STUDIES INVOLVING THREE SPECIES OF TICKS (ACARI: IXODIDAE) ARISING FROM AN OUTBREAK OF EQUINE PIROPLASMOSIS (*THEILERIA EQUI*) IN SOUTH TEXAS

A Dissertation

by

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ABSTRACT

Equine piroplasmosis (EP) is a tick-borne disease of equines caused by *Theileria equi*. In 2009, an outbreak occurred in Texas, USA. Known intrastadial vectors of EP include *Amblyomma mixtum* and *Dermacentor variabilis*. The status of *A. tenellum*, a common tick on equines in Texas remains unknown. Male ticks remain on hosts to blood feed and mate but may transfer from one host to another during close contact and mutual grooming. The research presented here arose from this outbreak, and included four lines of investigation: 1) a cross-mating and development study of two sympatric and morphologically similar ticks, *A. mixtum* and *A. tenellum*, 2) the use of phylogenetic techniques (parsimony, maximum-likelihood, and Bayesian) to parse their relationships with *A. maculatum* and *A. americanum* using four genes 12S, 16S, COI, and ITS2 and two concatenated datasets, 3) the role of host-to-host transfer of male *D. variabilis* might have in the transmission and maintenance of *T. equi* using agent-based modeling (ABM), and 4) the creation of a population matrix model (PMM) for *D. variabilis* that incorporated the life history of male ticks. The cross-mating study showed that one female of the *A. mixtum* × *A. tenellum* cross produced larvae likely due to parthenogenesis than hybridization. Overall differences occurred among crosses for all comparisons of life history events. For the phylogenetic analyses, all gene topologies for the four *Amblyomma* species were similar expect for the COI which showed poor resolution in branch support. Though the COI differed from the other genes, the concatenated datasets showed that it had little influence. Analyses revealed that *A. americanum* and *A. tenellum* are closely related. The ABM showed that the number of infected horses were influenced by the infection probability, and horses and ticks could maintain *T. equi* at low levels of infection and male transfer. The PMM showed a

low abundance of transferring males and that the off-host stages to on-host stages were most the sensitive to changes in the transition parameter. These stages may be targeted for management and control of tick populations.

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Contributors

This work was supervised by a dissertation committee consisting of my committee chair Dr. Pete Teel, Regents Professor and Interim Department Head of the Department of Entomology, Dr. William Grant, Professor, Department of Wildlife and Fishery Sciences, Dr. Craig Coates, Instructional Associate Professor and Associate Department Head for Academic Programs of the Department of Entomology, and Dr. Jeff Tomberlin, Professor, Department of Entomology. Each provided insight into the development of the research and made edits to this dissertation.

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model presented in Chapter 5. All other work conducted for the dissertation was completed by me independently.

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CHAPTER I

INTRODUCTION

Equine piroplasmosis is a tick-borne disease of horses and other equines such as mules, donkeys, and zebras, caused by two hemoprotozoans *Babesia caballi* (Nuttall and Strickland, 1910) and *Theileria equi* (Laveran, 1901) (formally *Babesia equi*). According to an estimate made by the Food and Agriculture organization of the United Nations about 90% of the global domestic equine populations live in areas where equine piroplasmosis is endemic. The following seven countries have been designated as being free of equine piroplasmosis: Australia, Canada, Great Britain, Ireland, Japan, New Zealand, and the United States. But recent outbreaks in the United States have threaten its status as being free of equine piroplasmosis and has the potential to have economic impacts and stricter regulations for movement from animals from state to state and also international travel. In a 2017 an economic impact report of the United States horse industry, a total of 7.2 million horses were estimated to be in the United States with Texas having the largest population (767,100; 9.4%) (American Horse Council 2017). The horse industry contributes approximately $50 billion to the US economy (American Horse Council 2017).

Equine piroplasmosis associated with *T. equi* in the United States was first diagnosed in Florida, in 1964, involving an infected horse with an infection of both *B. caballi* and *T. equi* (Ristic et al. 1964). In 1965, a thoroughbred horse in south Florida was diagnosed with only *T. equi* (Knowles et al. 1966). Two more cases of *T. equi* occurred that same year, including another case in Florida and one case involving an imported horse from Europe in New Jersey (Taylor et al. 1969). Prior to these dates, in 1959, it was assumed that *B. caballi* along with its

vector, *Dermacentor nitens* Neumann, 1897, was introduced in the United States by the importation of Cuban walking horses, into southern Florida (Sippel et al. 1962). The tick vectors for the horses that were infected with *T. equi* in 1964 and 1965 were not documented. Currently *D. nitens* is not a competent vector of *T. equi* (Stiller and Coan 1995). The infections of *B. caballi* in south Florida sparked the creation of an eradication program in 1962 lasting until 1978 (Coffman 1997). Once the discovery of *T. equi* was confirmed in Florida, efforts were made for its eradication as well.

Occasional cases of *T. equi* have occurred since the end of the eradication program but in 2008 and 2009 larger outbreaks occurred. In 2008, 20 horses in Florida were diagnosed with *T. equi*. These horses were a part of illegal racing and all were imported from Mexico (Short et al. 2012). Mode of transmission was not thought to be tick-borne, but rather through use of shared syringes. The largest outbreak, to date, of *T. equi* within the United States, occurred on a large ranch in south Texas, in 2009 (Scoles et al. 2011).

On this ranch a total of 360 horses were tested for *T. equi* of this population, 292 (81.1%) were seropositive (Scoles et al. 2011). Four species of ticks were collected from horses with *Amblyomma mixtum* Koch, 1844 (formally *A. cajennense* (Fabricius, 1787)) being the most numerous, followed by *A. maculatum* Koch, 1844, *Dermacentor variabilis* (Say, 1821), and *D. nitens*. The outbreak lead to an investigation (2009 – 2010) both in Texas and out of state yielding a total of 413 seropositive horses (Texas Animal Health Commission 2018). A total of 17 horses in 2012, in Kennedy County, were seropositive for *T. equi* (Texas Animal Health Commission 2018). In March 2013, the Texas Animal Health Commission (TAHC) began testing horses in Kleberg County after being designated as a high-risk area for *T. equi* (Texas Animal Health Commission 2013*a*). By May 2013, at total of 280 premises and 747 equines had

been tested with only 19 horses on six premises testing positive for *T. equi* (Texas Animal Health Commission 2013*b*). By the end of 2013 a total of 28 horses were found to be seropositive for *T. equi* and in addition the TAHC started testing equines in the neighboring county of Brooks (Texas Animal Health Commission 2014). Continual cases of equine piroplasmosis have occurred in Texas: six cases in 2014, 14 cases in 2015, and 15 cases in 2016 (Texas Animal Health Commission 2018). As of January 2007, to February 2018 a total of 16 cases of *T. equi* have been reported to the TAHC (Max Dow 2018). As of 17th of April 2018 a total of five cases of *T. equi* have been documented in Texas (Texas Animal Health Commission 2018).

The typical lifecycle of *T. equi* has been described in detail and can be broken down into four different stages of replication (Mehlhorn 1984; Moltmann et al. 1983; Zapf and Schein 1994*a, b*). The first stage of replication is schizogony which occurs within an infected equine host where peripheral blood mononuclear cells (a lymphocyte) are penetrated by sporozoites. Once inside these lymphocytes the sporozoites undergo asexual reproduction producing microschizonts and macroschizonts. The second stage of replication is merogony which starts when the micro- and macroshizonts give rise to merozoites which then infect erythrocytes undergoing further asexual reproduction producing more merozoites. These merozoites will rupture from erythrocytes where they can infect more erythrocytes and further replicate. Some of these merozoites will undergo some morphological changes, becoming more spherical, to form the gamonts. Gamogony, the third stage of replication starts when these gamonts are ingested by bloodfeeding adult ticks and subsequent development into “ray bodies”. After about four to six days from ingestion these ray bodies divide to form microgamonts and macrogamonts. Eventually the microgamonts and macrogoamonts fuse to become zygotes. Development will then take place within these zygotes forming kinetes which then penetrate the

epithelial cell lining of the midgut to be released within the hemocoel. These kinetes will then migrate through the hemocoel to the salivary glands where they will undergo meiosis to eventually form the sporozoites. The sporozoites are then transferred to an equine host while the tick is blood feeding.

Some infected equines can be asymptomatic with very low parasitemia levels of *T. equi* and may not be detectable with blood smears (Friedhoff and Soule 1996; Bashiruddin et al. 1999). These asymptomatic equines serve as reservoirs for *T. equi* having the ability to amplify *T. equi* in areas were efficient tick vectors occur allowing for the continual maintenance of *T. equi*. No self-limiting immune response of *T. equi* occurs; therefore after infection equines will remain as carriers throughout the life of the animal (Schein 1988). In addition, transplacental transmission of *T. equi* occurs which can lead to abortions, stillbirths, or severely anemic foals (Lewis et al. 1999; Phipps and Otter 2004). Acute equine piroplasmosis can typically be characterized by the following symptoms: fevers exceeding 40ºC, sweating, anorexia, malaise, dehydration, anemia, hemoglobin in the urine, congestion of the mucus membranes, rapid breathing and heart rate (Rothschild and Knowles 2007). An enlargement of the spleen (splenomegaly) may occur in subacute cases (De Wall 1992). Death can result if the animals are left untreated in both acute and subacute cases. A five to 10% mortality rate occurs among endemic horses but with naïve horses the morality rate can be higher than 50% (Maurer 1962; Rothschild and Knowles 2007). Chronic equine piroplasmosis does not show very specific symptoms like those of acute equine piroplasmosis. Malaise, poor performance and body condition, and mild cases of anemia (Rothschild 2013). These symptoms may potentially lead to equine piroplasmosis being misdiagnosed as equine infectious anemia.

As a result of the large 2010 equine piroplasmosis outbreak in Texas infected horses many of the horses were euthanized but some were entered into a treatment trial using imidocarb dipropionate with a dosage of 4.0 mg/kg every 72 hours with a total of four intramuscular injections (Ueti et al. 2012). A total of 25 horses were included within this trial with 24 subsequently being undetectable by PCR for *T. equi* and failed to transmit the pathogen to other horses via blood transfer. The one horses that remained infected after the original four doses was given a second treatment which then became undetectable for *T. equi.* Though this treatment will be beneficial, tick-vectors of *T. equi* are worldwide providing a continual source for *T. equi* infections.

Two modes of transmission have been confirmed with *T. equi*. The first mode of transmission is intrastadial which occurs when one stage of a tick acquires the pathogen and then will transmit the pathogen to a host while in that particular stage, usually occurring with adult males. The second mode of transmission, is transstadial, where transmission occurs between two stages. For example, a tick may acquire the pathogen during the nymphal stage, drop into the environment and molt in to the adult stage, attach to an uninfected horse and transmit to this host while in the adult stage. Transovarial passage is another mode of transmission in which the eggs of an infected female will lead to infected larvae of the next generation. This mode of transmission does not seem to occur with *T. equi,* however PCR positive eggs have been documented in *Haemaphysalis longicornis* Neumann, 1901, a new invasive tick to the United States of America (Ikadai et al. 2007).

A total of 26 species of ticks from six genera (*Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Hyalomma*, *Ixodes*, and *Rhipicephalus*) have been suspected in the transmission of *T. equi* worldwide (Scoles and Utei 2015). Only four species are suspected in the

transmission of *T. equi* in the United States: *A. mixtum*, *D. variabilis*, *H. longicornis*, and *Rhipicephalus (Boophilus) microplus* (Canestrini, 1888). *Amblyomma mixtum* was suspected in the 2010 outbreak in Texas and was later confirmed by transmission studies to be competent in intrastadial transmission but may not be an efficient vector (Scoles and Ueti 2013; Scoles et al. 2011). Experimental transmission studies of *D. variablis* have demonstrated intrastadial transmission but like *A. mixtum* may not be an efficient vector of *T. equi* (Scoles and Ueti 2013; Stiller and Coan 1995; Stiller et al. 1982; Stiller et al. 2002). One tick, *R. microplus*, may be considered to be one of the most efficient vectors of *T. equi* (Guimarães et al. 1997 and 1998; Ueti et al. 2003, 2005, and 2008). The number of *R. microplus* collected on horses in Texas is low, survey data from the Texas Animal Health Commission, from 2000 – 2016, show that only 45 collections *of R. microplus* were made. In the 2010 Texas outbreak no *R. microplus* were collected on the horses but past outbreaks of *R. microplus* have occurred on this ranch and possible remnants of populations could exist and not be reported. Lastly an asexually reproducing tick *H. longicornius* has recently been introduced within the United States (Rainey et al. 2018). No evidence by transmission studies have been conducted but there was one study on the competence of this species which also included the PCR evidence of *T. equi* in the eggs (Ikadai et al. 2007). More research must be conducted on this species as it may have a potential impact on the transmission of *T. equi* within the United States.

The aim of this introductory chapter was to provide a brief overview of the history of equine piroplasmosis (*T. equi*) within the United States, etiology, treatment, the vectors and their mode of transmission of *T. equi*. An understanding of these aspects is beneficial as the research that follows in the chapters of this dissertation arose from an outbreak of equine piroplasmosis in south Texas. More specifically this dissertation will cover: 1) a crossbreeding and development

study of two sympatric and morphologically similar ticks, *A. mixtum* and *A. tenellum*, 2) the use phylogenetic techniques to parse out the relationship of *A. mixtum* and *A. tenellum* and two other *Amblyomma* ticks, *A. americanum* and *A. maculatum*, within the United States, 3) the role host-to-host transfer of male *D. variabilis* might have in the spread of *T. equi* using agent-based modeling, and 4) the creation of a stage-based matrix model to investigate the population dynamics of *D. variabilis* which incorporates these transferring males.

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CHAPTER II

CROSS MATING OF TWO SYMPATRIC AND MORPHOLOGICALLY SIMILAR TICKS: *AMBLYOMMA MIXTUM* KOCH, 1844 AND *AMBLYOMMA TENELLUM* KOCH, 1844 (ACARI: IXODIDAE)

A species complex exists within *Amblyomma cajennense,* which encompasses a large geographic area extending from southern Texas to South America and extending off the mainland to the Caribbean Islands (Estrada-Peña et al. 2004, 2014). A reassessment of this species complex led to the redescription of *A. cajennense*, the validation of both *A. mixtum* and *A. sculptum* which were originally synonyms for *A. cajennense,* and three new species descriptions: *A. tonelliae, A. interandinum*, and *A. patinoi* (Beati et al. 2013, Nava et al. 2014*b*). Out of these six species, *A. mixtum* is of relative importance to this study as it is found within southern Texas extending into Mexico. In addition to the taxonomic changes of this species complex it was found that *A. imitator,* a tick that shares phenotypic similarities with *A. mixtum,* has been reverted to *A. tenellum* and thus *A. imitator* is now considered to be a junior synonym (Nava et al. 2014*a*). The distribution of *A. tenellum,* is limited to southern Texas on into Mexico and may extend further south into Central America as specimens have been collected from Honduras (Becklund 1959).

Both *A. mixtum* and *A. tenellum* occur within the same geographic range and may be in frequent contact with each other allowing for the rare opportunity of hybridization. Not only is there an overlap within their geographic ranges, an overlap exists with the utilization of hosts. Known host species utilized by both species included: cattle (Bovidae), equines (Equidae), deer (Cervidae: *Odocoileus*), opossum (Didelphidae), squirrel (Sicuridae), feral swine (Suidae),

peccary (Tayssuidae), dogs (Canidae), humans (Hominidae) and turkey (Phasianidae: *Melagris*) (Rivas 1984, Guzmán-Cornejo et al. 2011, Corn et al. 2016). Both *A. mixtum* and *A. tenellum* exhibit an overlap in seasonal activity and are active year-round in southern Texas. Additionally, both species are of medical importance as they have been known to vector *Rickettsia rickettsii*, and possibly they have potential to vector tick typhus rickettsia species (Billings et al. 1998, Oliveira et al. 2010, Parola et al. 2013). In relation to veterinary importance, *A. mixtum* has recently been implicated in an outbreak of equine piroplasmosis (etiological agent: *Theileria equi*) in south Texas (Scoles et al. 2011, Scoles and Ueti 2013). Within this outbreak area *A. tenellum* is known to be present but its status as a competent vector of equine piroplasmosis remains unknown.

No previous studies have investigated the possibility of interbreeding between *A. mixtum* and *A. tenellum* occurring neither in nature, nor in the laboratory. Other species of ticks are likely to hybridize but the fertility of the offspring is variable among species. Based on morphological data, Graham and Price (1966) suggested hybridization might occur occasionally or constantly between intermediate forms of *Rhipicephalus* (*Boophilus*) *annulatus* and *Rhipicephalus* (*Boophilus*) *microplus*. Later Graham et al. (1972) carried out laboratory cross-mating experiments and found the following: the size and eggs of females were not altered by cross mating, hatching rates were equal, F_1 generation appeared normal, the hatching rate between F_1 siblings was low, and backcrossing resulted in sterile males and a reduction in female fertility. Testes were absent or vestigial in hybrid males and females showed chromosomal abnormalities in ovaries (Newton et al. 1972). Thompson et al. (1981) went further with the *R. annulatus* and *R. microplus* experiments to test if the sterility would be maintained within in

successive generations. They found that some 4th generation hybrid males of backcrosses were fertile and by the 7th generation their fertility was equal to that of the control.

Besides the *R. annulatus* and *R. microplus* experiments other species have been investigated for the production of hybrids: *Hyalomma excavatum* and *H. marginatum* (Cwilich and Hadani 1963), *Dermacentor andersoni* and *D. variabilis* (Oliver et al. 1972), and *Ornithodoros* (Balashov 1970). Gladney and Dawkins (1973) conducted the first known crossbreeding experiment within the genus *Amblyomma*. Their study showed that the F_1 generations of *A. maculatum* × *A. americanum* had a reduction in fertility and some suffered malformations (legs absent or curly legs, intermediates between nymphs and adults, and gynadromorphism). Rechav et al. (1982) performed crosses of *A. hebraeum* and *A. variegatum* resulting in egg masses that were infertile (< 1% of eggs hatched).

The effects of hybridization on vector and host interactions are poorly understood. It is possible that hybridization in ticks may result in broader host preferences, enhanced physiological adaptations, and larger host ranges leading to a rapid increase in the transmission of the pathogens they carry. The purpose here to investigate if cross mating occurs between *A. mixtum* and *A. tenellum*. In addition to the investigation of hybridization, comparisons can be made of several life history events between *A. mixtum* and *A. tenellum* and their cross-mated pairs. Specifically, the following eight life history events were investigated: 1) success of engorgement, 2) drop-off duration, 3) engorgement weight, 4) success of egg-production, 5) egg-production duration, 6) egg mass weight, 7) egg-production efficiency, and 8) success of larvae production. Lastly, the relationship between: 1) egg mass weight and engorgement weight and 2) egg efficiency (%) and engorgement weight was investigated.

METHODS

Tick Colonies

The two species of ticks that were used in this study were obtained from laboratory colonies maintained by the Tick Research Laboratory at Texas A&M University, College Station, Texas, USA. The *A. mixtum* colonies were established from 60 – 100 founders collected on feral hogs (*Sus scrofa*) and CO_2 traps located at Welder Wildlife Center, Wilder Wildlife Conservation Foundation, Texas, USA (28.1211, -97.4419), which have been through 2 – 3 generations. While the *A. tenellum* colonies consisted of founding adults collected on CO_2 traps and from nymphs were collected from Laguna Atascosa National Wildlife Refuge, Texas, USA (26.2289, -97.3472). The nymphs obtained were fed on hens (*Gallus gallus domesticus*) to obtain adults. Theses adults were then placed on calves to feed and engorge resulting in 91 engorged females. This *A. tenellum* colony has only been through one generation. Ticks from each colony were fed separately from one-another to insure the likelihood that ticks would remain purebreds. Between feedings ticks were housed separately by species in incubators under the following conditions: approximately 20ºC, 85 – 90% relative humidity, and 14:10 (Light:Dark) photoperiod. All feedings of ticks were conducted adhering to the Texas A&M University IACUC animal use protocol (AUP) #2011-213.

Both tick species look very similar but can be distinguished by a few morphological characters. Females of *A. mixtum* and *A. tenellum* can be distinguished by 1) size as *A. mixtum* tends to be larger than *A. tenellum*, 2) the presence of chitinous tubercles located on the postero-internal angle of the festoons in *A. mixtum* and lacking in *A. tenellum*, and 3) and shape of the opening of the female external genitalia with *A. mixtum* having slit like openings and *A. tenellum* with a bulbous opening. The ornamentation on the scutum of both female species is similar with

slight variation in the pattern. Nava et al. (2014) found that the scutal punctations varies with *A. mixtum* having fewer and less dense punctations than that of *A. tenellum.* Males are harder to tell apart lacking real distinguishing characters other than size with *A. mixtum* larger than *A. tenellum* and a slight difference in ornamentation on the scutum. In addition, the spur on coxa III appears to be more acute in *A. tenellum* than in *A. mixtum* (Sundman 1966).

Cross mating

The following crosses were implemented on two calves (Calf 1 and Calf 2) approximately 136 – 181 kg: *A. mixtum* × *A. mixtum*, *A. tenellum* × *A. tenellum*, *A. mixtum* × *A. tenellum*, and *A. tenellum* × *A. mixtum.* From here on the names of species will be abbreviated as *A. mix* for *A. mixtum* and *A. ten* for *A. tenellum.* Previous history for the calves showed no treatment with acaricides and both were washed to insure uniformly clean surfaces. Along the back of each calf, two rows (right = Side 1 and left = Side 2) of four randomized locations were denoted to one of the four crosses per row (Figure 1). For each denoted area, a 20 × 20 cm square area of hair was clipped and a stockinette sleeve was glued to the adjoining circle of hair using livestock identification tag cement (Nasco). A total of 20 female and 20 male ticks were placed in each of the stockinette sleeves along the back of the calves. To ensure that the majority of the ticks made contact with the calves, the ticks sticking to the stockinette sleeve were tapped onto the skin. Then the stockinette sleeve was twisted and wrapped with rubber bands to insure no ticks could escape. Within the stockinette sleeves the ticks were allowed to feed, mate, and detach. The daily drop-off cycle of the engorged females was monitored over a 24-day period.

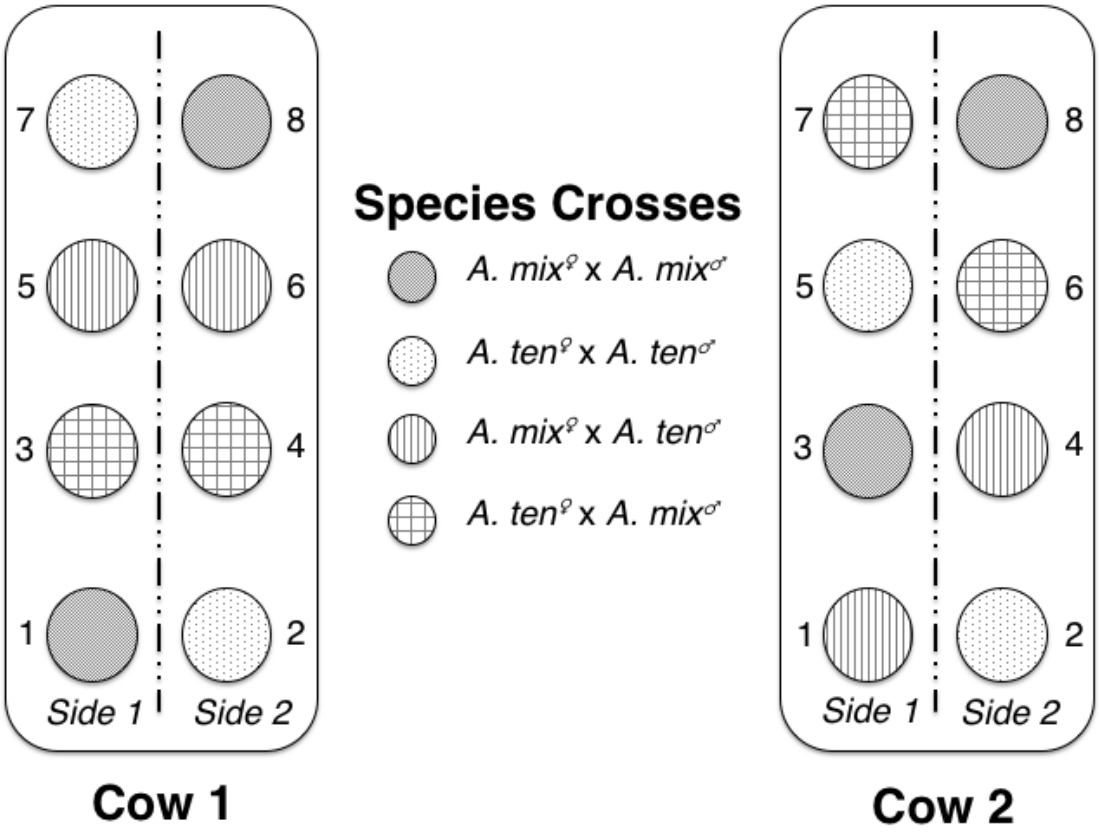

Figure 1. Species crosses. Two calves were divided into two sides a left (Side 1) and a right (Side 2). Within in these sides the four crosses were randomly assigned into four locations creating eight different positions on a single calf (Gray = *A. mix* x *A. mix*; Dotted = *A. tel* x *A. tel*; Vertical lines = *A. mix* X *A. tel*, and Checkered = *A. tel* X *A. mix*). Here *Amblyomma mixtum* is abbreviated as *A. mix* and *Amblyomma tenellum* is abbreviated as *A. ten*.

Once the engorged female ticks dropped the date of drop was recorded and each individual tick was weighted (mg) and placed in a collection vial. Those that were still attached at the end of the study on the 24th day were pulled off and excluded from this study as they did not represent the normal drop-off behavior of engorged females. Ticks were kept in an incubator

under optimal conditions approximately 20ºC, 85 – 90% relative humidity, and 14:10 (L:D) photoperiod. The preovipostion period of engorged ticks were followed for each individual tick until the first indication of oviposition. A total of 30 days were allowed for each tick to lay eggs. After that time the engorged females were removed from the vial and the egg masses were weighted (mg). The egg production efficiency ($\frac{Weight\ of\ egg\ mass}{Weight\ of\ engorged\ female} \times 100$) was then calculated for each individual tick (Bennett 1974).

Data analysis

All statistical analyses were performed using the software JMP® v.12.0.1 and SAS® v.9.4 (SAS Institute, Inc. Cary, NC) with statistical graphics created in R v.3.4.1 (R Foundation for Statistical Computing, Vienna, Austria, www.r-project.org). The first analysis was to test for differences in success of engorgement of each cross: 1) between each calf, 2) between the left and right side among each calf, and 3) between individual side compared from each calf. For these analyses we used the chi square goodness of fit test (χ^2). When expected values were less than five we applied the Fisher's exact test instead of the Pearson's test. The study utilized a randomized complete block design with a split plot treatment assignment: in which the two calves served as the blocks ($Calf$), species cross ($Cross$) as the first factor and side of calf ($Side$) as the second factor. In addition, an interaction term of cross and side was included to determine whether there was any influence of placement on the left or right side of the calves. The experimental unit for side was the left or right side of the calves and for species cross the position was the experimental unit. The statistical model for analysis in SAS® was $Y = Calf + Side + Position(Side) + Cross + Cross \times Side$ with Cow and $Position$ as random factors with $Position$ nested within $Side$ ($Postion(Side)$). I ran this model for each of the five following response variables: 1) drop-off duration, 2) engorgement weight, 3) egg production

duration, 4) egg mass weight, and 5) egg production efficiency. A post ad hoc Tukey's HSD test was conducted for all possible pairwise comparisons. For all statistical analyses the level of significance was: $\alpha = 0.05$. Lastly for each cross, data were pooled as a result of small sample size, in order for the following linear regressions to be made: 1) prediction of egg mass weight (mg) based on engorged tick weight (mg) and 2) prediction of egg production efficiency based on engorged tick weight (mg). Additionally, comparisons of slopes of the four regressions of each the crosses were made for the egg mass model ($EggMass = TickWeight + Cross + Cross * TickWeight$) and the egg production efficiency model ($EggProEff = TickWeight + Cross + Cross * TickWeight$). Outliers were noted and investigated for biological relevance and removed, models were then rerun in JMP.

RESULTS

Attachment

Attachment was monitored daily for each of the crosses until all females attached. After one day of infestation of *A. mix* × *A. mix* a total of 62 (88.57%) female ticks were attached where Calf 2 had a higher number of attachment with 37 (92.5%) attached while Calf 1 had a total of 25 (62.5%) females attached. By day two all 40 (100%) *A. mix* × *A. mix* females on Calf 2 were attached, but it another three days, to day five, for all females on Calf 1 to become attached (Table 1). The grouping of *A. mix* × *A. mix* was variable from cell to cell: one cell all attached in at least three main groupings, while two cells all in one group along the margin, and the last cell where five groupings occurred with three to four female ticks.

Table 1. The number of female ticks attached by day after infestation for each cross of the four crosses on two calves, total, and mean (± standard deviation) (*A. mix* = *Amblyomma mixtum*; *A. ten* = *Amblyomma tenellum*).

Cross	Calves	Number of Female Ticks Attached				
		Day 1 # (%)	**Day 2 # (%)**	**Day 3 # (%)**	**Day 4 # (%)**	**Day 5 # (%)**
A. mix × *A. mix*	Calf 1	25 (62.5)	36 (90)	38 (95)	39 (97.5)	40 (100)
	Calf 2	37 (92.5)	40 (100)	40 (100)	40 (100)	40 (100)
	Total	62 (88.57)	76 (95)	78 (97.5)	79 (98.75)	80 (100)
	Mean	31 ± 8.49	38 ± 2.83	39 ± 1.41	39.5 ± 0.71	40
A. mix × *A. ten*	Calf 1	35 (87.5)	39 (97.5)	40 (100)	40 (100)	40 (100)
	Calf 2	39 (97.5)	39 (97.5)	40 (100)	40 (100)	40 (100)
	Total	74 (92.5)	78 (97.5)	80 (100)	80 (100)	80 (100)
	Mean	37 ± 2.83	39	40	40	40
A. ten × *A. ten*	Calf 1	38 (95)	40 (100)	40 (100)	40 (100)	40 (100)
	Calf 2	34 (85)	39 (97.5)	40 (100)	40 (100)	40 (100)
	Total	72 (90)	79 (98.75)	80 (100)	80 (100)	80 (100)
	Mean	36 ± 2.83	39.5± 0.71	40	40	40
A. ten × *A. mix*	Calf 1	39 (97.5)	40 (100)	40 (100)	40 (100)	40 (100)
	Calf 2	38 (95)	40 (100)	40 (100)	40 (100)	40 (100)
	Total	77 (96.25)	80 (100)	80 (100)	80 (100)	80 (100)
	Mean	38.5 ± 0.71	40	40	40	40

One day after infestation of *A. ten* × *A. ten* a total of 72 (90%) female ticks were attached, of these 38 (95%) were from Calf 1 and 34 (85%) were from Calf 2. Attachment of female *A. ten* × *A. ten* was completed on day 2 for Calf 1 and on day 3 for Calf 2 (Table 1). The groupings of *A. ten* × *A. ten* were variable where at least two groups were found in two of the cells, one cell where all were attached along the margin of the cell, and one cell where no real groupings were observed.

For the *A. mix* × *A. ten* cross a total of 74 (92.5%) females were attached after one day of infestation. On Calf 1 attachment consisted of 35 (87.5%) females while there was 39 (97.5%)

females attached on Calf 2. For ticks on both calves, completion of attachment of the *A. mix* × *A. ten* occurred on day three (Table 1). The grouping of *A. mix* × *A. ten* was variable: having two cells ticks the majority of ticks in one central group, another cell where a single group occurred along the edge of the cell, and one cells containing two small groups.

The *A. ten* × *A. mix* cross had a total of 77 (96.25%) ticks attached on day one. For day one similar number of ticks were attached on each calf with Calf 1 having 39 (97.5%) attached and Calf 2 with 38 (95%) attached. Upon day two all ticks were attached for *A. ten* × *A. mix* for both calves (Table 1). The *A. ten* × *A. mix* females were variable in grouping with two cells showing no real apparent grouping pattern, one cell where they were all grouped together in one large mass along the edges of the cell, and another cell where there was only one group of six individuals.

Success to Engorgement

Ticks removed from study.—Normally once the female ticks reach full engorgement they drop off into the environment. In this study female ticks that were still attached on the last day of the study were physically removed and excluded from the study as they did not represent a normal feeding cycle. Normally females will become fully engorged and drop off the host. A total of 41 female ticks were removed: five (6.25%) females from the *A. ten* × *A. ten* cross were removed, 19 (23.75%) from the *A. mix* × *A. ten* cross, and 17 (21.25%) from the *A. ten* × *A. mix* cross (Table 2). No ticks from the *A. mix* × *A. mix* cross were physically detached as the majority fully engorged and normally dropped off the calves by the end of the study.

Table 2. The number and percentage of successful engorgement, mortality, and ticks removed from study by physical detachment for each cross of the four crosses on two calves, total, and mean (± standard deviation) (*A. mix* = *Amblyomma mixtum*; *A. ten* = *Amblyomma tenellum*).

Calf Number	*A. mix* × *A. mix*	*A. ten* × *A. ten*	*A. mix* × *A. ten*	*A. ten* × *A. mix*
		Engorgement		
1	34 (85%)	40 (100%)	23 (57.5%)	22 (55%)
2	28 (70%)	31 (77.5%)	9 (22.5%)	14 (35%)
Totals	62 (77.5%)	71 (88.5%)	32 (40%)	36 (45%)
Mean	31 ± 4.24	35 ± 6.36	16 ± 9.9	15 ± 5.66
		Mortality		
1	6 (15%)	0 (0%)	11 (27.5%)	16 (40%)
2	12 (30%)	4 (10%)	18 (45%)	11 (27.5%)
Totals	18 (22.5%)	4 (5%)	29 (36.25%)	27 (33.75%)
Mean	9 ± 4.24	2 ± 2.85	14.5 ± 4.95	13.5 ± 3.54
		Removed from Study (Physically Detached)		
1	0 (0%)	0 (0%)	6 (15%)	2 (5%)
2	0 (0%)	5 (12.5%)	13 (32.5%)	15 (37.5%)
Totals	0 (0%)	5 (6.25%)	19 (23.75%)	17 (21.25%)
Mean	0	2.5 + 3.54	9.5 ± 4.95	8.5 ± 9.19

Mortality.—While feeding some ticks died as a result of being crushed as a result of the calves rubbing against or laying against the stanchions and for others their cause of death was unknown. The highest mortality of ticks occurred within the mixed crosses with a total of 29 (36.25%) ticks in the *A. mix* × *A. ten* cross and 27 (33.75%) within the *A. ten* × *A. mix* cross. In the *A. mix* × *A. mix* cross 18 (22.5%) ticks had died (Table 2). The lowest morality was within the *A. ten* × *A. ten* cross with a total of 4 (5%) dead ticks.

Engorgement.—For both calves the cross of *A. ten* × *A. ten* had the highest success of engorgement among the crosses followed by *A. mix* × *A. mix* (Table 2). While the crosses of *A. mix* × *A. ten* and *A. ten* × *A. mix* were both similar for Calf 1 but slightly different for Calf 2. In

order to determine whether successful engorgement (= dropping off the host rather than being pulled off) for all crosses differed from Calf 1 and Calf 2 a chi square test was performed. The drop-off for all crosses between the calves did not significantly differ (N = 201, df = 3, χ^2 = 2.824, p = 0.4196). Next, we looked to see if the success of engorgement differed between the right side (Side 1) and the left side (Side 2) among each calf. There was no difference between the left and right side for both Calf 1 (N = 119, df = 3, χ^2 = 0.397, p = 0.9409) and Calf 2 (N = 82, df = 3, χ^2 = 5.600, *Fisher's Exact Test Table probability* = 0.000911, p = 0.1411). Lastly, I looked at each individual side and compared results from Calf 1 and Calf 2. There was no difference in the success of engorgement from Side 1 on Calf 1 and Side 1 of Calf 2 (N = 86, df = 3, χ^2 = 4.397, p = 0.2394). Nor was there a difference in Side 2 between Calves 1 and 2 (N = 115, df = 3, χ^2 = 1.525, p = 0.6765).

Drop-off duration.—From the time the ticks were placed on the calves, I monitored the duration of days it took for the ticks to naturally drop off the hosts. The mean drop-off duration for both the *A. mix* × *A. mix* and *A. ten* × *A. ten* crosses were similar ($\bar{x}$ = 10.6611 $\pm$ 2.0720 SD, $\bar{x}$ = 10.3333 $\pm$ 2.1030 SD respectively) with the earliest drop-off within eight days for both crosses and the longest drop-off of 20 days for *A. mix* × *A. mix* and 22 days for *A. ten* × *A. ten.* The mean drop-off for the mixed crosses was longer than that of pure crosses (*A. mix* × *A. ten*: $\bar{x}$ = 18.4688 $\pm$ 3.9918 SD; *A. ten* × *A. mix*: $\bar{x}$ = 21.1944 $\pm$ 2.6920 SD). The earliest drop-off for *A. mix* × *A. ten* occurred within 9 days and lasted up to 26 days while that of *A. ten* × *A. mix* the earliest drop-off occurred within 13 days and lasted up to 25 days.

The effect of $Cross$ was significant (df = 3, *Den_df* = 189, *F-value* = 201.71, p < 0.0001) but the effect of $Side$ was not significant (df = 1, *Den_df* = 4, *F-value* = 1.13, p = 0.3474) nor was the interaction of $Cross \times Side$ (df = 3, *Den_df* = 189, *F-value* = 1.82, p = 0.1441). The

Tukey's HSD of all possible pairwise contrasts revealed that the drop-off duration of all paired crosses were significantly different from one another ($p < 0.0001$) except for the pure strain pair of *A. mix* × *A. mix* and *A. ten* × *A. ten* ($p = 0.9545$) (Table 3).

Table 3. *P*-values for the pairwise cross comparisons using the Tukey's HSD test ($\propto = 0.05$, significance shown with and asterisk) for each of the life stage event (Drop-off duration, engorgement weight, egg production duration, egg mass weight, and egg production efficiency $[(\frac{Weight\ of\ egg\ mass}{Weight\ of\ engorged\ female} \times 100)]$). Abbreviations of *A. mix* = *Amblyomma mixtum* and *A. ten* = *Amblyomma tenellum*.

Cross Comparison	Drop-off Duration	Engorgement Weight	Egg Production Duration	Egg Mass Weight	Egg Production Efficiency
A. mix * *A. mix* – *A. ten* * *A. ten*	0.9545	< 0.0001*	0.0003*	0.0060*	0.6374
A. mix * *A. mix* – *A. mix* * *A. ten*	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	0.0354*
A. mix × *A. mix* – *A. ten* × *A. mix*	< 0.0001*	< 0.0001*	0.1218	< 0.0001*	0.1148
A. ten × *A. ten* – *A. mix* × *A. ten*	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	0.0003*
A. ten × *A. ten* – *A. ten* × *A. mix*	< 0.0001*	< 0.0001*	0.9833	< 0.0001*	0.0060*
A. mix × *A. ten* – *A. ten* × *A. mix*	< 0.0001*	0.0048*	< 0.0001*	0.6636	0.9291

Engorgement weight.—After the ticks dropped off, each was individually weighed (in mg). Engorgement weight varied within each cross as a result of the variability of the weight of the ticks when placed on the calves. The heaviest of the pure crosses was *A. mix* × *A. mix* with an average engorgement weight of 636.3945 mg (SD = 172.8443) and *A. ten* × *A. ten* with an average engorgement weight of 467.4134 mg (SD = 147.7916). The mixed species crosses showed about a 37% average reduction in engorgement weight compared to the pure crosses with the average engorgement weight of 272.1378 mg (SD = 122.0388) for *A. mix* × *A. ten* and 140.8508 mg (SD = 84.7617) for *A. ten* × *A. mix*.

For the engorgement weights of ticks, the effect of $Cross$ was significant (*df* = 3, *Den_df* = 188, *F-value* = 88.49, $p < 0.0001$) but neither the effect of $Side$ (*df* = 1, *Den_df* = 4, *F-value* = 3.27, $p = 0.1443$) nor the interaction of $Cross \times Side$ were significant (*df* = 3, *Den_df* = 188, *F-value* = 0.23, $p = 0.8727$). According to the Tukey's HSD test all possible pairwise combinations were significant with similar *p* values of < 0.0001 except for the pair containing *A. ten* × *A. mix* and *A. mix* × *A. ten* with a *p* value of 0.0048 (Table 3).

Eggs

Success of egg production.—A total of 66 (91.7%) ticks of the *A. ten* × *A. ten* cross produced eggs while those of the *A. mix* × *A. mix* cross only 55 (88.7%) ticks produced eggs. The mixed species crosses showed a lower number of egg production with the cross of *A. mix* × *A. ten* with 21 (65.6%) producing eggs and a total of 16 (44.4%) ticks from the *A. ten* × *A. mix* cross.

Egg production duration.—I monitored the number of days it took for each individual tick to deposit eggs. The mean egg laying duration for *A. mix* × *A. mix* was 21.4727 days (SD = 4.3156) with the earliest onset of production occurring within 15 days and upwards to 42 days.

The pure cross of *A. ten* × *A. ten* was similar with a mean of 25.3788 days (SD = 5.2029) with the range of production occurring much earlier than *A. mix* × *A. mix* starting within 2 days with the longest duration of 48 days. Surprisingly *A. mix* × *A. tel* had a shorter mean of duration than all the other crosses ($\bar{x}$ = 15.7000 $\pm$ 7.0270 SD) with a duration range of 6 to 30 days while *A. ten* × *A. mix* was similar to the pure crosses ($\bar{x}$ = 24.5333 $\pm$ 2.6690 SD) ranging from 20 to 30 days.

The duration of egg production varied significantly among $Cross$ (*df* = 3, *Den_df* = 143, *F-value* = 24.33, $p < 0.0001$) while $Side$ was not significant (*df* = 1, *Den_df* = 4, *F-value* = 7.15, $p = 0.0555$). Additionally, there was a significant interaction among $Cross \times Side$ (*df* = 3, *Den_df* = 143, *F-value* = 2.89, $p = 0.0374$). The Tukey's HDS test showed that only two of the combination crosses (*A. mix* × *A. mix* – *A. ten* × *A. mix* and *A. ten* × *A. ten* – *A. ten* × *A. mix*) were not significantly different while all others had p values < 0.0001 except for the *A. mix* × *A. mix* – *A. ten* × *A. ten* with a *p* value of 0.003 (Table 3).

Egg mass weight.—After 30 days of oviposition the female ticks were removed and each egg mass was weighed (in mg). The heaviest mean egg mass weights were found among the pure crosses with *A. mix* × *A. mix* having a mean of 431.5318 mg (SD = 136.6488) and *A. ten* × *A. ten* with a mean of 354.3918 mg (SD = 122.9102). While the mean egg masses for the mixed crosses were smaller with about a 35% reduction in the average egg weight mass. The average egg mass weight for *A. mix* × *A. ten* was 157.0019 mg (SD = 102.9245) and the *A. ten* × *A. mix* cross showed the lightest average egg weight mass of 117.3356 mg (SD = 70.1153).

The mean egg mass weight varied significantly between $Cross$ (*df* = 3, *Den_df* = 145, *F-value* = 41.87, $p < 0.0001$) but was not significant with $Side$ (*df* = 1, *Den_df* = 4, *F-value* = 1.03, $p = 0.3671$) nor was there a significant difference with the interaction of $Cross \times Side$ (*df* = 3,

Den_df = 145, *F-value* = 0.22, $p = 0.8818$). For the pairwise comparisons using the Tukey's HSD test all comparisons were significant except for the pair involving *A. ten* × *A. mix* and *A. mix* × *A. mix* ($p = 0.6636$). The majority of the significant pairs had a p value < 0.001 while the pair involving the pure strains *A. ten* × *A. ten* and *A. mix* × *A. mix* had a p value of 0.0060 (Table 3).

Egg mass weight in relation to engorgement weight.—A total of five outliers were removed from the linear regression analyses (Figure 2). The first two outliers removed belonged to the *A. mix* × *A. mix* cross, which included one tick having an engorgement weight of 759.23 mg and an egg mass weight of 94.77 mg. While the second outlier was a tick with an engorgement weight of 910 mg and an egg mass weight of 220.21 mg. Two outliers also occurred in the *A. tel* × *A. tel* cross with one tick having an engorgement weight of 296.78 mg with a 16.34 mg egg mass weight and the second tick having an engorgement weight of 459.03 mg and an egg mass weight of 167.26 mg. The last outlier removed occurred in the *A. tel* × *A. mix* cross with an engorgement weight of 179.91 mg and an egg mass weight of 16.34 mg. The removal of these influential outliers increased the fit for each of the crosses. All crosses had relatively high fits, with three of the crosses having a R^2 higher than 0.90 (*A. tel* × *A. tel*, *A. mix* × *A. tel*, and *A. tel* × *A. tel*) (Figure 3). The *A. mix* × *A. mix* cross seemed to show more variability in the engorgement weight and egg mass weight with ticks having mid engorgements weights with lower egg mass weights. The overall model for differences between slopes and intercepts of the linear regression model was significant, indicating differences among the four crosses ($p < 0.001$). A significant difference was found among the four intercepts of each cross (*df* = 3; Sum of Squares = 19226.72; *F*-ratio = 3.2779; $p = 0.0228$) as well differences among the four slopes (*df* = 3; Sum of Squares = 19720.21, *F*-ratio = 3.3620; $p = 0.0205$).

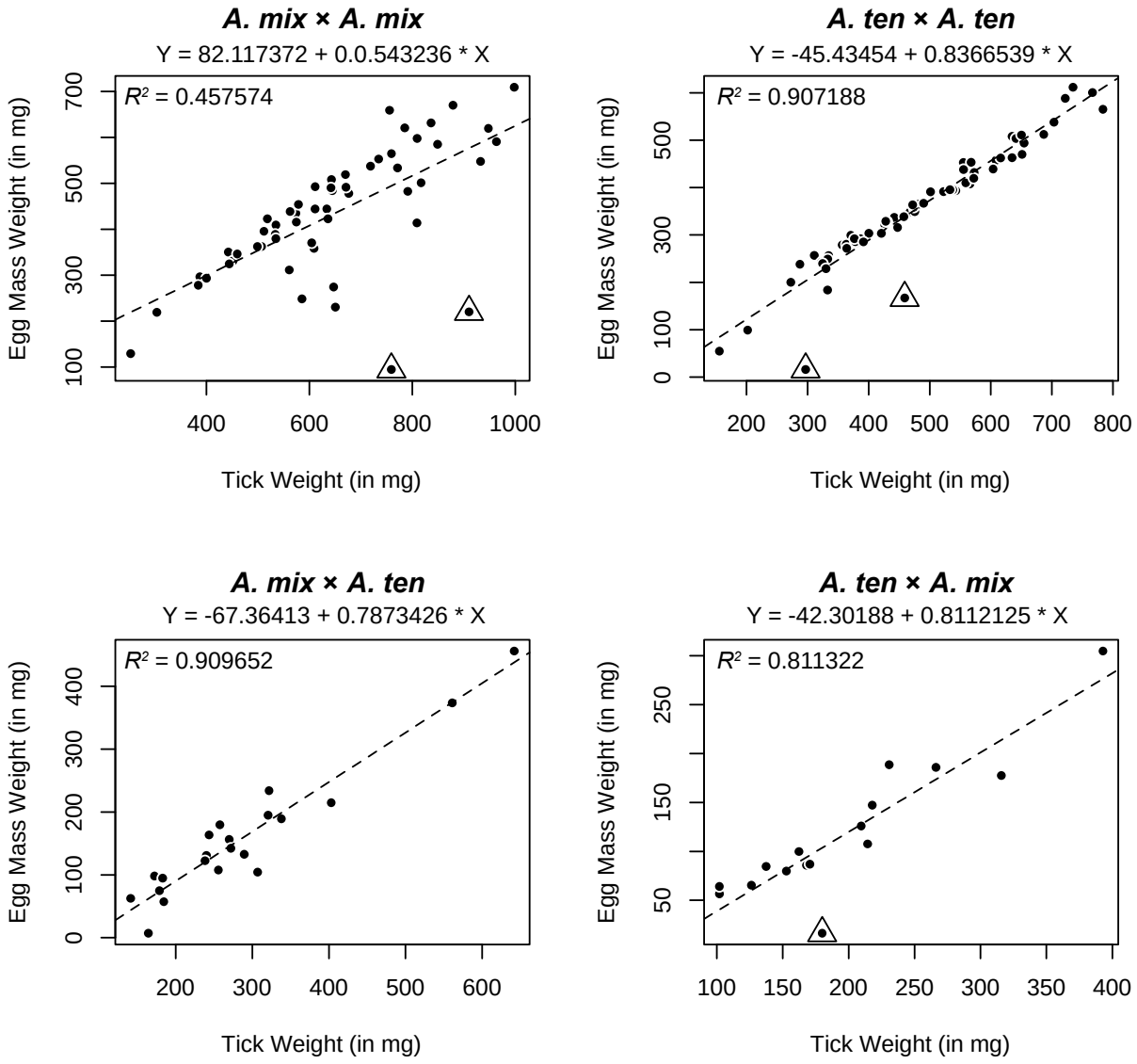

Figure 2. Egg mass weight of all four crosses, in relation to engorgement weight using linear regression models with the inclusion of outliers. R^2 values are reported in the corner of each graph. Here *Amblyomma mixtum* is abbreviated as *A. mix* and *Amblyomma tenellum* is abbreviated as *A. ten*. Outliers are shown with triangles.

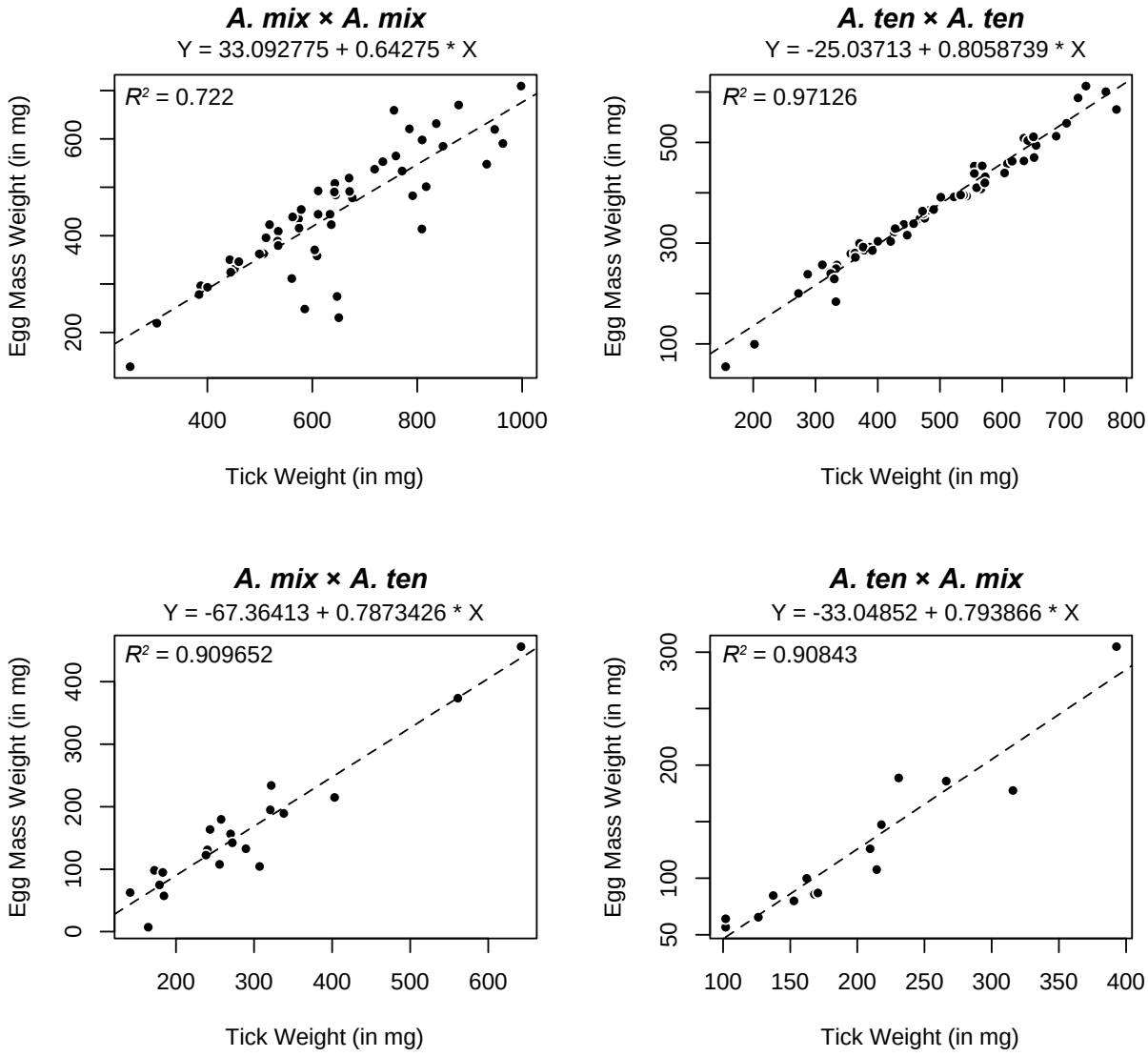

Figure 3. Egg mass weight of all four crosses, in relation to engorgement weight using linear regression models. R^2 values are reported in the corner of each graph. Here *Amblyomma mixtum* is abbreviated as *A. mix* and *Amblyomma tenellum* is abbreviated as *A. ten*. Outliers removed see Figure 2 for outliers.

Egg production efficiency.—Once the weight for each individual tick and that of their egg masses were acquired we were able to calculate the egg production efficiency ($\frac{Weight\ of\ egg\ mass}{Weight\ of\ engorged\ female} \times 100$) which is a measure of the total body mass put into production of eggs. The largest mean egg production efficiency belonged to the pure crosses with the *A. ten* × *A. ten* cross with an average of 72.96% (SD = 11.9469) ranging between 5.5058% and 83.2641% while that of the *A. mix* × *A. mix* cross was 67.8772% (SD = 14.1923) with the range of percentages between 12.4824% and 87.2234%. Both the mixed species crosses were similar with *A. mix* × *A. ten* exhibiting a mean egg production efficiency of 51.6971% (SD = 15.8686) with a range between 4.3740% and 72.6954% and that of the *A. ten* × *A. mix* cross having a mean of 57.5027% (SD = 16.0457) with the percentages ranging between 9.0823% and 81.7579%.

The mean egg production efficiency was significant for the $Cross$ (*df* = 3, *Den_df* = 145, *F-value* = 6.95, *p* = 0.0002) but was not significant for $Side$ (*df* = 1, *Den_df* = 4, *F-value* = 0.20, *p* = 0.6800) nor was the mean egg production significant for the interaction of $Cross \times Side$ (*df* = 3, *Den_df* = 145, *F-value* = 0.24, *p* = 0.8665). The Tukey's HSD all pairwise test indicated that three of the six pairwise comparisons were significant (Table 3).

Egg efficiency in relation to engorgement weight.—The same outliers were removed as those from the egg mass in relation to engorgement weight linear regression model (Figure 4). The two outliers in the *A. mix* × *A. mix* cross that were removed consisted of one tick with an engorgement weight of 759.23 mg and an egg efficiency of 12.48238347% and the second engorged tick weighting 910 mg with an efficiency of 24.1989011%. Within the *A. tel* × *A. tel* cross two outliers were removed with one engorged tick weighting 296.78 and an egg efficiency of 5.5057618% and the other tick with an engorgement weight of 459.03 mg with an egg

efficiency of 36.43.77056. Lastly only one outlier was removed in the *A. tel* × *A. mix* with an engorgement weight of 179.91 mg and an egg efficiency of 9.082318937%. For the *A. tel* × *A. tel* and *A. tel* × *A. mix* crosses the removal of the outlier improved the fit of the model slightly but for the *A. mix* × *A. mix* cross the fit was actually decreased. The model fits for all the four crosses were low just under a R^2 value of 0.30, showing a low relationship between tick engorgement weight and egg efficiency (Figure 5). The overall model for differences between slopes and intercepts of the linear regression model, for tick engorgement weight and egg efficiency, was significant, indicating differences among the four crosses ($p < 0.001$). A significant difference was found among the four intercepts of each crosses ($df = 3$; Sum of Squares = 770.8442; F-ratio = 2.8457; $p = 0.0398$) as well in the differences among the four slopes ($df = 3$; Sum of Squares = 1687.6195, F-ratio = 6.2302; $p = 0.0005$).

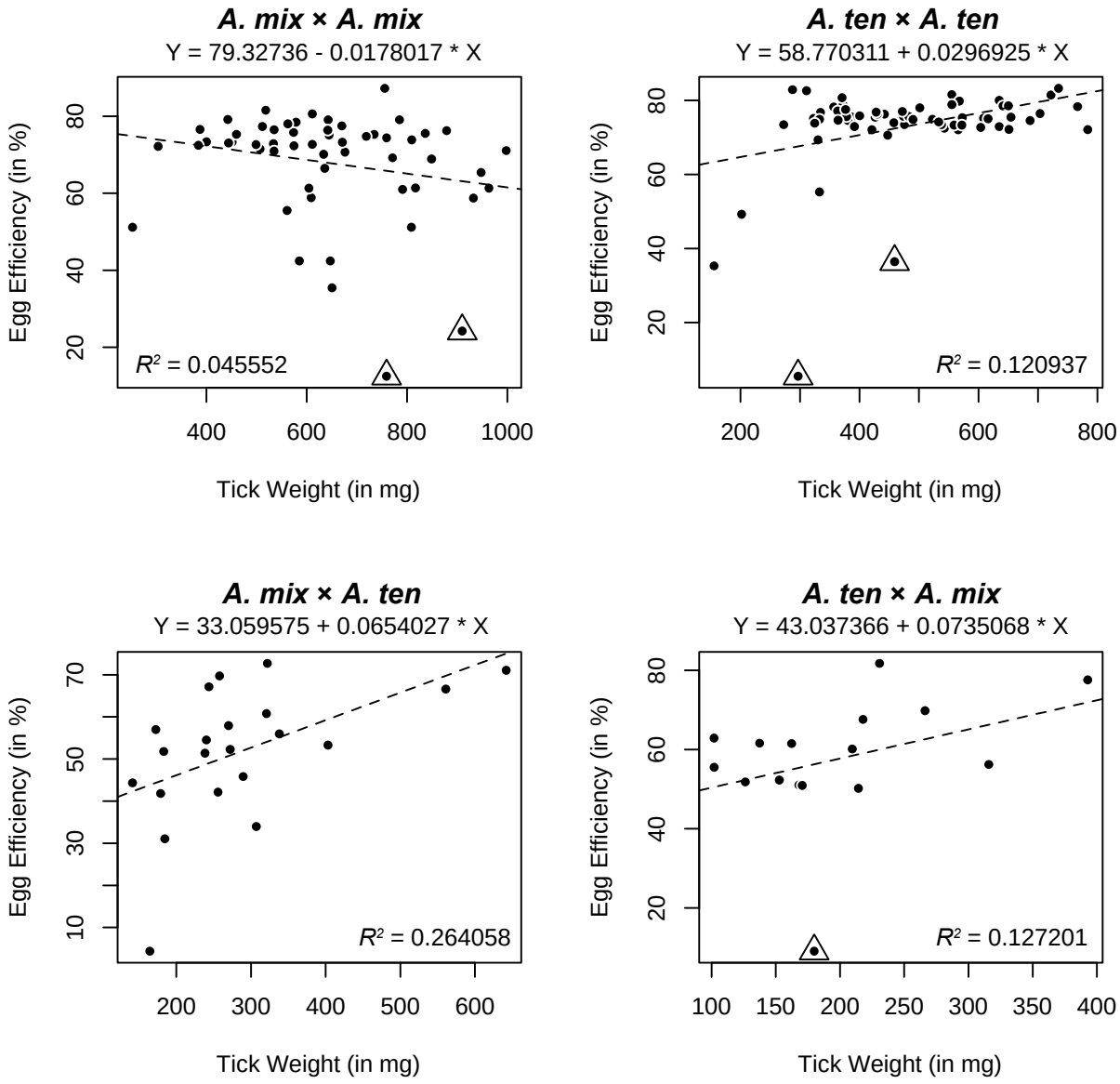

Figure 4. Egg efficiency for all four crosses, in relation to engorgement weight using linear regression models with the inclusion of outliers. Here *Amblyomma mixtum* is abbreviated as *A. mix* and *Amblyomma tenellum* is abbreviated as *A. ten*. R^2 values are reported in the corner of each graph. Outliers are shown with triangles.

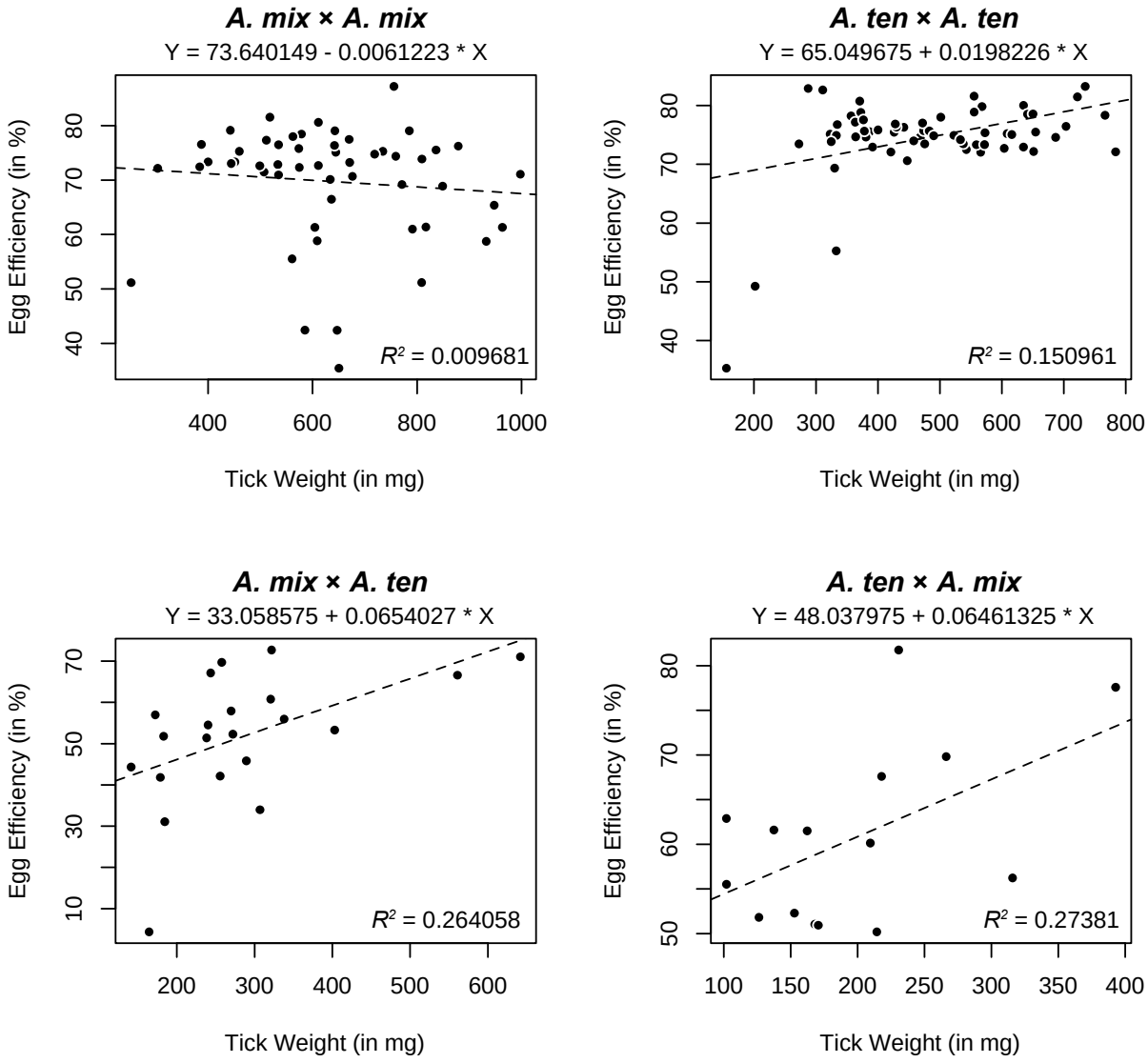

Figure 5. Egg efficiency for all four crosses, in relation to engorgement weight using linear regression models. R^2 values are reported in the corner of each graph. Here *Amblyomma mixtum* is abbreviated as *A. mix* and *Amblyomma tenellum* is abbreviated as *A. ten*. Outliers removed see Figure 4 for outliers

Larvae

Hatch percentage.—Out of the 66 egg batches produced from the *A. ten* × *A. ten* cross only 55 produced larvae (83.3%). Percentage wise the *A. mix* × *A. mix* cross was similar with 46 females producing larvae out of the 55 that produced eggs (83.6%). Only one of the 21 egg batches of the *A. mix* × *A. ten* produced larvae (4.8%). The total number of larvae was small compared to the full strains with only 10 larvae being counted. No larvae were produced from the 16 egg clusters produced by the *A. ten* × *A. mix* cross. Larvae production of the pure strain crosses were also similar between the two calves: 1) for the *A. mix* × *A. mix* cross a total of 24 females produced larvae were from Calf 1 and 22 females from Calf 2 produced larvae while 2) similar numbers of females that produced larvae for *A. tel* × *A. tel* were found with a total of 29 females from Calf 1 and 26 females from Calf 2.

Total duration period and incubation period.—The duration of the time of hatch was only noted from nine *A. mix* × *A. mix* females, six females from the *A. tel* × *A. tel* cross, and one female from the *A. mix* × *A. tel* cross. The total duration period (TDP) includes the time from once a female dropped after full engorgement to the time of hatch. The average TDP for both the pure strains crosses were similar with *A. mix* × *A. mix* with an average TDP of 64 days (6.78233 SD) and 65.8333 days for *A. tel* × *A. tel* (7.6267095 SD). The TDP for the one *A. mix* × *A. tel* was 83 days close to the maximum of the crosses with a total of 79 days. The incubation period (IP) included the time from the first sign of oviopostioning to the time of hatch. Both pure strain crosses were similar with an average IP of 43.667 days for *A. mix* × *A. mix* (3.968627 SD) and with the *A. tel* × *A. tel* cross of 39.8333 days (6.4316924 SD). The IP for the *A. mix* × *A. tel* was longer with a total of 62 days whereas the maximum IP days for the *A. mix* × *A. mix* cross was 51 days and 49 days for the *A. tel* × *A. tel* cross.

DISCUSSION

I looked at the engorgement period for both pure and mixed crosses and found that pure strains were similar in their engorgement period but different from that of the mixed crosses. *Amblyomma mixtum* exhibited a wider range in the number of days of engorgement of 8 to 22 days than had previously been found of 6 to 12 days (Drummond and Whetstone 1975). The ranges in this study are similar to the findings that on rabbits *A. mixtum* fed for a period of 6 to 15 days and 6 days on cattle (Piña et al. 2017). Gunn and Hilburn (1991) determined the mean engorgement period for *A. mixtum*, that were fed in the absence of males, lasted between 20.1 and 29.4 days. The mixed crosses appear within this range with *A. mix* x *A. ten* occurring slightly earlier with 18.5 days. Labruna et al. (2011) also showed that longer periods of engorgement occurred in South American using different species of *Amblyomma* in the absence of males often times two or three times that of a normal engorgement duration.

The engorgement weights of all four crosses differed with heavier engorgement weights for the pure and lighter weights for the mixed crosses. Differences among weights between *A. mixtum* and *A. tenellum* can be explained by differences in sizes with *A. mixtum* generally being larger than *A. tenellum* (Kohls 1958). The mean weight of engorgement of *A. mixtum* was similar to that found by Drummond and Whetstone (1975) with means of 681 mg and 639 mg. In addition, the ranges of weights for *A. mixtum* in this study had a higher and lower range of engorgement weights compared to Drummond and Whetstone (1975) ranges of 497 – 836 mg and 457 – 831 mg and to 327 – 414.6 mg (Gunn and Hilburn 1991). In this study the ranges for *A. mixtum* were surprisingly different from those of Piña et al. (2017) in which *A. mixtum* fed on cattle showing a mean engorgement weight of 237 mg with a range of 229.5 to 244.5 mg while those ticks that fed on rabbits showed more similar results to this study with higher engorgement

weights (mean 550.6 mg and range 462 – 612.3 mg). This low engorgement weight on cattle maybe due to the small sample size used by Piña et al. (2017) of only 20 ticks compared to my total of 62 *A. mixtum* among the pure cross.

Previous studies have shown that the preoviposition period of *A. mixtum* to be shorter compared to what was exhibited in this study. Drummond and Whetstone (1975) found that the average preoviposition among ticks that were disturbed and undisturbed during feeding were very similar with 6.23 days (range = 3 – 8 days) and 6.29 days (*N* = range = 5 – 10 days) respectively. A lower average occurred in one rearing of *A. mixtum* of 4.67 days (*N* = 6, range = 3 – 8 days) and a higher average in another rearing 7.16 days (*N* = 19, range = 0 – 24 days) (Gunn and Hilburn 1991). The average preoviposition of *A. mixtum* that were fed on rabbits was 5.5 days (range = 4 – 8 days) (Piña et al. 2017). The long duration of the preoviposition in this study may be a result of temperature as it may play some role in the duration by influencing metabolic rates for egg development (Nagar, Srivastava and Varma 1964). All the previous studies mentioned used temperatures of 25 – 27ºC whereas in this study I used a temperature of 20ºC. Additionally, the relative humidity varied from mine of 85 – 90% compared to the others studies of 65%, 80%, and >80%. Simulation of daylength in this study of 14:10 varied with all previous studies using 12:12.

The egg mass weight of the pure strains differed from one another and among the mixed species crosses. For *A. mixtum* the ranges of egg mass weights were similar to those found by Gunn and Hilburn (1991) with ranges of 33.5 to 439.2 mg and 11.6 to 166.8 mg. My maximum and minimum range differed from theirs with a maximum of 709.19 mg and minimum of 94.77 mg. These ranges also appear higher than those found by Piña et al. (2017) with those *A. mixtum* feeding on rabbits with a range of 259.4 to 332.2 mg and those fed on cattle with a range of

144.5 – 148.9 mg. Those on cattle greatly differed from mine which may be due to the sample of size of 20 individuals. The average egg mass weights of *A. mixtum* were similar to those found by Drummond and Whetstone (1975) of 418.134 mg and 394.902 mg and those found by Gunn and Hilburn (1991) of 414.6 mg and 334.4 mg. Lower ranges of *A. mixtum* were found in by Piña et al. (2017) with 302.5 mg on rabbits and 146.7 mg on cattle.

The egg production efficiency (EPE) for *A. mixtum* presented in this study was similar to two prior studies. Drummond and Whetstone (1975) found similar results of an average among two groups of *A. mixtum* 1) among females that were disturbed daily during feeding 61.4% EPE (*N* = 34, Range: 49 – 69.9%) and 2) undisturbed females 61.8% EPE (*N* = 61, Range: 31.4 – 71.7%). The average EPE of in this study was similar to these groups but exhibited a lower range of 12.48% and a higher range of 83.26%. Further similarities were found by Piña et al. (2017) who fed *A. mixtum* on rabbits with an average EPE of 55% (Range: 54.3 – 56.1%) and on cattle with average EPE of 61.9%. In this study the EPE of *A. mixtum* and *A. tenellum* were not significantly different. These two ticks of various sizes but still proportionally put in the same amount of engorgement weight into egg production. Regardless of weight both species seemed to put in the same EPE with the majority occurring about 60%.

Parthenogenesis, the ability to produce offspring without fertilization of the eggs, is well documented in hard ticks. *Amblyomma rotundatum* (originally *A. agatum*) was the first tick to be known to exhibit parthenogenesis (Aragao 1912). Since this discovery more species of hard ticks have been found to be able to reproduce parthenogenetically not just within the genus *Amblyomma* but within *Dermacentor*, *Haemaphysalis*, *Hyalomma* and *Rhipicephalus* (*Boophilus)*. I believe that hybridization did not occur in the one batch of eggs produced by the *A. mixtum* × *A. tenellum* cross but was rather a result of parthenogenesis. Gunn and Hilburn

(1991) found that *A. mixtum* was able to reproduce parthenogenetically under laboratory conditions using inbred and colony ticks. Within in these inbred lines of ticks they found a higher rate of successful parthenogenesis compared to that of the colony ticks which they contribute inbreeding to a greater frequency of genes for parthenogenesis. Genes for parthenogenesis have been noted to vary within sympatric and allopatric populations of ticks (Oliver et al 1973, Oliver 1981). Such an example of this is *Haemaphysalis longicornis* where parthenogenetic populations exclusively occur in Australia, New Zealand, and the northern Japanese island of Hokkaido and the northern region of Honshu while they can be found sympatrically with the bisexual race on the Japanese islands of Honshu and Kyushu, Korea, and areas of northeastern China (Oliver 1989, Kiszewski et al 2001). It is possible that in this situation gene variation could exist in *A. mixtum* as Gunn and Hilburn (1991) have found variation among chromosomes among different lines. Natural populations of ticks may be under different selection pressures that might allow for bisexual and parthenogenetic reproduction to occur. What advantage parthenogenesis may have in *A. mixtum* needs further study as the survival of larvae is low with about 10 larvae being produced under laboratory conditions. Additionally, why do some parthenogenetic ticks like *H. longicornis* become more distributed than that of normally producing bisexual ticks and what role this may have in it being an invasive species currently introduced to the United States? As well as what role does parthenogenetic ticks have in maintaining pathogens within the environment?

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CHAPTER III

PHYLOGENETIC RELATIONSHIP BETWEEN TWO SYMPATRIC MORPHOLOGICALLY SIMILAR TICKS *AMBLYOMMA MIXTUM* KOCH, 1844 AND *AMBLYOMMA TENELLUM* KOCH, 1844 (ACARI: IXODIDAE)

The phylogenetic relationship between the two sympatric and morphologically similar ticks *Amblyomma mixtum* Koch, 1844 (formally *A. cajennense* (Fabricius, 1787)) and *Amblyomma tenellum* Koch, 1844 (formally *A. imitator* Kohls, 1958) [these taxonomic name changes are discussed later] has been researched over a period of 75 years (1944 to currently 2019). Spanning the early use of morphological characters to isozymes, to karyotypes, and to the use of several genes for molecular phylogenetic analyses.

Cooley and Kohls (1944) found that while preparing descriptions for the genus *Amblyomma* occurring in the United States that many individuals of *A. mixtum* had nubs on the ventral scutes while some males had conspicuous extensions of the ventral plaques. According to Kohls (1958), *A. tenellum* females can be distinguished from *A. mixtum* on the basis of two morphological characters: 1) lack of chitinous tubercles on the region of the posterinternal angle of the festoons, originally referred to as the nubs (Cooley and Kohls 1944) and 2) the shape of the opening of the external genitalia with *A. mixtum* having slit like openings and *A. tenellum* with a bulbous opening. For males, Kohls utilized size and markings on the scutes to distinguish males of the two species. In addition to these characters, Sundman (1966) determined that males of the two species could be distinguished by differences in the margins of the scutum occurring at the region of the scapulae. The margins of *A. mixtum* appear to be broadly rounded while those of *A. tenellum* are more angulated. Sundman also found that the spur on the third coxa was

more acute in *A. tenellum* than in *A. mixtum*. Body shape and size along with the length of the ventral tubercles, originally the extensions of the ventral plaques found by Cooley and Kohls (1944), were used by Jones et al. (1972) to distinguish the males of *A. mixtum* and *A. tenellum*.

Hilburn et al. (1989) examined isozyme phenotypes of both *A. mixtum* and *A. tenellum* and proposed the question whether there were genetic characters that could distinguish the two species and whether hybridization could occur. Based on their analyses a total of eight proteins were diagnostic. They suggested it was not very likely that the two species would be sufficiently compatible to produce hybrids. Mating studies were being conducted at the time of their research but results of whether or not they were successful in hybridization have not been published.

Karyotyping studies were conducted by Gunn and Hilbrun (1995). They found that the X chromosome of *A. mixtum* was extremely subacrocentric (chromosomal arms unequaled) and appeared to be identical to the X chromosomes noted in *A. tenellum*. Based on the karyotypes the two species could be distinguished by the presence of a biarmed pair of autosomes (chromosome 6). They indicated that *A. mixtum* and *A. tenellum* are related based on the morphology of the X chromosome. In addition, the autosomes showed divergence between the two species, with no similarity noted in the noncentromeric C-band-positive regions.

As mentioned earlier, *A. mixtum* was formally known as *A. cajennense* and was originally part of a species complex. This species complex was first suggested in two crossmating studies conducted with *A. cajennense* from several geographic areas in Brazil (Labruna et al. 2011) and Argentina (Mastropalo et al. 2011). Beati et al. (2013) investigated the phylogenetic relationships within this species complex using one nuclear and three mitochondrial gene sequences. They found that this complex was composed of six distinct genetic units each

associated with a unique habitat type. After the revelation of these six distinct genetic units a reassessment of the taxonomic status was conducted by Nava et al. (2014*b*) which lead to formalization of descriptions for the three new species (*A. tonelliae*, *A. interandinum*, and *A. patinoi*), a redescription of *A. cajennense*, and the validation of both *A. mixtum* and *A. sculptum* Berlese, 1888, which were originally synonyms for *A. cajennense*. A taxonomic species name change also occurred for the formally named *A. imitator*, which has now been reverted to *A. tenellum* with *A. imitator* being considered a junior synonym (Nava et al. 2014*a*).

Recently two phylogeographic studies have been conducted, one involving only the *Amblyomma* (Seabolt 2016) and the other with additional genera of ticks (Beati and Klompen 2019). These two studies have provided insight into the relationship of *A. mixtum* and *A. tenellum* to some North American species of ticks but these studies were limited to the use of one genetic marker (18SrDNA). It is the purpose here to investigate the phylogenetic relationship of *A. mixtum* and *A. tenellum* to two species of North American *Amblyomma* using a suite of four commonly used molecular markers.

METHODS

Taxa Selection

A total of four species of *Amblyomma* found within North America were used in this study: 1) *A. americanum*, 2) *A. maculatum*, 3) *A. mixtum*, and 4) *A. tenellum*. In this study *Dermacentor albipictus* (Packard, 1869) was used as an outgroup. Four commonly available genes were selected as molecular markers: 1) 12S ribosomal DNA (12SrDNA), 2) 16S ribosomal DNA (16SrDNA), 3) cytochrome c oxidase subunit (COI) and 4) internal transcribed spacer 2 (ITS2). These selected genes have been used as markers for DNA barcoding (Lv et al. 2014). All but one of the sequences needed for this study were obtain from GenBank (Table 4). For *A.*

tenellum there was no deposit of the 16SrDNA gene therefore molecular procedures were performed in the laboratory to obtain this sequence.

Table 4. Taxa and genes used in this study with GenBank accession number, sequence size, and locality.

Gene	GenBank Accession #	Sequence Length	Locality
Amblyomma americanum			
12SrDNA	AF150050	340	USA
16SrDNA	L34314	402	Texas, USA
COI	KX360420	658	Florida, USA
ITS2	AF548538	1144	Oklahoma, USA
Amblyomma maculatum			
12SrDNA	KX772751	324	Texas, USA
16SrDNA	KU284933	409	Georgia, USA
COI	KX360379	658	Not Available
ITS2	KU285092	1025	Georgia, USA
Amblyomma mixtum			
12SrDNA	JX987841	347	Texas, USA
16SrDNA	KT820359	405	Ecuador
COI	KY595139	657	Columbia
ITS2	KF527295	809	Texas, USA
Amblyomma tenellum			
12SrDNA	EU791615	338	Not available
16SrDNA	None	334	Texas, USA
COI	KX360351	657	Not available
ITS2	JN866910	819	Not available
Dermacentor albipictus			
12SrDNA	AF150041	337	Not available
16SrDNA	GU968860	402	Alberta, Canada
COI	GU968843	658	Alberta, Canada
ITS2	KP236454	820	Missouri, USA

Abbreviations: 12SrDNA = 12S ribosomal gene; 16SrDNA = 16S ribosomal gene; COI = cytochrome c oxidase gene; ITS2 = internal transcribed spacer 2; and USA = United States of America.

Tick Dissection and DNA Extraction

Six *A. tenellum* female ticks were used for dissection and removal of the salivary glands and midgut while visiting Washington State University, Pullman, Washington, USA. Ticks were placed dorsally on a small rectangular piece of dental wax and covered with phosphate-buffered saline solution. Once placed the ticks were then cut along the bottom of the idiosoma and along the sides creating a flap. This flap was then lifted to expose the internal organs after which the salivary and midgut was removed. To 10 ml of cell lysis buffer 100 µl of proteinase K was added and mixed thoroughly. Afterwards 100 µl of the lysis solution was aliquoted into individual 1.5 ml centrifuge tubes. Into these filled microcentrifuge tubes the dissected salivary glands and midguts were placed separately in the lysis solution and allowed to incubate overnight at 55ºC. Upon the next day 400 µl of cell lysis buffer containing 70 µg/ml of glycogen was added to the incubated tubes and mixed well. Once thoroughly mixed 200 µl of protein precipitation solution was added and vortexed for 10 seconds and then centrifuged for 5 minutes at 14,000 xg. Tubes were then set on ice for 10 minutes and then centrifuged for 5 minutes at 14,000 xg. The tubes were set on ice for another 10 minutes and centrifuged for 3 minutes at 14,000 xg. The supernatant was immediately transferred to a new 1.5 ml centrifuge tube and 500 µl of isopropanol was added and mixed by gently inverting the tube approximately 30 times. After mixing the tubes were placed in a centrifuge set for 5 minuets at 14,000 xg. The isopropanol was gently poured off. The remaining DNA pellet was washed with 500 µl of 70% ethanol and centrifuged for 2 minutes at 14,000 xg. The 70% ethanol was then poured off leaving behind the DNA pellet and allowed to air dry overnight. Upon the next day the DNA pellet was resuspended by adding 50 µl of DNA hydration solution.

Primers and PCR Amplification

For *A. tenellum* the 16SrDNA was not databased within GenBank, therefore this sequence had to be amplified. Tick specific primers used by Black and Piesman (1994) for the 16SrDNA gene were used: 16S+1F (5'-CTGCTCAATGATTTTTTAAATTGCTGTGG-3') and 16S–1R (5'-CCGGTCTGAACTCAGATCAAGT-3'). A PCR master mix for one 32 µl reaction was used containing 24.62 µl of water, 3.20 µl of 10X PCR buffer with MgCl, 0.64 µl of each oligonucleotide primers, 0.64 µl of dNTP mix (10mM), 0.26 µl of FastStart Taq (Sigma-Aldrich), and 2 µl of template DNA. The PCR was performed in a Mastercycler® Personal thermocycler (Eppendorf, Hamburg, Germany) with one initial denaturing cyc1e of 2 minutes at 95ºC, followed by 10 cycles of denaturing for 1 minute at 92ºC, annealing for 1 minute 48ºC, and extension for 1 minute and 30 seconds at 72ºC. After the 10 cycles an additional 32 cycles were run: denaturing for 1 minute at 92ºC, annealing for 25 seconds at 54ºC, and extension for 1 minute and 50 seconds at 72ºC. Lastly a final extension was carried out for 10 minutes at 72ºC. Thermocycler was then set to hold at 4ºC until samples were removed.

Agarose Electrophoresis

One gram of agarose was added to a 300 ml beaker with 100 ml of 1X Tris/Borate/EDTA (TBE) and then swirled to thoroughly mix. Mixture was placed in microwave and heated on high for 30 seconds. After 30 seconds the flask was removed and swirled then placed back in the microwave for 30 seconds more. This process was repeated using 10 seconds of microwaving until the solution became clear. The solution was allowed to cool for about 5 minutes and once cooled 12 µl of SYBR Green 1 dye was added and then thoroughly mixed. The gel mixture was poured into the gel box with casting comb in position and allowed to cool until opaque (~30 min). Once cool the gel was covered with 1X TBE. A piece of parafilm was cut and placed on

counter surface. On this parafilm 1.5 ml of gel loading dye was pipetted into rows with the number of dots corresponding to the number of PCR samples that were loaded into gel. Onto each of these dots 3 µl of PCR product was pipetted mixed by pipetting up and down before loading into individual gel wells. In addition, 10 µl of a ready to use 50 bp DNA step ladder (DirectLoad™) (Sigm-Aldrich) and 5 µl of a ready to use 100 bp DNA step ladder (DirectLoad™) (Sigma-Aldrich) was added to gel wells. The gel was run at 80 volts for approximately 75 minutes then visualized.

Sequencing

A 20 µl sequencing reaction was created with the following components: 2 µl of Big Dye (BigDye Terminator v3.1), 3 µl of Big Buffer (5X), 1 µl of primer, 12 µl of water, and 2 µl of template DNA. The PCR was performed in a Mastercycler® Personal thermocycler (Eppendorf, Hamburg, Germany) with an initial denaturing cycle of 1 minute at 96ºC followed by 26 cycles of denaturing for 10 seconds at 96ºC, annealing for 12 seconds 50ºC, and extension for 4 minutes at 60ºC. Thermocycler was then set to hold at 4ºC until samples were removed. The cleanup of the sequencing reactions utilized the Performa® DTR Gel Filtration Cartridges (EdgeBio). Sequences were sequenced at Washington State University, Pullman, Washington, USA.

Alignment

Sequences were aligned using the widely used phylogenetic alignment program ClustalX (v.2.1) with the multiple alignment mode selected (Larkin 2007). This program utilizes the progressive method for multiple sequence alignment allowing for more sequences to be analyzed. The generalized steps of the program process involve: preforming a pair-wise alignment for all sequences then applying alignment scores in order to produce an approximate phylogenetic tree by neighbor-joining which then guides the multiple sequence alignment

process. The aligned sequences were then imported into Mesquite (v.3.51) (Maddison and Maddison 2018) and saved as a nexus file (.nex), a common input file for phylogenetic programs.

Phylogenetic analysis

The homogeneity of base frequencies among each of the four genes was evaluated using a chi-square goodness-of-fit test in PAUP* (v.4.0b10) (Swofford 2000). Gaps were treated as missing for all phylogenetic analyses. Each of the individual genes were analyzed separately. In addition to the separate genes two concatenated data sets were created using the program SequenceMatrix (v.1.8) (Vaidya et al. 2011): 1) mtDNA which included the 12SrDNA, 16SrDNA, and the COI genes, 2) mtDNA+ITS2. A total of three phylogenetic methods involving maximum parsimony, maximum likelihood, and Bayesian were conducted with the previous data sets.

The first phylogenetic analysis utilized the method of maximum parsimony (MP) which evaluates the simplest tree with fewest evolutionary changes that are required by the inputted data. Because of the number of taxa being less than 11, an exhaustive search was able to be run in PAUP* (Swofford and Sullivan 2009). The more taxa in an analysis the slower the runs will be and often times require a different type of heuristic search such as tree bisection-reconnection (Swofford and Sullivan 2009). Bootstrapping analyses of 1,000 replicates were performed in order to determine branch support for the MP analyses. The MP trees and all subsequent trees from analyses were visualized and rooted to the outgroup (*D. albipictus*) using the program FigTree (v.1.4.0) (Rambaut 2012). Three tree support measures were taken for each of the parsimony analyses. The first was the consistency index (CI) which is a measure of the amount of homoplasy on a tree which can be calculated by $CI = 100 *$

$minimum\ number\ of\ changes/tree\ length$. The CI ranges from one to zero where a value of 1 means no homoplasy. The second tree support measure is the retention index (RI) which is the proportion of taxa whose states do not evolve more than once and is calculated by $RI = 100 * (\max changes - tree\ length)/(\max changes - \min changes)$. The last measure of tree support was the homoplasy index (HI) which can be calculated by $HI = 1 - CI$.

Before continuing on with the other methods of phylogenetic analyses, each of the individual genes and the concatenated data sets were evaluated upon the best fit of various nucleotide substitution models using jModelTest v.2.1.7 (Darriba et al. 2012). A total of 88 substitution schemes were evaluated by using an optimized maximum likelihood base tree for likelihood calculations and by performing a base tree search using the best fit of either nearest-neighbor-interchange (NNI) or subtree-pruning-regrafting (SPR). The corrected Akaike information criterion (AICc) was used as an estimator of the best fitting model of nucleotide substitution. For each of these best fitted models a PUAP* block of line code was written by jModelTest with all parameters needed to perform further maximum likelihood and Bayesian phylogenetic analyses.

The second phylogenetic analysis was that of maximum likelihood which is different than that of maximum parsimony by requiring an explicit model of sequence evolution and a hypothesis is formulated (Schmidt and von Haeseler 2009). The hypothesis with the higher probability of giving rise to the observed data (DNA sequences) is the preferred one aka the higher the likelihood. Formulated hypotheses typically include different tree topologies, the branch lengths, and the set parameters of the nucleotide substitution model (Schmidt and von Haeseler 2009). An exhaustive search was run in PAUP* to find the most likely tree with parameters set for each individual model of nucleotide substitution specific for each of the data

sets. Assessment of branch support was accomplished by bootstrapping with 1,000 replicas. Upon bootstrapping a 50% majority-rule consensus tree was conducted.

The final phylogenetic analysis utilized Bayesian inference which was implemented using MrBayes v.3.2.6 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). Bayesian inference of phylogenies is closely related to that of maximum likelihood methods but differs in using a prior, generally tree topologies. This prior is updated to a posterior probability distribution by using data (observations) in terms of DNA sequences and some model of nucleotide substitution (Ronquist et al. 2009). The posterior probability distribution typically cannot be calculated analytically; to overcome this is to estimate the posterior probability distribution using Markov chain Monte Carlo (MCMC) sampling (Ronquist et al. 2009). The ultimate goal of MCMC is to move randomly in tree and parameter space so that it will settle down and converge into an equilibrium distribution of trees and parameter values. The "top model" of nucleotide substitution for each of the genes and concatenated genes datasets that was selected by jModelTest was incorporated in the analyses. A total of two runs with each of the runs having four chains were concurrently run for Bayesian analyses with 10,000 generations with a sampling frequency of 10 iterations. A burn-in was used to discard the first 25% of the samples totaling 250 samples removed. Lastly a 50% majority-rule consensus tree was conducted and for each tree branch posterior probabilities were recorded and labeled.

RESULTS

Mitochondrial genes

12S ribosomal DNA (12SrDNA).—The following mean base compositions across all taxa were: adenine (A) 41.52% $\pm$ 0.47 standard deviation (SD), cytosine (C) 9.19% $\pm$ 1.04 SD, guanine (G) 14% $\pm$ 0.62 SD, and thymine (T) 35.29% $\pm$ 0.48 SD. Overall mean number for

base pairs (bp) for this gene was 337.2 bp $\pm$ 8.35 SD. For the 12SrDNA gene there was no significant difference in the chi-square test of homogeneity of base frequencies across all five taxa (χ^2 = 2.1, *df* = 12, *p* = 1).

Alignment of the 12SrDNA sequences of the five taxa resulted in a total of 363 characters. The first phylogenetic analysis of maximum parsimony contained 32 parsimony-informative characters. An exhaustive search evaluated a total of 15 trees retaining only one with the best tree length score of 135 (CI = 0.90, RI = 0.56, HI = 0.10). The most parsimonious tree for the 12SrDNA gene is shown in Figure 6. All *Amblyomma* species are clustered in a monophyletic clade with *D. albipictus* as the outgroup. A bootstrap analysis revealed a 93.6% of branch support for the terminal node containing *A. americanum* and *A. tenellum* and a branch support of 84.93% for the node containing these two species with *A. mixtum*.

For the maximum likelihood analysis of the 12SrDNA gene, a model was selected by using the lowest corrected Akaike information criterion score (AICc) in jModelTest. The selected model was the transversion model with a gamma distribution (TVM + G) (Table 5). A log-likelihood score computed for the resulting tree was 1022.99. Overall the topology and the branch support using bootstrapping were similar to that of the parsimony analysis (Figure 7). The TVM+G model, the same that was used for the maximum likelihood, was used for the Bayesian analysis. The log-likelihood score from the Bayesian reconstructed tree was very similar to that of the maximum likelihood analysis (1025.46). The topology of the tree was identical to that of the parsimony and maximum likelihood analysis but differed in their branch support by having higher values (Figure 8).

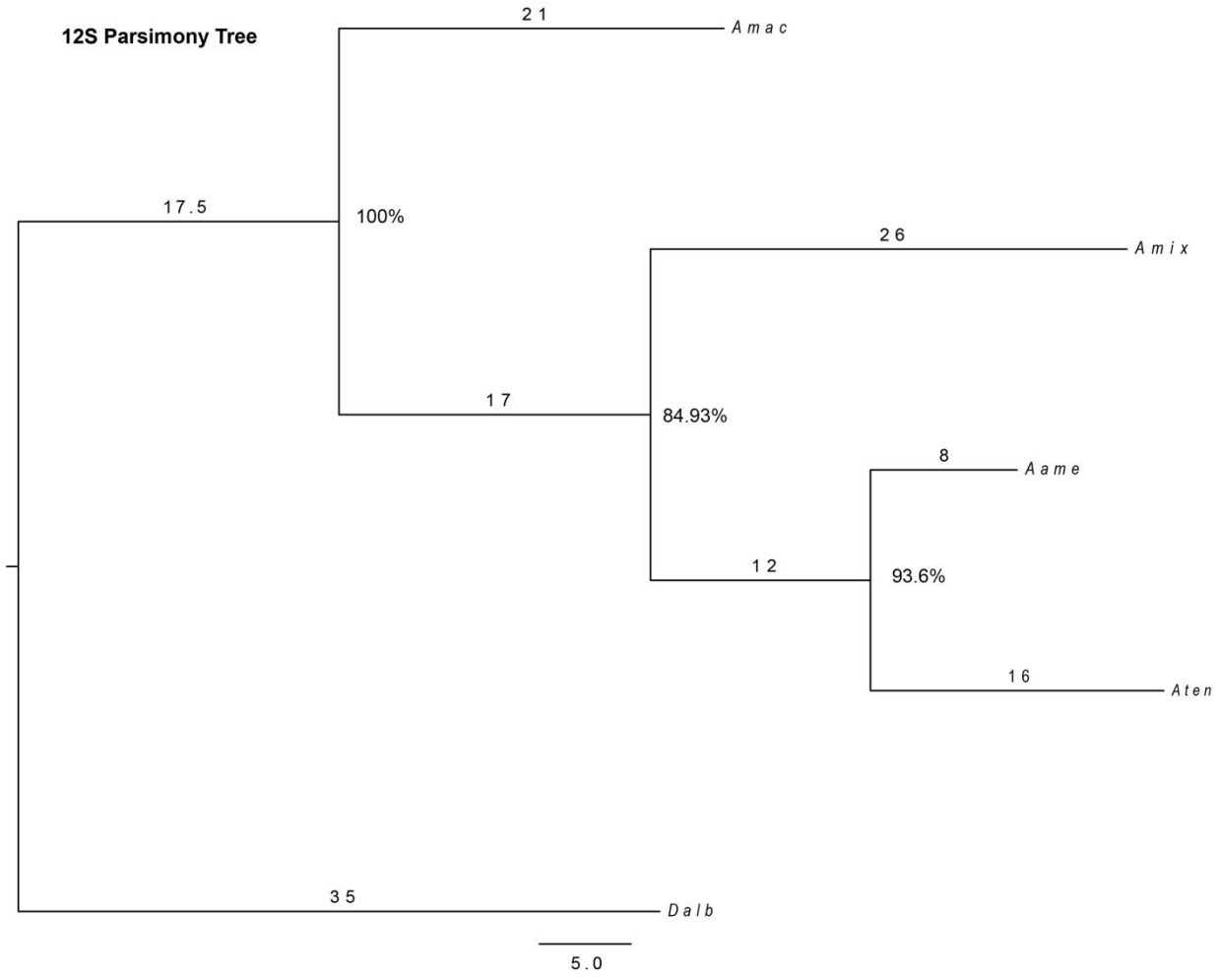

Figure 6. 12S ribosomal gene (12SrDNA) maximum parsimony tree. Species names are abbreviated: *A. ame = Amblyomma americanum*, *A. mac = Amblyomma maculatum*, *A. mix = A. mixtum*, *A. ten = Amblyomma tenellum*; and *D. alb = Dermacentor albipictus*. Branch lengths are shown above each branch represent the number of expected substitutions per site and branch support by bootstrapping is shown by the percentage value next to each tree node.

Table 5. Substitution models for maximum likelihood and Bayesian analyses.

Gene	Model	A>C	A>G	A>T	C>G	C>T	G>T	*α*	I	%A	%C	%G	%T
12SrDNA	TVM+G	0.31	6.04	3.45	0.28	6.04	1	0.47	0	39.8	10.4	13.4	36.4
16SrDNA	TVM+G	2683.22	7883.54	7303.87	0.49	7883.54	1	0.32	0	39.4	9.4	15	36.2
COI	GTR+I	1	1532.6	1727.01	440.60	4747.67	1	Equal	0.63	30.6	17	14.4	38
ITS2	GTR+G	0.80	1.74	1.32	0.48	3.18	1	2.64	0	20.2	28.3	34.9	16.6
mtDNA	GTR+G	8.74	100.22	88.85	15.12	186.51	1	0.24	0	35.8	12.8	14.3	37.1
mtDNA+ITS2	GTR+I	1.09	3.65	4.25	1.88	5.86	1	Equal	0.43	28.7	19.8	23.4	28.1

Abbreviations: 12SrDNA = 12S ribosomal gene; 16SrDNA = 16S ribosomal gene; COI = cytochrome c oxidase gene; ITS2 = internal transcribed spacer 2; mtDNA = concatenated mitochondrial genes; TVM + G = transversion model + gamma distribution; TVM+I = transversion model + invariable sites; GTR + I = general time reversal mode = invariable; GTR + G = general time reversal mode + gamma distribution; $x > y$ = represents mutations from nucleotide x to nucleotide y; α = shape of gamma distribution; and base frequencies in percent: A = adenine; C = cytosine; G = guanine; and T = thymine.

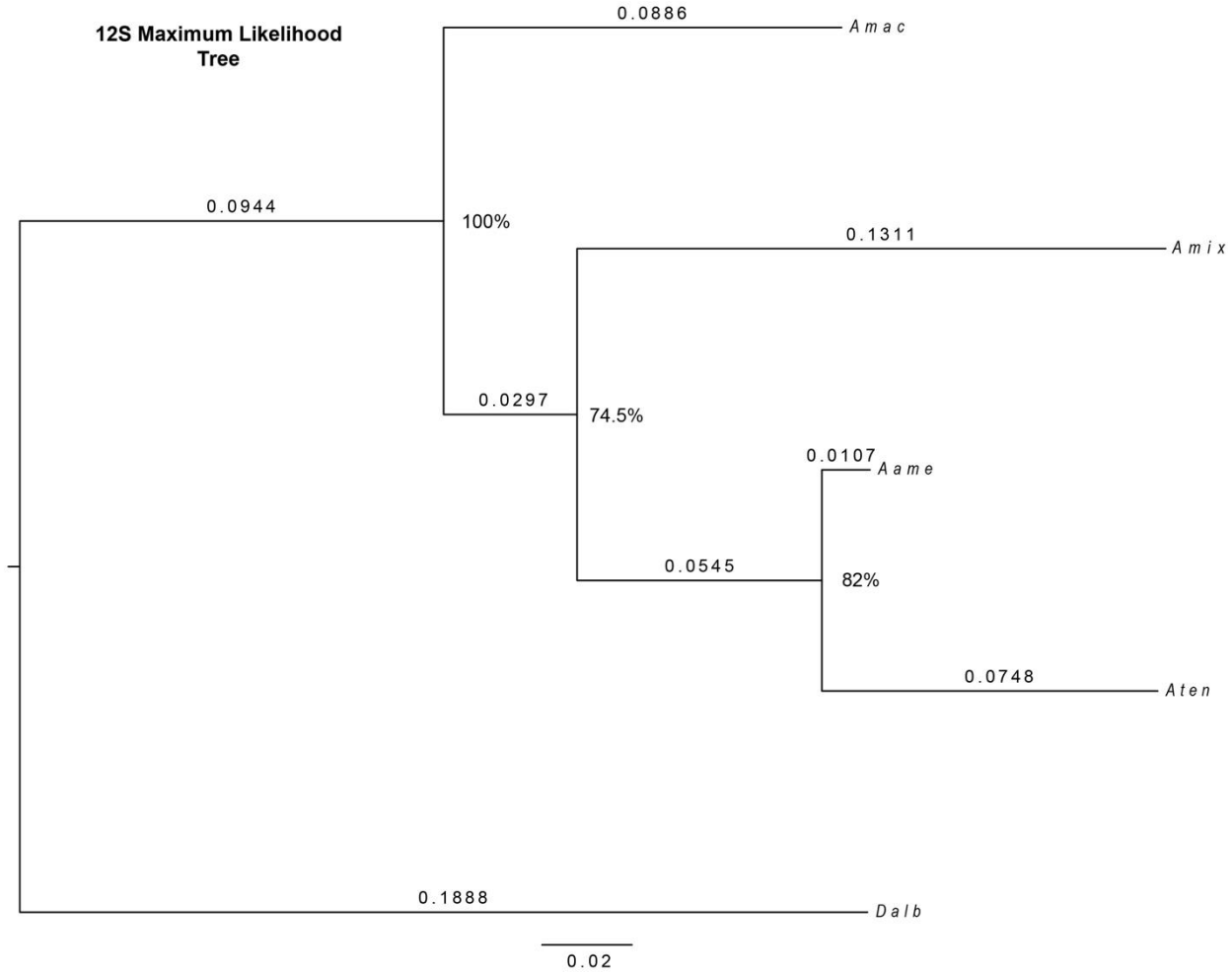

Figure 7. 12S ribosomal gene (12SrDNA) maximum likelihood tree. Species names are abbreviated: *A. ame = Amblyomma americanum*, *A. mac = Amblyomma maculatum*, *A. mix = A. mixtum*, *A. ten = Amblyomma tenellum*; and *D. alb = Dermacentor albipictus*. Branch lengths are shown above each branch represent the number of expected substitutions per site and branch support by bootstrapping is shown by the percentage value next to each tree node.

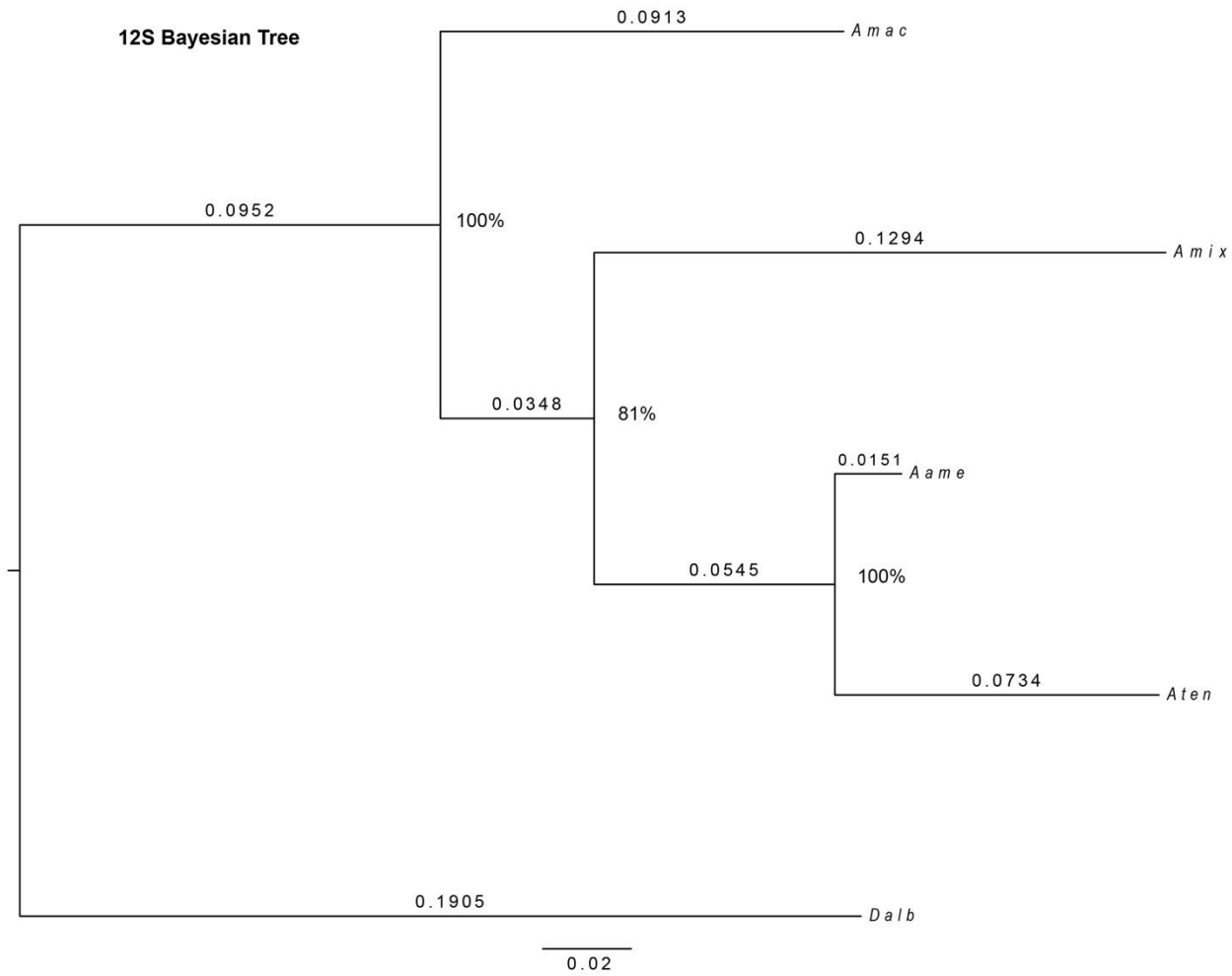

Figure 8. 12S ribosomal gene (12SrDNA) Bayesian tree. Species names are abbreviated: *A. ame = Amblyomma americanum, A. mac = Amblyomma maculatum, A. mix = A. mixtum, A. ten = Amblyomma tenellum*; and *D. alb = Dermacentor albipictus*. Branch lengths are shown above each branch represent the number of expected substitutions per site and branch support by the posterior probabilities shown by the percentage value next to each tree node.

16S ribosomal DNA (16SrDNA).—The following mean base compositions across all taxa were: 40.47% $\pm$ 1.24 SD for A, 8.86% $\pm$ 0.78 SD for C, 14.78% $\pm$ 0.93 SD for G, and 35.89% $\pm$ 1.01 SD for T. Overall mean number for base pairs (bp) for this gene was 401.8 bp (*SD* = 44.52). For the 16SrDNA gene there was no significant difference in the chi-square test of homogeneity of base frequencies across all five taxa (χ^2 = 3.18, *df* = 12, *p* = 0.99).

Alignment of the 16SrDNA sequences of the five taxa resulted in a total of 462 characters. The first phylogenetic analysis of maximum parsimony contained 38 parsimony-informative characters. An exhaustive search evaluated a total of 15 trees retaining only one with the best tree length score of 172 (CI = 0.88, RI = 0.45, HI = 0.12). The 16SrDNA parsimony analysis was similar in topology of the 12SrDNA parsimony with a monophyletic grouping of the *Amblyomma* but exhibited lower branch support with bootstrapping (Figure 9). The terminal taxa containing *A. americanum* and *A. tenellum* had a branch support value of 55% while these two with *A. mixtum* had a support of 73%.

The model with the lowest AICc score for the maximum likelihood analysis of the 16SrDNA gene was the TVM+G (same as the 12SrDNA) (Table 5). The computed log-likelihood score for the resulting maximum likelihood tree was 1284.34. Overall the topology and the branch support using bootstrapping was very similar to that of the parsimony analysis (Figure 10).

The TVM+G model of the 16SrDNA, the same that was used for the maximum likelihood, was used for the Bayesian analysis. The computed log-likelihood for the Bayesian reconstructed tree was very similar to that of the maximum likelihood analysis (1287.16). The topology of the tree was identical to that of the parsimony and maximum likelihood analysis but differed in their branch support by having higher values (Figure 11).

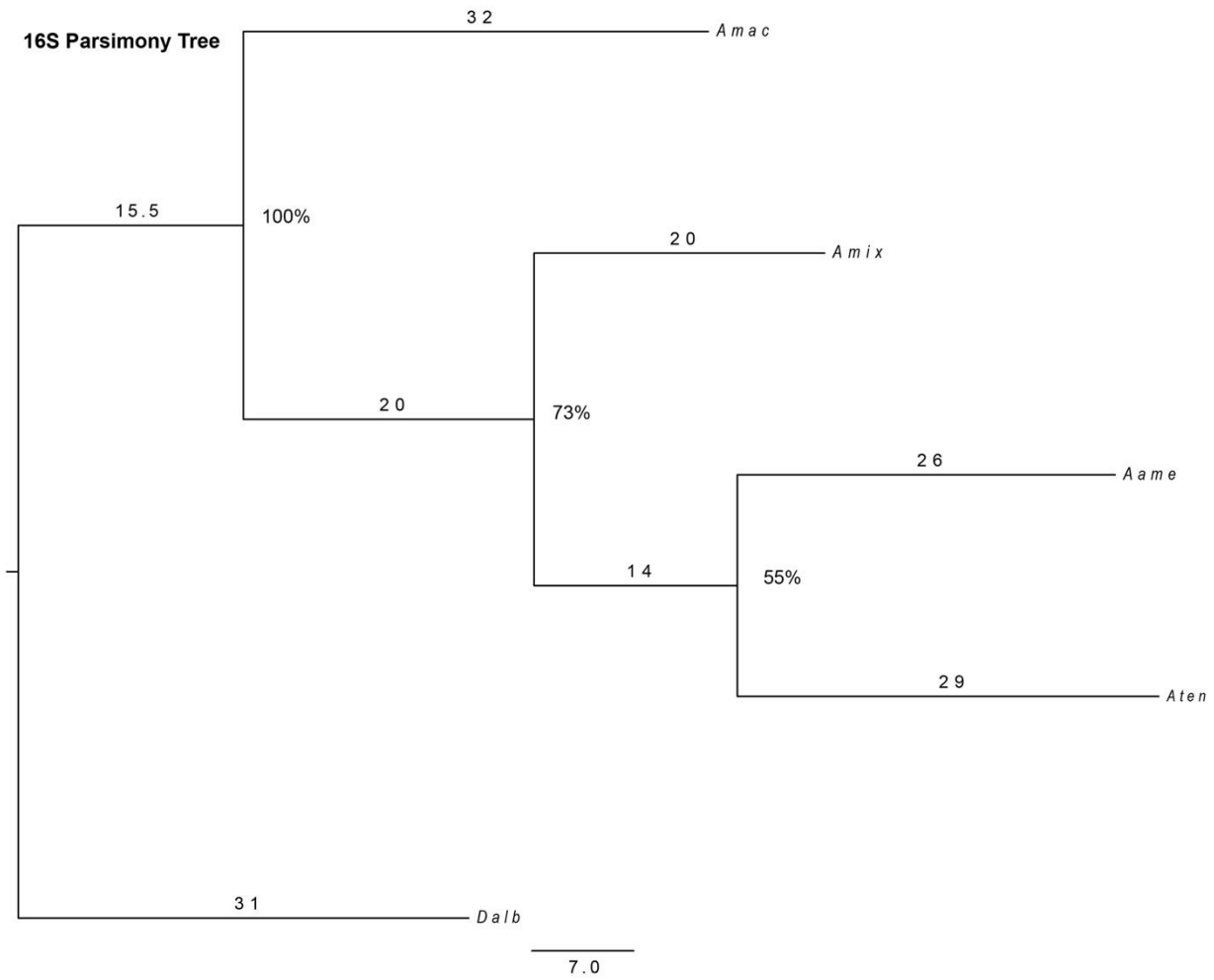

Figure 9. 16S ribosomal gene (16SrDNA) maximum parsimony tree. Species names are abbreviated: *A. ame = Amblyomma americanum*, *A. mac = Amblyomma maculatum*, *A. mix = A. mixtum*, *A. ten = Amblyomma tenellum*; and *D. alb = Dermacentor albipictus*. Branch lengths are shown above each branch represent the number of expected substitutions per site and branch support by bootstrapping is shown by the percentage value next to each tree node.

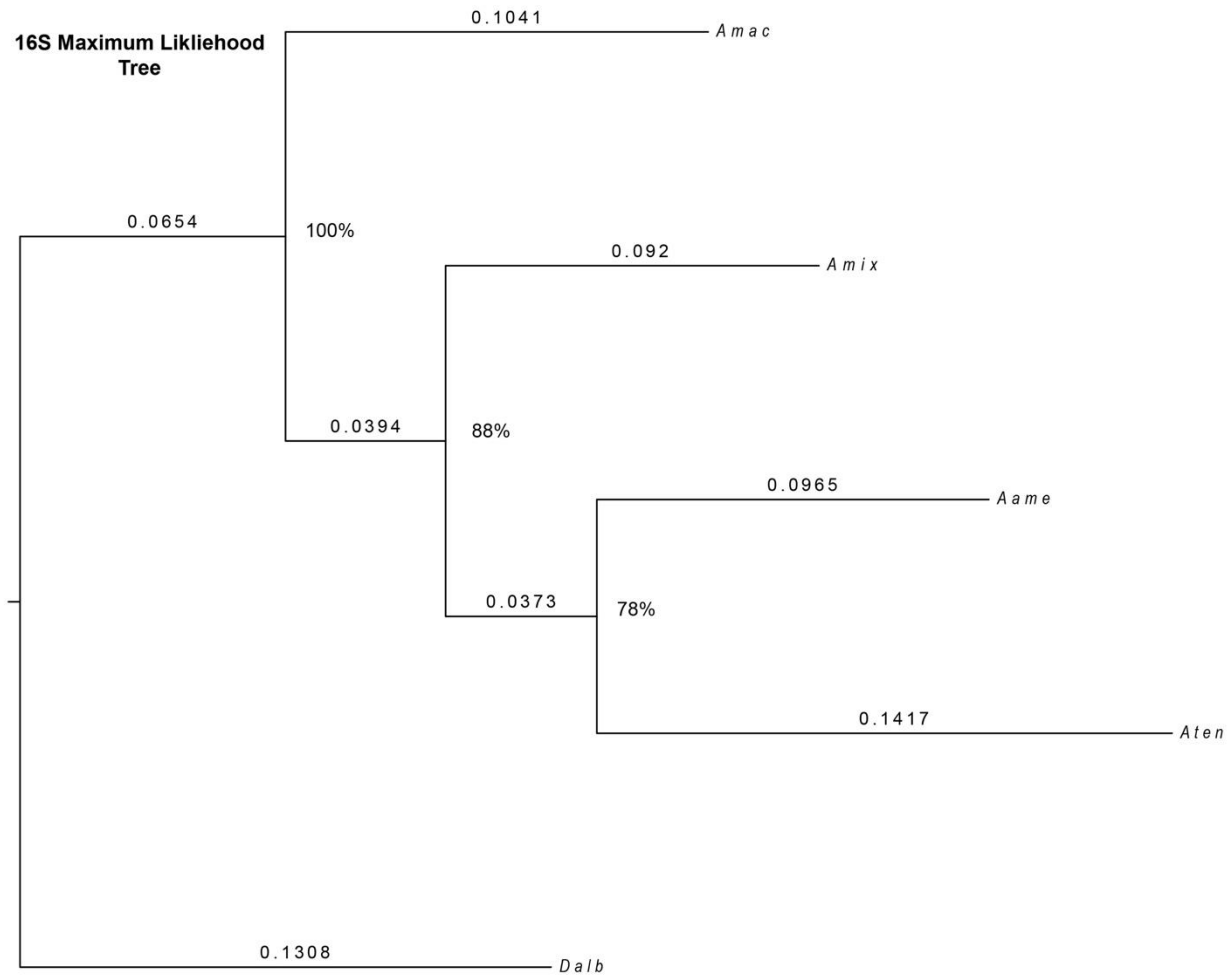

Figure 10. 16S ribosomal gene (16SrDNA) maximum likelihood Tree. Species names are abbreviated: *A. ame = Amblyomma americanum*, *A. mac = Amblyomma maculatum*, *A. mix = A. mixtum*, *A. ten = Amblyomma tenellum*; and *D. alb = Dermacentor albipictus*. Branch lengths are shown above each branch represent the number of expected substitutions per site and branch support by bootstrapping is shown by the percentage value next to each tree node.

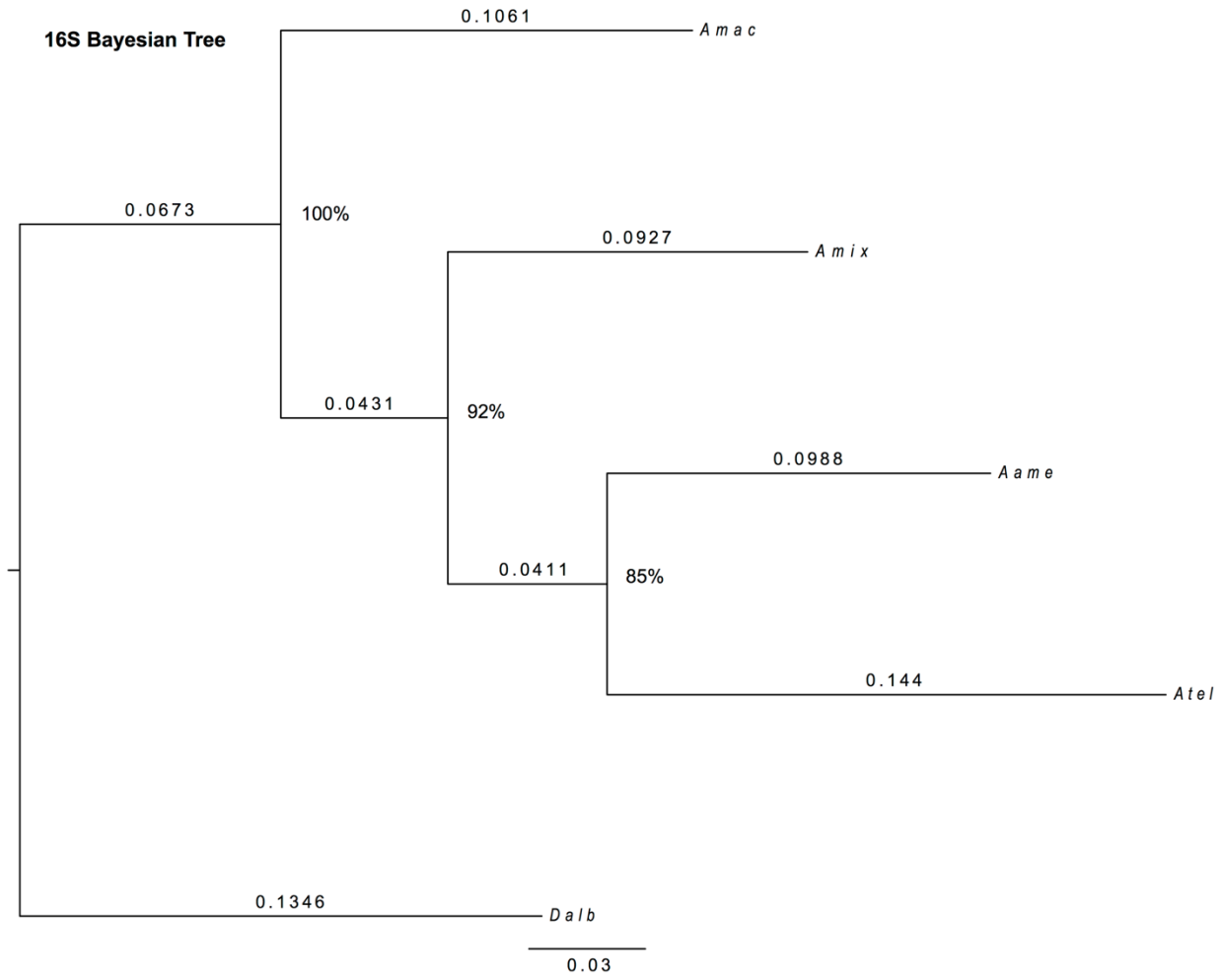

Figure 11. 16S ribosomal gene (16SrDNA) Bayesian tree. Species names are abbreviated: *A. ame = Amblyommaamericanum*, *A. mac = Amblyomma maculatum*, *A. mix = A. mixtum*, *A. ten = Amblyomma tenellum*; and *D. alb = Dermacentor albipictus*. Branch lengths are shown above each branch represent the number of expected substitutions per site and branch support by the posterior probabilities shown by the percentage value next to each tree node.

Cytochrome C Oxidase Subunit (COI).—The following mean base compositions across all taxa were: 29.33% $\pm$ 0.94 SD for A, 17.73% $\pm$ 1.11 SD for C, 14.68% $\pm$ 0.67 SD for G, and 38.26% $\pm$ 0.66 SD for T. Overall mean number for base pairs (bp) for this gene was 657.6 bp (*SD* = 0.55). For the COI gene there was no significant difference in the chi-square test of homogeneity of base frequencies across all five taxa (χ^2 = 3.71, *df* = 12, *p* = 0.99).

Alignment of the COI sequences of the five taxa resulted in a total of 661 characters. The first phylogenetic analysis of maximum parsimony contained 82 parsimony-informative characters. An exhaustive search evaluated a total of 15 trees retaining only one with the best tree length score of 320 (CI = 0.82, RI = 0.29, HI = 0.18). The tree topology based on parsimony of the COI gene did reveal a monophyletic group with the *Amblyomma* but location of taxa upon this tree differed from that of all other genes using parsimony (Figure 12). Differences in topology included: 1) grouping *A. maculatum* with *A. tenellum* instead *A. americanum* and *A. tenellum*, 2) *A. mixtum* being the less similar while others tree showed and *A. maculatum* to be the least similar among the *Amblyomma*. Additionally, the relationships of taxa showed poor resolution with very low bootstrapping values of branch support.

For the COI maximum likelihood analysis, the general time reversal with a proportion of invariable sites (GTR+I) was chosen based on the AICc score (Table 5). A log-likelihood score computed for the resulting tree was 2118.63. The topology was similar to that of the parsimony analysis but with higher branch support (Figure 13). Compared to all other genes the topology was not similar showing different taxa relationships.

For the Bayesian analysis of the COI gene the GTR+I model was used (Table 5). The likelihood score computed from the Bayesian reconstructed tree was similar to that of (2221.79).

Both the Bayesian and maximum likelihood showed identical topologies but differed in the Bayesian analysis having higher branch support (Figure 14).

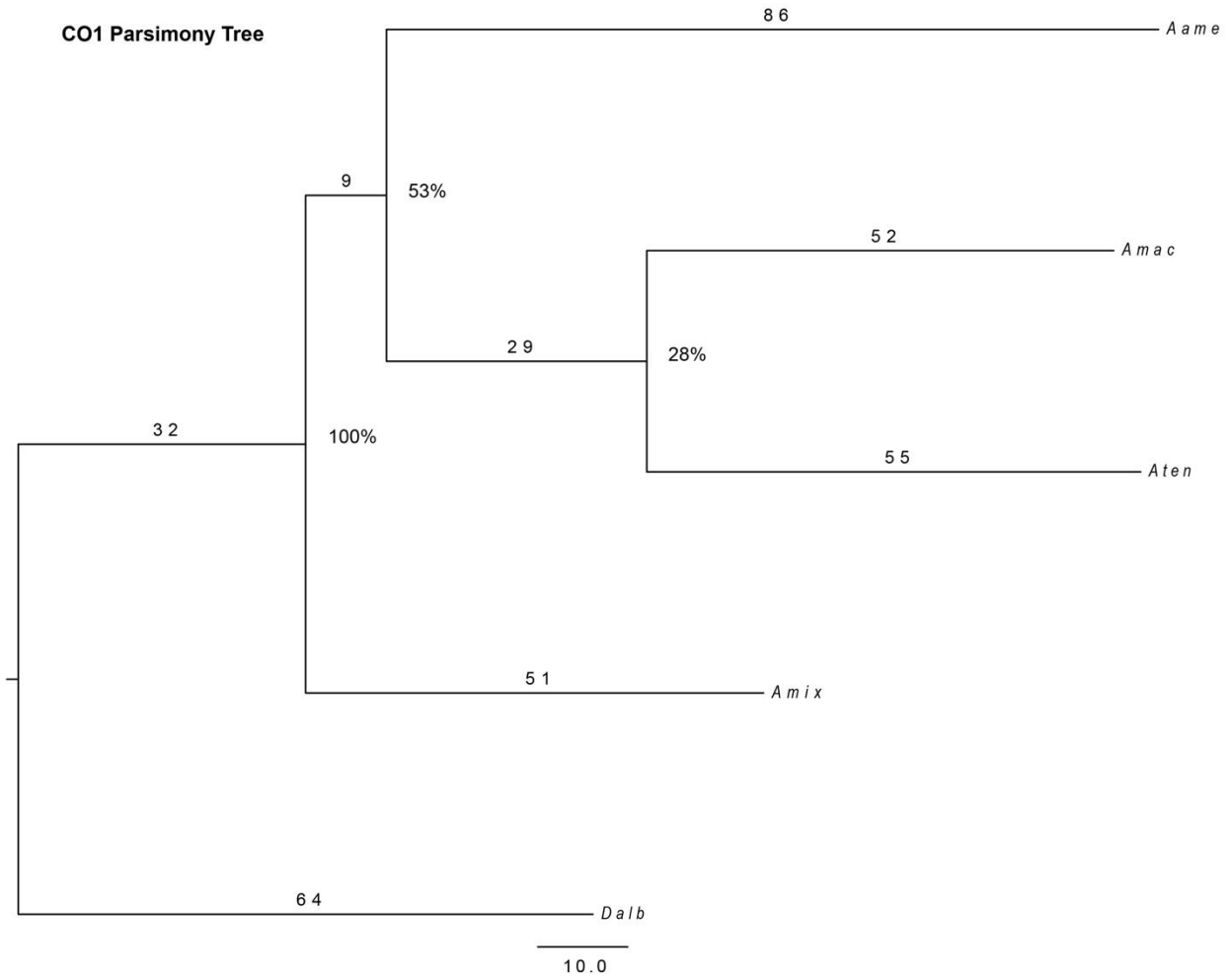

Figure 12. Cytochrome c oxidase gene (COI) maximum parsimony tree. Species names are abbreviated: *A. ame* = *Amblyomma americanum*, *A. mac* = *Amblyomma maculatum*, *A. mix* = *A. mixtum*, *A. ten* = *Amblyomma tenellum*; and *D. alb* = *Dermacentor albipictus*. Branch lengths are shown above each branch represent the number of expected substitutions per site and branch support by bootstrapping is shown by the percentage value next to each tree node.

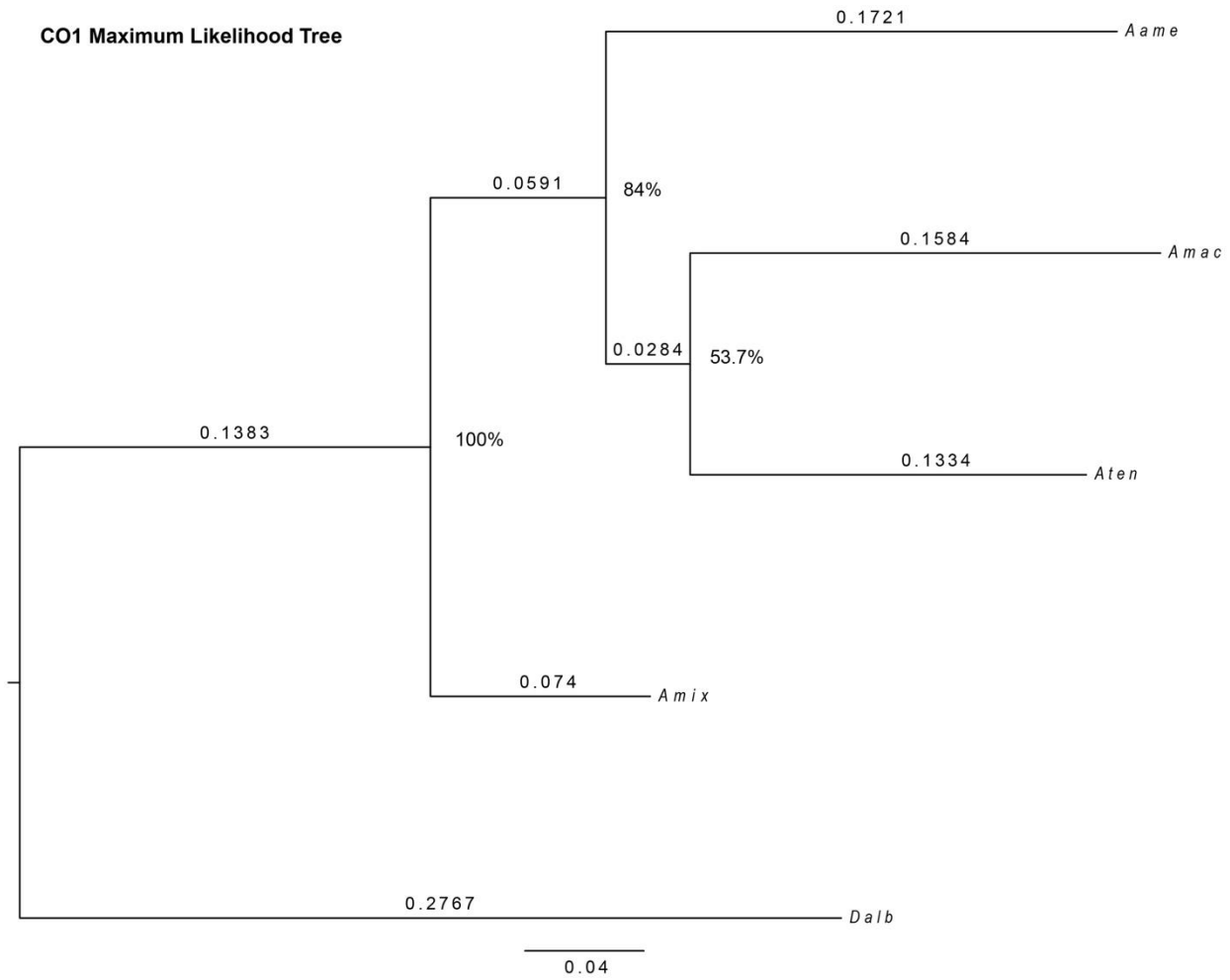

Figure 13. Cytochrome c oxidase gene (COI) maximum likelihood tree. Species names are abbreviated: *A. ame* = *Amblyomma americanum*, *A. mac* = *Amblyomma maculatum*, *A. mix* = *A. mixtum*, *A. ten* = *Amblyomma tenellum*; and *D. alb* = *Dermacentor albipictus*. Branch lengths are shown above each branch represent the number of expected substitutions per site and branch support by bootstrapping is shown by the percentage value next to each tree node.

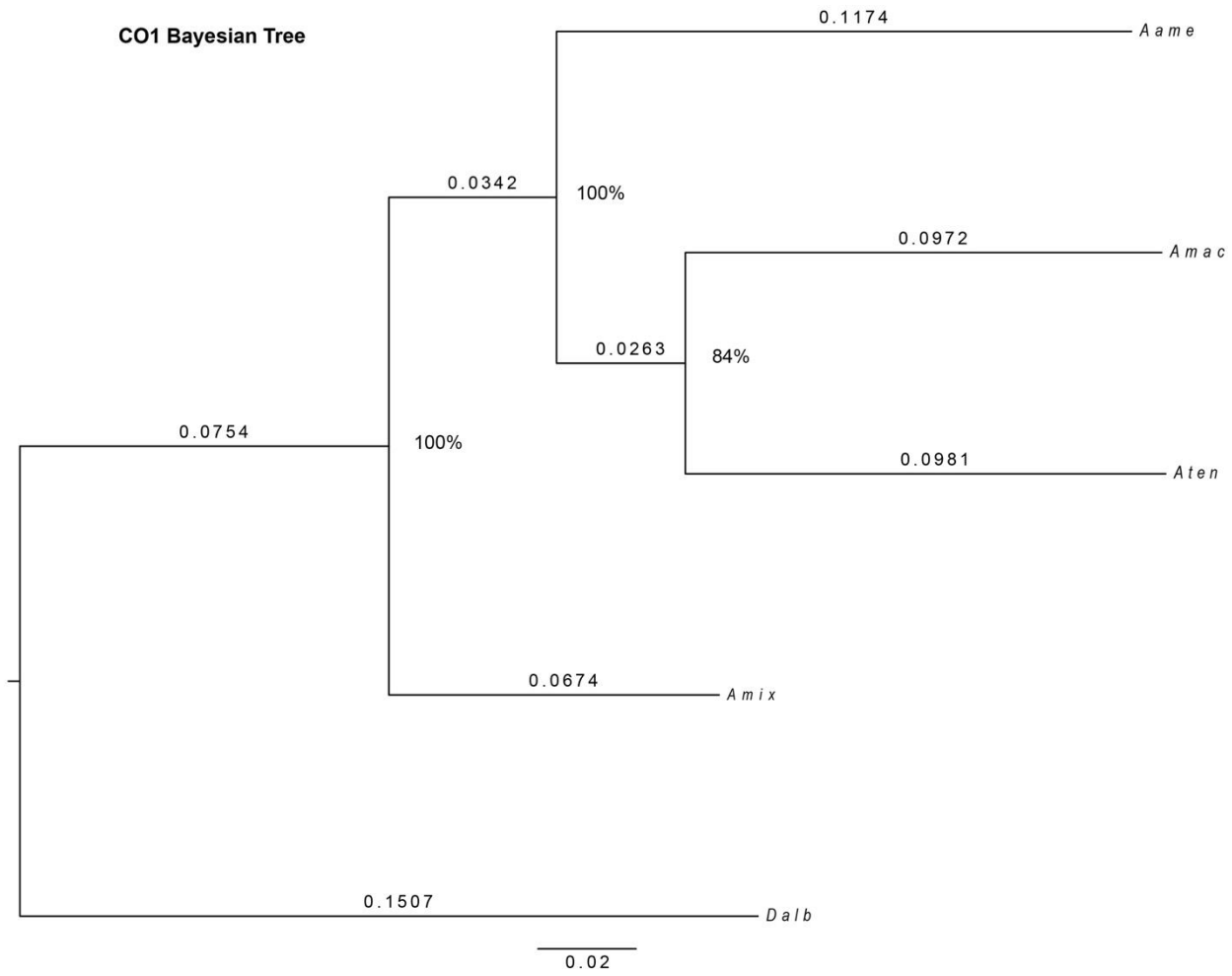

Figure 14. Cytochrome c oxidase gene (COI) Bayesian tree. Species names are abbreviated: *A. ame = Amblyomma americanum, A. mac = Amblyomma maculatum, A. mix = A. mixtum, A. ten = Amblyomma tenellum*; and *D. alb = Dermacentor albipictus*. Branch lengths are shown above each branch represent the number of expected substitutions per site and branch support by the posterior probabilities shown by the percentage value next to each tree node.

Nuclear gene

Internal transcribed spacer 2 (ITS2).—The following mean base compositions across all taxa were: 19.21% $\pm$ 0.94 SD for A, 28.86% $\pm$ 0.84 SD for C, 36.6% $\pm$ 2.14 SD for G, and 15.33% $\pm$ 2.36 SD for T. Overall mean number for base pairs (bp) for this gene was 657.6 bp (*SD* = 0.55). For the ITS2 gene there were no significant differences in the chi-square test of homogeneity of base frequencies across all five taxa (χ^2 = 19.92, p = 0.07).

Alignment of the ITS2 sequences of the five taxa resulted in a total of 1157 characters. The first phylogenetic analysis of maximum parsimony contained 81 parsimony-informative characters. An exhaustive search evaluated a total of 15 trees retaining only one with the best tree length score of 617 (CI = 0.97, RI = 0.75, HI = 0.03). The ITS2 gene parsimony reconstruction revealed the same topology as those of both 12SrDNA and 16SrDNA, but differed from the COI (Figure 15). Branch supports using bootstrapping were very high.

The model with the lowest AICc score for the maximum likelihood analysis of the ITS2 gene was the TVM+G (same as the 12SrDNA) (Table 5). The computed log-likelihood score for the resulting maximum likelihood tree was 3706.88. Overall the topology and the branch support using bootstrapping was very similar to that of the parsimony analysis (Figure 16).

For the Bayesian analysis of the ITS2 gene the GTR+G model was used, the same one used in the maximum likelihood analysis (Table 5). The likelihood score computed form the Bayesian reconstructed tree was similar to that of (3712.45). Both the Bayesian and maximum showed identical topologies but differed in the Bayesian analysis having higher branch support (Figure 17).

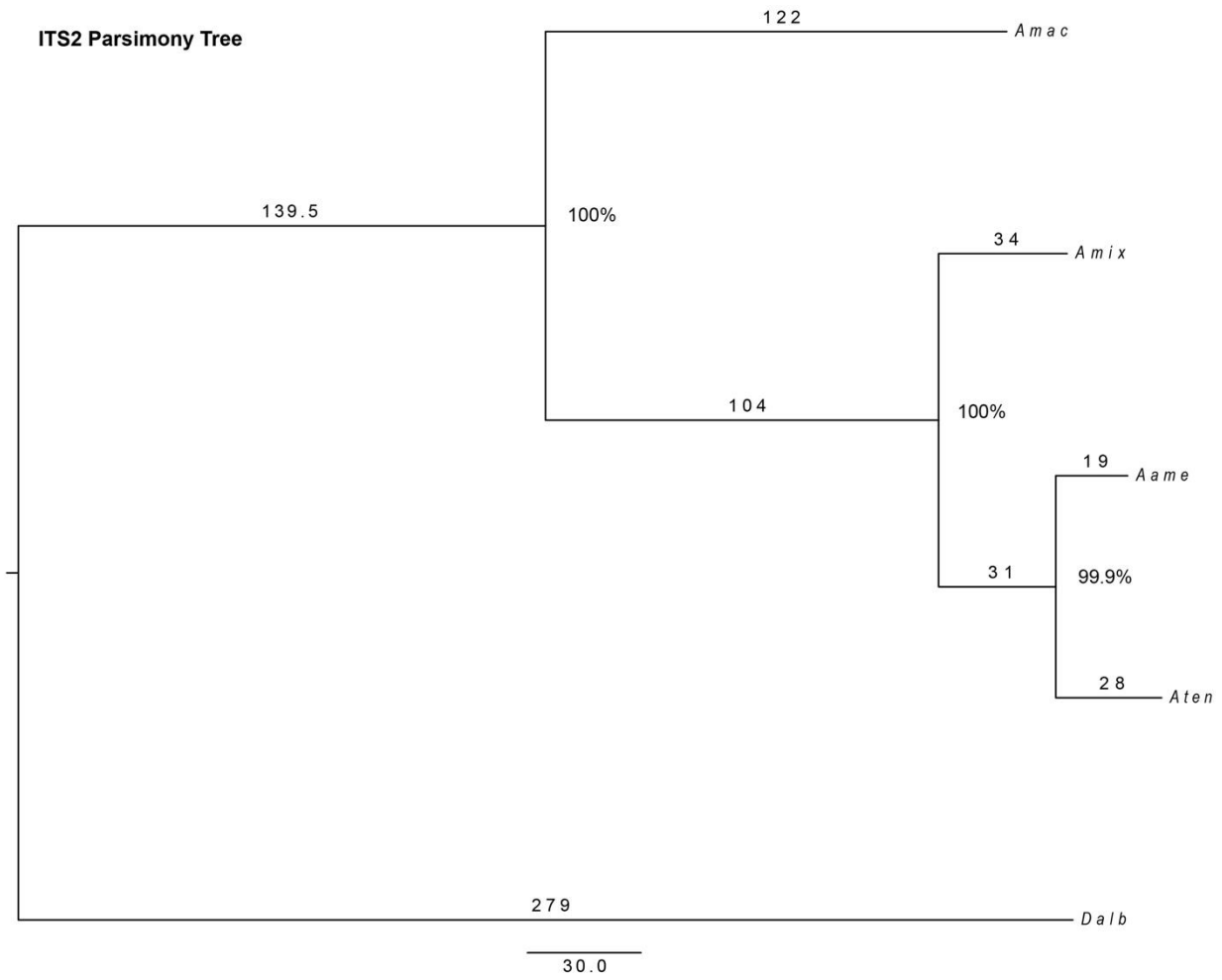

Figure 15. Internal transcribed spacer 2 gene (ITS2) maximum parsimony tree. Species names are abbreviated: *A. ame* = *Amblyomma americanum*, *A. mac* = *Amblyomma maculatum*, *A. mix* = *A. mixtum*, *A. ten* = *Amblyomma tenellum*; and *D. alb* = *Dermacentor albipictus*. Branch lengths are shown above each branch represent the number of expected substitutions per site and branch support by bootstrapping is shown by the percentage value next to each tree node.

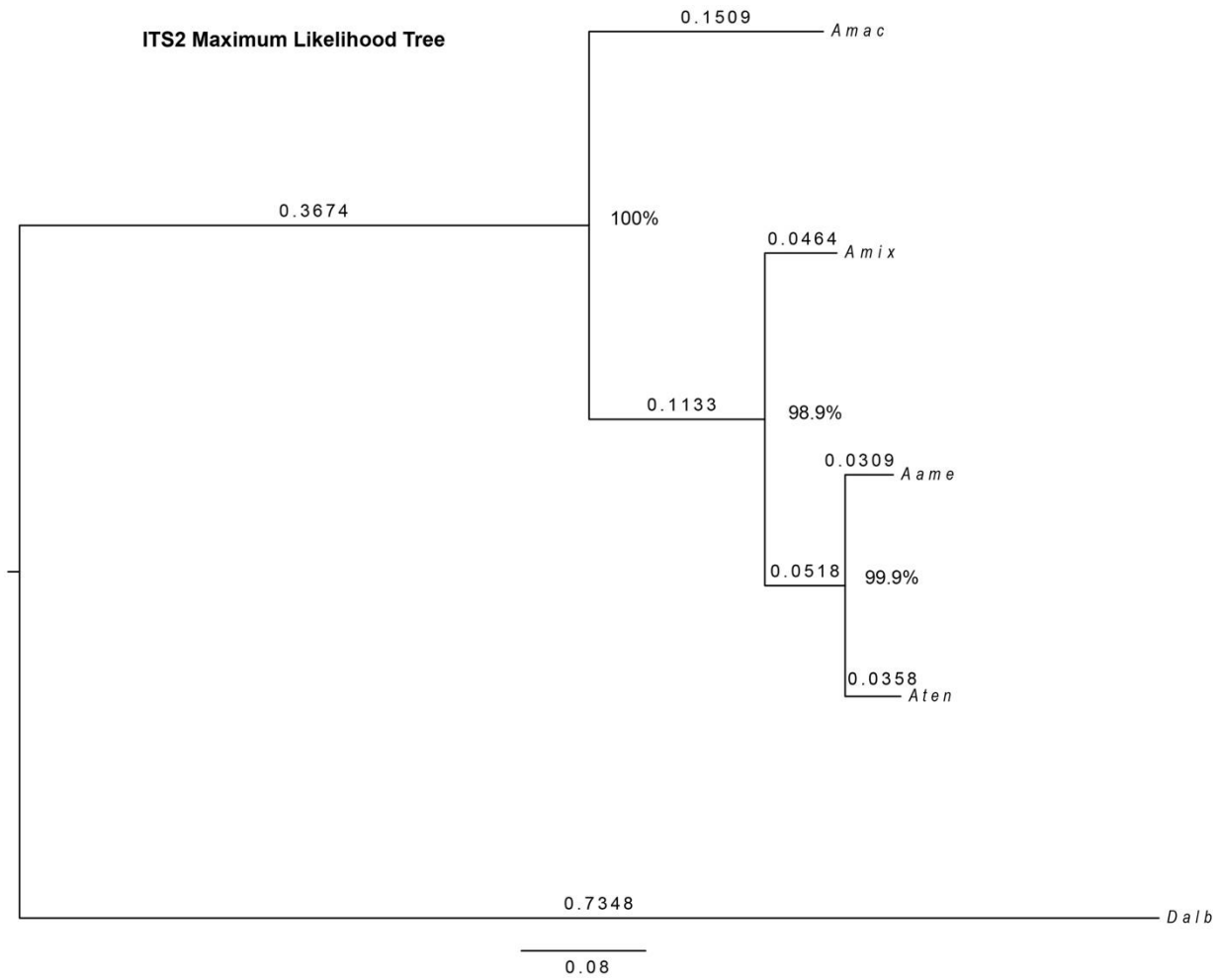

Figure 16. Internal transcribed spacer 2 gene (ITS2) maximum likelihood tree. Species names are abbreviated: *A. ame = Amblyomma americanum*, *A. mac = Amblyomma maculatum*, *A. mix = A. mixtum*, *A. ten = Amblyomma tenellum*; and *D. alb = Dermacentor albipictus*. Branch lengths are shown above each branch represent the number of expected substitutions per site and branch support by bootstrapping is shown by the percentage value next to each tree node.

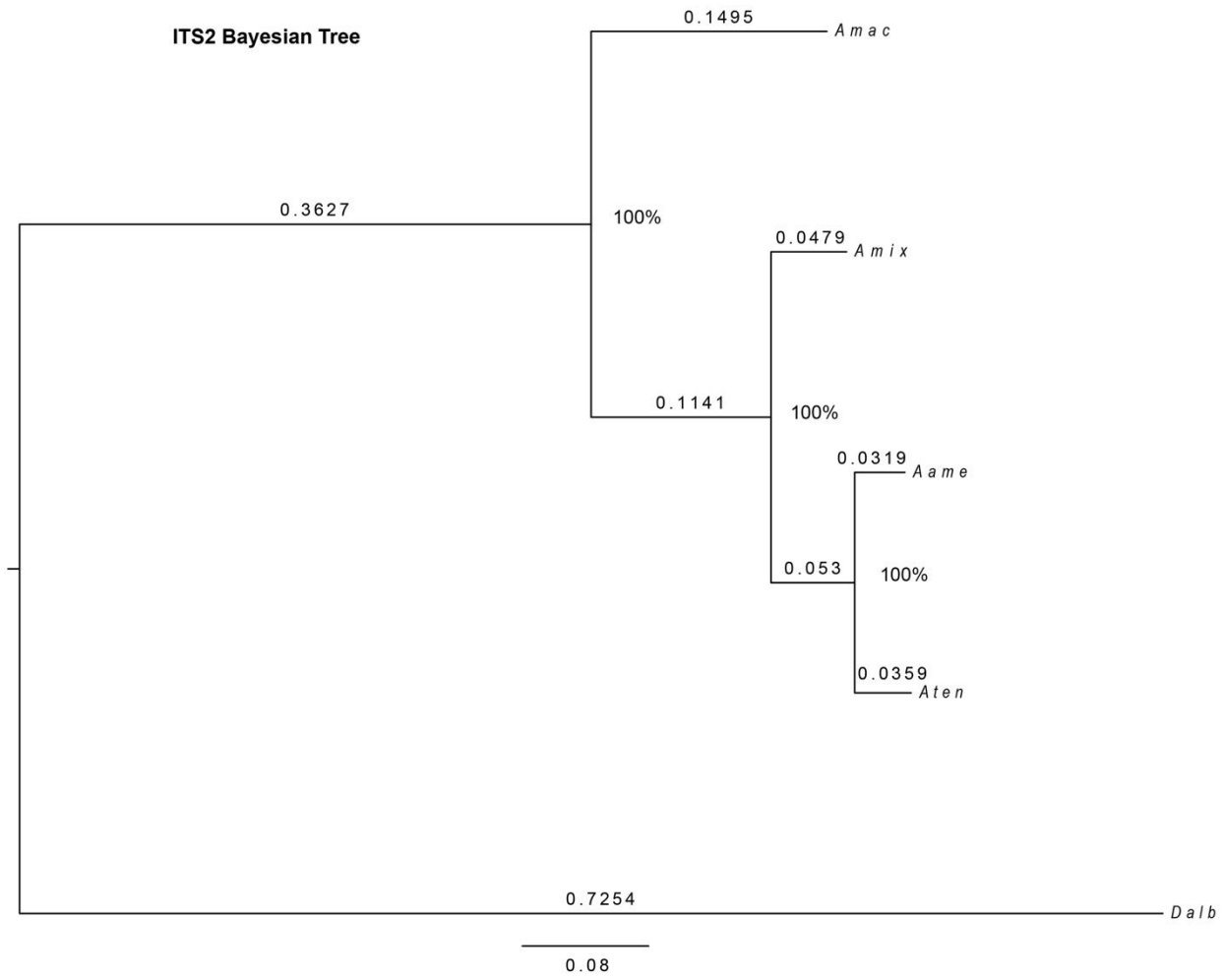

Figure 17. Internal transcribed spacer 2 gene (ITS2) Bayesian tree. Species names are abbreviated: *A. ame = Amblyomma americanum*, *A. mac = Amblyomma maculatum*, *A. mix = A. mixtum*, *A. ten = Amblyomma tenellum*; and *D. alb = Dermacentor albipictus*. Branch lengths are shown above each branch represent the number of expected substitutions per site and branch support by the posterior probabilities shown by the percentage value next to each tree node.

Concatenated datasets

Mitochondrial DNA (mtDNA).—A concatenated dataset for the mtDNA genes (12SrDNA, 16SrDNA, COI) was made resulting in a total of 1486 characters. The maximum parsimony analysis of this concatenated dataset contained 152 parsimony-informative characters. An exhaustive search evaluated a total of 15 trees retaining only one with the best tree length score of 634 (CI = 0.84, RI = 0.23, HI = 0.16). The most parsimonious tree for the mtDNA concatenated dataset is shown in Figure 18. This parsimony reconstruction revealed the same topology as those of both 12SrDNA and 16SrDNA but differed from the COI. The addition of the COI though having a different tree topology and the relative lower bootstrap values did not seem to affect the topology of the tree, but rather affect the analysis by producing lower branch support values. This was especially so with the *A. mixtum* branch node connected with both *A. americanum* and *A. tenellum.*

For the maximum likelihood analysis of this concatenated dataset, the GTR+G model was used (Table 5). The computed log-likelihood score for the resulting reconstructed maximum likelihood tree was 4487.85 (Figure 19). As with the parsimony analysis of this dataset, the COI did not affect the topology of the tree, but did seem to affect the branch supports values.

The GTR+G model was used for the Bayesian analysis which resulted in the reconstructed maximum likelihood tree having a computed log-likelihood value of 4491.82 (Table 5) (Figure 20). This log-likelihood value was very similar to that of the maximum likelihood analysis. As with the previous two phylogenetic analysis the only effect of the COI gene seemed to be that it produced lower branch support values for the branch node of *A. mixtum* to the connection of the *A. americanum* and *A. tenellum*.

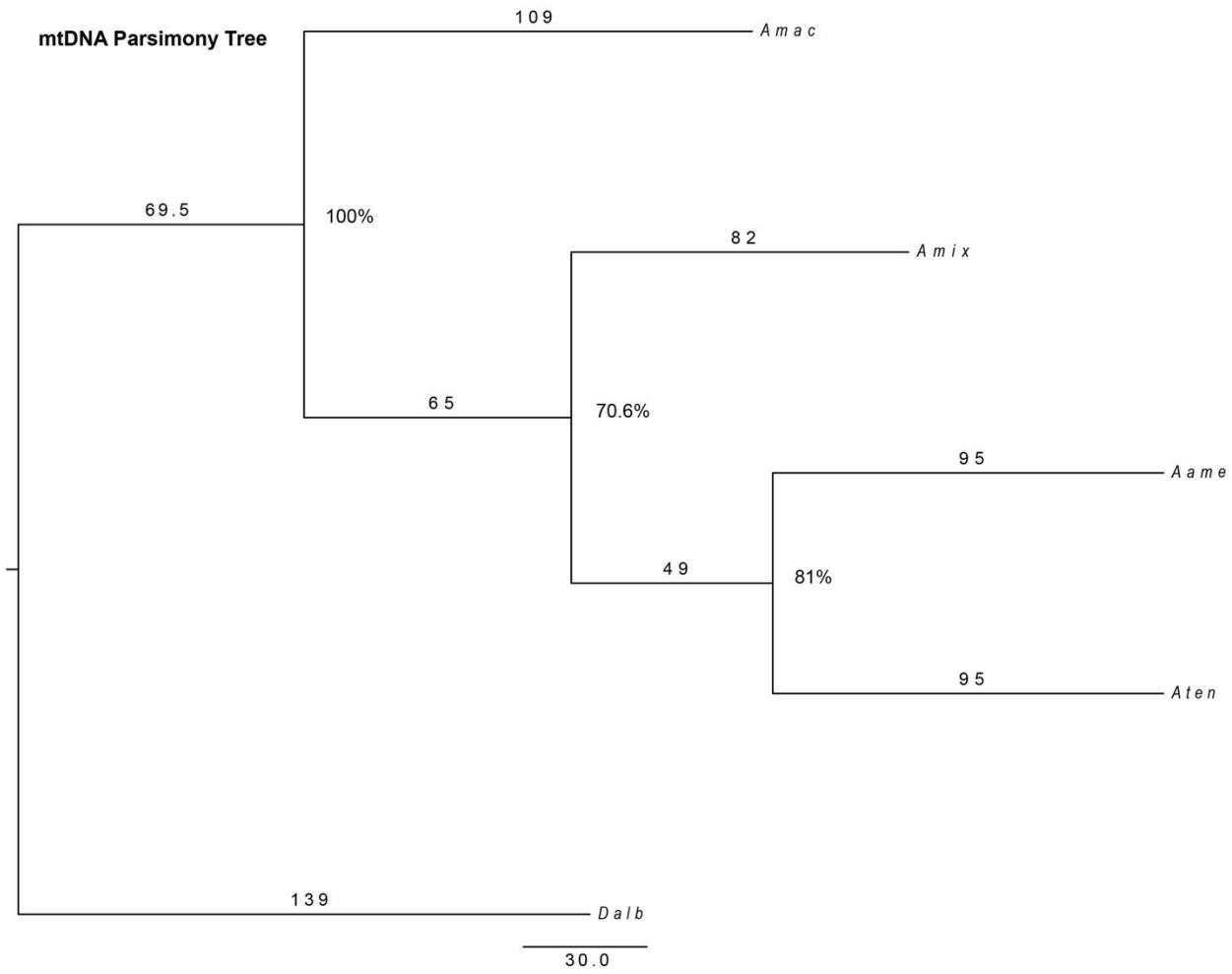

Figure 18. Concatenated mitochondrial gene (mtDNA) maximum parsimony tree. Species names are abbreviated: *A. ame = Amblyomma americanum, A. mac = Amblyomma maculatum, A. mix = A. mixtum, A. ten = Amblyomma tenellum*; and *D. alb = Dermacentor albipictus*. Branch lengths are shown above each branch represent the number of expected substitutions per site and branch support by the posterior probabilities shown by the percentage value next to each tree node.

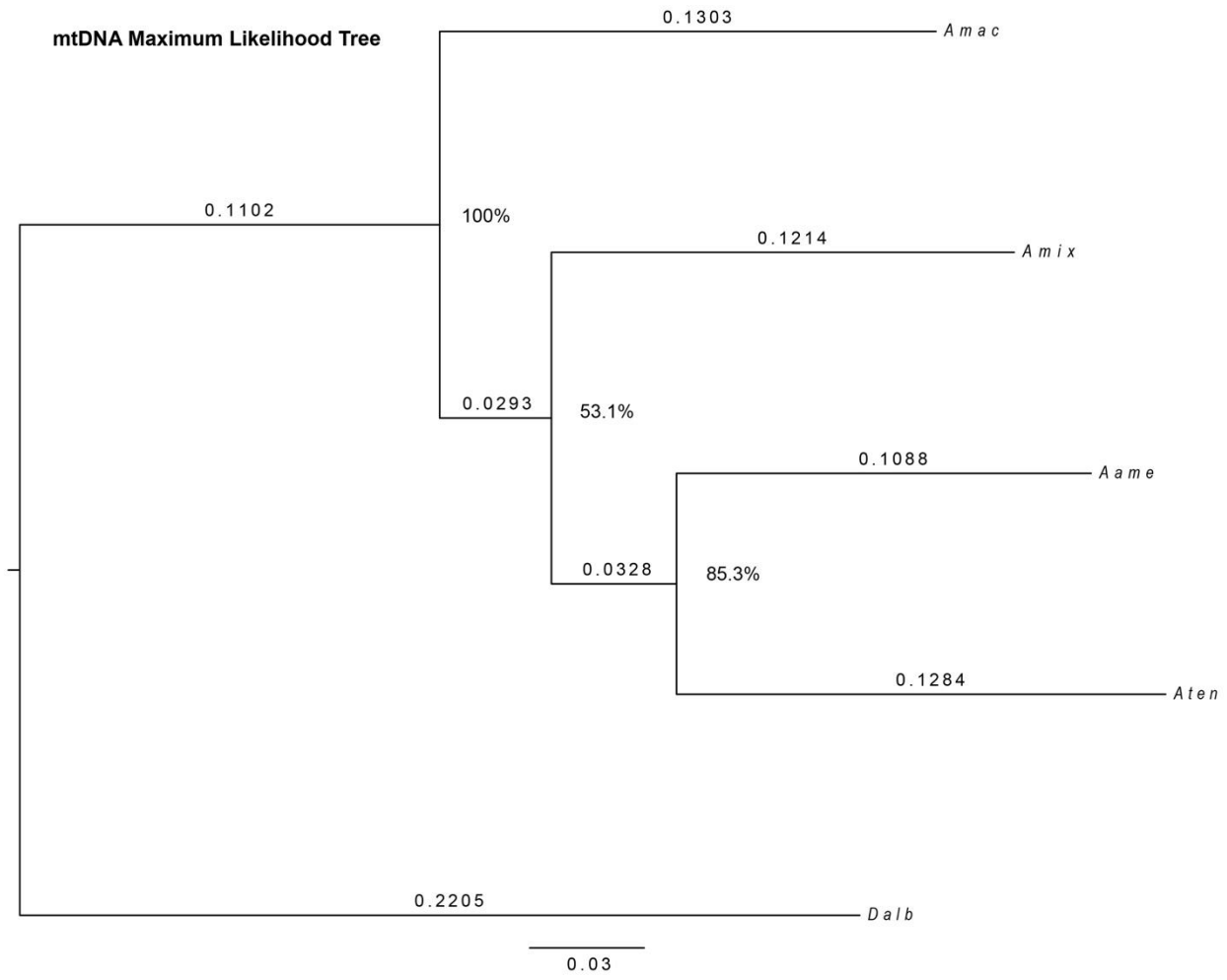

Figure 19. Concatenated mitochondrial gene (mtDNA) maximum likelihood tree. Species names are abbreviated: *A. ame = Amblyomma americanum*, *A. mac = Amblyomma maculatum*, *A. mix = A. mixtum*, *A. ten = Amblyomma tenellum*; and *D. alb = Dermacentor albipictus*. Branch lengths are shown above each branch represent the number of expected substitutions per site and branch support by the posterior probabilities shown by the percentage value next to each tree node.

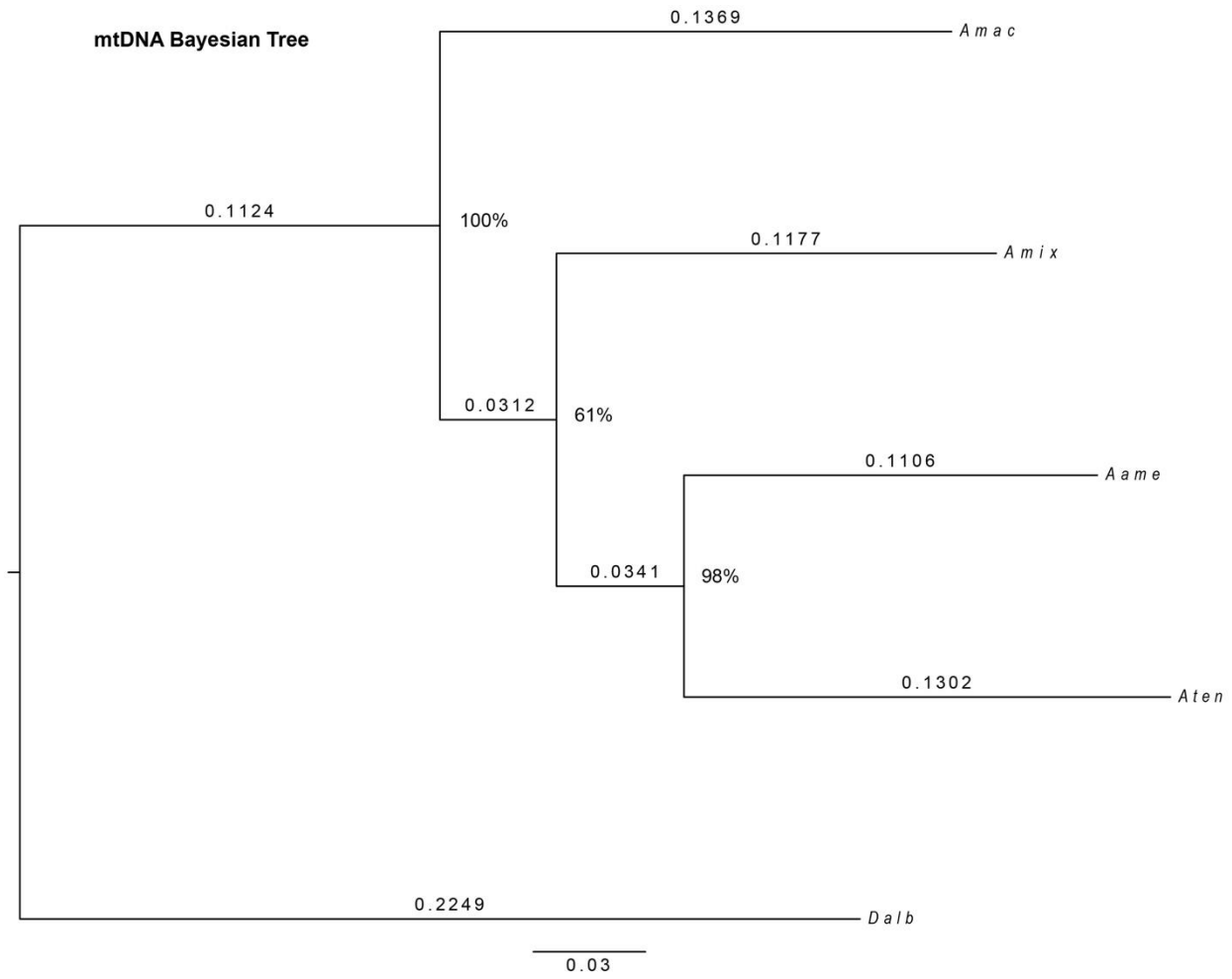

Figure 20. Concatenated mitochondrial gene (mtDNA) Bayesian tree. Species names are abbreviated: *A. ame* = *Amblyomma americanum*, *A. mac* = *Amblyomma maculatum*, *A. mix* = *A. mixtum*, *A. ten* = *Amblyomma tenellum*; and *D. alb* = *Dermacentor albipictus*. Branch lengths are shown above each branch represent the number of expected substitutions per site and branch support by the posterior probabilities shown by the percentage value next to each tree node.

Mitochondrial DNA + Nuclear DNA (mtDNA+ITS2).—A second concatenated dataset was created which combined both the mitochondrial genes (12SrDNA, 16SrDNA, COI) and one nuclear DNA gene (ITS2). This concatenation resulted in 2634 characters, out of this 233 were parsimony-informative characters. An exhaustive search evaluated a total of 15 trees retaining only one with the best tree length score of 1251 (CI = 0.90, RI = 0.49, HI = 0.10). The most parsimonious tree for the mtDNA+ITS2 concatenated dataset is shown in Figure 21. Parsimony results were similar to the mtDNA concatenated dataset but with higher branch support values. The COI gene seemed to have no effect on the topology of the most parsimonious tree. Nor did the COI have an effect on the branch support of the branch containing *A. mixtum* connected to the *A. americanum* and *A. tenellum*.

The maximum likelihood analysis for the mtDNA+ITS2 concatenated dataset utilized the GTR+I substitution model (Table 5). The computed log-likelihood score for the resulting reconstructed maximum likelihood tree was 8676.53 (Figure 22). Results of the reconstructed maximum likelihood tree were similar to the parsimony analysis. Also these results were consistent with the mtDNA concatenated dataset, but having higher branch support values.

For the Bayesian analysis of the mtDNA+ITS2 concatenated dataset, the GTR+I was used, the same as that used in the maximum likelihood analysis (Table 5). The computed likelihood score of the reconstructed Bayesian tree was similar to that of the maximum likelihood score (8756.15) (Figure 23). As with the previous two phylogenetic analysis of this dataset there was no difference in the tree topology nor in branch support.

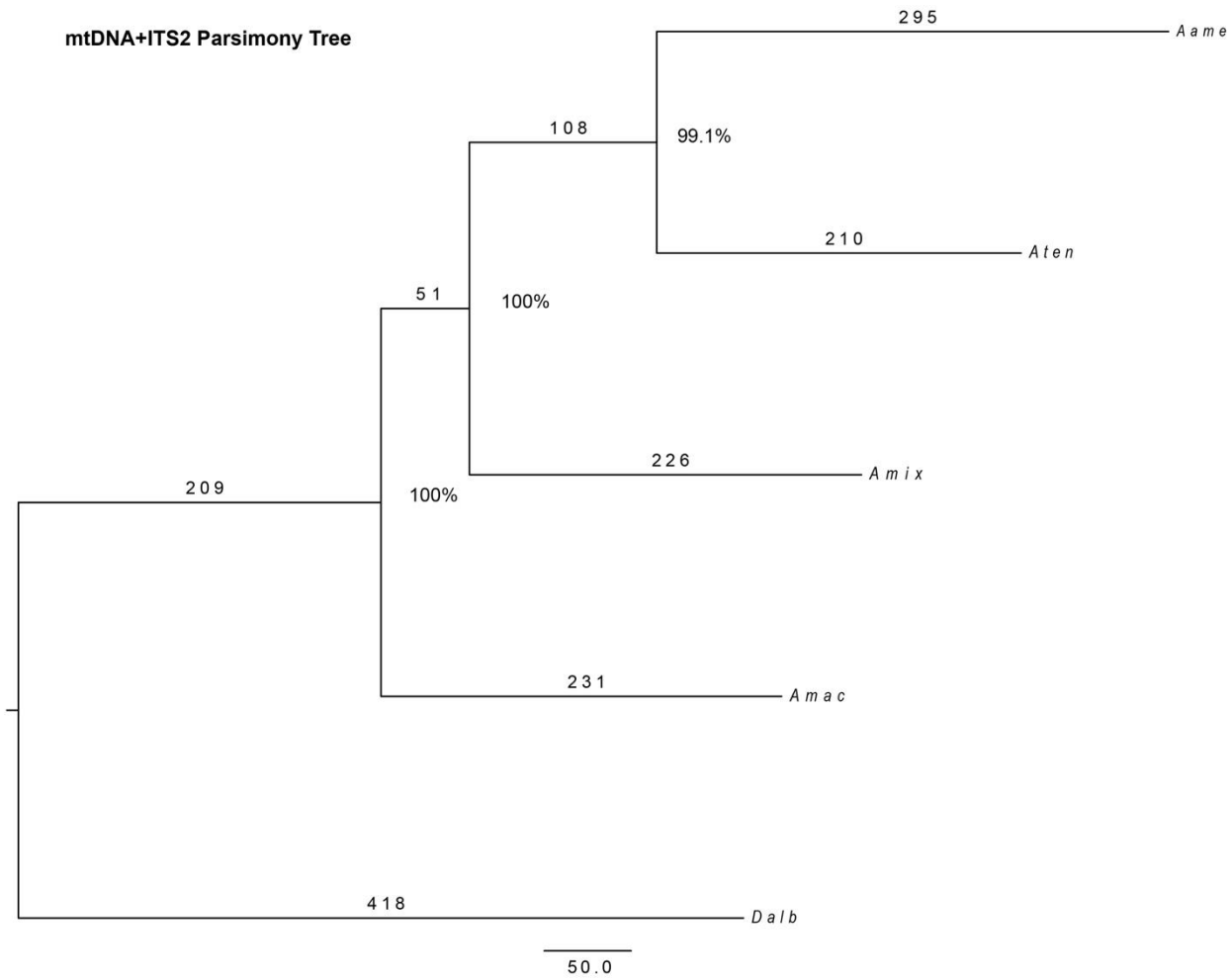

Figure 21. Concatenated mitochondrial genes and the internal transcribed spacer 2 gene (mtDNA+ITS2) maximum parsimony tree. Species names are abbreviated: *A. ame* = *Amblyomma americanum*, *A. mac = Amblyomma maculatum*, *A. mix = A. mixtum*, *A. ten = Amblyomma tenellum*; and *D. alb = Dermacentor albipictus*. Branch lengths are shown above each branch represent the number of expected substitutions per site and branch support by bootstrapping is shown by the percentage value next to each tree node.

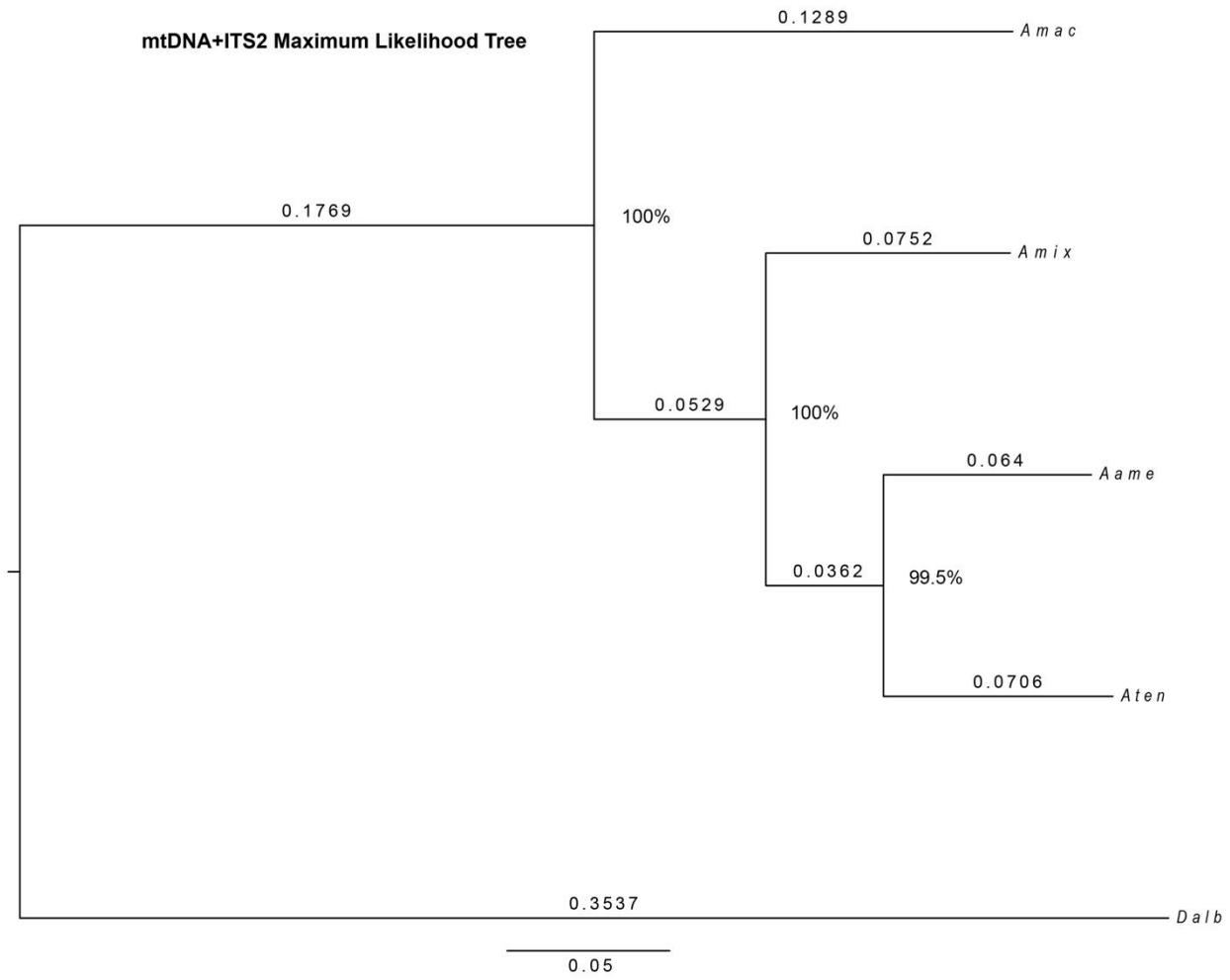

Figure 22. Concatenated mitochondrial genes and the internal transcribed spacer 2 gene (mtDNA+ITS2) maximum likelihood tree. Species names are abbreviated: *A. ame* = *Amblyomma americanum*, *A. mac = Amblyomma maculatum*, *A. mix = A. mixtum*, *A. ten = Amblyomma tenellum*; and *D. alb = Dermacentor albipictus*. Branch lengths are shown above each branch represent the number of expected substitutions per site and branch support by bootstrapping is shown by the percentage value next to each tree node.

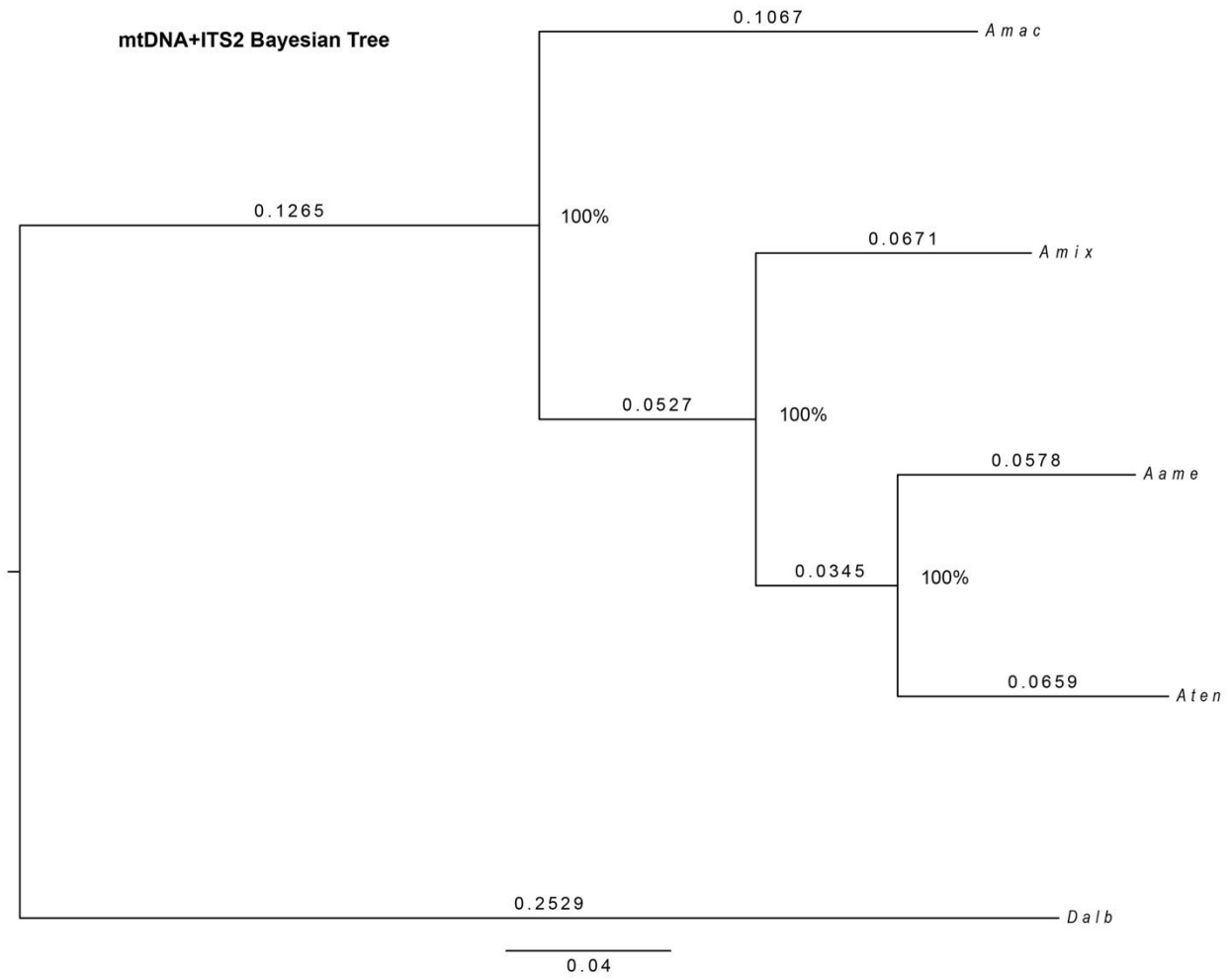

Figure 23. Concatenated mitochondrial genes and the internal transcribed spacer 2 gene (mtDNA+ITS2) Bayesian tree. Species names are abbreviated: *A. ame = Amblyomma americanum*, *A. mac = Amblyomma maculatum*, *A. mix = A. mixtum*, *A. ten = Amblyomma tenellum*; and *D. alb = Dermacentor albipictus*. Branch lengths are shown above each branch represent the number of expected substitutions per site and branch support by the posterior probabilities shown by the percentage value next to each tree node.

DISCUSSION

The purpose of this study was to investigate the phylogenetic relationship of *A. mixtum* and *A. tenellum* to two species of North American *Amblyomma* using a suite of four commonly used molecular markers. The phylogenetic analysis using the datasets of this study revealed that *A. americanum* and *A. tenellum* are more closely related than they are to *A. mixtum* and *A. maculatum*. In the previous chapter it was assumed that *A. tenellum* and *A. mixtum* were closely related leading to the possibility that they must be able to hybridize. This assumption was made before the completion of this chapter. From that study, larvae were produced from one batch of eggs from a single female cross (*A. mixtum* × *A. tenellum*) but it was likely they were produced by parthenogenesis rather than by hybridization providing further evidence that these two species are not closely related enough to produce hybrids. Based on this study the two tick species *A. mixtum* and *A. tenellum* have unique fixed characters among distinct genetic sequences providing further evidence that these two species are not closely related enough to possibly produce hybrids.

This study did not include all known species of *Amblyomma* but only four species. Therefore it must be made clear that phylogenetic relations shown in this study are only using these four taxa of *Amblyomma*. Ideally it would have been better to have all taxa represented but due to the limitation of publicly available sequences this could not be done in this present study. But studies have been published using the four *Amblyomma* species used in this study allowing for comparisons (Beati et al. 2013; Beati et al. 2019). The same relationship of the branch containing *A. mixtum* with both *A. americanum* and *A. tenellum* was found by Beati et al. (2013) who used two of the same genes in this study (12SrDNA and ITS2). Additionally, their results from two other mitochondrial genes, the COII (cytochrome c oxidase subunit 2) and D-Loop

(displacement loop), showed the same relationship among the three taxa. Not included in the Beati et al. (2013) study was *A. maculatum* therefore no comparisons could be made about its relationship to the other three *Amblyomma* species.

While a later study by Beati et al. (2019) used a different gene (18SrDNA), they showed conflicting relationships compared to this study. It was shown in their study that *A. tenellum* and *A. maculatum* were both grouped together within a single branch. Additionally, *A. americanum* was placed outside of this branch. These topological features were identical to that of the COI gene trees in this study (Figure 7 – 9). No inference on the relationship of these taxa could be made with *A. mixtum* as it was not included in their study. Different genes for phylogenetic analysis can yield conflicting branching patterns, often termed gene tree discordance (Degnan and Rosenberg 2006; 2009). Gene tree discordance has been found in a recent phylogenetic study investigating the species complex among *A. parvum* Aragão 1908. In this study, trees reconstructed from 6 different genes (12SrDNA, 16SrDNA, COI, COII, DL, and ITS2) showed an overall difference in topology between each gene tree (Lado et al. 2016). To overcome gene tree discordance, it has been suggested to select only molecular data that contains limited nonphylogenetic signals (Jeffroy et al. 2006). In these studies, it may possible that the COI and the 18SrDNA genes may not have provided enough phylogenetic signals for looking at the relationships of *Amblyomma* taxa. The concatenation of combining these COI with the others in this study seemed to be a good approach than individually looking at single gene trees.

More understanding of this gene tree discordance needs to be investigated especially as phylogenetic analyses use whole genomes for reconstructions of relationships. More data in phylogenomic analysis may lead to more nonphylogenetic signals leading to incorrect and misleading trees (Delsuc et al. 2005). Further studies need to be conducted in order to

understand the phylogenetic relationships for *Amblyomma*. One such study would be to combine the robustness of Beati's et al. (2019) study using the 18SrDNA gene, which included a good representation of *Amblyomma,* with all the genes used in this study.

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CHAPTER IV

SIMULATION OF HOST-TRANSFERRING ADULT MALE TICKS (*DERMACENTOR VARIABILIS* (SAY, 1821)) (ACARI: IXODIDAE) IN THE TRANSMISSION OF EQUINE PIROPLASMOSIS (*THEILERIA EQUI*)

Males ticks (Acari: Ixodidae) are known to remain on the host after females have dropped off into the environment continuing to feed multiple times and search for mates; but the duration of this is not well known. The possibility of males transferring from hosts may occur in close proximity while horses are involved in mutual grooming. Cases of male transmission, among cattle have been well-documented with male *Dermacentor andersoni* Stiles, 1908 and the pathogen *Anaplasma marginale* (Anthony and Roby 1966; Potgieter 1979; Kocan et al. 1996). The studies of both Stiller et al. (1989) and Lysyk (2013) provided some insight into the frequency of male transfer of *D. andersoni* but no studies have addressed male transfer occurring in *D. variabilis* (Say, 1821).

Equine piroplasmosis is a tick-borne disease of horses and other equines such as mules, donkeys, and zebras, caused by two hemoprotozoans *Babesia caballi* (Nuttall & Strickland, 1910) and *Theileria equi* (formally *Babesia equi*). An outbreak of *T. equi* occurred in south Texas, in 2009 where a total of 360 horses were tested and of those 292 horses were seropositive for *T. equi* (81.1%) (Scoles et al. 2011). *Dermacentor variabilis* were collected off of infected horses from this outbreak. Experimental transmission studies of *D. variablis* have demonstrated intrastadial transmission but indicated it may not be an efficient vector of *T. equi* (Scoles and Ueti 2013; Stiller and Coan 1995; Stiller et al. 2002).

Models are useful tools for problem solving and answering questions about a particular system. Experiments alone cannot capture all systems because they are too complex and usually develop slowly (Railsback and Grimm 2012). Disease systems are one such problem that require the use of models because of the complexities that exist within them. They involve environmental effects that drive hosts and vector populations and involve interactions with each other on a landscape; these in turn can affect pathogen transmission. Additionally the events of male transmission of a pathogen and the transfer of male ticks from host-to-host is not thoroughly understood and the frequency at which these event could occur are hard to capture in field studies. Models can help evaluate the role that transferring male ticks might have in the transmission of pathogens. Presented here is an agent-based model that simulates the spatial-temporal dynamics of *D. variabilis* while incorporating transferring adult males to see what role these transferring males have in the transmission and maintenance of *T. equi*. More specifically, to evaluate the likelihood that male transfer could lead to the seroprevalences of *T. equi* as high as those observed in the 2009 outbreak of equine piroplasmosis in south Texas, USA.

BACKGROUND INFORMATION OF PREVIOUS TICKS MODELS

Haile and Mount (1987) developed a computer simulation for *Amblyomma americanum* (Linnaeus, 1758). In this stage structured model they simulated the effects of environmental conditions on the population dynamics of *A. americanum*. They would later go on to develop similar models for three other tick species (Ixodidae): 1) *D. variabilis* (Mount and Haile 1989), 2) *Rhipicephalus microplus* (Canestrini, 1888) (Mount et al. 1991), and 3) *Ixodes scapularis* Say, 1821 (Mount et al. 1997). Wang et al. (2012) modified the model of Haile and Mount (1987) by making it spatially explicit and agent-based. Agent-based models, can be defined as models containing agents that are unique and autonomous which can interact with each other and their

environment (Railsback and Grimm 2012). Wang et al. (2016*a*) later included a hypothetical infectious agent in to their model. Later a model was developed for *R. microplus* and its hosts deer and cattle on a landscape representing south Texas, USA (Wang et al. 2016*b*). In this current model the work of Wang et al. (2012; 2016*a* and 2016*b*) was utilized. From Wang et al. (2012) the basic structure of model was obtained and modified by fitting the parameters from Mount and Haile (1989) for *D. variabilis*. Implementation of a pathogen in the systems utilized the rules for pathogen transmission from Wang et al. (2016*a*). From Wang et al. (2016*b*) the habitat preferences of deer (used here for large hosts) and cattle (used here for horses) and habitat proportions for building their landscape were utilized in this model.

MODEL DESCRIPTION

The following model description follows the protocol ODD (Overview, Design concept, and Details) suggested by of Grimm et al. (2006; 2010).

Purpose

The purpose of this model was to simulate the spatial-temporal dynamics of *D. variabilis* while incorporating transferring adult males to see what role these males have in the transmission and maintenance of *T. equi* within a population of horses. The major outcome of this model was to investigate how changes in the infection probability and the probability of male transfer lead to the seroprevalence of *T. equi* (80% prevalence) in the 2009 outbreak of equine piroplasmosis in south Texas, USA.

Entities, state variables, and scales

Entities in the model included: 1) 400, square-shaped, 30 m x 30 m (0.09 ha/cell; total area = 36 ha) habitat cells that were arrayed on a flat surface with the allowance of world-wrapping from top to bottom and from left to right, and 2) four classes of mammalian hosts:

small-sized hosts, medium-sized hosts, large-sized hosts, and horses. The numbers of hosts varied after initialization of the model due to the population dynamics but horses stayed at a fixed population of 10 individuals throughout the entire duration of the simulations.

The state variables of the habitat cells included: 1) a location based on x and y coordinates, 2) habitat patch type (mesquite savanna, mixed-brush savanna, and open grassland), 3) current numbers of hosts, and 4) current numbers of tick eggs, host seeking larvae, engorged larvae, host seeking nymphs, engorged nymphs, host seeking male and female adults, and engorged female adults. The state variables of the individual hosts included: 1) location (x and y coordinates) of the hosts both their center of their activity range and current location, 2) habitat type of current location (mesquite savanna, mixed-brush savanna, and open grassland), 3) habitat preferences which is a value of 0 (low preference) and 1 (high preference), 4) size radius of activity range, 5) number of larvae, nymphs, and adults they can carry, 6) the current number of on-host ticks for each individual stage (larvae, nymphs, and adults), 7) age in weeks, and 8) a maximum longevity in weeks. The host class of horses had a few more state variables that only pertained to them: 1) infection status (infected or not infected) and 2) pathogen transmission rate from horses to adult male ticks.

The global variables represented by environmental conditions of temperature (ºC), daylength (hr), and saturation deficit (mbar). These environmental conditions were updated on a weekly a basis over a five-year period.

The model's temporal and spatial scales were determined on three factors: 1) the ecology of the of the ticks and hosts involved, 2) the amount detail of available information, and 3) the constraints of computational considerations. Mount and Haile (1989) used a weekly time step which was able to provide sufficient representation of environmental conditions of temperature,

daylength, and saturation deficit and their effects on the various stages of off-host ticks and egg development. Additionally, the use of a weekly time step allowed for sufficient representation of seasonal changes in the density of the host population (Schauber and Ostfeld 2002). The five-year period allowed for sufficient time for the distribution and abundance of ticks to respond to environmental conditions. A spatial resolution of approximately 0.1 ha has been by used Wang et al. (2012), which was useful enough for the representation of modeling processes and to obtain model results.

Process overview and scheduling

The model was programmed and simulated in NetLogo v.5.2 (Wilensky 1999, http://ccl.northwestern.edu/netlogo/). Simulation results were exported as text files (.txt) to Excel© (Microsoft, 2003), Google Sheets (Google LLC, 2018), and R (R Core Team 2018) for statistical analyses and graphical representation of data. The first step of each simulation was to initialize the system: 1) by reading in a fixed landscape, 2) creating hosts communities, and 3) placing eggs in the environment. Additional manual inputs on the user's interface were initialized: 1) setting the infections rates from tick-to-horse and from horse-to-tick, 2) setting the maximum distance for male-transfer to occur, 3) setting the maximum distance from lead horse, and 4) setting the probability of male transfer, and 5) randomly designate a single horse to be infected. Next Netlogo read an input file containing a time-series of environmental data over a five-year period. Following this Netlogo executed four submodels: 1) adjustment of environmental conditions and survival rates, 2) adjustment of host densities, 3) process of the tick life cycle, and 4) male transfer. All these submodels were run 10 times during a simulated week. At the end of each week the program wrote several text files with value summaries of: 1) the environmental conditions, 2) the density of off-host ticks, 3) the number infected and

uninfected male ticks on horses, 4) the number of other stages of ticks on hosts, and 5) the number of infected horses.

Design concepts

Basic principles.—This model was designed to take in account the lifecycle of *D. variabilis*, which is influenced by environmental conditions, the heterogeneity of a landscape, and the composition of the host community. In this model the environmental variability of nature was represented as an input file containing a time series over a five-year period. The landscape was characterized into three habitat types commonly found in south Texas. Host communities were categorized into four classes: 1) small-size, 2) medium-sized, 3) large-sized, and 4) horses. These hosts move about the landscape picking up and dropping off ticks into the environment. Changes in these factors in the lifecycle of *D. variabilis* can also affect its ability in the maintenance of pathogens, such as *T. equi*.

Emergence.—The spatial and temporal patterns in the abundance of ticks in each of the various life stages emerged as system-level properties from: 1) a set of equations that described the off-host tick development and survival rates and 2) a set of rules that governed the movements of hosts. The development and survival rates of off-host ticks were dependent on the environmental data. Movements of hosts within the simulated landscape were dependent on the distribution of the type of habitat as well on the preferred habitat type of the hosts.

Adaptation.—The individual hosts' behaviors were fixed by a set of rules, therefore individuals did not possess adaptive traits.

Objectives.—Individual hosts in the model did not adapt their behavior to achieve given objectives.

Learning.—In this model individual hosts did not learn as result of changes in their behavior from past experiences.

Prediction.—Individual hosts in this model did not make predictions of future conditions nor were able to judge the consequences of their specific behaviors.

Sensing.—Individual landscape cells were able to sense ("aware of") their habitat type, which affected the off host survival rates of ticks in each lifecycle stage that were located in the cells. Individual hosts in this model were able to sense: 1) the location of the center of their activity range, 2) the size of their activity range, 3) their habitat preferences, 4) the proportions of landscape cells of each habitat that were within their activity range, and 5) the maximum number of larvae, nymphs, and adults they could carry. Both the landscape cells and individual hosts did not require explicit rules on how sensing occurred. But the host class of horses did require an explicit rule on how the proximity of the closest horses was obtained.

Interaction.—An interaction between the landscape cells and individual hosts cells existed involving the collection and dispersal of ticks in each life stage category among the landscape cells that were within the activity ranges of individual hosts. There was also an interaction among horses where males would transfer to the closest horses based on a proximity distance.

Stochasticity.—During the initialization of the model, the center of the activity range of each individual host was probabilistically selected and placed the hosts in that center of their habitat cell based on their habitat preferences. During simulations of the model, another probabilistic selection was made and placed the hosts in another habitat cell based on its habitat preferences. Horses did not have a center point for movement within a habitat cell but rather were allowed to move to any location in the cell. Both these movement created stochastic results

in the distribution of hosts available. Additionally, this stochasticity in movement affected the proximity of horses in the model. Pathogen transmission from both tick-to-horse and horse-to-tick was also probabilistically selected based on a rate of transmission (1%, 0.5%, 0.25%, and 0.1%). Male transfer, males moving to one horse to the other, was also probabilistically selected based on five rates (100%, 75%, 50%, 25%, and 1%).

Collectives.—This model grouped individual hosts into four collectives: 1) small-sized hosts, 2) medium-sized hosts, 3) large-sized hosts, and 4) horses. The individuals within each group shared common attributes which included habitat preferences, size of activity range, and relative number of larvae, nymphs, and adults carried.

Observation.—This model recorded the weekly values for: 1) the number of eggs, host seeking larvae, engorged larvae, host seeking nymphs, engorged nymphs, host seeking adults, and engorged adult females in each habitat cell, and 2) on host larvae, nymphs, adult females and males, and transferring adult males (both infected and uninfected) for each hosts. The summary outputs included: 1) the total number and mean of horses infected per week, 2) the total number and mean of uninfected and infected transferring males per week, 3) total duration of infection (time, in weeks, from 1st horse to infected to last infected horses), 4) the first increase in the number of infected horses (time, in weeks, from 1st infected horse to 2nd infected horse, and 5) the cumulative number of infected horse weeks (IHW) (calculated by a summation index equation: $IHW = (\#\ of\ infected\ horses\ in\ week\ 1) \times (week\# = 1) + (\#\ of\ infected\ horses\ in\ week\ 2) \times (week\# = 2)$ ...). These summary outputs were evaluated over different probabilities for infection (1%, 0.5%, 0.25%. and 0.1%) as well as different probabilities in male transfer (100%, 75%, 50%, 25%, and 1%).

Initialization

A total of 572 hosts were created: 360 small-sized hosts, 180 medium-sized hosts, 22 large-sized hosts, and 10 horses. The values of small and medium host came from Wang et al. (2012). In this model large-sized host were assumed to be deer. A deer density found by Kie and Bowyer (1999) of 0.6175 individuals per hectare was used to calculate the density of large-sized hosts in the model ($36\ ha\ \times\ 0.6175\ =\ 22\ hosts$). The value of 10 horses represented a typical group size found in nature (Feist and McCullough 1976). Habitat preferences for small and medium sized hosts were all equal for each of the three habitat types (33%). Large hosts were assigned the following habitat preferences: 20% mesquite, 40% mixed-brush, and 40% grassland (Wang et al. 2016*b*; McMahan and Inglis 1974; Cohen et al. 1989; Cooper et al. 2008). The habitat preferences for horses were assigned as follows: 30% mesquite, 10% mixed-brush, and 60% grasslands (Wang et al. 2016*b*). These values were originally assigned to cattle by Wang et al. (2016*b*) but it was assumed that horses would have similar habitat preferences. None of the hosts were initialized with ticks attached nor where ticks placed in the environment instead 833 eggs were placed in each cell for a total of 30,000 eggs in the system. In order to start the infection of *T. equi*, one horse was randomly selected to be infected, no ticks nor eggs were infected during initialization of the model.

The model of Mount and Haile (1989) focused mainly on medium sized hosts, which included dogs, with no inclusion of large hosts. They used the following classification with minimum and maximum carry loads of on hosts ticks: 1) for 100% adults feeding on dogs with threshold density points of 60 and 240 adult ticks per hosts and 2) for 50% of adults feeding on dogs with threshold density points of 32 and 130. It was assumed that these values did not represent the system in south Texas where dogs do not make up the majority of hosts. Therefor

estimates had to be made in order to obtain the maximum number of ticks in each class of hosts they could carry; which required a literature search (Anderson and Magnarelli 1980; Cooney and Burgdorfer 1974; Sonenshine 1972; Sonenshine and Stout 1971; Tugwell and Lancaster 1962; Koch and Dunn 1980; Clymer et al. 1970; Carroll and Schmidtmann 1986; Bishop and Trembley 1945). From this literature search hosts species for *D. variabilis* were first classified as small-size, medium-sized, or large-sized hosts. Next the stage of tick that was found on the host was recorded (larvae, nymph, or adults). These were then imported into JMP® in order to calculate the count and probability of occurrence of ticks in a given stage for each individual host classification (Table 6). For example, a total of 41 occurrences of adult ticks were found on large-sized hosts with a given probability of 98% (Table 6). The occurrence probabilities listed in this table were used for calculations to estimate the maximum number of ticks a host could carry. But first it was assumed that each successive host stage could carry twice as many ticks (small-sized = 50 ticks; medium-sized = 100 ticks; large-sized = 200 ticks). Next an assumption was made that medium sized host could carry a total of 65 adult ticks. This assumption was made by dividing the maximum value of 130 for 50% dogs given by Mount and Haile (1989) by half to represent a population that just included wildlife and no dogs. This allowed for calculations using the probabilities in Table 6 for the estimate of the maximum number of ticks a host could carry to be made. For example, for finding the number of nymphs that can be carried on medium-sized hosts the calculation would be the product of the amount of total ticks (medium-sized = 100) and the probability listed in Table 1 for medium-sized hosts ($100 \times 0.21 = 21\ nymphs\ carried\ on\ medium-sized\ animals$). All results from these calculations are shown in Table 7.

Table 6. Probabilities acquired from a literature search that were used to calculate the maximum tick loads a hosts in a particular size category could carry.

Host	Stage	Count	Probability
Large	Adult	41	98%
Large	Nymph	1	2%
Large	Larvae	0	0%
Medium	Adult	49	65%
Medium	Nymph	16	21%
Medium	Larvae	11	14%
Small	Adult	7	10%
Small	Nymph	24	38%
Small	Larvae	33	52%

Table 7. Maximum tick loads hosts.

Small-sized Hosts (Total = 50)	Medium-Sized Hosts (Total = 100)	Large-sized Hosts (Total = 200)
Adults = 5	Adults = 65	Adults = 196
Nymphs = 19	Nymphs = 21	Nymphs = 4
Larvae = 26	Larvae = 14	Larvae = 0

Input data

Climatic conditions.—Daily temperature and humidity for Corpus Christi, Texas, USA were obtained from 2008 to 2012 from the National Weather Service. Daily day lengths were obtained from United States Naval Observatory (USNO 2015). These daily values were then calculated into mean weekly data. These environmental data were used for the parameterization of baseline climatic conditions of the model (Figure 24).

Landscape-heterogeneity.—In this model a hypothetical landscape was read into Netlogo to insure a fixed landscape over all simulations. This hypothetical landscape, representing south Texas, was created based on three habitat proportions used by Wang et al. (2016*b*); who derived their proportions based on the characterization of habitat from two studies in south Texas (Archer et al. 1988; McMahan and Inglis 1974). These proportions of habitat used in Wang et al. (2016*b*) and this study were: 1) 30% of mesquite, 2) 30% of mixed-brush, and 3) 40% open grassland. In this model these proportions resulted in 10.89 ha of mesquite (120 cells), 10.8 ha of mixed-brush (121 cells), and 14.31 ha of open grassland (159 cells) (Figure 25).

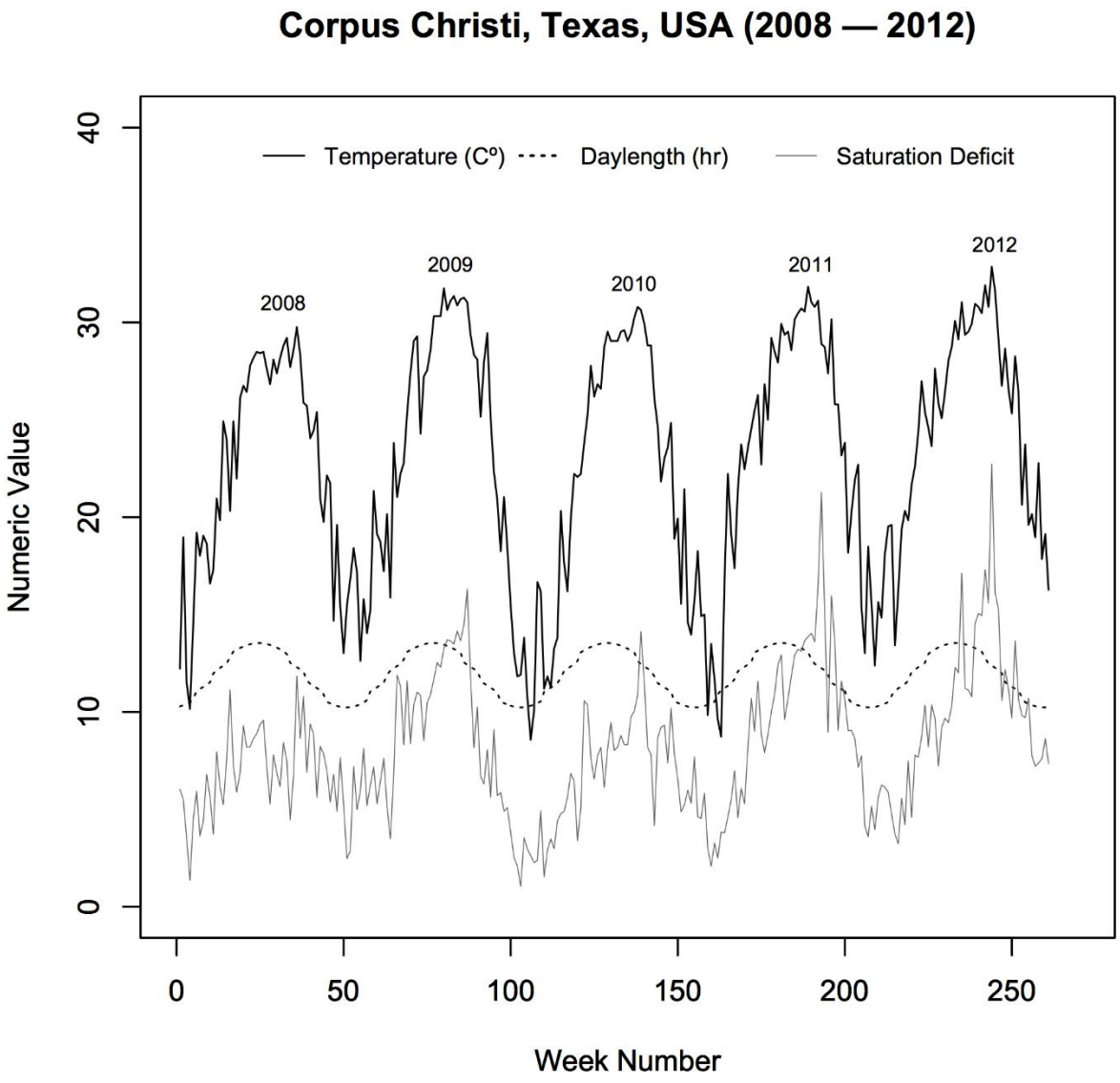

Figure 24. Climatic data used in model simulations. Five-year (2008 – 2012) time series representing weekly temperature (solid thick black line, in ºC), daylength (dashed line, in hours), and saturation deficit (light gray line, in millibars) for Corpus Christi, Texas, USA.

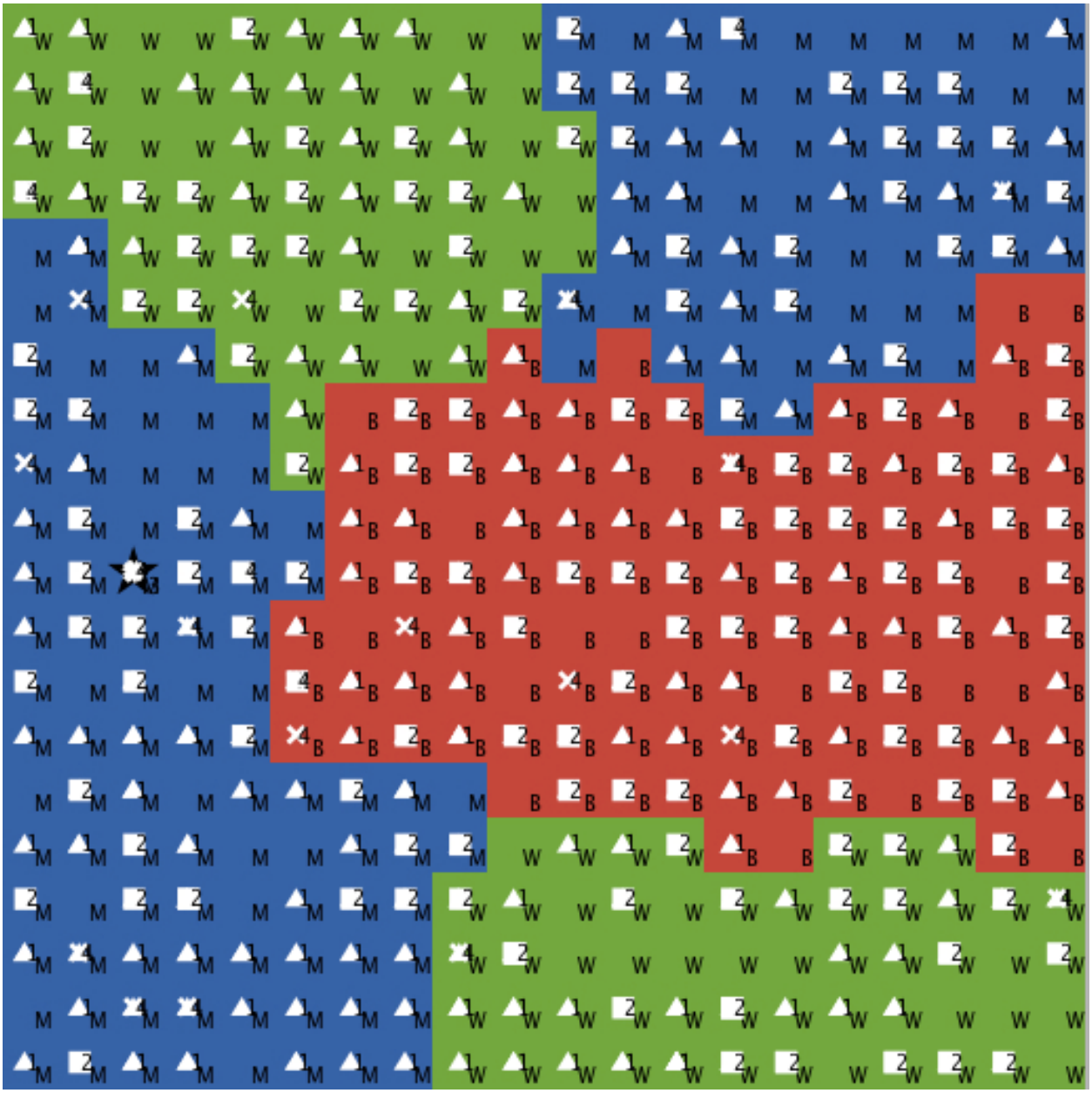

Figure 25. Hypothetical landscape that represents south Texas used in model simulations (36 ha). Habitat types and their proportions included: mesquite 1) 30% of mesquite (green), 2) 30% of mixed-brush (red), and 3) 40% open grassland (blue). On this landscape host were distributed randomly (small-sized hosts = triangles; medium-size host = squares; large-sized host = x's ; and horses = points surrounded by yellow circle).

Submodels

Adjustment of environmental conditions and survival rates.—The temperature, saturation deficit, and daylength were adjusted each week according to the time series data corresponding with the environmental input file. These values were used to recalculate the off-host developmental rates and survival rates for each individual tick stage within a given habitat type (Appendix A). In addition, these values were also used to recalculate host-seeking rates of these off-host ticks (Appendix A). Parameterization of these rates were based on a suite of equations developed by Mount and Haile (1989) from a previous simulation model of *D. variabilis* (Appendix A).

Adjusting host densities.—The adjustment of host densities for small, medium, and large host utilized a curve which represented the seasonal fluctuations in host density. This curve was generated by Wang et al. (2012) following (Schauber and Ostfeld 2002) which used the following equation: $H_i = H_{i-1} \times \exp[-d_H \times \cos\left(\frac{\pi \times i}{26}\right)]$, where H_i was the density of hosts in week number i ($1 \leq i \leq 52$) and d_H was used to determine the range of densities over fluctuating host populations within a year then sets the turnover rate of the population (Wang et al. 2012). The host density for horses stayed constant at 10 individuals throughout the model simulation. Removal of hosts were selected at random and the hosts that were added were assigned to random home cells. Death of hosts was represented by each individual host having a maximum age and once this max was exceeded the hosts were removed from the population and replaced by new hosts who were assigned to random home cells. No horses were removed from the model as result of mortality.

Process of the tick life cycle.—This model utilized the same life stages in the life cycle of *D. variabilis* following Mount and Haile (1989). One modification of their model included the

addition of two stages of male ticks: 1) males before they transferred (Adult Stage 20) and 2) males that have transferred (Adult Stage 21). Oliver (1972) found that *D. variabilis* had a sex ration of 1:1. Therefore a 1:1 sex ratio was added into this model to represent the male and female ticks. For every week, the model first calculated: 1) the number of on-host ticks (larvae, nymphs, adults both females and males) that would each drop off into the habitat cells, 2) the number of all stages that would survive within each habitat cell, 3) the number of eggs, host-seeking, and engorged ticks that would progress to the next life stage, 4) the number of host-seeking larvae, nymphs, adults that encounter and attach to each host (small, medium, large, and horses) from habitat cells, 5) the number of males on the host that would be transferred if in close proximity of horses (see next section), and 6) the number of engorged females adults that would oviposit in each habitat cells.

Development rates of eggs and engorged ticks were calculated as functions of cumulative degree-weeks (CDW) of temperature with a minimum developmental threshold of temperature (DT), 10ºC for eggs and 9ºC for engorged ticks. Survival rates of all stages were calculated as functions of the average weekly saturation deficit and temperature as well as an effect of the type of habitat. The host-finding rates were calculated as functions of the average weekly temperature and daylength. All these calculations followed equations developed by Mount and Haile (1989) for *D. variabilis* (Appendix A).

The collection and distribution of ticks was represented as functions of: 1) body size, 2) activity range size, and 3) habitat preferences of the hosts. It was assumed that the larger the hosts the more ticks a host could carry. It was also assumed that the earlier the life stage of a tick, such as larvae, the more likely they would be to attach to small-sized hosts. An equation, developed by Wang et al. (2012), was used to calculated the proportion of total ticks picked up

by hosts from each habitat cell: $D = \frac{HP_i \times N_i}{\sum_{i=1}^{j}(HP_i \times N_i)}$, D_i was the proportion of the total number of ticks within an individual life stage, each stage calculated separately, that are collected by a single hosts from a habitat cell i, while N represents the number of host-seeking ticks from a habitat cell i, and HP_i was the habitat preference of the host for the habitat type in represented in cell i (Wang et al. 2012).

Male transfer.—Transfer of adult male ticks was dependent on the distance between horses. This maximum distance in which male transfer could occur was set at 1 m which was used to simulate generalized mutual grooming events. Individual horses were aware of which horses were closest to them and those horses that were within the range of 1m or less were able to transfer ticks. The number of ticks that transferred from one horse to another was based on the probability of male transfer. The model summed up the number of male adult ticks on the horse then took the product of this and the probability of male transfer. This resulting product was the number of ticks that were transferred to the closest horse. Several simulations were run at five different male transfer probabilities: 100%, 75%, 50%, 25%, and 1%. Males adult ticks were assumed to have the same on hosts survival rates as adult females. Only, male ticks were infected with *T. equi*, simulations of four different infection rates were conducted (1%, 0.5%, 0.25%, and 0.1%).

MODEL EVALUATION

To evaluate the model, I compared seasonal dynamics of on-host ticks simulated by my model to those simulated by the model of Mount and Haile (1989). To do this I used time series (1949 – 1953) of environmental variables (temperature, saturation deficit, and day length) representative of the conditions of Jacksonville, Florida, USA simulated by Mount and Haile (1989). Results indicated similar trends with some variability which was likely due to the

stochasticity of my model (Figure 26). Overall the model structure and the functional relationships in the model were assumed to be a reasonable representation of Mount and Haile (1989). After this evaluation the environmental data from Corpus Christi, Texas, USA was added to the model.

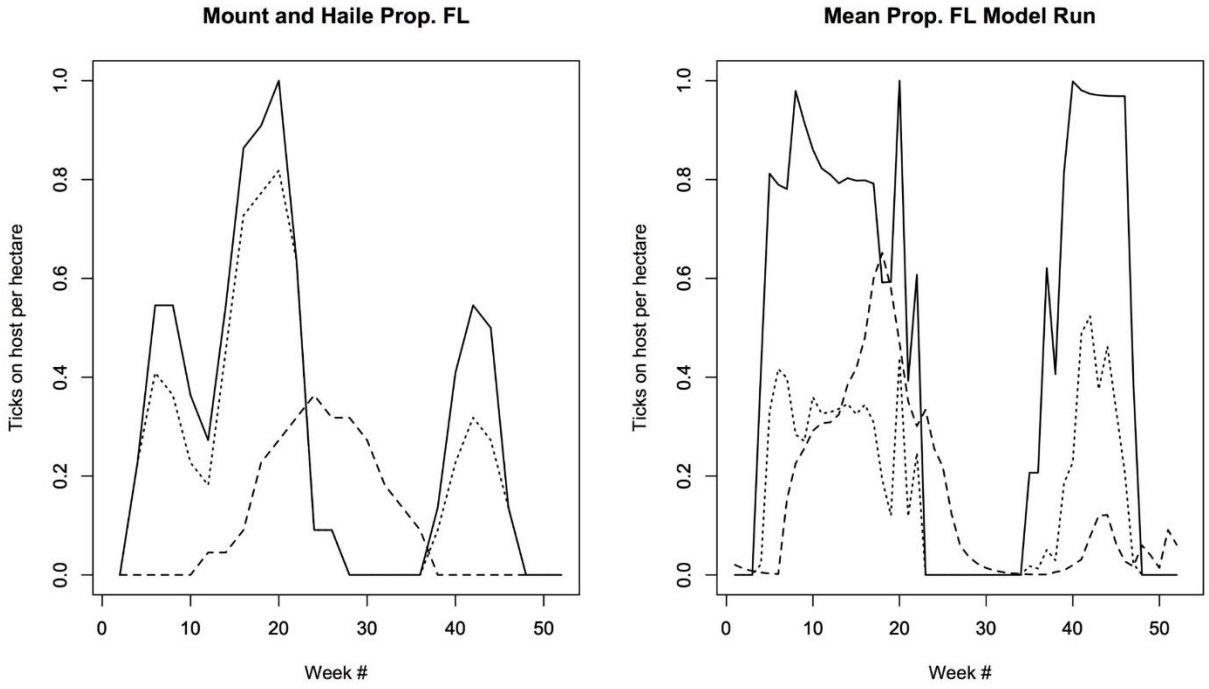

Figure 26. Model evaluation results comparisons. The proportions of ticks on-hosts per hectare that were calculated from Mount and Haile (1989) using environmental data from 1949 – 1953 (left). The mean proportions of ticks on host per hectare simulated results of this model using the weather data from 1949 – 1953 (right). Representation of tick stages are as follows: solid line = larvae, dotted lined = nymphs, and dashed line = adults. Results indicated similar trends but with some variability which was likely due to the stochasticity of the NetLogo model.

To evaluate the rules that governed male transfer, I tracked a set of host attributes: 1) their proximity to one another, 2) the total number of on-host adult male ticks both infected and non-infected on each host, and 3) the total number of infected and non-infected male ticks that had transferred . I started the model with two horses for ease of tracking these attributes. I would run the model one time-step at a time and at each time step NetLogo would report back these attributes. This allowed me to insure: 1) that males were transferring at the set distance of 1 m or less and 2) that the proper proportions of adult male ticks would move from horse to horse based on the set probability of male transfer (100%, 75%, 50%, 25%, and 1%). After the rules that governed male transfer where observed to function properly additional horses were added.

The infection probability of this model had to be evaluated. This process was started by adjusting the infection probability until at least one horse did not become infected over the course of the simulation model. Results from this evaluation showed that this change did not happen at a whole percentage, as no change was observed at 1%, but rather around 0.5%. Therefore, an evaluation of three decimal infection probabilities of 0.5%, 0.25%, and 0.1% were investigated. Each of these showed reduced number of horses. After this evaluation process these three infection probabilities plus the 1% probability were used for simulation runs.

MODEL APPLICATION

This model was used to simulate the spatial-temporal dynamics of *D. variabilis* to evaluate the potential role of transferring adult males have in the transmission and maintenance of *T. equi* within a population of horses. Five replicate stochastic (Monte Carlo) simulations of each of the 20 possible combinations of five male transfer probabilities (100%, 75%, 50%, 25%, and 1%) and four infection probabilities (1%, 0.5%, 0.25%, and 0.1%) ($5 \times 5 \times 4 =$

100 *simulations*). During each simulation, I monitored: 1) the number of horses infected and 2) the number of infected and non-infected transferred males on horses. I compared the number of horses infected across all combinations of male transfer probabilities and infection probabilities as well as the number of transferred males across those combinations. I evaluated 1) the first increase in the number of infected horses which was the time in weeks from the first infected horse to the second infected horse and 2) the total duration of infection the time period from the first infected to the last infected horse at the end of the simulation.

RESULTS

Number of infected horses

All 10 horses became infected at the infection probability of 1% regardless of the change in the male transfer probability (Figure 27). Under the 0.5% infection probability the number of infected horses slightly dropped but the number of horses was still very high ranging from seven to 10 (Figure 28). It was not untill the 0.25% infection probability when the number horses dropped below seven (Figure 29). This infection probability of 0.25% showed a lot of variability likely due to the stochasticity of the model. The last infection probability of 0.1% showed that the majority of the number of horses infected was three or less (Figure 30). Overall it seems that the infection probability had more of an effect on the number of infected horses than the male transfer probability. One would expect the number of infected horses to gradually reduce as the male transfer probability was lowered but that is not the case here where the number of infected horses seem to be within the same range regardless of the male transfer probability. Since the model only used a set of fixed percentages for male transfer a heatmap function was conducted in R that extrapolated between these fixed values. Only the averages of each of the five simulations for all possible combinations of male transfer and infection probabilities are

presented which showed a sinuous curve with two peaks one at 100% male transfer and the other at 25% male transfer and under 0.25% infection probability (Figure 31). In addition, as noted before the variability can be seen in this average map; as a result another heatmap was generated which included the range values of the number of infected horses to show where this variability would occur within these extrapolated percentages (Figure 32). Results from this showed the variability occurring within the same area as the peaks in the average heatmap with a range of about four to six horses infected.

Overall the time from the first infected horse to the second infected horse occurred early at 100% male transfer, with a time period less than one year. As the male transfer probability was lowered from 100% to 1% the time of infection between the first and second horses generally increased (Figure 33). To get an overall all sense of the infection I looked at the total duration, which was the time from the first infected horse to the last infected horse at the end of the simulation. Result of this exhibited a bell shape curve, where at 100% male transfer the duration was short then gradually increased reaching an apex around 50% then declining (Figure 34). These results seemed reasonable as the more likely infected males are to transfer, the quicker the infection would occur. Next, I accumulated the number of infected horses per week creating a cumulative number of infected horse weeks (see equation under Observations in the Design Concept section). Simulation results showed that when the male transfer probability was incrementally reduced from 100% to 1%, the cumulative number of infected horse weeks were also reduced (Figure 35). Results of this indicated that less infection occurred at lower male transferring probabilities than at higher probabilities.

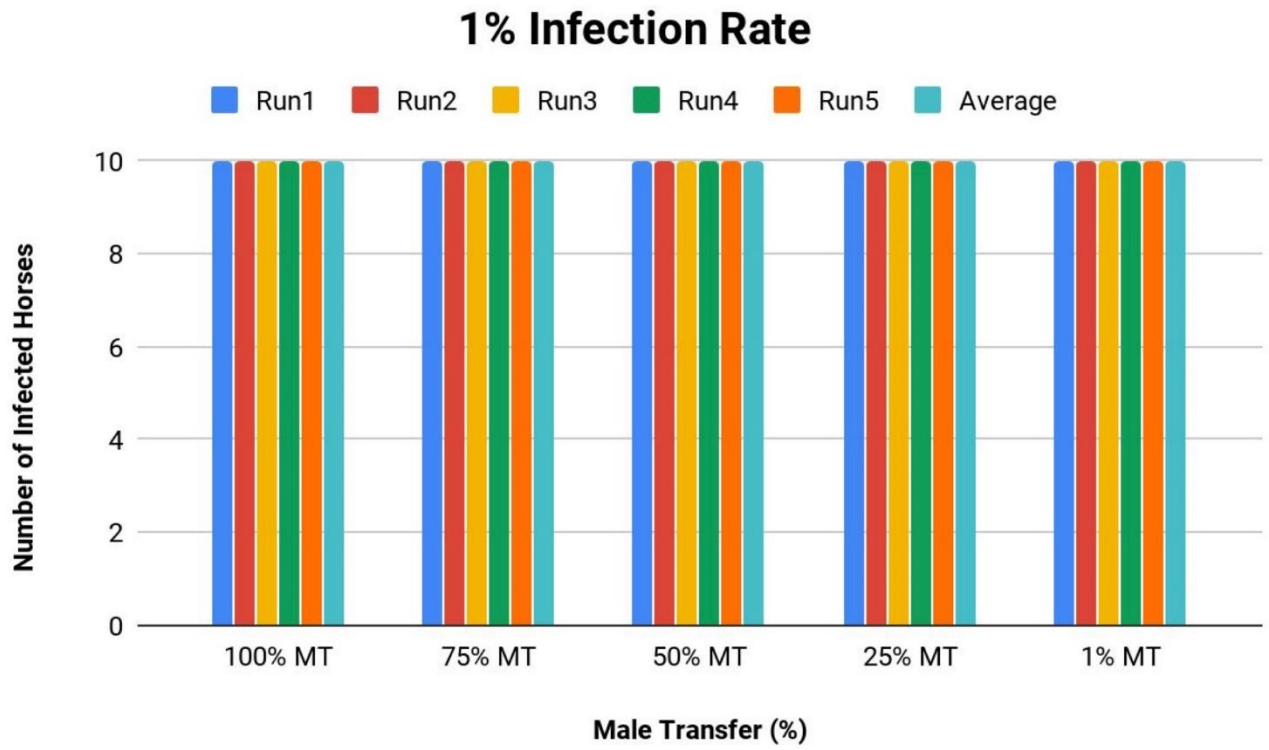

Figure 27. Simulation results of the number of infected horses with a simulated infection probability of 1%. A total of five runs were conducted for each probability of male transfer (100%, 75%, 50%, 25%, and 1%). Included is the average of these five runs. Standard deviation for all these averages were 0.

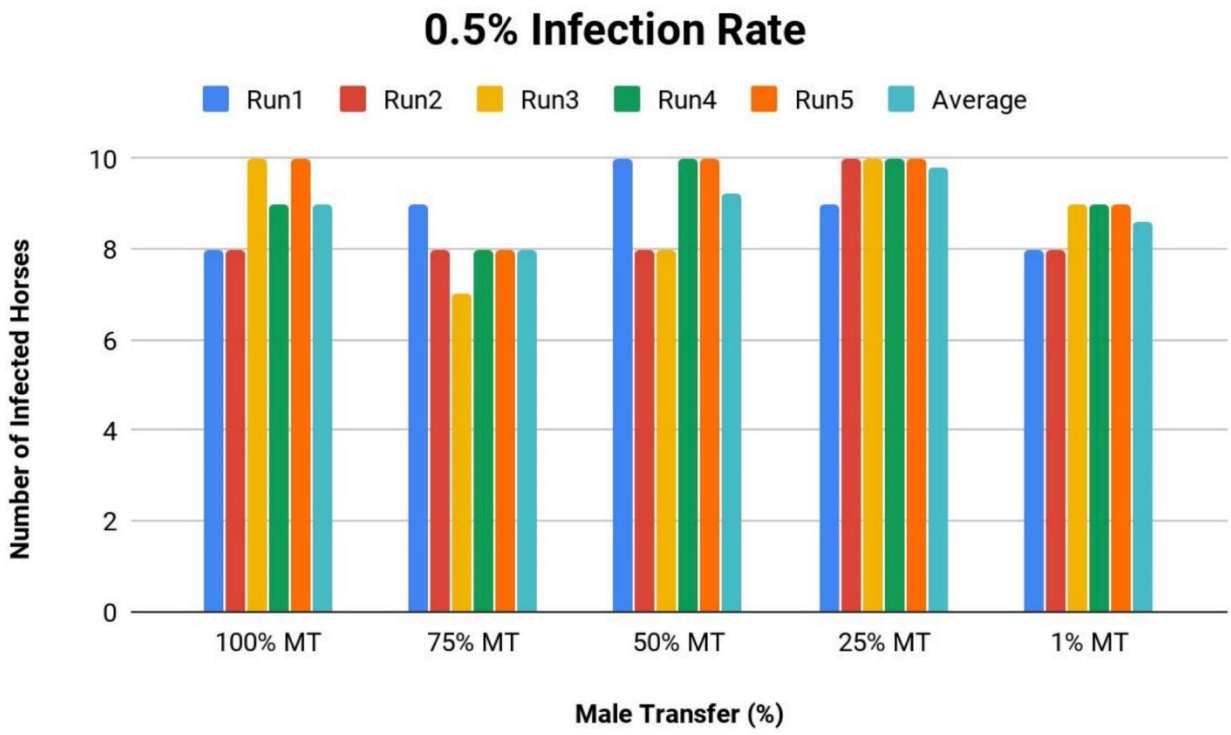

Figure 28. Simulation results of the number of infected horses with a simulated infection probability of 0.5%. A total of five runs were conducted for each probability of male transfer (100%, 75%, 50%, 25%, and 1%). Included is the average of these five runs. Standard deviation for all these averages were: 100% ($\pm$ 1); 75% ($\pm$ 0.71); 50% ($\pm$ 1.1); 25% ($\pm$ 0.45); and 1% ($\pm$ 0.55).

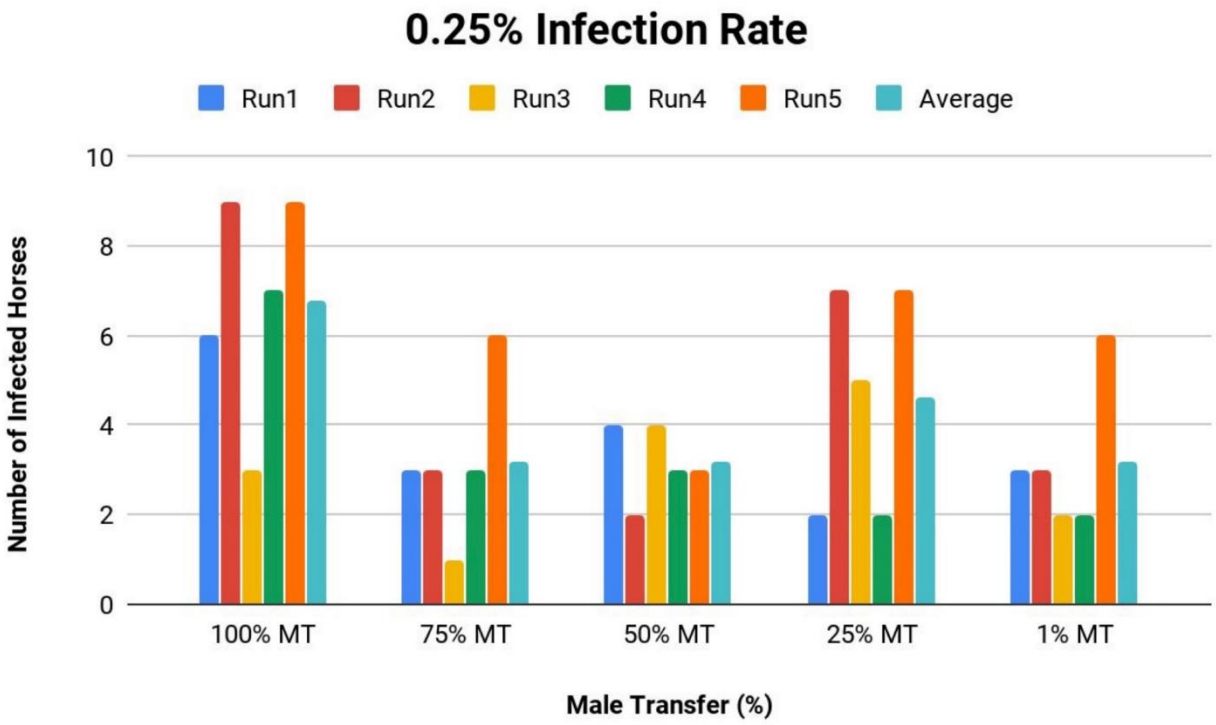

Figure 29. Simulation results of the number of infected horses with a simulated infection probability of 0.25%. A total of five runs were conducted for each probability of male transfer (100%, 75%, 50%, 25%, and 1%). Included is the average of these five runs. Standard deviation for all these averages were: 100% (± 2.49); 75% (± 1.79); 50% (± 0.84); 25% (± 2.51); and 1% (± 1.64).

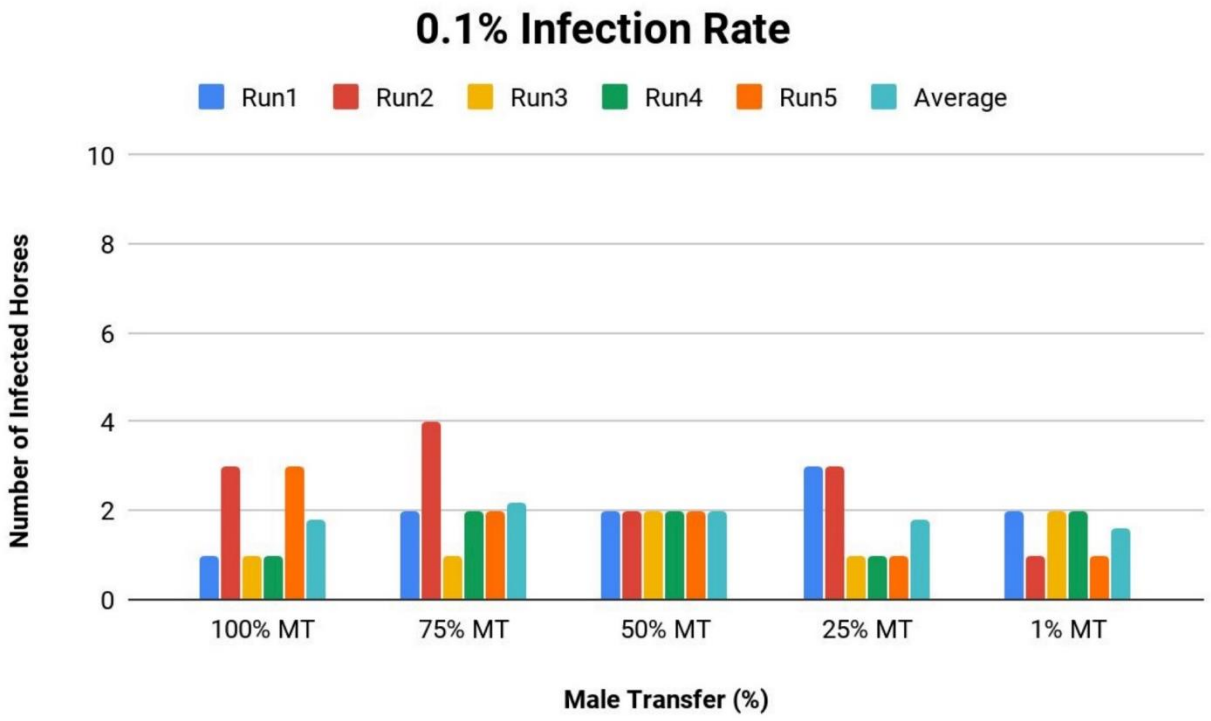

Figure 30. Simulation results of the number of infected horses with a simulated infection probability of 0.1%. A total of five runs were conducted for each probability of male transfer (100%, 75%, 50%, 25%, and 1%). Included is the average of these five runs. Standard deviation for all these averages were: 100% (± 1.1); 75% (± 1.1); 50% (± 0); 25% (± 1.1); and 1% (± 0.55).

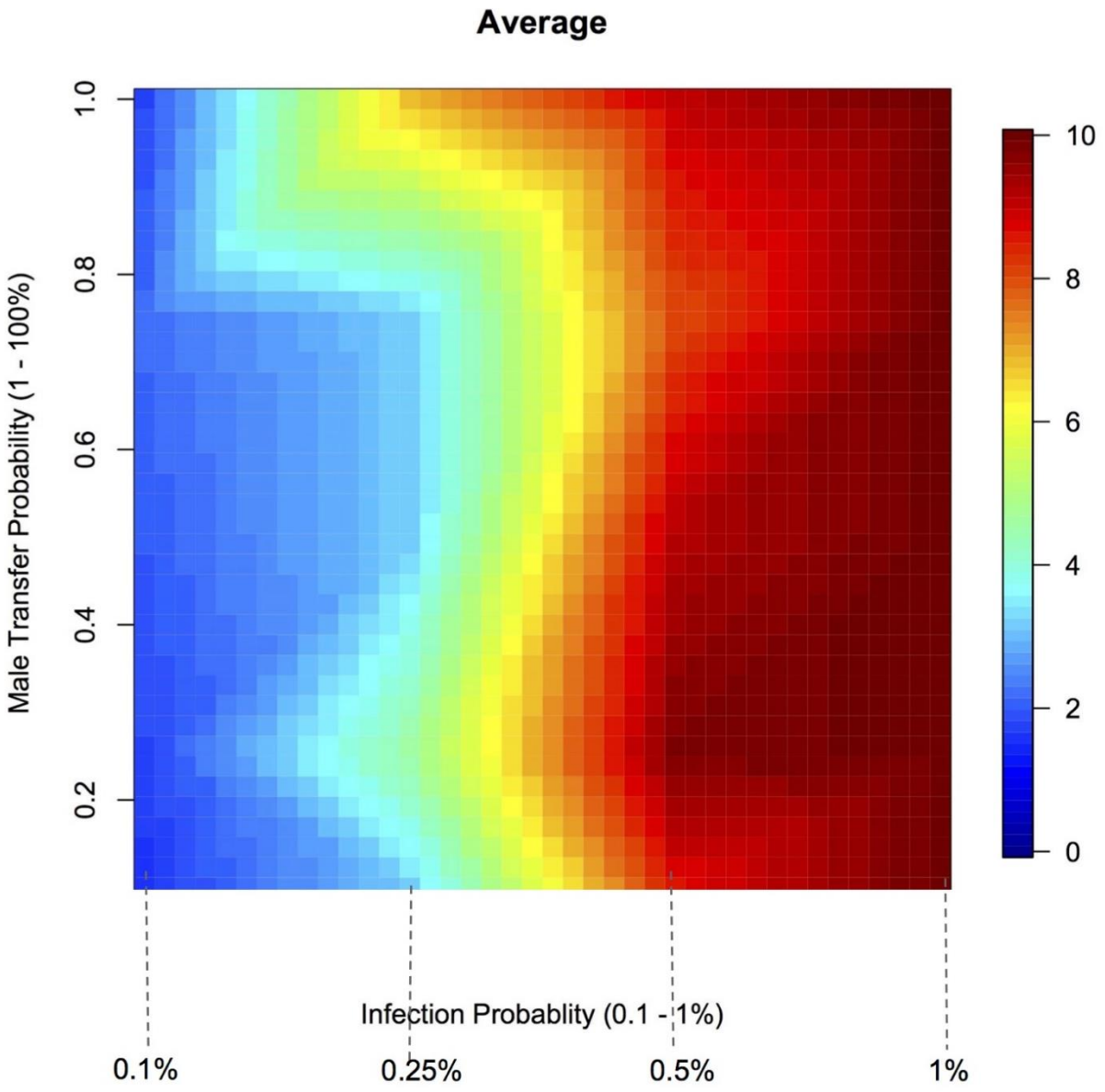

Figure 31. Average heatmap. Shown here is a heatmap with extrapolated probabilities of male transfer and infection probability. The square pixels are color coded indicating the number of horses infected.

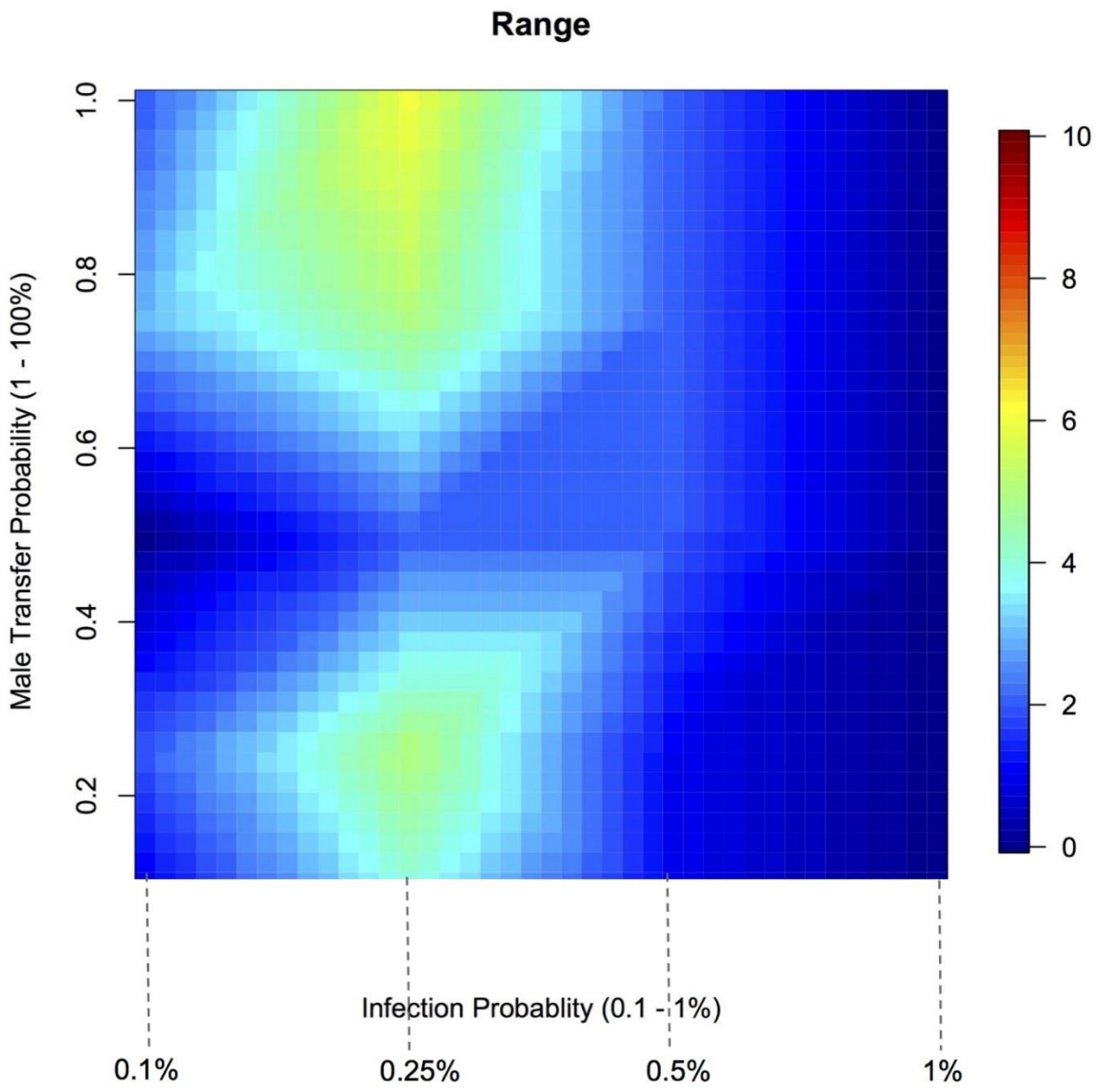

Figure 32. Range heatmap. Shown here is a heatmap with extrapolated probabilities of male transfer and infection probability that show the variability in the number of infected horses. The square pixels are color coded indicating the number of horses infected.

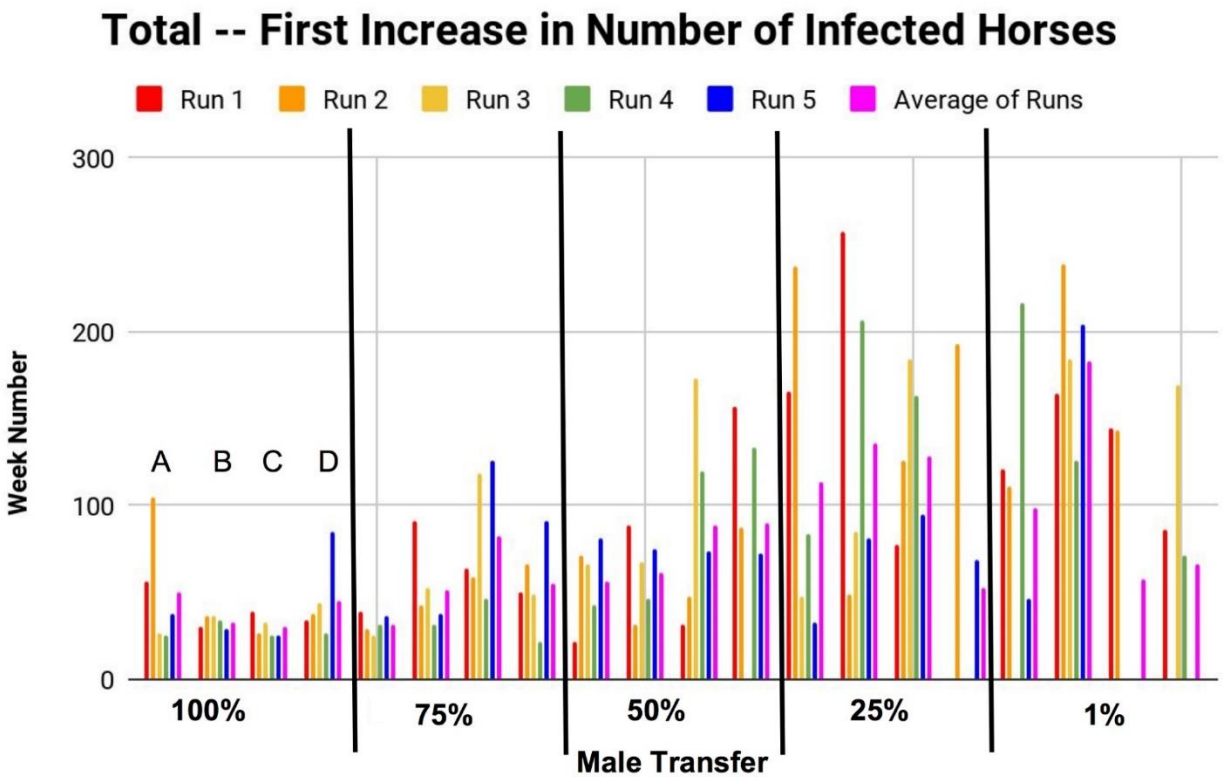

Figure 33. First increase in the number of infected horses. The first increase in the number of infected horses is defined as the amount of time (in weeks) from the 1st infected horse to the onset of infection of the 2nd horse. The letter above the bars represents infection probability (A = 1%; B = 0.5%; C = 0.25%; and D = 0.1%); this repeats across the graph. Pattern observed shows an general upward tick in the number of weeks as the male transfer probability is increased.

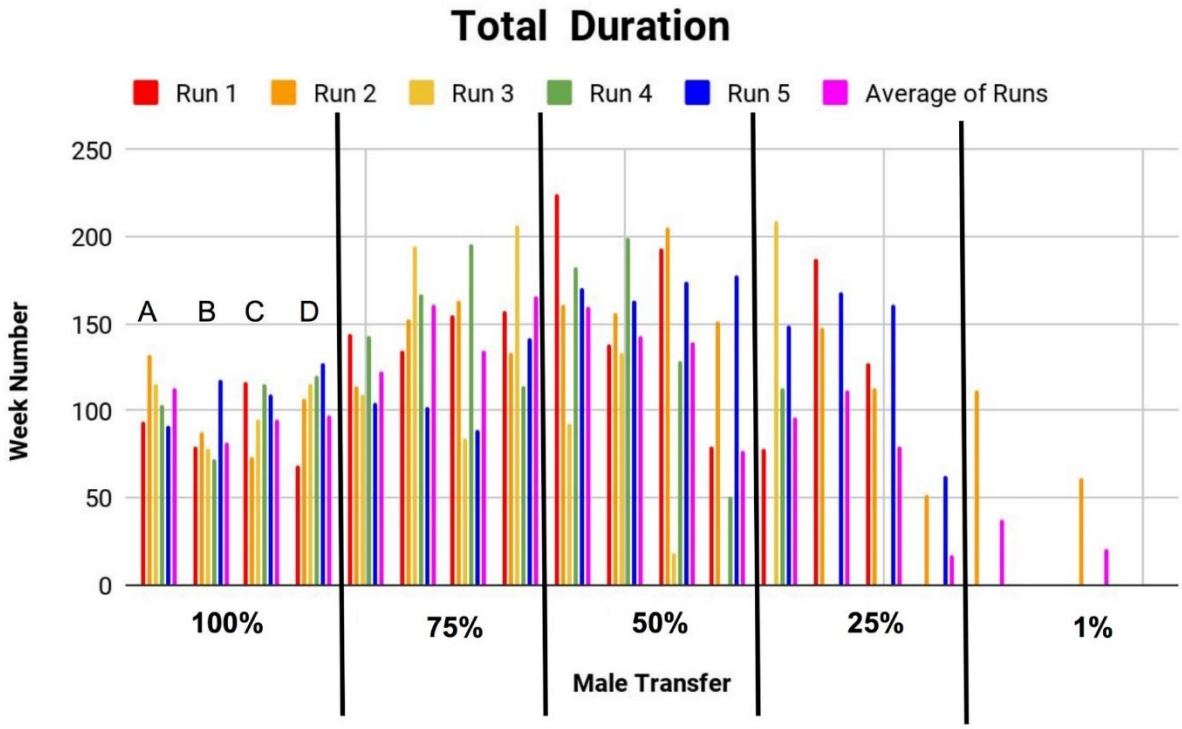

Figure 34. Total duration of infection. The total duration of infection can be defined as the amount of time (in weeks) from the 1St infected horses to the last infected horses (at end of simulation run). The letter above the bars represents infection probability (A = 1%; B = 0.5%; C = 0.25%; and D = 0.1%); this repeats across the graph. Pattern observed shows a bell-shaped curve with duration fizzling out at 1% male transfer; a result of no horses getting infected beyond that first horse infected upon initialization of the model.

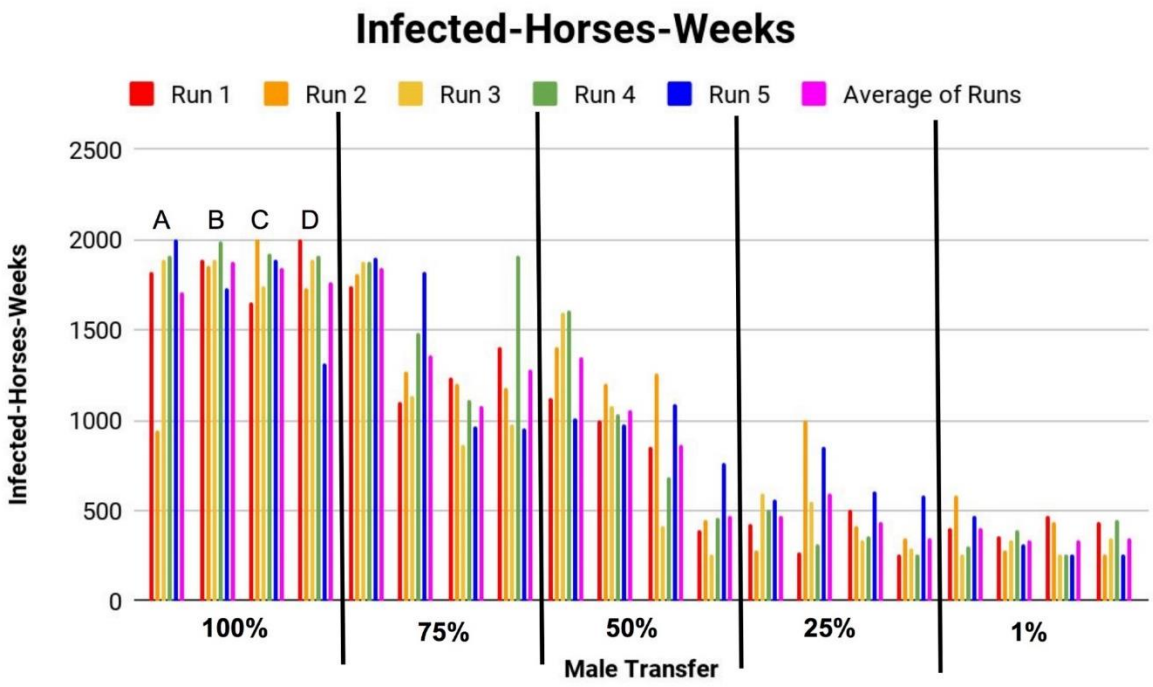

Figure 35. The cumulative number of infected horse weeks (IHW). This is a product summation index calculated by the equation: $IHW = (\#of\ infected\ horses\ in\ week\ 1) * (week\ \#) + (\#of\ infected\ horses\ in\ week\ 2) * (week\ \#) \ldots (\#of\ infected\ horses\ in\ week\ 260) * (week\ \#)$. The letter above the bars represents infection probability (A = 1%; B = 0.5%; C = 0.25%; and D = 0.1%); this repeats across the graph. Pattern observed shows a downward trend in the cumulative number of infected horse weeks from higher male transfer probabilities to lower transfer probabilities.

Transferring male ticks

Netlogo tracked the number of transferring adult male ticks that were non-infected and infected (life stages = Adult21 and IAdult20 respectively) in each of the simulations. From these summaries the average number of total transferring male ticks were calculated then graphed to show the trend over the five-year time series (Appendix B). This allowed for the visualization of the seasonal dynamics of the transferring male ticks. In addition, it revealed that the total number transferring males were in low abundance. Infected transferring males were less than that of the non-infective transferring males. Even though the abundance of infected ticks was low, horses were still able to get infected.

DISCUSSION

This model addressed a knowledge gap in the role that transferring males play in the transmission and maintenance of *T. equi* within a population of horses. The model was spatially explicit, agent based, and stochastic and was not meant to make precise predictions, but rather, serve as a useful tool for exploring the complex nature of intrastadial transmission among male *D. variabilis*. The results of this model showed: 1) that the number of infected horses were influenced by the infection probability, 2) adjustments of the infection probability and the male transfer probability affected the population of males that transferred and their role in the transmission of *T. equi*, and 3) both horses and ticks could maintain the pathogen with infected horses at very low levels of infection and male transfer.

This model also showed that variability existed across the simulation runs such that high peaks would occur in the number of infected horses under low probabilities of male transfer and infection. This may be explained by the movement rules given to horses which moved the animals at random throughout the simulated landscape based upon their habitat preferences.

This stochastic function could have led to horses being in closer contact leading to more horses becoming infected. Most of the variability seemed to occur mainly with an infection probability of 0.25%. Further investigation with additional simulation runs may help understand why this particular percentage would give such a result and why there was not much variability in the other percentages.

This model revealled possible infection rates and male transfer rates that might help explain the 2009 outbreak of *T. equi*, in which an overall seroprevalence of 81.1% was observed (Scoles et al. 2011). Here the model showed that the prevalence of the majority of horses infected reached below 80% prevalence under the simulation with an infection rate of 0.25% and a male transfer probability of 100%. It is unlikely that the male transfer probability of ticks in nature would be as high as 100%. Stiller et al. (1989) found that up 2.6% of male *D. andersoni* ticks placed on hosts transferred to another host while Lysyk (2013) found that up to 1.7% of male *D. andersoni* placed on cattle transferred to another host. No values of the transmission of *T. equi* exist, here the infection probability of *T. equi* was assumed to be low. This model has shown that *D. variabilis* alone can sustain the pathogen in a system, but in nature other tick species may be involved in the transmission of *T. equi*. One such tick is *R. microplus*, a one host tick, who is an efficient vector of *T. equi* (Guimarães et al. 1997 and 1998; Ueti et al. 2003, 2005, and 2008). This model can be updated with the inclusion of *R. microplus* with *D. variabilis*. Such a model would be the first to incorporate the mechanics of a three-host tick and one-host tick in a model system. Future studies are planned to take on this task of a multi-species tick model with transmission of intrastadial adult males.

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CHAPTER V

A POPULATION MATRIX MODEL FOR THE AMERICAN DOG TICK (*DERMACENTOR VARIABILIS* (SAY, 1821)) (ACARI: IXODIDAE) WITH THE INCLUSION OF HOST TRANSFERRING MALES

Dermacentor variabilis (Say, 1821), the American dog tick, is a three-host tick in the family Ixodidae. In this life-cycle engorged female adults deposit eggs into the environment that hatch into host-seeking larvae. Host-seeking larvae attach to small-sized hosts, take a blood meal and drop off into the environment where they molt into host-seeking nymphs. Host-seeking nymphs follow the same pattern of larvae in attachment except they attach to small and medium sized hosts. Nymphs blood feed and then drop off into the environment molting into host-seeking adults. Host-seeking adults seek medium to large sized hosts. Attachment and initial blood feeding is required for both male and female ticks before spermatophore development and ova maturation, respectively (Kiszewski et al. 2001). Sexually mature male ticks detach to mate with attached females on the hosts. Mated females complete blood engorgement and drop off into the environment to oviposite. Males are known to remain on the host after females have dropped, repeating bouts of blood feeding and mate searching until death (Hooker et al. 1912). The duration of this time on the host is not well known. Males may also transfer from one host to another during close proximity contact and during mutual grooming events (Little et al. 2007; Lysyk 2013). How often this occurs is not well known.

Intrastadial transmission occurs when one stage of a tick acquires a pathogen from an infected host, and then transmits the pathogen to a naïve host while still in that particular stage usually occurring with adult males. Because male ticks remain on their hosts for some period of

time, and may move to other hosts there exists the possibility that male-host transfers could lead to higher transmission of pathogens. Well-documented cases of male host transfer of *D. andersoni* and transmission of *Anaplasma marginale* have provided some insights into intrastadial male transmission (Anthony and Roby 1966; Potgieter 1979; Kocan and Stiller 1992; Kocan et al. 1996). Stiller et al. (1989) found that 2.6% of male *D. andersoni* ticks placed on cattle transferred while Lysyk (2013) found that 1.7% of male *D. andersoni* placed on cattle transferred. No known studies have examined adult male host transfer in *D. variabilis*.

The role of male ticks as vectors of piroplasmosis has been suggested by Robinson (1982) with *Theileria annulata* and Sergent et al. (1945). Equine pirpoplasmosis is a tick-borne protozoal disease of horses and other equids. The disease is caused by two haemoprotozoan parasites, *Theileria equi* and *Babesia caballi*. In 2009, an outbreak of *T. equi* occurred in south Texas (Scoles et al. 2011) in which *D. variabilis* was one of several ixodid ticks found on infected horses. *Dermacentor variabilis* has been shown to transmit the pathogen (*T. equi*) experimentally and naturally via intrastadial transmission but is not judge to be an efficient vector (Scoles and Ueti 2015 and 2013; Stiller and Koan 1995; Stiller et al. 1995; Stiller 2002).

Field and laboratory studies have provided information about the population dynamics of *D. variabilis*. Such studies have looked at: 1) egg production under various environmental conditions (Campbell and Harris 1979; Dodds et al.1969), 2) survival rates of various life stages (Hooker et al. 1912; Bishopp and Smith 1938; Smith et al. 1946; and Sonenshine 1972), 3) population biology (McEnroe 1985; Harman et al. 1984), and 4) seasonal activity (McEnroe W. D. 1971, 1974 and 1975; Smith et al. 1941). Mount and Haile (1989) utilized these studies in the development of a computer simulation model focusing on the population dynamics of *D.*

variabilis. Their study did not include individual stages for male ticks nor for transferring male ticks.

Past matrix models of ixodid ticks represented male and female adults as a single stage (Sandberg et al. 1992; Dobson et al. 2011). This grouping of the sexes may not fully represent the life history of ixodid ticks. Male ticks warrant their own stage as their dynamics are different from females. Males can remain on hosts for longer periods of time and can move from host to host. The objective of this study was to develop a population matrix model for *D. variabilis* that incorporated the life history of transferring male ticks under the climatic conditions of south Texas. This model differed from the agent-based model in the previous chapter in that it was represented in a matrix format, which allowed for the calculation of the asymptotic population growth rate (λ), stable stage distribution, and stage-specific reproductive values, as well as the sensitivities and elasticities of λ to stage-specific fecundity, growth, and survival rates.

METHODS

Stage class model

A stage-classified model is one form of a population matrix model where the population is divided into unequal stage groups and is commonly used when age of an individual is unknown (Lefkovitch 1965). Stage-classified models assume using stages rather than ages (age-classified models) is a better predictor of a population's demographics (Caswell 2001). This model used several predefined developmental stages of *Dermacentor variabilis* following Mount and Haile (1989) for the creation of a stage classified model.

Dermacentor variabilis was divided into 12 stage classes: (1) eggs, (2) host-seeking larvae, (3) on-host larvae, (4) engorged larvae, (5) host-seeking nymphs, (6) on-host nymphs, (7) engorged nymphs, (8) host-seeking adults, (9) on-host male adults, (10) transferring males, (11)

on-host female adults, and (12) engorged female adults (Figure 36). These classifications were based on those used by Mount and Haile (1989) with a slight change of creating a 1:1 sex ratio for the inclusions of males. Additionally, there was a stage added representing males transferring from one host to another. An arrow between each stage represented the probability of surviving and transitioning to the next stage (Figure 36). Some of the stages had self-loops which represented the probability of surviving and remaining in a given stage. The reproductive term or fertility term, was defined as fecundity. In this model fecundity was calculated by the product of the number of eggs per female and the female's survival rate using temperature (ºC) and the saturation deficit (mbar). On the life cycle graph fecundity was shown by a dashed arrow going from the last stage of engorged females to eggs (Figure 36). A population projection model was constructed as: $n(t+1) = An(t)$, where A is the population projection matrix, n is the stage-class vector, and t is time. This construction yielded a 12 * 12 matrix (Table 8).

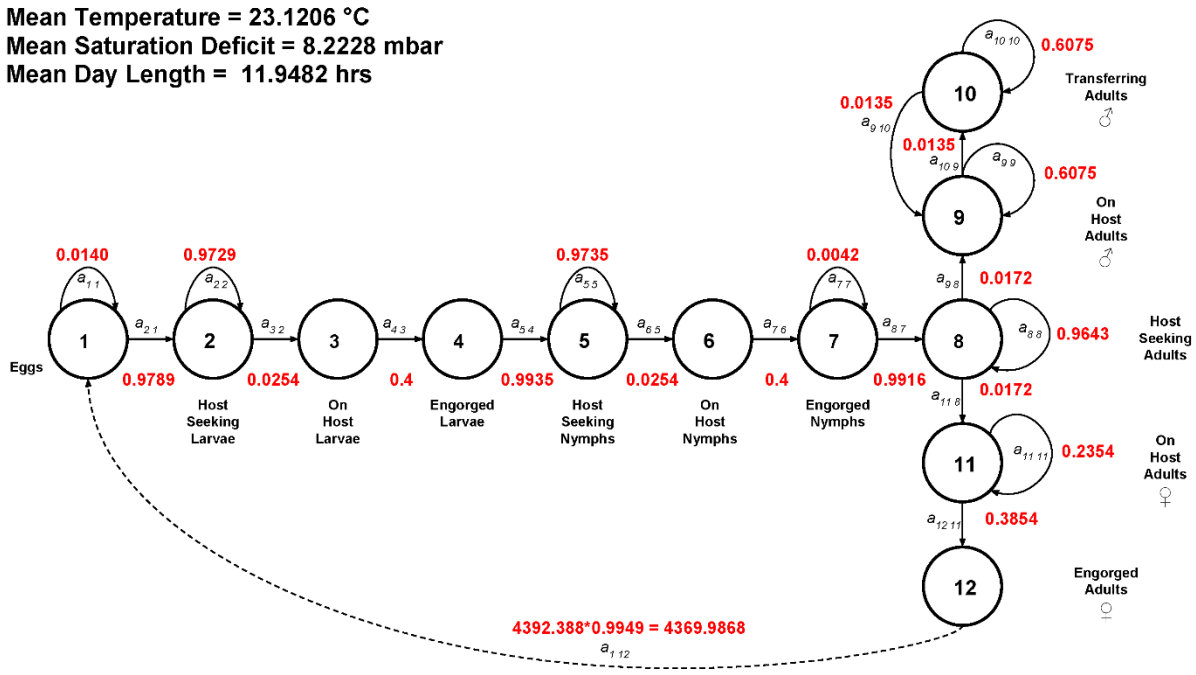

Figure 36. The life-cycle graph for *Dermacentor variabilis* indicating numbers and names of stages (black), and transition rates among stages (red).

Table 8. Stage-class population matrix for *Dermacentor variabilis* based on the life-cycle graph persented in Figure 36.

0.0140	0	0	0	0	0	0	0	0	0	0	4369.9868
0.9789	0.9729	0	0	0	0	0	0	0	0	0	0
0	0.0254	0	0	0	0	0	0	0	0	0	0
0	0	0.4	0	0	0	0	0	0	0	0	0
0	0	0	0.9935	0.9735	0	0	0	0	0	0	0
0	0	0	0	0.0254	0	0	0	0	0	0	0
0	0	0	0	0	0.4	0.0042	0	0	0	0	0
0	0	0	0	0	0	0.9916	0.9643	0	0	0	0
0	0	0	0	0	0	0	0.0172	0.6075	0.0135	0	0
0	0	0	0	0	0	0	0	0.0135	0.6075	0	0
0	0	0	0	0	0	0	0.0172	0	0	0.2354	0
0	0	0	0	0	0	0	0	0	0	0.3854	0

Demographic parametrization

Environmental data.—The fecundity and survival rates of each individual stage of *D. variabilis* were based on environmental data. Daily temperature and saturation deficit for Corpus Christi, Texas, USA were obtained for 2008 – 2012 from the National Weather Service. Mean weekly day length was calculated using daily day lengths obtained from the United States Naval Observatory (USNO 2015). From these daily data the mean weekly temperature (T = 23.1206ºC), mean weekly saturation deficit (SD = 8.2228 mbar), and the mean weekly daylength (DL = 11.9482 hr) were calculated across this five-year time series. Corpus Christi was chosen because it was the closest city to the outbreak of equine piroplasmosis (*T. equi*). Values of demographic parameters are indicated in the life-cycle graph in Figure 36.

Egg stage.—In order to calculate egg survival I used a quadratic equation from Mount and Haile (1989) with mean temperature and mean saturation deficit (Table 9, Equation 2; resulting value equaled = 0.9929). An incubation period of three weeks was assumed. To

represent this incubation period I raised the egg survival that was obtained from Mount and Haile's equation to the third power, the number three representing the number of weeks (0.9929^3). This resulted in a value of 0.9789 that represented $a_{2\,1}$ of the life cycle graph (Figure 36). Next I calculated the mortality of eggs: 1 – 0.9789 = 0.0071. This resulting mortality was used to calculated the self-loop stage of $a_{1\,1}$: 1 – 0.9789 – 0.0071 = 0.0140 (Figure 36).

Host-seeking tick stages.—Survival rates for host-seeking stages were based on the equations from Mount and Haile (1989; Table 9; Equations: 3, 4, 7, 8, 11, and 12). For host-seeking stages there were two equations that had different survival rates based on age (e.g. Table 9; larvae = equations 3 and 4; nymphs = 7 and 8; and adults = 11 and 12). I used the average of these equation by each stage resulting in host seeking larvae having a survival rate of 0.9983, host-seeking nymphs of 0.9989, and host-seeking adults of 0.9987. Mount and Haile also developed an equation to represent the host-finding rates of each host-seeking stage as a function of temperature and daylength (Mount and Haile 1989; Table 9, larvae, nymphs, and adults, equations 5, 9; and 13, respectively). It was assumed that the host finding rate of larvae and nymphs were the same; which was determined to be 0.0254 from the equations from Mount and Haile. And adults had a host finding rate of 0.0344.

Table 9. The equations from Mount and Haile (1989) that were used for obtaining the transition rates for the matrix model of *Dermacentor variabilis*.

Equation #	Stage Class	Equations
1	Fecundity (F)	$-11588.3+1278.7*T-25.4181*T^2$
2	Eggs (S_E)	$(-0.00024770*T^2+0.010899*T+0.880111)*(-0.00011008*SD^2+0.00008137*SD+1)$
		Larvae
3	Host Seeking ($S1_L$)	$(-0.0000685*T^2+0.003014*T+0.966846)*(-0.00004315*SD^2+0.00017529*SD+0.9998625)$
4	Host Seeking ($S2_L$)	$(-0.00006164*T^2+0.002712*T+0.970168)*(-0.00007178*SD^2+0.00048075*SD+0.9992038)$
5	Host Finding Rate (HF_L)	$(-0.00818182*T^2+0.2454546*T-0.8409091)*(-0.02153798*DL^4+1.082504*DL^3-20.22092*DL^2+166.5624*DL-510.8353)$
6	Engorged (SE_L)	$(-0.00021692*T^2+0.0095445*T+0.8950105)*(-0.00012258*SD^2+0.00023933*SD+1)$
		Nymphs
7	Host Seeking ($S1_N$)	$(-0.00007532*T^2+0.00314*T+0.963546)*(-0.0000814*SD^2+0.00052552*SD+0.9991561)$
8	Host Seeking ($S2_N$)	$(-0.0.00002279*T^2+0.010028*T+0.889692)*(-0.00019869*SD^2+0.00112314*SD+0.9984188)$
9	Host Finding Rate (HF_N)	$(-0.00818182*T^2+0.2454546*T-0.8409091)*(-0.02153798*DL^4+1.082504*DL^3-20.22092*DL^2+166.5624*DL-510.8353)$
10	Engorged (SE_N)	$(-0.0001269*T^2+0.0055845*T+0.9385705)*(-0.00010876*SD^2+0.00045429*SD+0.999616)$
		Adults
11	Host Seeking ($S1_A$)	$(-0.0000135*T^2+0.000594*T+0.993466)*(-0.00002997*SD^2+0.000245*SD+0.9995346)$
12	Host Seeking ($S2_A$)	$(-0.00007977*T^2+0.00351*T+0.96139)*(-0.000067*SD^2+0.00037169*SD+0.9994912)$
13	Host Finding Rate (HF_A)	$(-0.00505102*T^2+0.2323469*T-1.67199)*(-0.02427784*DL^4+1.230494*DL^3-23.20363*DL^2+193.1549*DL-599.3686)$
14	Engorged (SE_A)	$(-0.0001573*T^2+0.006921*T+0.923869)*(-0.00013086*SD^2+0.0005243*SD+0.9996048)$

On-host tick stages.—A density-dependent survival rate was used by Mount and Haile (1989) for larvae, nymphs, and adults. Mount and Haile assumed the same survival rate for both larvae and nymphs which was divided into three categories: (1) when on-host tick density was less than 10 the survival rate was 0.55, (2) when density was greater than 40 the survival rate was 0.25, and (3) when density was between 10 and 40 survival was calculated as: $-0.01 \times D + 0.65$ ($D = density\ of\ ticks\ on\ host$). I assumed a value of 0.4, the average of the maximum and the minimum survival rates, for on-host survival larvae ($a_{4\,3}$) and nymphs ($a_{7\,6}$) (Figure 36).

Mount and Haile (1989) also divided on-host survival of adults into three categories: (1) when on-host tick density was less than five the survival rate was 0.7416, (2) when density was greater than 20 the survival was 0.5, and (3) when density was between five and 20 survival was calculated as: $-0.0161 \times D + 0.8221$. I assumed a value of 0.6208, the average of the maximum and the minimum survival rates, for weekly survival of on-host adults for both males and females. On-host females must stay on the host for two weeks to mate and become fully engorged. In order to represent this in the model, the weekly on-host survival of 0.6208 was raised to the second power, the number two representing the number of weeks (0.6208^2). This resulted in a value of 0.3854 that represented $a_{12\,11}$ of the life cycle graph (Figure 36). Next I calculated the mortality of on-host males and females: 1 – 0.6208 = 0.3792. This mortality was used along with the value of 0.3854 to calculate the self-loop that occurred at $a_{11\,11}$ = 1 – 0.3854 – 0.3792 = 0.2354 (Figure 36).

Engorged tick stages.—For engorged survival rate of larvae, nymphs, and adults, I used three quadratic equations based on temperature and saturation deficit (Mount and Haile 1989; Table 9; Equations 6, 10, and 14, respectively). Based on the calculations from theses equations

I obtained the following survival rates: 9.9935 for engorged larvae ($a_{5\,4}$), 0.9958 for nymphs, and 0.9949 for adult females. In this model, nymphs took two weeks to molt into adults. In order to represent this in the model, the weekly engorged nymph survival of 0.9958 was raised to the second power, the number two representing the number of weeks (0.9958^2). This resulted in a value of 0.9916 that represented $a_{8\,7}$ of the life cycle graph (Figure 36). Next I calculated the mortality of engorged nymphs: 1 – 0.9916 = 0.0042. This mortality was used along with the value of 0.9916 to calculate the self-loop that occurred at $a_{7\,7}$ = 1 – 0.9916 – 0.0042 = 0.0042 (Figure 36). For female engorged adults the survival was combined with the fecundity (see fecundity section).

Male tick stages.—Two stages were created specifically to represent males: (1) on-host adult males and (2) transferring males. Stiller et al. (1989) found that 2.6% of *D. andersoni* males that were placed on cattle transferred while Lysyk (2003) found that 1.7% of *D. andersoni* males transferred. In order to obtain the transfer probability for males from host to host the average of these two studies was calculated ($\bar{x}$ = 0.0215; 2.15%), assuming that *D. variabilis* would be similar to *D. andersoni* in male transfer. It was also assumed that on-host males and transferring males had the same survival rate as on-host females of 0.6208 and a mortality rate of 0.3792 (see on-host tick stages section). For adult males, I calculated two transition rates 1) from on-host adult males to transferring adult males ($a_{10\,9}$) and 2) from transferring adult males to on-host adult males ($a_{9\,10}$). This was done by taking the product of the survival rate of on-host adult males (0.6208) and the male transfer rate from host to host (0.0215): 0.6208 * 0.0215 = 0.0135 (Figure 36). The mortality was used along with the value of 0.0134 to calculate the self-loop that occurred at $a_{10\,10}$ = 1 – 0.3792 – 0.0134 = 0.6075 (Figure 36).

Fecundity.—For parameterizing the fecundity, I used a quadratic equation (Mount and Haile 1989: Table 8; Equation 1). This equation was based on a regression study of egg production by female *D. variabilis* at various temperatures (Campbell and Harris 1979). Assuming temperature and mean saturation deficit used for this model, weekly fecundity was 4392.388 eggs/female. Next, I calculated the transition rate from engorged adults to eggs ($a_{1\,12}$), this was done by taking the product of the survival rate of engorged females (0.9949; see engorged stages section) and the fecundity calculated by the equations from Mount and Haile (4392.388): 0.9949 * 4392.388 = 4369.9868 (Figure 36).

Analyses of the population projection matrix

I calculated: 1) the asymptotic population growth rate, 2) stable stage-class distribution, and 3) stage-specific reproductive values, as well as conducting sensitivity and elasticity analyses of the population projection matrix (Caswell 2000). The asymptotic population growth rate represents the finite weekly population growth rate. The stable stage-class distribution represents the proportion of the population in each stage class. The stage-specific reproductive value represents the contribution that a female in stage class will make to the future population.

The sensitivity analysis indicates how sensitive λ is to changes in population parameters (fecundity, growth, or survival rates). The elasticity (proportional sensitivity) analysis indicates how λ changes in response to proportional changes in each of the population parameters (Crouse et al. 1987).

RESULTS

Analyses of the population projection matrix yielded λ = 1.0931 ($r = log(\lambda)$ = 0.0387). Host seeking larvae were the most abundant and transferring males were the least abundant of all stages. The stable stage-class distribution showed high proportions of individuals in the host-

seeking larvae stage, followed by the stages that included eggs, host-seeking nymphs, and on-host larvae, with all other stages comprising less than 1% (Table 10). Analysis of the stage-specific reproductive values showed that engorged adult females contributed the most to the future population, followed by on-host adult females (Table 10). On-host nymphs, engorged nymphs, and host-seeking adults also had some potential to contributing to the future population (Table 10). The sensitivity analysis revealed three high values representing host-seeking to on-host for larvae, nymphs, and female adults (Figure 37). In addition, the self-loops of the host-seeking stages showed moderate sensitivity values (Figure 37). The elasticity analysis revealed that the self-loops of the host-seeking stages had the largest proportional influence on the population growth rate (Figure 38).

Table 10. Stable stage-class distribution and stage-specific reproductive values for *Dermacentor variabilis* population.

Stage #	Stage Class	Stable Stage-class Distribution	Stage-specific Reproductive values
1	Eggs	0.1001	1.0000
2	Host-Seeking Larvae	0.8154	1.1023
3	On-Host Larvae	0.0189	5.2161
4	Engorged Larvae	0.0069	14.2542
5	Host-Seeking Nymphs	0.0532	15.6831
6	On-Host Larvae	0.0012	79.9502
7	Engorged Nymphs	0.0050	218.4813
8	Host-Seeking Adults	0.0035	239.9169
9	On-Host Adults ♂	0.0001	0.0000
10	Transferring Adults ♂	0.0000	0.0000
11	On-Host Adults ♀	0.0001	1796.4160
12	Engorged Adults ♀	0.0000	3997.8421

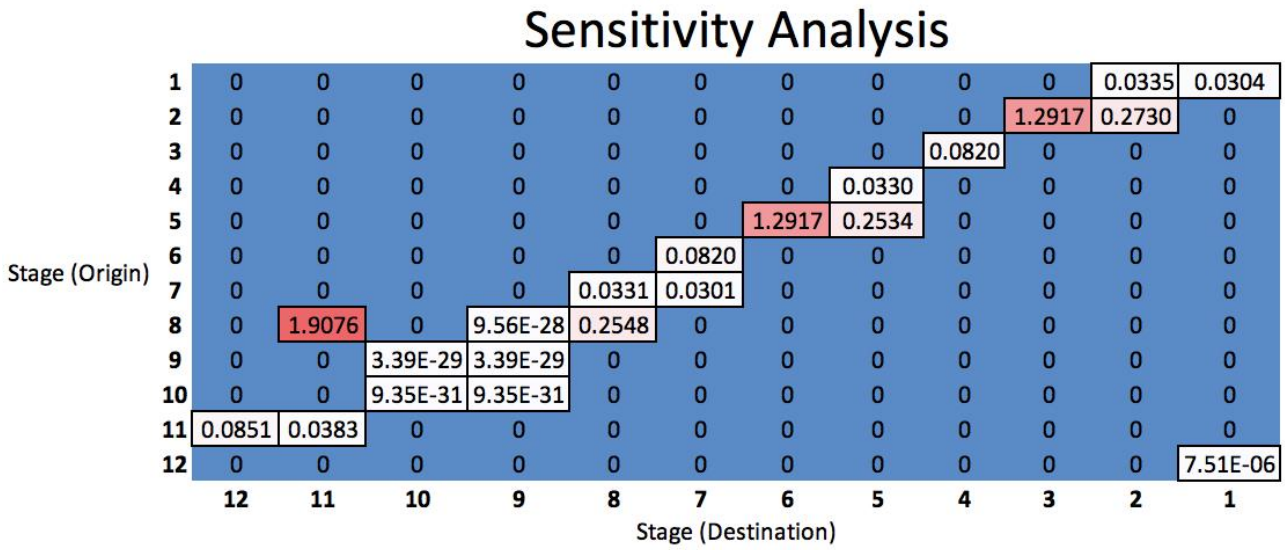

Figure 37. Sensitivity analysis values for the projection of the matrix model for *Dermacentor variabilis*. Host-seeking stages to on-host stages showed sensitivity to changes in the parameter indicated by darker red boxes. For stage-class names for each corresponding stage number refer to Figure 36.

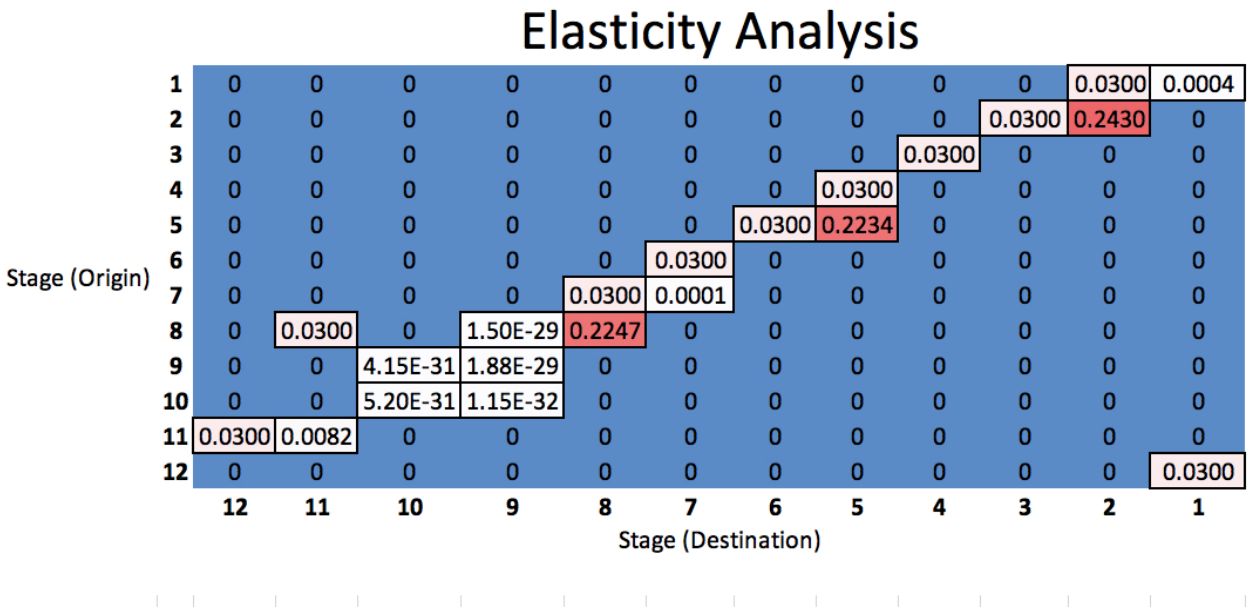

Figure 38. Elasticity analysis values for the projection of the matrix model for *Dermacentor variabilis*. The self-loops of the host-seeking stages had the largest elasticity values, indicated by darker red boxes, which have a large proportional influence the population growth of *D. variabilis*. For stage-class names for each corresponding stage number refer to Figure 36.

DISCUSSION

The results of this matrix model showed a low abundance of transferring males. This same pattern was seen in the previous chapter where transferring males were also low in abundance. This low abundance of transferring males may reflect real systems. Stiller et al. (1989) found that up 2.6% of male *D. andersoni* ticks placed on cattle transferred while Lysyk (2013) found that up 1.7% of male *D. andersoni* placed on cattle transferred hosts. Though the abundance of male transferring ticks may be low, they can still play a possible role in intrastadial transmission. Further work must be done to investigate intrastadial transmission of male ticks and what role these ticks play in the maintenance of pathogens.

The sensitivity analysis of matrix models are useful tools in conservation biology allowing one to make decisions on the best way to improve the status of a population by identifying which stages are the most vulnerable (Heppell et al. 2000). These models can also be used to make decisions on how to best manage and control pests and invasive species (Bock et al. 2016; Morris et al. 2011). In this model, the transition of host seeking stages to on host ticks, for all life stages of *D. variabilis,* was shown to be the most sensitive when this transition rate was changed thus affecting the population growth. The analysis indicated that these off-host stages of host-seeking ticks would be the best stages to target in the management and control of *D. variabilis*. Such management practices would involve habitat modification which could consist of periodic prescribed burns, clearing brush, and use of herbicides. Tick survival of these host-seeking ticks are dependent on the conditions of the microclimate such as humidity and temperature. This was the case in this model where survival rates of ticks were driven by temperature and the saturation deficit. Habitat modification has been shown to reduce tick densities but many of these methods spark a regrowth of vegetation that will attract mammalian herbivore hosts which can reintroduce ticks into the environment (Meyer et al. 1982; Sonenshine and Mather 1994). Animal control of hosts could also be used but may be costlier and harder to implement in large habitats with numerous hosts (Meyer et al. 1982). Further studies should look into modeling these control measures that target these off-host tick stages to see what affect they might have on the population of *D. variabilis*.

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CHAPTER VI

CONCLUSIONS AND FUTURE STUDIES

First I set out to investigate cross mating between *Amblyomma mixtum* and *A. tenellum,* conducted by allowing the following four combinations of adult ticks to blood feed on two calves: *A. mixtum* × *A. mixtum, A. tenellum* × *A. tenellum, A. mixtum* × *A. tenellum,* and *A. tenellum* × *A. mixtum*. Only one female of the *A. mixtum* × *A. tenellum* cross produced larvae and may have been due to parthenogenesis in the absence of mating, rather than hybridization. Further studies are warranted on *A. mixtum* to determine what role these parthenogenetic ticks might have in the dynamics of pathogen transmission and how common parthenogenesis is nature. The present study also allowed for the documentation of the laboratory development of each individual cross. Overall differences occurred among crosses for all comparisons with the mixed crosses being very different from the pure crosses. These differences illustrate what happens when female ticks do not find a potential mate; which in this study resulted in longer attachment, a reduced engorgement weight, and higher mortality. This study only tracked the development of *A. mixtum* and *A. tenellum* up to the larvae stage. Much remains unknown about the drop off period and molting period of the nymphs and the adults; future studies of these developmental periods must be conducted. Completion of this would provide a good understanding of the development of both *A. mixtum* and *A. tenellum,* and could aid in field studies, modeling or other endeavors that require their known life histories.

Second I set out to investigate the phylogenetic relationship of *A. mixtum, A. tenellum, A. americanum,* and *A. maculatum*. This was done by using three mitochondrial genes 12S ribosomal gene, 16S ribosomal gene, cytochrome oxidase I gene, and a nuclear gene the internal

transcribed spacer 2; as well as two concatenated datasets of the mitochondrial genes, and mitochondrial genes plus the one nuclear gene. Analyses employed three phylogenetic approaches: maximum parsimony, maximum likelihood and Bayesian. All gene topologies of the four *Amblyomma* species were similar except for that of the COI gene which showed poor resolution in branch support (gene tree discordance). Though the COI differed from the other genes the concatenated data sets showed little influence of the COI gene. Overall these phylogenetic analyses, using these genes and these taxa, revealed that *A. americanum* and *A. tenellum* are more closely related than they are to *A. mixtum* and *A. maculatum*. This study serves as a starting point for more robust studies. Ideally, more taxa of *Amblyomma* with more genes should be included to obtain a more thorough understanding of the relationships within *Amblyomma*. But how many genes and how informative these genes are, is a question for debate especially since phylogenetic analyses are moving toward using whole genomes. More data in phylogenomic analyses may lead to more non-phylogenetic signals leading to incorrect and misleading trees. Further investigations are required before accepting the accuracy of whole genome analyses.

Third I set out to investigate the role of transferring male ticks (*Dermacentor variabilis*) in the transmission and maintenance of *Theileria equi* by using an agent based model. This model showed: 1) that the number of infected horses were influenced by the infection probability, 2) adjustments of the infection probability and the male transfer probability affected the population of males that transferred and their role in the transmission of *T. equi*, and 3) both horses and ticks could maintain the pathogen even at low probabilities of male transfer and infection. The model showed that *D. variabilis* alone can sustain the pathogen in the simulated system but in nature other tick species, such as *Rhiphicephalus microplus*, may be involved in

the transmission of *T. equi*. Future modeling approaches must take on the task of a multi-species tick model with transmission of intrastadial adult males, as the landscapes of south Texas are home to potential vectors in the genera *Dermacentor*, *Amblyomma* and *Rhipicephalus*. But uncertainties still remain that should be resolved beforehand. The first is that, though mutual grooming has been well documented and described, no studies have determined a frequency in which this activity occurs, including influences of herd size and season. Second we do not have any data on the transmission rate of *T. equi*. Third, though we know which species of ticks occur on horses we do not know the frequency in which these species can be found on horses nor the tick burden horses could handle. Lastly male transfer of ticks has been poorly studied for any tick species. Conducting further studies to unravel these uncertainties will greatly help in understanding the role of male ticks in the transmission and maintenance of pathogens.

Last I set out to develop a population matrix model for *D. variabilis* that incorporated the life history of transferring male ticks. This matrix model showed a low abundance of transferring males that may reflect real systems. Though the abundance of males transferring from host to host may be low, they can still play a role in intrastadial transmission of *T. equi* and the system maintenance of this pathogen. Sensitivity analyses revealed that the host-seeking stages to on-host ticks for larvae, nymphs, and adults were the most vulnerable to changes in the transition rate. These stages are the best to target in the management and control through habitat modification. Tick survival of these host-seeking ticks is dependent on the conditions of the habitat microclimate, thus if habitat modification were made it would lower the survival of ticks reducing the population of *D. variabilis*. But uncertainties remain, how frequent and how best to manage this, and is one technique or multiple techniques better. Habitat modification alone may not be the sole solution, as these methods spark a regrowth in vegetation that attracts herbivore

hosts which can reintroduce ticks back into the environment. One approach would be an integrated tick management program that uses strategies that include numerous habitat management tactics rather than one that align with the overall land management goals of the property and incorporate a comprehensive program involving tick suppression both in the environment and on animals. The studies presented in this dissertation are only the beginning and more research must be conducted on these topics which will likely lead to more questions to be answered.

APPENDIX A

MODEL EQUATIONS

Model equations that represent the effect of environmental conditions on off-host tick development and survival rates for each tick stage in each of the three habitats, rates of host-seeking ticks, and fecundity rates. (Adapted from Mount and Haile 1989).

1. Fecundity = $(eggs/engorged\ female)$
    - $F = -11588.3 + 1278.87 \times T - 23.4181 \times T^2$
2. Eggs
    - Survival rates for three habitats:
        i. $S1_E = \begin{bmatrix} Mesquite = 0.9825581 \\ Mixed\ Brush = 0.9592828 \\ Grassland = 0.9171487 \end{bmatrix}$
    - Survival rate effects for eggs (SRE_E); T = temperature; SD = saturation deficit
        i. $SRE = (-0.00024770 \times T^2 + 0.010899 \times T + 0.88011) \times (-0.0001108 \times SD^2 + 0.00008137 \times SD + 1)$
3. Host-Seeking Larvae
    - Survival rates for three habitats:
        i. $1 - 40\ weeks\ old \quad S1_{HSL} = \begin{bmatrix} Mesquite = 0.9969413 \\ Mixed\ Brush = 0.9921140 \\ Grassland = 0.9783304 \end{bmatrix}$
        ii. $> 40\ weeks\ old \quad S2_{HSL} = \begin{bmatrix} Mesquite = 0.9311392 \\ Mixed\ Brush = 0.9267952 \\ Grassland = 0.9074803 \end{bmatrix}$
    - Survival rate effects for host seeking larvae (SRE_{HSL})
        i. $1 - 40\ weeks\ old \quad SRE_{S1\,HSL} = (-0.00006858 \times T^2 + 0.003014 \times T + 0.966846) \times (-0.00004315 \times SD^2 + 0.00017529 \times SD + 0.9998625)$
        ii. $>\ 40\ weeks\ old \quad SRE_{S2\,HSL} = (-0.0000614 \times T^2 + 0.002712 \times T + 0.970168) \times (-0.00007178 \times SD^2 + 0.00048075 \times SD + 0.9992038)$
4. Host-Finding Rate for Host Seeking Larvae
    - DL = Daylength
    - $H_L = (-0.00818182 \times T^2 + 0.2454546 \times T - 0.8409091) \times (-0.02153798 \times DL^4 + 1.082504 \times DL^3 - 20.22092 \times DL^2 + 166.5624 \times DL - 510.8353)$
5. On Host Larvae Survival
    - D = density of ticks/host
    - When $D < 10$: $S_L = 0.55$
    - When $10 \leq D < 40$: $S_L = 0.40$
    - When $40 \leq D$: $S_L = 0.25$

6. Engorged Larvae
    - Survival rates for three habitats:
        i. $SE_L = \begin{bmatrix} Mesquite = 0.9835758 \\ Mixed\ Brush = 0.9631911 \\ Grassland = 0.9193043 \end{bmatrix}$
    - Survival rate effects for host seeking larvae (SE_L)
        i. $SRE_L = (-0.00021692 \times T^2 + 0.0095445 \times T + 0.8950105) \times (-0.00012258 \times SD^2 + 0.00024933 \times SD + 1)$
    - Cumulative degree-weeks: $CDW \geq 42$
    - Minimum developmental threshold temperature: $DT = 9$
    -
7. Host-Seeking Nymphs
    - Survival rates for three habitats:
        i. $1 - 40\ weeks\ old \quad S1_{HSN} = \begin{bmatrix} Mesquite = 0.9969413 \\ Mixed\ Brush = 0.9921140 \\ Grassland = 0.9783304 \end{bmatrix}$
        ii. $> 40\ weeks\ old \quad S2_{HSN} = \begin{bmatrix} Mesquite = 0.9311392 \\ Mixed\ Brush = 0.9267952 \\ Grassland = 0.9074803 \end{bmatrix}$
    - Survival rate effects for host seeking nymphs (SRE_{HSN})
        i. $1 - 40\ weeks\ old \quad SRE_{S1\ HSN} = (-0.00007532 \times T^2 + 0.003314 \times T + 0.963546) \times (-0.0000814 \times SD^2 + 0.00052552 \times SD + 0.9991561)$
        ii. $> 40\ weeks\ old \quad SRE_{S2\ HSN} = (-0.00002279 \times T^2 + 0.010028 \times T + 0.889692) \times (-0.00019869 \times SD^2 + 0.00112314 \times SD + 0.9984188)$
8. Host-Finding Rate for Host-Seeking Nymphs
    - $H_N = (-0.00818182 \times T^2 + 0.2454546 \times T - 0.8409091) \times (-0.02153798 \times DL^4 + 1.082504 \times DL^3 - 20.22092 \times DL^2 + 166.5624 \times DL - 510.8353)$
9. On Host Nymphs
    - When $D < 1$: $S_N = 0.55$
    - When $1 \leq D < 4$: $S_N = 0.40$
    - When $4 \leq D$: $S_N = 0.25$
10. Engorged Nymphs
    - Survival rates for three habitats:
        i. $SE_N = \begin{bmatrix} Mesquite = 0.9830865 \\ Mixed\ Brush = 0.9711609 \\ Grassland = 0.9366675 \end{bmatrix}$
    - Survival rate effects for host seeking adults (SRE_{EA})
        i. $SRE_{EN} = (-0.00012692 \times T^2 + 0.0055845 \times T + 0.9385705) \times (-0.00010876 \times SD^2 + 0.00045429 \times SD + 0.999616)$
    - $CDW \geq 42$
    - $DT = 9$
11. Host Seeking Adults
    - Survival rates for three habitats:

i. $1-60\ weeks\ old\quad S1_{HSA}=\begin{bmatrix} Mesquite=0.9992437 \\ Mixed\ Brush=0.9982923 \\ Grassland=0.9910756 \end{bmatrix}$

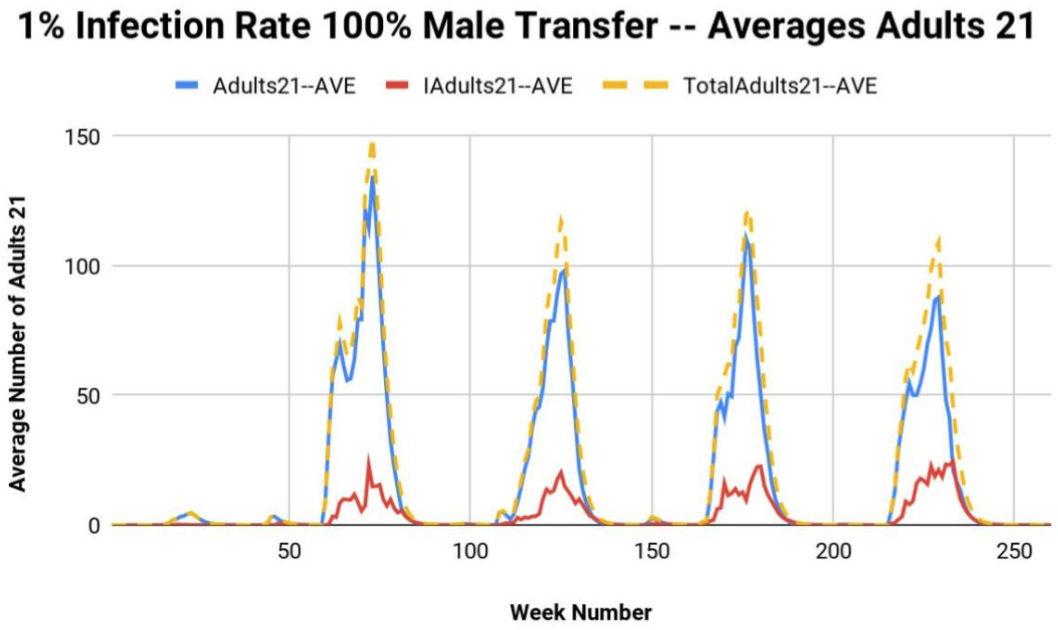
- Survival rate effects for host seeking larvae (SRE_{HSL})
  i. $1-60\ weeks\ old\quad SRE_{S1\,HSA}=(-0.0000135\times T^2+0.000594\times T+0.993466)\times(-0.00002997\times SD^2+0.000245\times SD+0.9995346)$

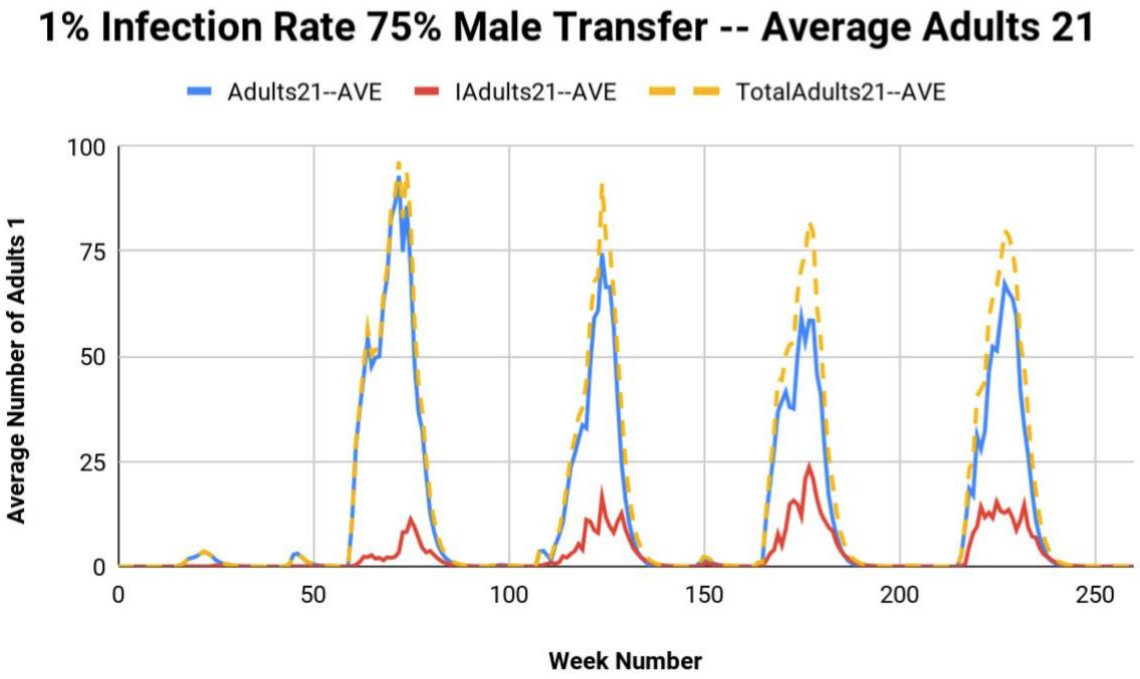
12. Host-Finding Rate for Host-Seeking Adults
    - $H_A=(-0.00505102\times T^2+0.24323469\times T-1.67199)\times(-0.02427784\times DL^4+1.230494\times DL^3-23.20363\times DL^2+193.1549\times DL-599.3686)$
13. On Host Adults
    - When $D<5$: $S_A=0.7416$
    - When $5\le D<20$: $S_A=0.6208$
    - When $20\le D$: $S_A=0.5$
14. Engorged Adults
    - Survival rates for three habitats:
      i. $SE_A=\begin{bmatrix} Mesquite=0.9882457 \\ Mixed\ Brush=0.9734650 \\ Grassland=0.9315355 \end{bmatrix}$
    - Survival rate effects for host seeking adults (SRE_{EA})
      i. $SRE_{EA}=(-0.00015730\times T^2+0.006921\times T+0.923869)\times(-0.00013086\times SD^2+0.0005243\times SD+0.9996048)$
    - $CDW\ge 15$
    - $DT=9$

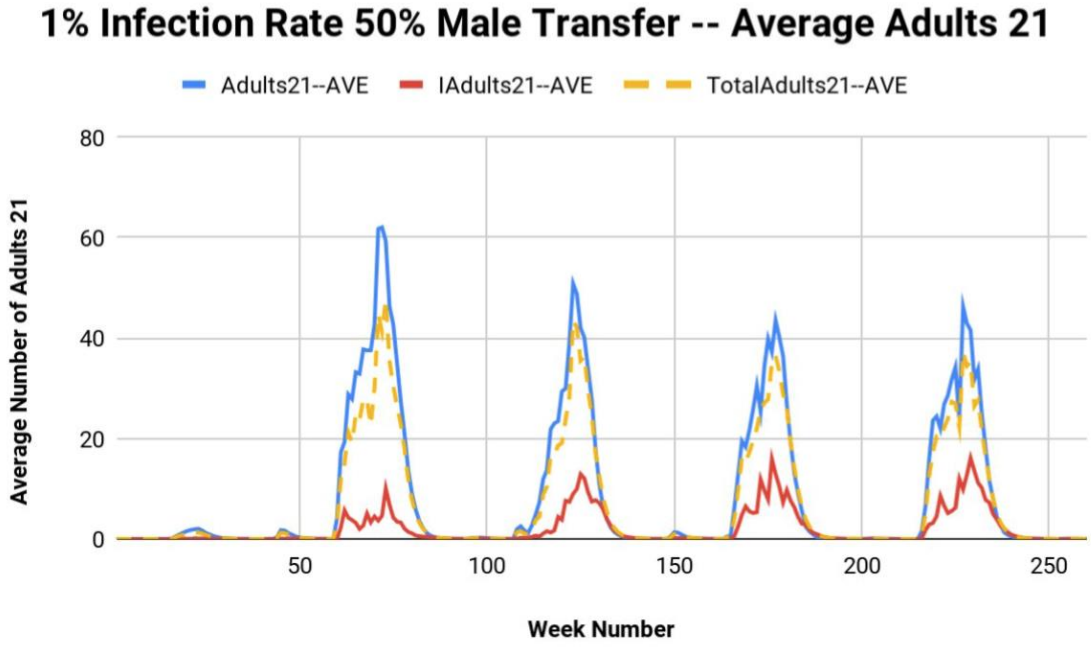
APPENDIX B

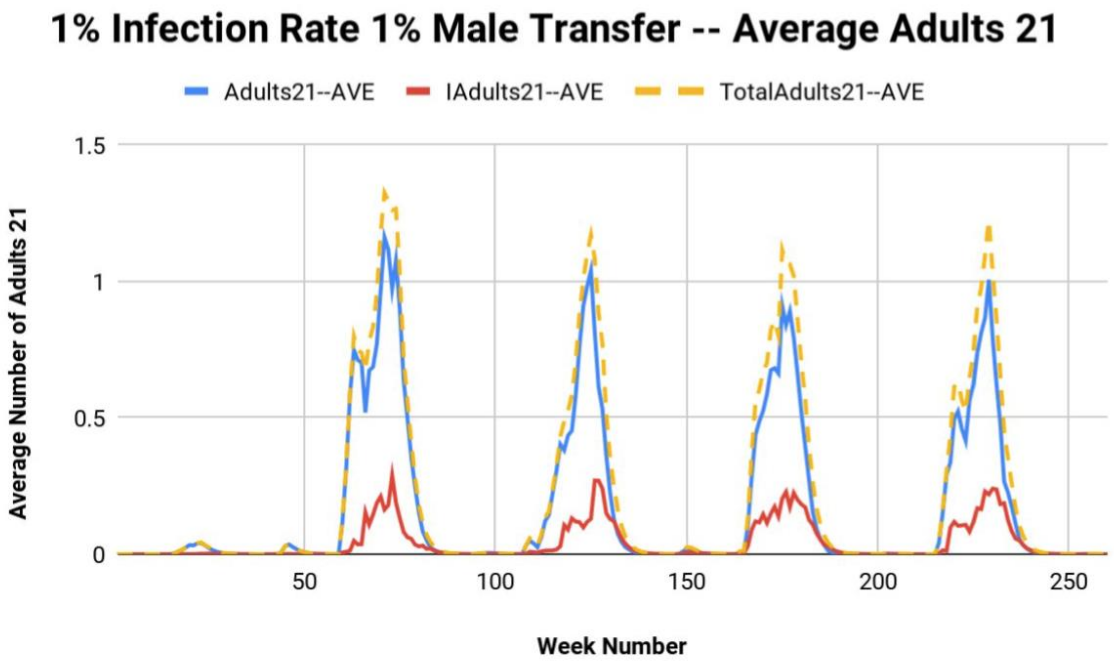
FIGURES OF SIMULATION RESULTS FOR THE AVERAGE TOTAL NUMBER OF TRANSFERRING MALES

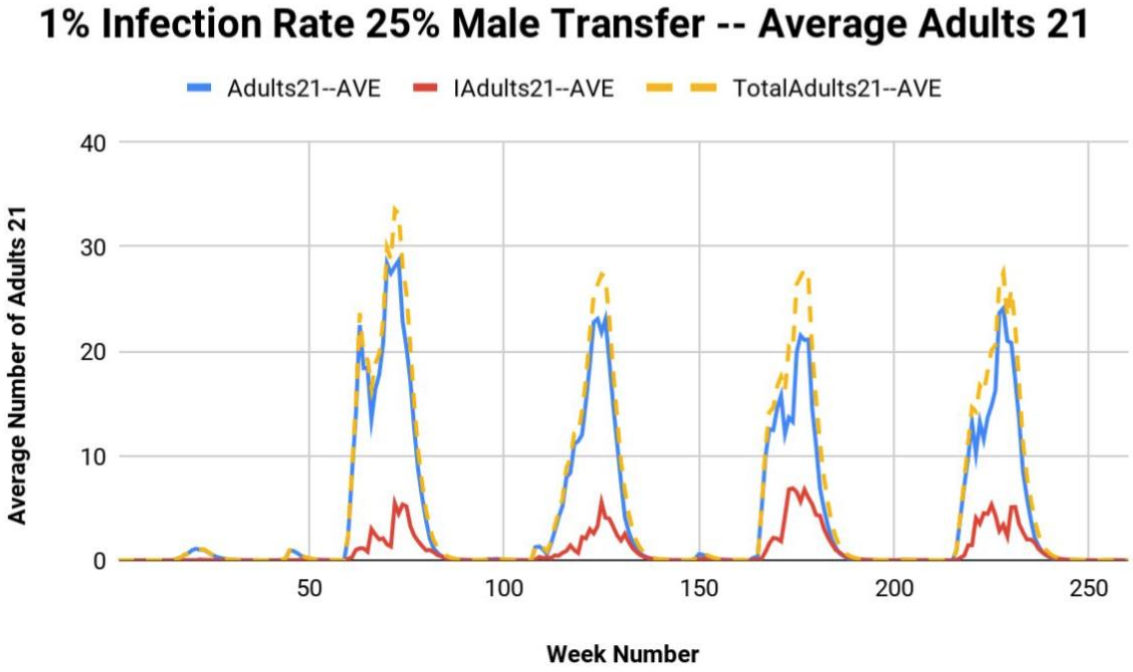
Simulation results of the average total number of transferring (TotalAdults21; Yellow) that are both non-infective (Adults21-AVE; Blue) and infective (IAdults21-Ave; Red). At combinations of infection probabilities (1%, 0.5%, 0.25%, and 0.1%) and male transfer probabilities (100%, 75%, 50%, 25%, 1%).

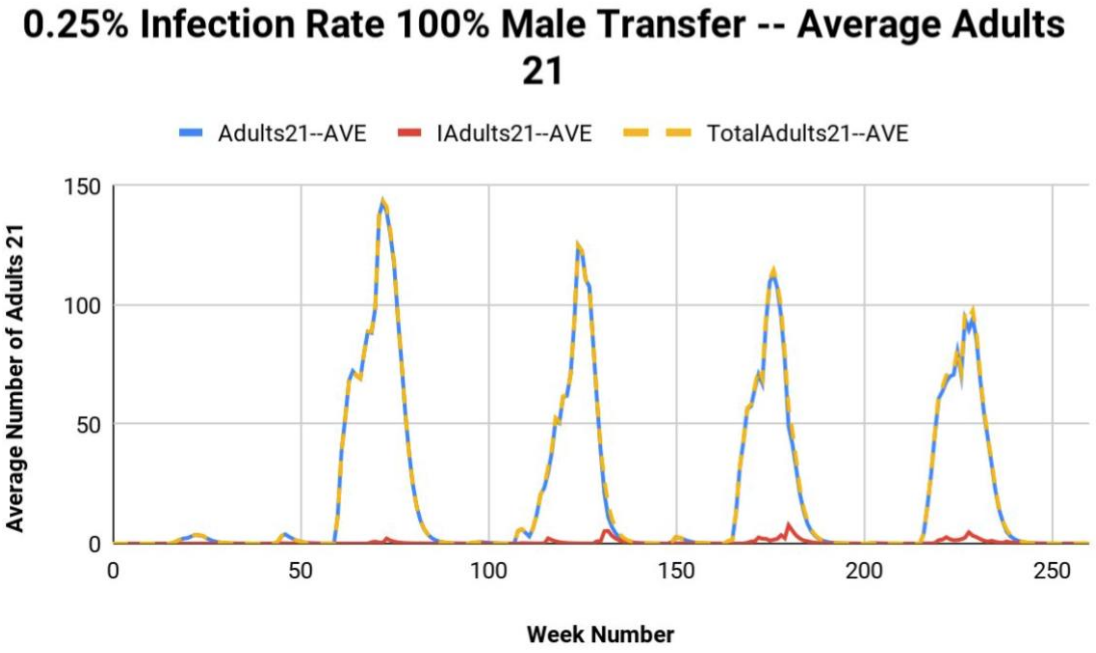



1% Infection Rate 75% Male Transfer -- Average Adults 21
Adults21--AVE
IAdults21--AVE
TotalAdults21--AVE
Average Number of Adults 1
100
75
50
25
0
0
50
100
150
200
250
Week Number


1% Infection Rate 50% Male Transfer -- Average Adults 21
Adults21--AVE
IAdults21--AVE
TotalAdults21--AVE
Average Number of Adults 21
80
60
40
20
0
50
100
150
200
250
Week Number


1% Infection Rate 1% Male Transfer -- Average Adults 21
Adults21--AVE
IAdults21--AVE
TotalAdults21--AVE
Average Number of Adults 21
1.5
1
0.5
0
50
100
150
200
250
Week Number


1% Infection Rate 25% Male Transfer -- Average Adults 21
Adults21--AVE
IAdults21--AVE
TotalAdults21--AVE
Average Number of Adults 21
40
30
20
10
0
50
100
150
200
250
Week Number


0.25% Infection Rate 100% Male Transfer -- Average Adults 21
Adults21--AVE
IAdults21--AVE
TotalAdults21--AVE
Average Number of Adults 21
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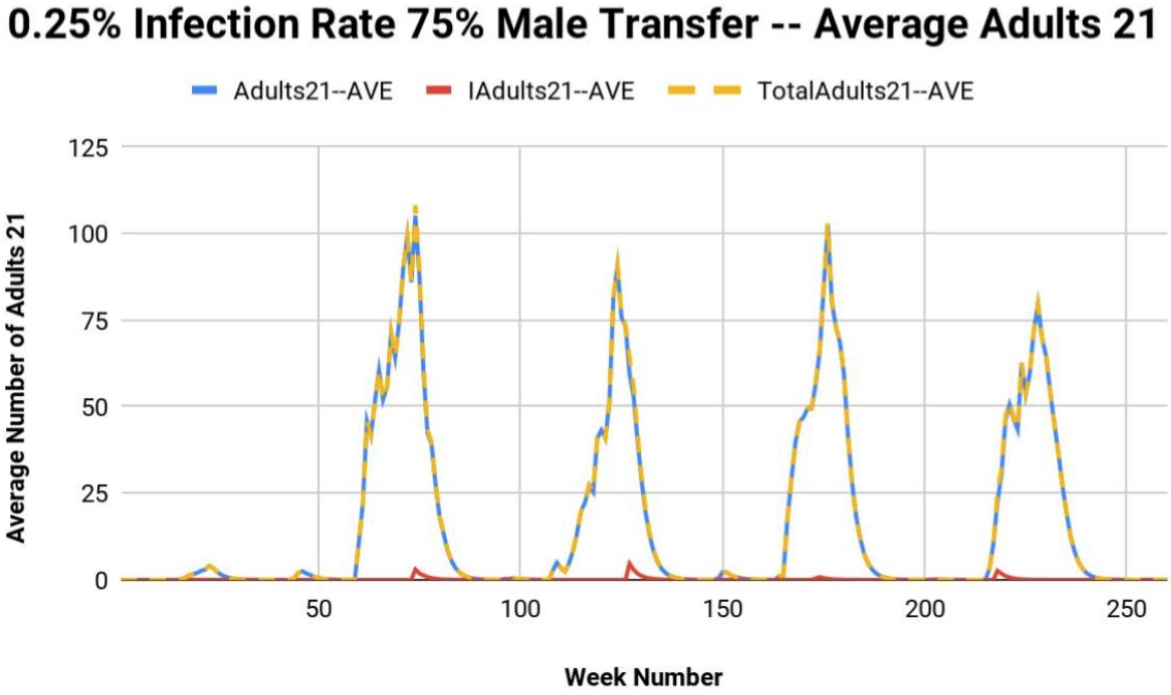
100
50
0
0
50
100
150
200
250
Week Number


0.25% Infection Rate 75% Male Transfer -- Average Adults 21
Adults21--AVE
IAdults21--AVE
TotalAdults21--AVE
Average Number of Adults 21
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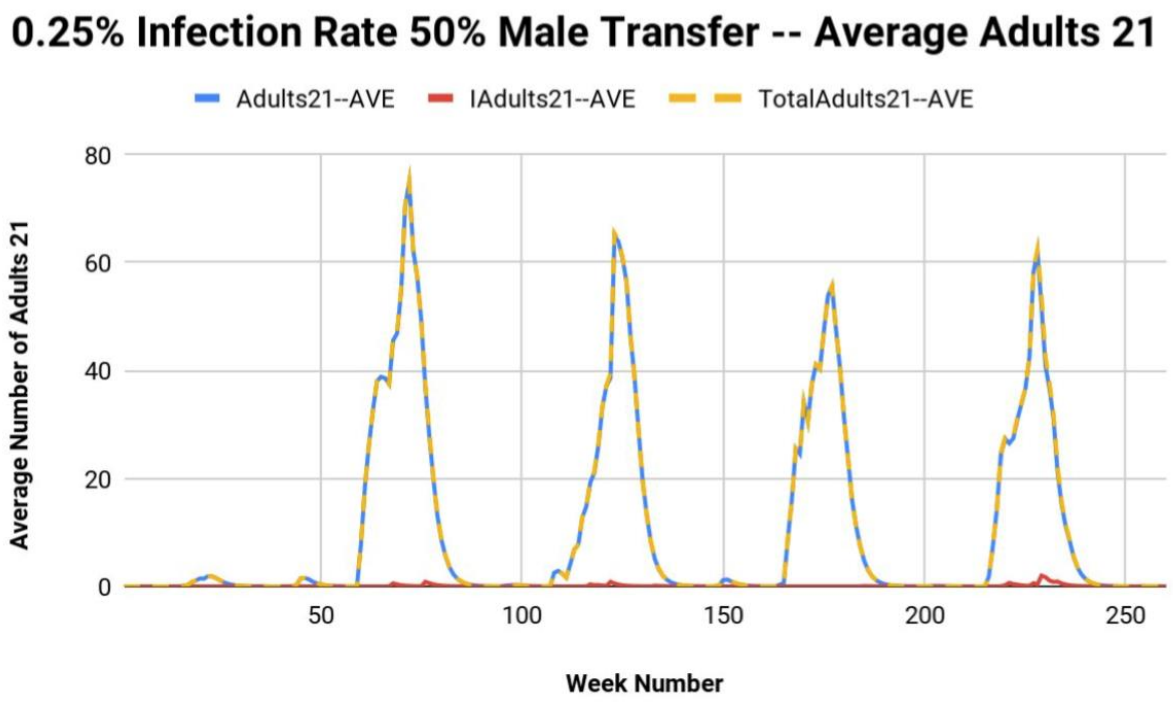
100
75
50
25
0
50
100
150
200
250
Week Number


0.25% Infection Rate 50% Male Transfer -- Average Adults 21
Adults21--AVE
IAdults21--AVE
TotalAdults21--AVE
Average Number of Adults 21
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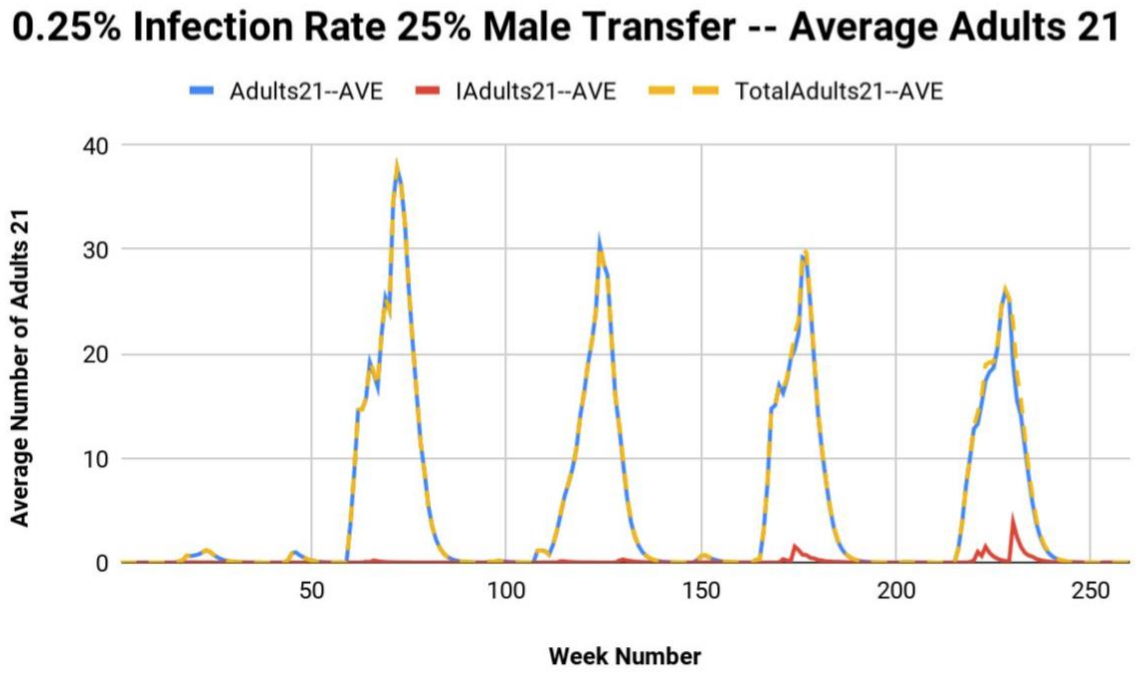
20
0
50
100
150
200
250
Week Number


0.25% Infection Rate 25% Male Transfer -- Average Adults 21
Adults21--AVE
IAdults21--AVE
TotalAdults21--AVE
Average Number of Adults 21
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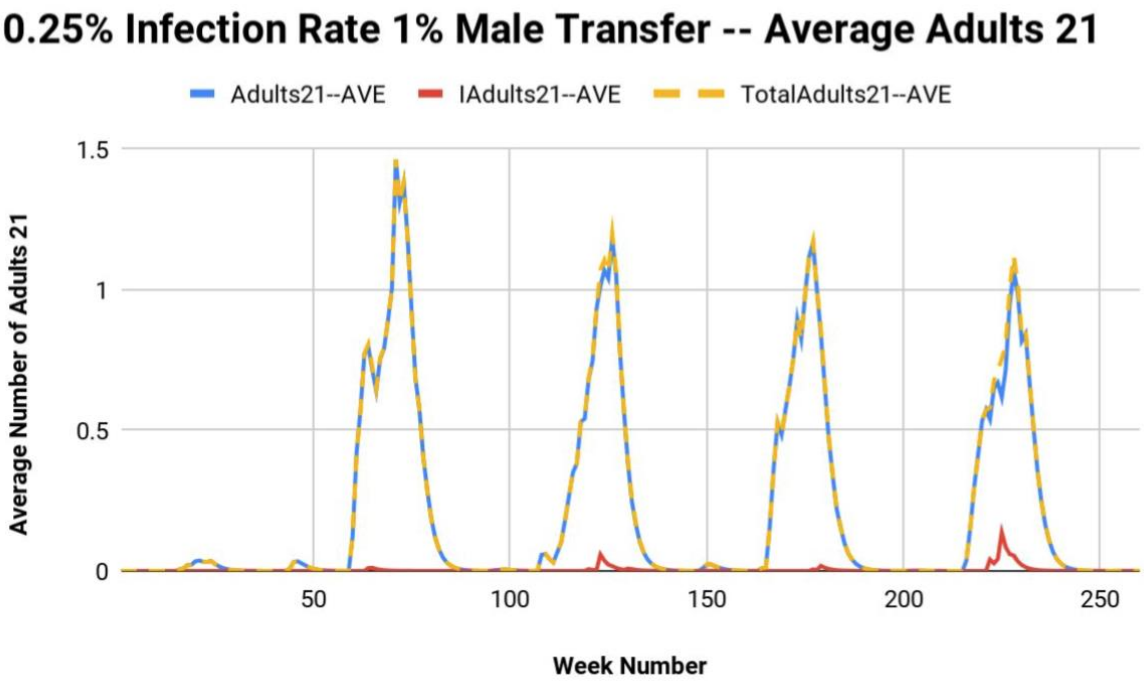
30
20
10
0
50
100
150
200
250
Week Number


0.25% Infection Rate 1% Male Transfer -- Average Adults 21
Adults21--AVE
IAdults21--AVE
TotalAdults21--AVE
Average Number of Adults 21
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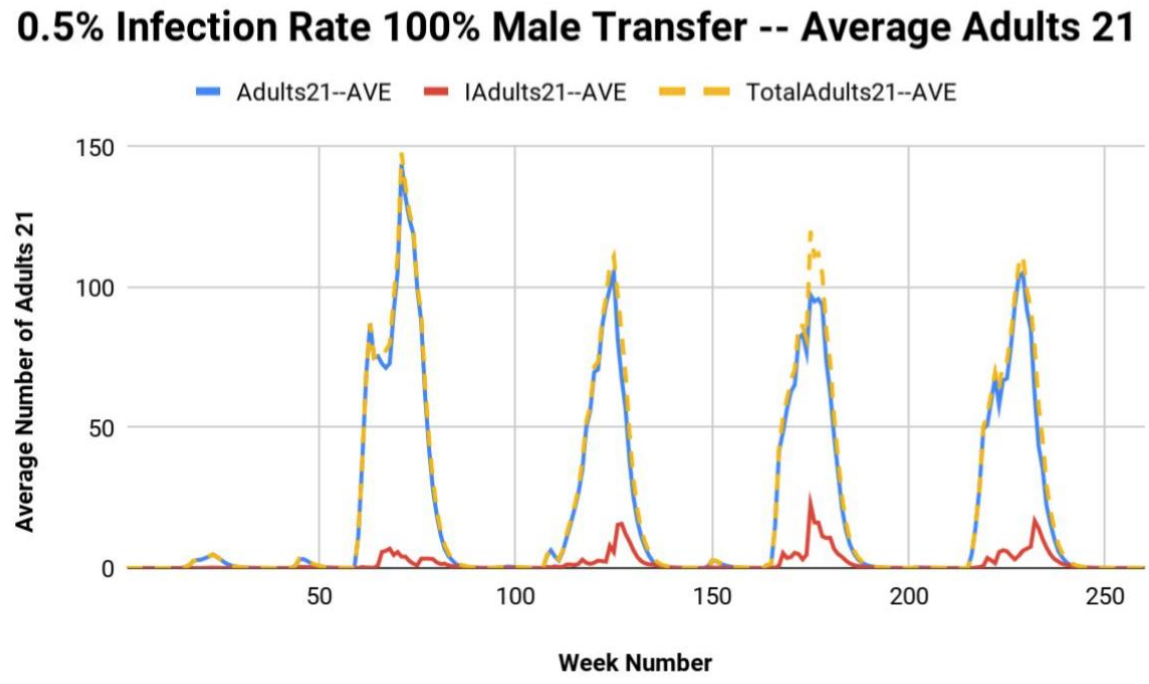
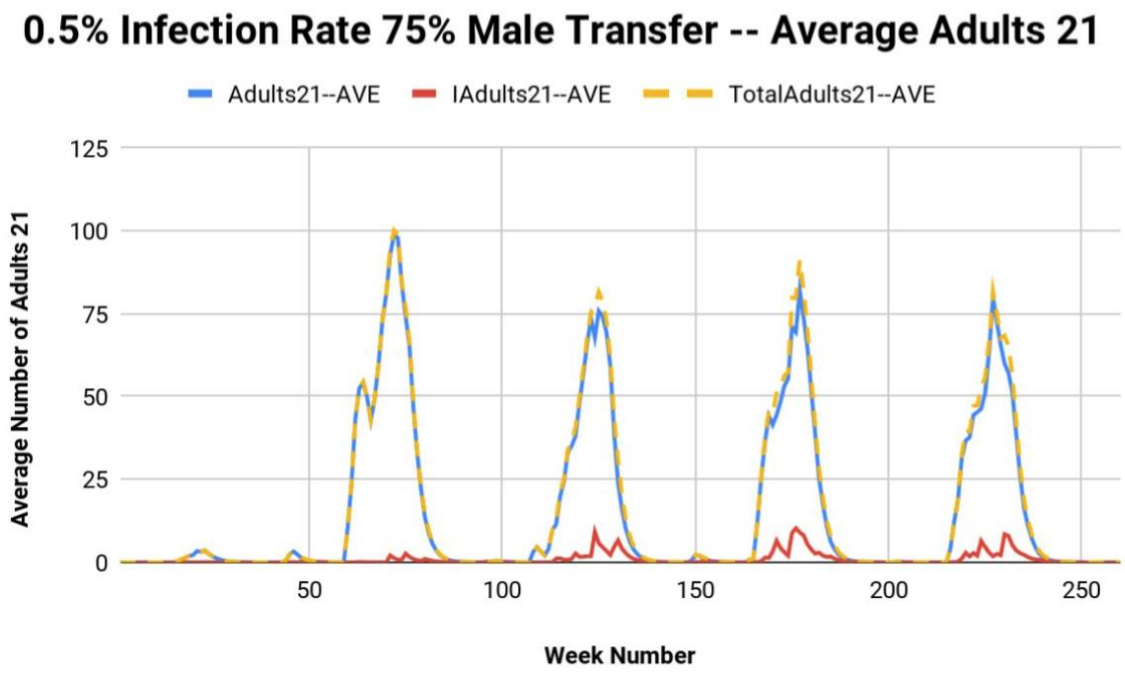
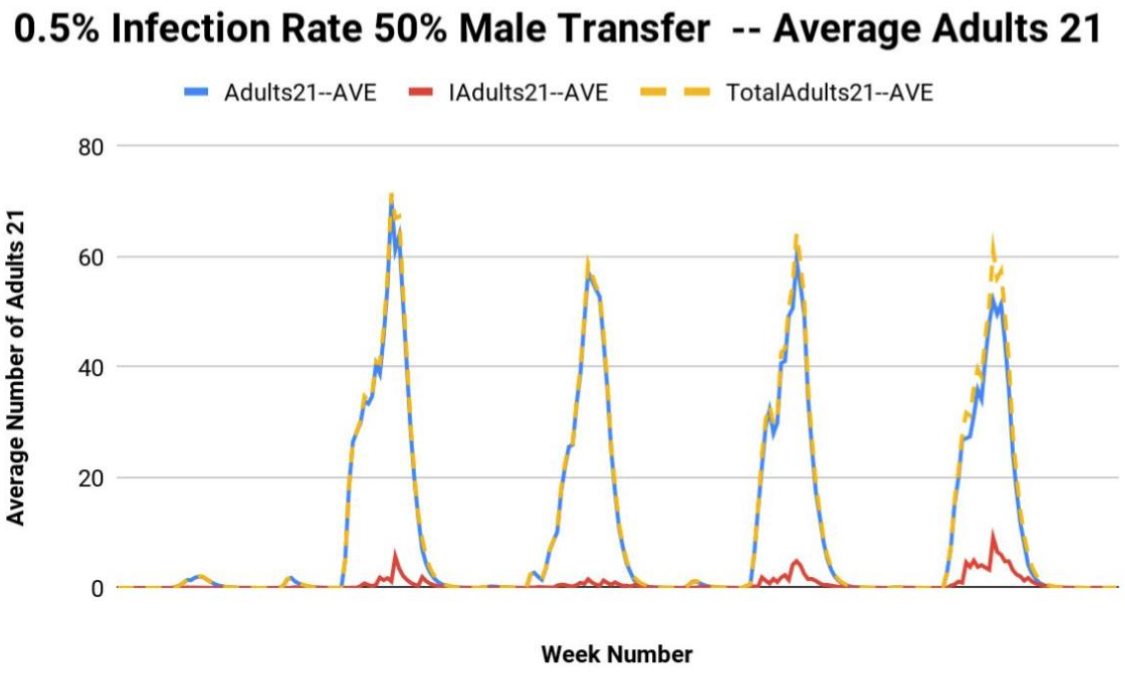
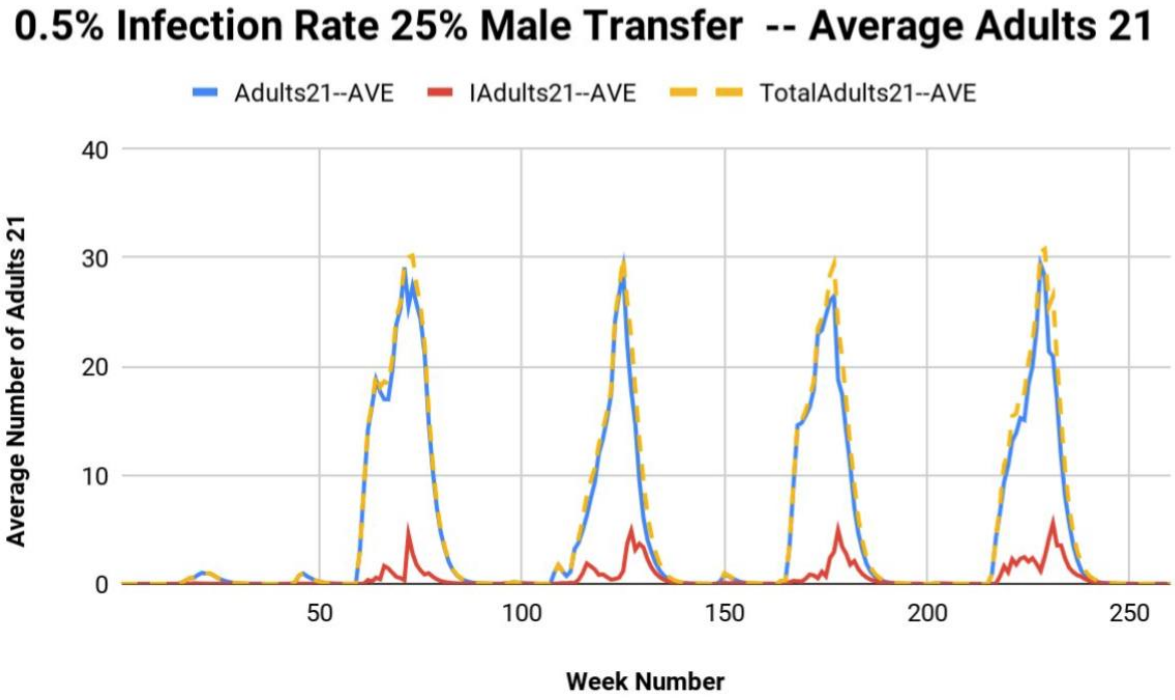
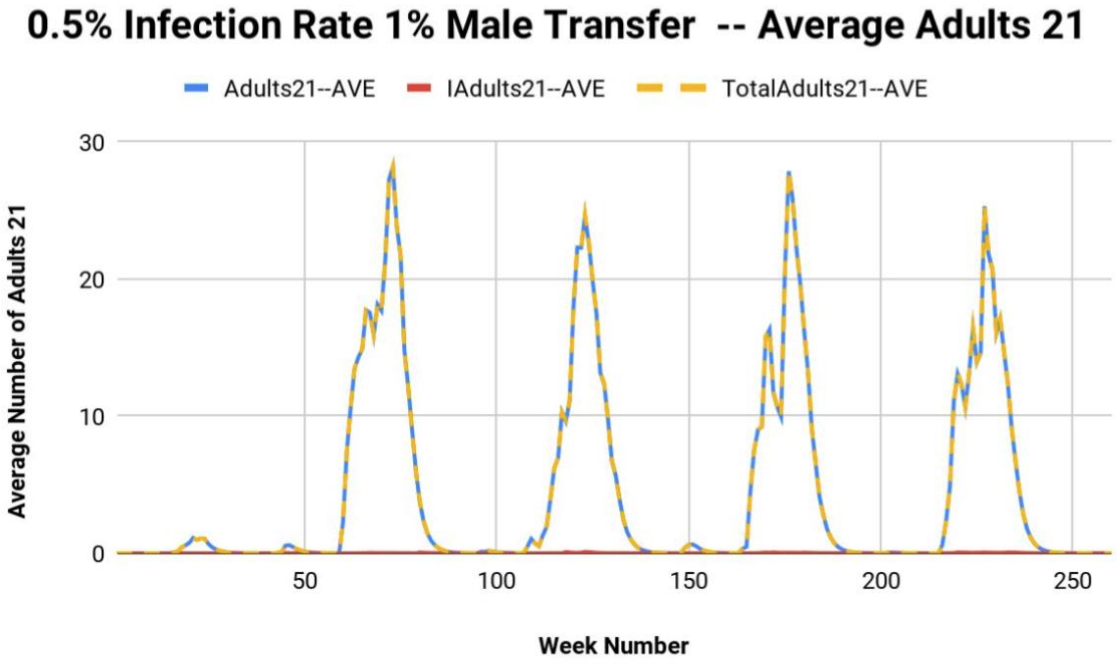
1
0.5
0
50
100
150
200
250
Week Number


0.5% Infection Rate 100% Male Transfer -- Average Adults 21
Adults21--AVE
IAdults21--AVE
TotalAdults21--AVE
Average Number of Adults 21
150
100
50
0
50
100
150
200
250
Week Number


0.5% Infection Rate 75% Male Transfer -- Average Adults 21
Adults21--AVE
IAdults21--AVE
TotalAdults21--AVE
Average Number of Adults 21
125
100
75
50
25
0
50
100
150
200
250
Week Number


0.5% Infection Rate 50% Male Transfer -- Average Adults 21
Adults21--AVE
IAdults21--AVE
TotalAdults21--AVE
Average Number of Adults 21
80
60
40
20
0
Week Number


0.5% Infection Rate 25% Male Transfer -- Average Adults 21
Adults21--AVE
IAdults21--AVE
TotalAdults21--AVE
Average Number of Adults 21
40
30
20
10
0
50
100
150
200
250
Week Number


0.5% Infection Rate 1% Male Transfer -- Average Adults 21
Adults21--AVE
IAdults21--AVE
TotalAdults21--AVE
Average Number of Adults 21
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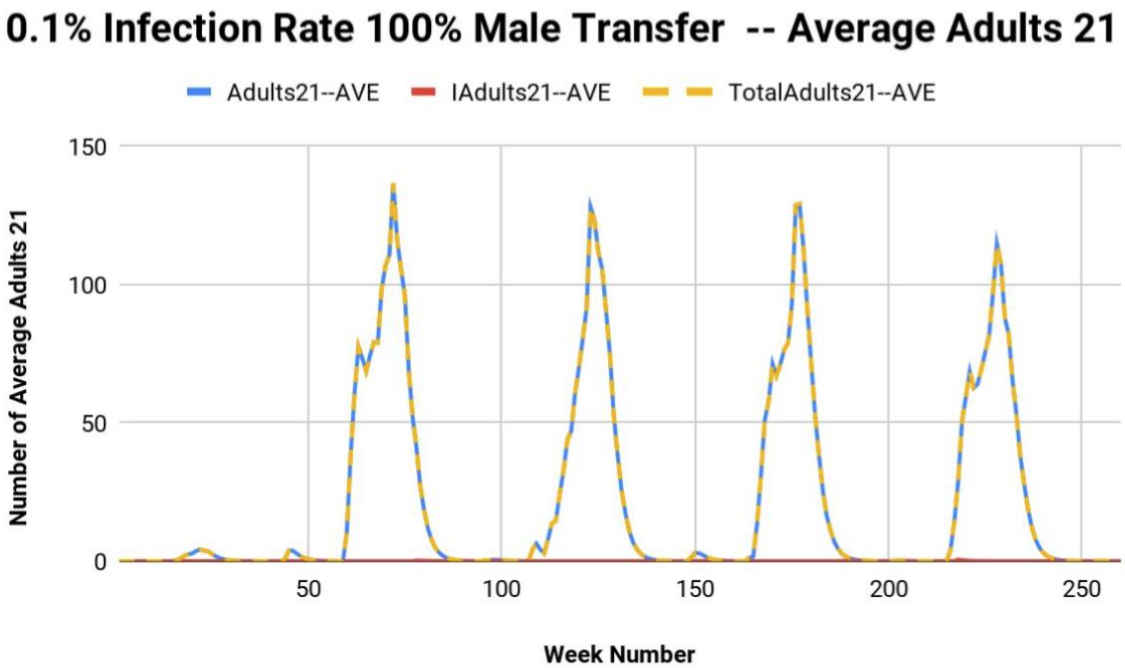
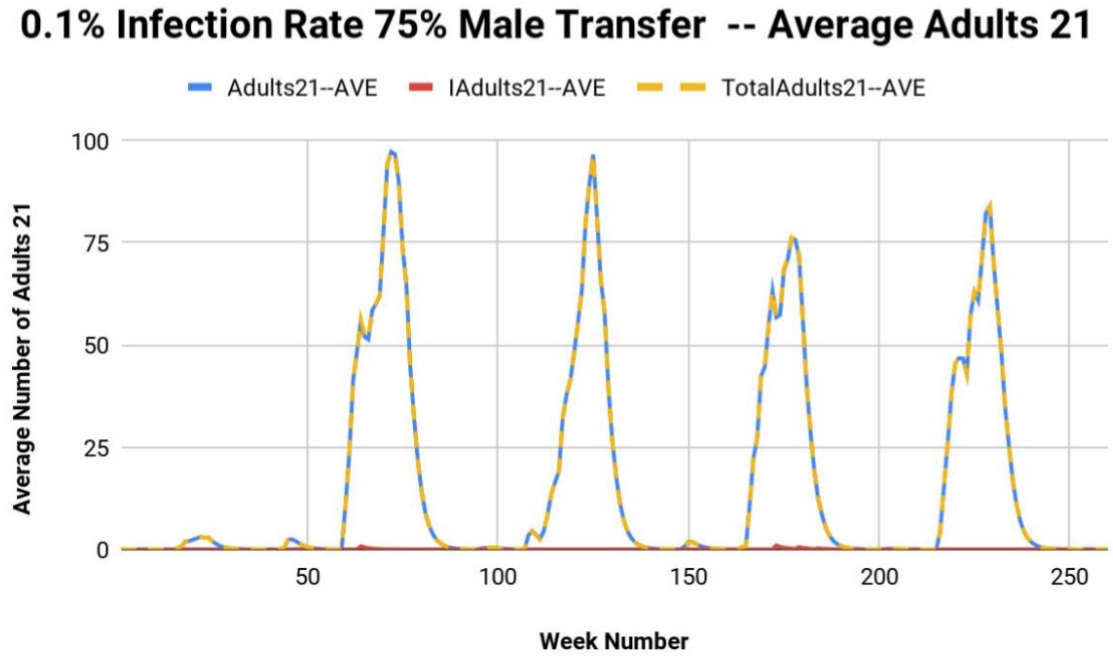
20
10
0
50
100
150
200
250
Week Number


0.1% Infection Rate 100% Male Transfer -- Average Adults 21
Adults21--AVE
IAdults21--AVE
TotalAdults21--AVE
Number of Average Adults 21
150
100
50
0
50
100
150
200
250
Week Number


0.1% Infection Rate 75% Male Transfer -- Average Adults 21
Adults21--AVE
IAdults21--AVE
TotalAdults21--AVE
Average Number of Adults 21
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75
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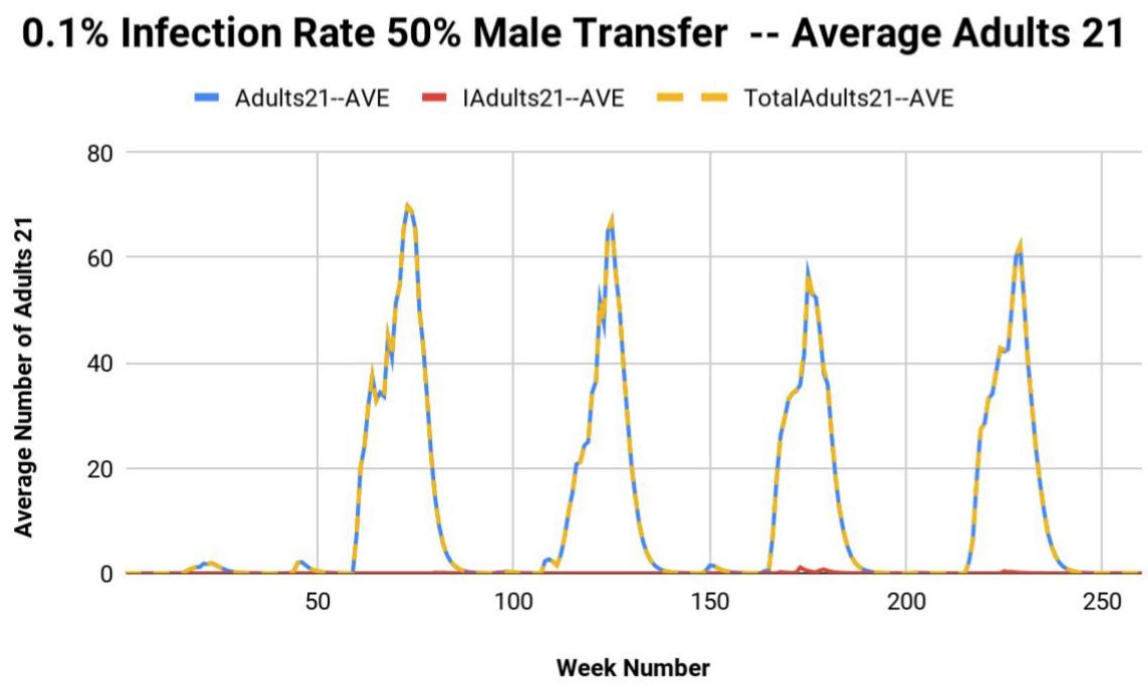
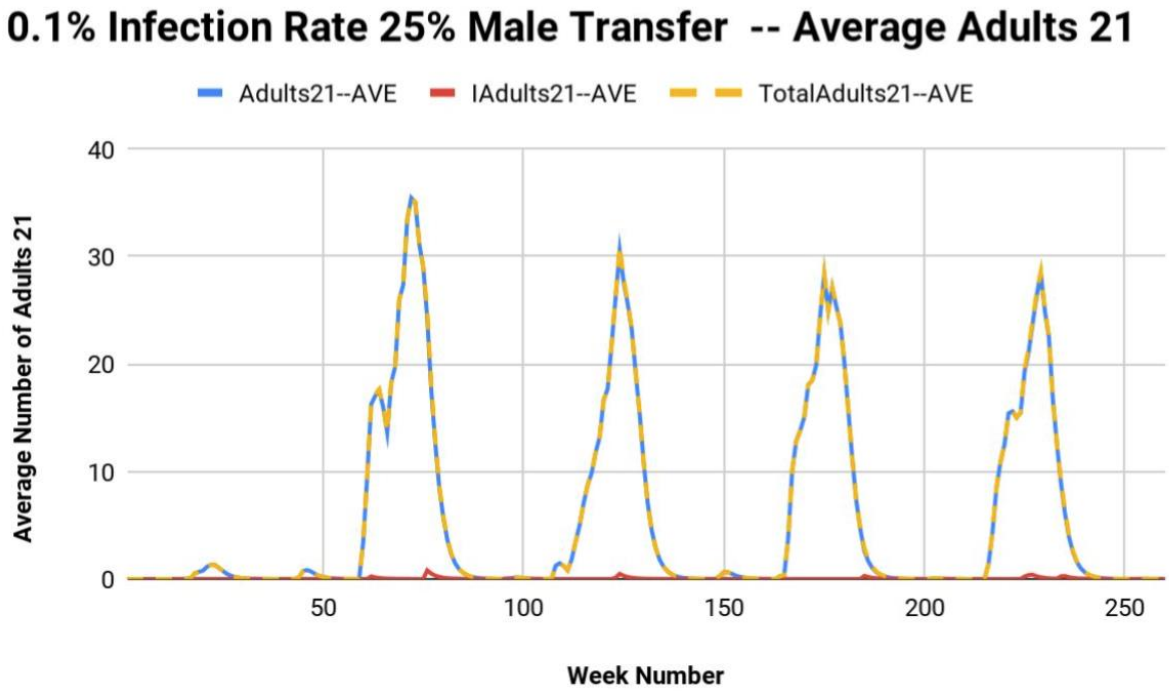
25
0
50
100
150
200
250
Week Number


0.1% Infection Rate 50% Male Transfer -- Average Adults 21
Adults21--AVE
IAdults21--AVE
TotalAdults21--AVE
Average Number of Adults 21
80
60
40
20
0
50
100
150
200
250
Week Number


0.1% Infection Rate 25% Male Transfer -- Average Adults 21
Adults21--AVE
IAdults21--AVE
TotalAdults21--AVE
Average Number of Adults 21
40
30
20
10
0
50
100
150
200
250
Week Number