks was used to determine when to start searching for fallen engorged female ticks. The cattle pen was

Fig. 2. (A) Vial of 500 questing larvae to be glued to the heifers for release, (B) Tag cement used to glue the vials of questing tick larvae to the heifers, (C) The heifers being run into the working chute for the questing tick larvae to be released on them, (D) Glue is placed on the vial of questing larvae prior to being placed on the top of the heifer’s shoulder, (E) Questing tick larvae vial being placed on the top of the shoulder, (F) The heifers in the pen after they were manually infested with *Rhipicephalus annulatus* and *R. microplus* questing larvae.

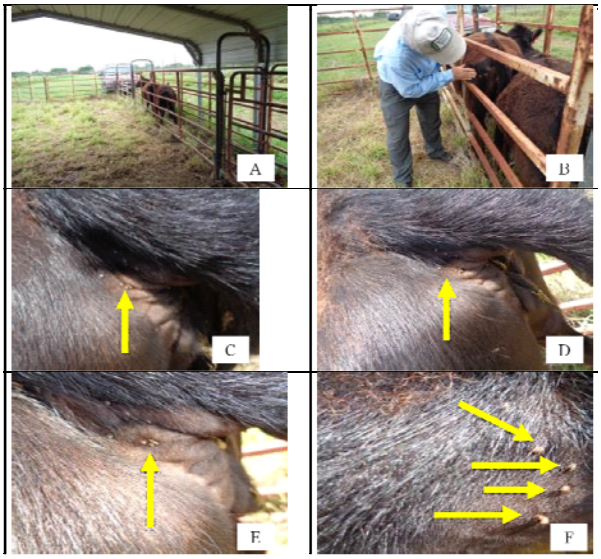

Fig.3. (A) Both heifers in the working chute where they were examined for tick development, (B) Checking the anal region of the heifer for ticks feeding on the tender tissue (C, D, E, F) and taking daily pictures to aid in the observation of cattle fever tick development on cattle.

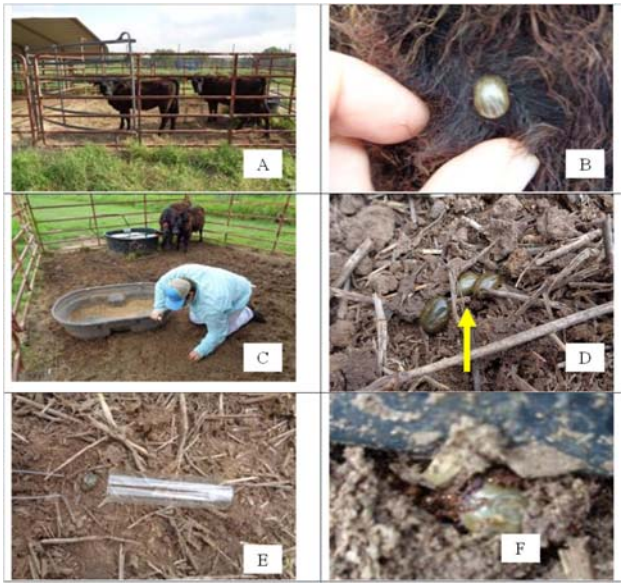

Fig. 4. (A) The two heifers in the reduced pen for the duration of the tick drop to minimize the area needed to search for ticks. (B) Nearly engorged *Rhipicephalus microplus* on the side of one of the heifers; (C) Searching the pen for fallen engorged female ticks; (D) Fallen engorged female ticks found in the chute; (E) The field collection vial used to store the ticks in until we are back in the lab; (F) engorged female tick found in the pen at the edge of the water trough, and being preyed upon by desert fire ants *Solenopsis aurea* (Wheeler) (Hymenoptera: Formicidae).

reduced in size on 24 May 2015, which was the time when *R. microplus* engorged females were starting to drop from the cattle (Fig. 4A). The pen size was reduced to aid in collection of the fallen engorged females. Starting on 26 May 2015 fallen engorged females were collected by searching around the pen and or chute for the fallen females (Fig. 4C-F). Most of the fallen ticks were collected in the pen shortly after the animals were moved.

Fallen fully engorged ticks found in the pen were placed in collection vials, closed with cotton plugs, labeled with the date and species and then transported to the lab (Fig. 5A). Collection of fallen engorged female ticks continued for seven calendar days, with the exception of one day where severe weather was imminent, and for the safety of the collectors, no collection occurred. Vials were placed into Ziploc bags that were also labeled with the date and area of collection. All vials containing *R. microplus* or *R. annulatus* were placed in an aquarium container with saturated NaCl slurry to maintain humidity at 70% RH and held at 27°C (Fig. 5B). Vials were checked daily by removing each vial from the Ziploc bags to visually determine if the ticks were alive, ovipositing, dead, or had been parasitized. Vials with eggs were checked daily for their eclosion. Once questing larvae eclosed, they were available for use in the outdoor time-lapse photography studies of predation.

Fig. 5. (A) Vials of engorged ticks collected from the pens; (B) terrarium with vials of engorged CFT is in a temperature controlled room that is kept at 27°C; (C) removal of questing larvae on leaf after one-week exposure; (D) questing larvae held in vial for emer-

Time-Lapse Photography of Questing Cattle Fever Tick Larvae**.** The questing behavior of cattle fever ticks was captured using a Brinno TLC200Pro, time-lapse camera (Brinno; Taipei City, Taiwan) (Fig. 6C). One vial of questing larvae from the collected engorged females was released on a bunch of buffelgrass (*Pennisetum cilare*) that was spaced from other foliage to ensure the larvae stayed within the field of view for the camera (Fig. 6A-B). Upon release the time-lapse camera was placed on the ground with the lens facing the underside of the leaves (Fig. 6E). At night, one red LED light bar (Birddog Distributing, Bozeman, MT) with a standard car battery was placed near the study site to provide additional lighting so the behavior of questing larvae could be photographed at night (Fig. 6D, F). The time lapse pictures were reviewed later in the laboratory to identify the predators and time of activity.

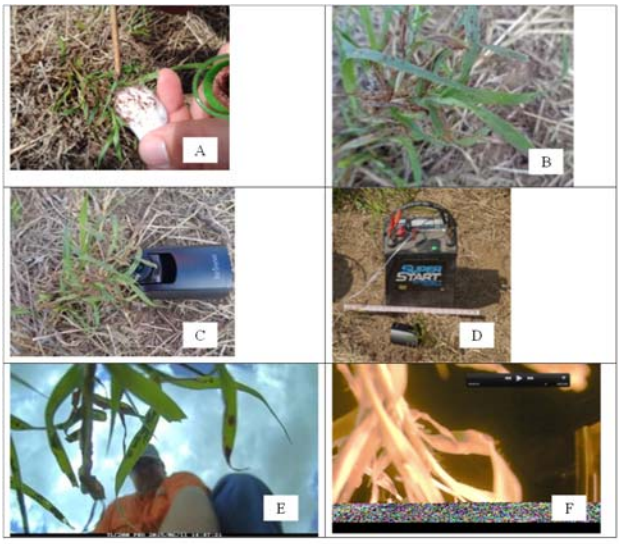

Fig. 6. (A) Release of questing larvae onto test plant. (B) Questing larvae on topside of leaf. (C) Time lapse photography camera for observing predation of questing larvae on plant. (D) Battery and LED red light used for night time observation. (E) Day time photo of larvae on underside of leaf. (F) Night time observation of larvae with spider in background.

Soil assays for entomopathogenic nematode infecting CFT. To develop methods for detection of entomopathogenic nematodes, soil was collected from locations at the CFTRL within CFT host animal bedding areas and from wallows frequented by mammal hosts of CFT (Bedding & Akhurst, 1974) (Fig. 7A,B). A fully engorged female CFT was placed in the moist soil in sealed 1 liter Ziploc® bags for 72 hours in darkness at 27°C to allow for infective juvenile nematodes to reach and penetrate the ticks (Fig. 7°C). Following exposure to the moist soil, CFT were removed and held at 27°C for 3 weeks in sealed Petri dishes. CFT were then dissected to look for the presence of infective juvenile nematodes. An example of juvenile entomopathogenic nematodes collected from a fly species) is shown in Fig 7D. Ticks were dissected open to expose their body contents. Body contents were mixed in saline solution and viewed under a compound microscope. Juvenile nematodes are generally small, thread-like, and wriggle.

Fig 7. Collection of soil for nematode assays from (A) tika deer bedding areas (India). (B) Water buffalo emerging from wallow (Philippines). (C) Fully engorged cattle fever ticks placed on moist soil in Ziploc to assay for nematodes. (D) Entomopathogenic nematodes in ovaries of a fly.

RESULTS AND DISCUSSION

CFT Infested Cattle Exposure Technique. No evidence of parasitism by insect parasitoids was detected from the 99 fully engorged females, (80 *R. microplus* and 19 *R. annulatus*) collected in the study. This suggests that there are no parasitoids in Edinburg, Texas, which might control the tick populations. Absence of host-specific natural enemies is consistent with CFT being an exotic organism in its introduced range. The tick parasitoid *Ixodiphagous hookeri* is known from Texas and has been reared from several tick species, including *Rhipicephalus sanguineus* Latreille, but has never been collected from *R. microplus* or *R. annulatus* (Larson 1937). Collatz et al. (2011) found that *I. hookeri* in Germany only parasitized tick species that were native to the region. They hypothesized that *Ixodiphagous* spp. are adapted to the local climate and tick fauna. *Ixodiphagous* spp. from the native ranges of *R. microplus* and *R. annulatus* are therefore more likely to parasitize these tick species in

Texas/Mexico where they are invasive.

Parasitoid surveys should be conducted at multiple locations throughout the CFT native ranges, including protected natural areas where native bovid and cervid hosts exist. Sampling for parasitoids should occur throughout the year to determine seasonal patterns of the parasitoids. (For example, *Ixodiphagous mysorensis* from India is most often collected in the spring before the summer monsoon.) Other olfactory cues from the host animal's hide, dung and/or urine may be useful to attract parasitoids. Olfactory compounds from wild hosts such as deer, guar, water buffalo and nilgai may be more attractive than those from domesticated cattle and should also be investigated.

It is critically important to use cattle in these studies that have no history of insecticide or acaricide use. Parasitoids, especially parasitic Hymenoptera may be very sensitive to pesticides. Parasitoid mortality could occur when adults are searching on an animal treated with a topical acaricide, or to larvae when they hatch inside fallen engorged females that have a lethal dose of systemic acaricides. Additionally, fields or pens that have been treated with either an insecticide or an acaricide should be avoided when trying to collect parasitoids. Pastures with treated herds may also affect the local population of parasitoids, therefore collections are optimal near natural habitat where untreated wild CFT hosts occur.

Time-Lapse Photography of Questing Cattle Fever Tick Larvae**.** Pictures of the time lapse camera showed that the questing larvae remained on the lower leaf surface and that predation was low. During 120 hours of observation we detected a few ants and spiders that appeared to be preying on the questing larvae. Thus, CFT larvae do not appear to have any specialized predators or parasitoids in its invasive range. Time lapse photography may detect specialized natural enemies of CFT in their native ranges. Predators or parasitoid species that are commonly detected should be collected and observed in vials with larvae to confirm their, identity, biology and ultimately their potential as biological control agents.

No entomopathogenic nematodes were collected from the CFT in the soil assays. The soil used in the studies came from areas in CFTRL pastures where cattle frequently bed down or rest in the shade. However, these were not wallows or habitats that may be more optimal for entomopathogenic nematodes. Future surveys should focus on wallows in the native range of CFT with a long history of large animal contact.

In summary, the methods described here allow for systematic investigation of CFT natural enemies in their native ranges. Natural enemies will need to be further evaluated for their environmental safety and potential efficacy to control the CFT where it is invasive. Natural enemies should be reared on CFT in the native range before shipment to quarantine facilities in the USA. Special precautions will be needed to exclude livestock pathogens associated with the CFT in its native ranges before importation to quarantine facilities.

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