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Reproductive incompatibility between *Amblyomma maculatum* Koch (Acari: Ixodidae) group ticks from two disjunct geographical regions within the United States

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Abstract

The *Amblyomma maculatum* Koch group of ixodid ticks consists of three species: *A. maculatum*, *A. triste*, and *A. tigrinum*. However, since Koch described this group in 1844, the systematics

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Conflicts of interest/Competing interests

The authors declare no conflicts of interest or competing interests.

Declarations

Ethics approval

All animal studies were performed according to the protocol 2904LEVRABC approved by the Institutional Animal Care and Use Committee (IACUC) of the Centers for Disease Control and Prevention.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

Not applicable.

Code availability

Not applicable.

of its members has been the subject of ongoing debate. This is especially true of *A. maculatum* and *A. triste*; recent molecular analyses reveal insufficient genetic divergence to separate these as distinct species. Further confounding this issue is the discovery in 2014 of *A. maculatum* group ticks in southern Arizona, USA that share morphological characteristics with both *A. triste* and *A. maculatum*. To biologically evaluate the identity of *Amblyomma maculatum* group ticks from southern Arizona, we analyzed the reproductive compatibility between specimens of *A. maculatum* group ticks collected from Georgia and southern Arizona, USA. Female ticks from both Arizona and Georgia were mated with males from both the Georgia and Arizona *Amblyomma* populations, creating 2 homologous and 2 heterologous F1 cohorts of ticks: GA ♀/GA ♂, AZ ♀/AZ ♂, GA ♀/AZ ♂, and AZ ♀/GA ♂. Each cohort was maintained separately into the F2 generation with F1 females mating only with F1 males from their same cohort. Survival and fecundity parameters were measured for all developmental stages. The observed survival parameters for heterologous cohorts were comparable to those of the homologous cohorts through the F1 generation. However, the F1 heterologous females produced F2 egg clutches that did not hatch, thus indicating that the Arizona and Georgia populations of *A. maculatum* group ticks tested here represent different biological species.

Keywords

Amblyomma maculatum ; *Amblyomma triste* ; *Rickettsia parkeri* ; Arizona; hybridization

Introduction

The taxonomic relationships among the *Amblyomma maculatum* tick group have been contested and dynamic for more than 175 years since Koch initially described several species belonging to this group (Koch 1844, Kohls 1956). Former members include *Amblyomma rubripes* Koch, 1844, *Amblyomma ovatum* Koch, 1844, *Amblyomma parvitarsum* Neumann, 1901, *Amblyomma neumanni* Ribaga, 1902, and multiple other taxa which have since been collapsed in synonymy (Kohls 1956, Camicas et al. 1998). The most recent analysis defines only three valid species: *A. maculatum* Koch, 1844, *Ambylomma triste* Koch, 1844, and *Amblyomma tigrinum* Koch, 1844 (Estrada-Peña et al. 2005). While *A. tigrinum* is morphologically distinct, *A. triste* and *A. maculatum* are extremely difficult to separate morphologically and are distinguished by relatively few diagnostic features, though geographical data has historically proven useful in separating these species due to their largely allopatric distributions (Estrada-Peña et al. 2005, Mertins et al. 2010).

Amblyomma maculatum are aggressive human biters and are the primary vectors in the United States of *Rickettsia parkeri*, the causative agent of an emerging rickettsiosis that causes a disease of humans similar to but typically milder than that caused by *Rickettsia rickettsii*, the causative agent of Rocky Mountain spotted fever (RMSF) (Paddock et al. 2008, Teel et al. 2010, Paddock and Goddard 2015). To date, no known fatalities have been reported from infection with *R. parkeri*. All stages of *A. maculatum* will parasitize humans (Goddard 2002, Paddock and Goddard 2015, Portugal and Goddard 2016), and *R. parkeri* infects adult *A. maculatum* at frequencies that generally range from 20% to 40% in the United States (Fornadel et al. 2011, Varela-Stokes et al. 2011, Wright et al. 2011, Ferrari et

al. 2012, Jiang et al. 2012, Nadolny et al. 2014, Pagac et al. 2014, Trout Fryxell et al. 2015, Lee et al. 2016, Mays et al. 2016, Allerdice et al. 2017).

Amblyomma triste are Neotropical ticks most commonly reported from Argentina, Brazil, Uruguay, and Venezuela (Guglielmone et al. 2006, Venzal et al. 2008, Nava et al. 2011, Melo et al. 2015) and are considered important vectors of *R. parkeri* in Argentina and Uruguay (Conti-Díaz et al. 2009, Portillo et al. 2013) (Venzal et al. 2004, Silveira et al. 2007, Nava et al. 2008, Romer et al. 2011, Melo et al. 2015). Until recently, the northernmost collections of *A. triste* have been reported from the Mexican states of Coahuila and Sonora (Guzmán-Cornejo et al. 2006). In 2010, Mertins et al. reported the recovery of an *A. triste* tick from cattle entering the United States from Mexico. A subsequent examination of archival tick specimens identified as *A. maculatum* and submitted to the United States Department of Agriculture (USDA) as part of the National Tick Surveillance Program revealed 15 specimens with morphological features of *A. triste.* An additional 12 new records of *A. triste* were submitted from several counties in Arizona, Texas, and Coahuila between 2004 – 2009 (Mertins et al. 2010).

In 2016, two cases of *R. parkeri* rickettsiosis were described in patients who had acquired tick bites in a mountainous region in southern Arizona that abuts the United States-Mexico border (Herrick et al. 2016). Ticks identified as *A. triste* based on their scutal ornamentation, leg armatures, and festoons (Jones 1972, Mertins et al. 2010, Guzmán-Cornejo et al. 2011, Martins et al. 2014) were associated with both cases. Subsequent field surveys identified *R. parkeri*-infected *Amblyomma* ticks from this region and other proximate sites in southern Arizona, New Mexico (Allerdice et al. 2017, Hecht et al. 2020), and west Texas (Paddock et al. 2020). These specimens were noted to possess morphological features of both *A. maculatum* and *A. triste*, precluding a definitive morphological assignment to either species (Allerdice et al. 2017, Lado et al. 2018). A recent evaluation of mitochondrial and nuclear gene sequences of these and other members of the *A. maculatum* group suggests that *A. triste* and *A. maculatum* are not sufficiently divergent to separate as species (Lado et al. 2018). This study by Lado et al. further identified 4 morphotypes comprising specimens defined as *A. maculatum* or *A. triste*, based on subtle morphological differences among populations from North, Central and South America. Based on this analysis, *Amblyomma* ticks from southern Arizona represent a unique morphotype, distinct from type specimens of *A. maculatum* sensu stricto (s. s.) and *A. triste* s. s.

To further characterize the identity of the *Amblyomma maculatum* group ticks in the southwestern United States, we performed a crossbreeding experiment to evaluate the reproductive compatibility of these ticks with *A. maculatum* s. s, to better determine if these represent the same or separate biological species.

Materials and Methods

Tick collection

Questing adult *A. maculatum* s. s. were collected from Panola Mountain State Park in Rockdale County, Georgia, USA on 5 July and during 18–19 July 2017. Questing adult *A. maculatum* group ticks (heretofore designated as *A. maculatum* sensu lato (s. l.))

were collected from San Pedro Riparian National Conservation Area in Cochise County, Arizona, USA on 14 July 2017. Ticks were collected as questing specimens from vegetation using flannel cloth flags and were transported live to CDC, where they were identified morphologically using standard taxonomic keys (Estrada-Peña et al. 2005). Specimens were rinsed with a solution of water and liquid dish soap and separated by sex into individual 12 × 75 mm polystyrene tubes in groups of 10 as previously described (Levin and Schumacher 2016).

Crossbreeding Assessments

All animal studies were performed according to the protocol 2904LEVRABC approved by the Institutional Animal Care and Use Committee of the Centers for Disease Control and Prevention and the previously published manual for maintenance of ixodid ticks (Levin and Schumacher 2016). Throughout the study, individual naïve 4-month-old female New Zealand white rabbits (*Oryctolagus cuniculus*) were utilized to feed each cross. The rabbits were infested at the same time and were housed in separate cages in the same room to minimize potential environmental variances. Infested rabbits were checked daily for dead, detached, or replete ticks. There was no co-feeding of different crosses on the rabbits, and experimental rabbits were humanely euthanized after the completion of each feeding.

For the initial F0 adult cross, groups of 24 unfed virgin *Amblyomma* females from Georgia or Arizona were placed in individual stockinette feeding bags on the shaved dorsa of the rabbits along with 24 males from either Georgia or Arizona, with one bag per rabbit. This created two homologous (GA ♀/GA ♂ and AZ ♀/AZ ♂) and two heterologous (GA ♀/AZ ♂ and AZ ♀/ GA ♂) breeding crosses. This parental F0 generation of ticks was allowed to feed to repletion, at which point detached replete females were weighed individually, and the duration to engorgement was recorded for each female. Attached males were removed with forceps on the day the last replete female detached. The detached males and all previously collected dead ticks were cleaned with water and liquid dish soap to dislodge any external contaminants and isolated in individual cryovials for morphological analysis.

Replete gravid females were placed in individual 11.1 ml polystyrene containers and maintained in a high humidity incubator (Levin and Schumacher 2016) under identical conditions of 90 % humidity, 22 ℃, and a 16/8 light/dark photoperiod. The females were monitored daily to assess for oviposition. The pre-oviposition period was determined by calculating the number of days between the day the replete females detached and the day oviposition started. Eggs were removed daily from the ovipositing females and placed into a separate 11.1 ml polystyrene tube which was maintained in the same incubator with the laying females. At the end of oviposition, egg mass weight corresponding to each female was recorded. To quantify the ability of each replete female to convert its bloodmeal to eggs, a bloodmeal conversion index (BMCI), was calculated by dividing the weight of the complete egg mass by the engorgement weight of the corresponding replete female. Evidence of larval eclosion for each egg clutch was monitored daily, and an incubation period was calculated as the difference between the date the female began oviposition and the date the first larvae were detected in the clutch. Hatching success was estimated as

previously described (Drummond et al. 1973) and recorded as successful for those egg clutches that exhibited over 90% eclosion.

For the larval feedings, four entire F1 larval cohorts from each of the F0 parental crosses were placed in separate labeled stockinette bags on naïve rabbits. Larval cohorts were selected for placement based on highest observed percentage of hatching. Larvae were fed in parallel on separate rabbits in individual cages in the same room under identical conditions, as described previously. Replete and dead larvae were removed daily using a vacuum pump system (Levin and Schumacher 2016). The first 2000 replete larvae in each cohort to detach were cleaned with water and liquid dish soap and separated into groups of 100 specimens into each of twenty 11.1 ml polystyrene tubes. Larvae were monitored daily and molting success for each larval cohort was recorded. If mold appeared at any point during the molt, the affected tube was removed and placed in a separate incubator under the same conditions as the non-moldy larvae. Moldy larvae were subsequently excluded from analyses of the molting success.

Molted F1 nymphs were allowed to feed on rabbits as described previously. Cohorts of 350 F1 nymphs each were placed in three separate bags on naïve rabbits, comprising a total of 1,050 nymphs per rabbit. Nymphs from each cross were fed in parallel on separate rabbits in individual cages in the same room under identical conditions. Replete nymphs were removed daily by vacuum pump. The replete nymphs were cleaned with water and liquid dish soap and separated into groups of 10 in separate 12 × 75 mm polystyrene tubes. Feeding success was evaluated as the percentage of nymphs that fed to repletion in each bag. Replete nymphs were monitored daily for eclosion, and molting success for each nymphal cohort was recorded.

Twenty-four resultant F1 females from each cross were placed on a separate naïve rabbit paired with 24 males from the same cross to assess their fertility and fecundity. Ticks were placed at approximately 24 days post-molt. Males and females were selected from separate genetic lineages within crosses to limit potential inbreeding. Females were allowed to feed to repletion, and survival parameters of feeding duration, engorgement weight, BMCI, preoviposition period, and hatching success of the F2 eggs were measured as described above. Males were removed after females had fully engorged and were subsequently cleaned with water and liquid dish soap and placed into individual cryovials for morphological analysis.

Morphology

Morphological analysis of the experimental ticks was performed qualitatively. The gross morphologies of the adult F0 parental ticks as well as the adult F1 homologous cohorts and hybrid ticks were analyzed using a Zeiss Stemi 305 dissecting microscope and compared to the four morphotypes presented in Lado et al. 2018. Prominent characters were assessed against the descriptions provided by Lado et al., with a primary focus on the tibial armatures on legs II-IV of both male and female specimens as well as the spiracular plates in male ticks.

Statistical Analyses

Linear regression was used to evaluate differences among crosses for the continuous fecundity variables: female feeding duration, engorgement weight, preoviposition period, egg mass, and minimum incubation period to eclosion. Crosses were compared using differences of mean (95% CIs) estimated from the regression fits and utilizing sandwich estimators of variance in multiple comparisons to account for heteroscedasticity (Bretz et al. 2011).

Logistic regression was used to evaluate differences among crosses for binary fecundity variables: female feeding success, percentage of replete females ovipositing, hatching success, and female-to-male ratio. Models were fit using the Firth correction, and sandwich estimators were used to account for heteroscedasticity in multiple comparisons of differences (95% CIs) between log odds.

To evaluate differences in blood meal conversion indices (BMCI), which range 0 to 1, an arcsine transformation was used on the outcome BMCI before employing linear regression to evaluate differences among crosses.

Data were also collected on larvae and nymphs: larval molting success, nymphal feeding success, and nymphal molting success. Logistic regression fit using the Firth correction was used to evaluate differences among F1 generation crosses, and sandwich estimators of variance were used in multiple comparisons to account for heteroscedasticity among F1 crosses.

Results

F0 → F1 Generations

The F0 cohorts fed successfully on their respective rabbits. For both homologous cohorts, 23/24 female ticks fed to repletion; one female from the Georgia homologous cohort only partially fed before being removed on day 15 post infestation and one female from the Arizona homologous cohort died four days post infestation. Biological parameters for these four cohorts were very similar, though females from AZ completed their repletion approximately two days faster than those from GA, regardless if they mated with homologous or heterologous males (Table 1). There was no significant difference in duration to engorgement among F0 cohorts based on the origin of the female and irrespective of the origin of the male, or among engorgement weight of the F0 females across the four cohorts (range 0.84 – 0.94 g). Similarly, there were no significant differences in weights among egg clutches from F0 females, which ranged from 0.50 g – 0.55 g. The blood meal conversion indices for these ticks ranged from 55.7 – 58.7 % and were not significantly different for any of the cohorts. The GA ♀/AZ ♂ egg clutches hatched approximately two days more quickly (34.3 days) than the reciprocal cross (36.5 days), which did not vary significantly from the homologous cohorts (Table 1).

Molting success of the fed F1 larvae from all four cohorts was 93.5 – 98.0% (Table 2). The molting successes of the heterologous cohorts did not differ from each other or from the homologous cohorts, however the two homologous cohorts showed a significant difference

in molting, with a greater degree of molting success in the AZ ♀/AZ ♂ cohort (Table 2). Nymphal feeding success was similar for three of the four F1 cohorts, however the nymphs from the Georgia F1 homologous cohort fed at a much lower success rate (56.3%) than the other three (83.7 – 88.7%). The molting success of the F1 nymphs was greater than 95% (range 95.4 – 99%) for all but the F1 Georgia homologous cohort (94.9%), and the ratio of females to males ranged from 1.19 in the Arizona F1 homologous cohort to 1.71 in the F1 AZ ♀/GA ♂ cohort (Table 2).

F1 → F2 Generations

The first attempt to cross the F1 adult hybrids in February 2018 ended early due to two of the experimental rabbits developing a clostridial infection unrelated to the crossbreeding study. The data collected in this feeding were subsequently excluded from the statistical analyses in Table 1. The rabbits infested with the AZ ♀/AZ ♂ F1 homologous cohort and the GA ♀/AZ ♂ F1 hybrid cohort were euthanized nine days after tick placement. At the time of euthanasia, nine replete females had been collected from the AZ ♀/AZ ♂ F1 homologous cohort and no replete females had been collected from the GA ♀/AZ ♂ F1 hybrid cohort. These ticks as well as all partially engorged ticks removed from these two rabbits were cleaned and separated into individual 11.1 ml polystyrene tubes to allow for oviposition. All nine of the fully engorged ticks from the AZ ♀/AZ ♂ F1 homologous cohort laid eggs, as did 13 of the 16 partially engorged females removed from the euthanized rabbit. All of these egg clutches hatched at a rate of >90%. Eight of the partially engorged ticks from the GA ♀/AZ ♂ F1 hybrid cohort laid a small clutch of eggs, however none of the eggs from this cohort hatched.

The remaining two healthy rabbits infested with the GA ♀/GA ♂ F1 homologous cohort and the AZ ♀/GA ♂ F1 hybrid cohort continued in the experiment. Seven of the ticks from the GA ♀/GA ♂ F1 homologous cohort came off the rabbit into the cage and were damaged when the rabbit broke its hobble and scratched the bag open eight days into the experiment. None of these seven ticks laid eggs. The remaining 17 females of the GA ♀/GA ♂ homologous cohort fed to repletion and laid egg clutches, all of which hatched at a rate of >90%. Twenty of the 24 AZ ♀/GA ♂ F1 hybrid cohort females fed to repletion. The remaining four female ticks were removed on day 17 post infestation. All 20 of the replete hybrid females laid eggs. No hatched larvae were detected until day 52 post oviposition, when 4 individual F2 larvae were detected in a single egg batch. These 4 hybrid larvae were sluggish and died within 24 hours after hatching.

The F1 adult feeding was repeated in late March 2018 with 20 pairs of ticks per rabbit as opposed to the 24 pairs that were fed in February. Only data collected during this second attempt were used to calculate the statistics in Table 1. Additionally, F1 GA ♀ /AZ ♂ ticks were excluded from all generation-cross group analyses with the exception of adult female feeding success. The low sample size of this generation-cross group following feeding lacks sufficient information to yield reliable results.

All of the females from the GA ♀/GA ♂ F1 homologous cohort fed to repletion within an average of 10.4 days and laid egg clutches which hatched at a rate of >90%. The females from the AZ ♀/AZ ♂ F1 homologous cohort all fed to repletion within an average of 10.1

days, and 19/20 laid egg clutches which hatched at a rate of >90%. The single female in this cohort that did not oviposit was damaged during cleaning and then discarded. There was no significant difference between the feeding duration of the F1 homologous cohorts as was seen in the F0 feeding; the F1 AZ ♀/AZ ♂ homologous cohort fed similarly to both the F0 and F1 GA ♀/GA ♂ homologous cohorts (Table 1). The egg clutches for the Georgia and Arizona F1 homologous cohorts weighed an average of 0.57 and 0.55 g respectively and were not significantly different from any of the other F0 or F1 cohorts.

The GA ♀/AZ ♂ F1 hybrid cohort began engorging at approximately the same time as the homologous cohorts, however most of the ticks stopped engorging by day 7 post infestation. The cuticle of the attached partially engorged ticks appeared dry and leathery. All attached females on the rabbit were paired with a male, however only two replete females were collected: one on day 12 post infestation and one on day 13. On day 16 post infestation, the rabbit housing these ticks was humanely euthanized and 18 partially engorged ticks were removed. The two replete females and 6/18 partially engorged females from this cohort laid egg clutches. However, none of these F2 hybrid eggs hatched (Table 1).

Adult ticks from the AZ ♀/GA ♂ F1 hybrid cohort appeared to feed better than the reciprocal hybrid cohort, with 14/20 replete females detaching within two weeks post infestation; however, this cohort fed to repletion at an average of 12.1 days, a significant difference of approximately two days longer when compared to the homologous cohorts. The remaining six partially engorged females were removed from this rabbit when it was euthanized on day 16 post infestation. All 14 of the replete females and 3/6 of the partially engorged females from this cohort laid egg clutches. However, none of these F2 hybrid eggs hatched (Table 1).

A final attempt was made in June 2018 to determine if the GA ♀/AZ ♂ F1 hybrid cohort would engorge. Ten female and 10 male F1 hybrid ticks were placed on a naïve rabbit. Because this was an attempt to qualitatively verify whether or not these ticks would engorge, there was no homologous control group fed at the same time. The ticks were visually inspected once attached to ensure that the females were paired with males. After 14 days, no females had fed to repletion, and all of the attached partially engorged females appeared pale and leathery. The rabbit was humanely euthanized on day 15 post infestation, and the partially engorged ticks were removed and saved for morphological analysis.

Morphological evaluation

All homologous adults from the F0 generation (24 ♀ and 24 ♂ from each cross) and 80 from each F1 cohort (40 ♀ and 40 ♂ from each cross) were examined using a dissecting microscope and compared to the morphotypes described in Lado et al. (Lado et al. 2018). The F0 adults were analyzed post feeding. The descriptions of the morphotypes for each homologous cohort generally matched the respective observed morphology of the ticks. The tibial armatures on legs II-IV of the ticks from Arizona were consistently of unequal thickness. The Arizona male spiracular plates were consistently comma shaped, and the female specimens all contained a central brown spot that reached the posterior margin of the scutum (Figure 1, d – f). The *A. maculatum* s. s. ticks from Georgia were uniformly consistent with classic descriptions of this species, with tibial armatures on legs II-IV of equal thickness, comma shaped spiracular plates in males, and central brown spots that often

do not reach the posterior margin of the scutum (Figure 1, a – c) (Kohls 1956, Camicas et al. 1998, Estrada-Peña et al. 2005, Lado et al. 2018).

For the hybrid cohorts, examination of 80 F1 adult hybrid ticks (40 ♀ and 40 ♂) from each cross revealed a mix of morphotypes and rarely matched 100% with any single group. Among the 80 female AZ ♀/GA ♂ F1 hybrids examined, 67 (83.8% (74.1, 90.3%, $\alpha = 0.05$)) had a brown central scutal area that reached the posterior margin of the scutum as in the description for the Arizona *A. maculatum* s. l. (morphotype III) (Figure 1, j), and 13 (16.3% (15.9,16.5)%, $\alpha = 0.05$)) demonstrated scutal coloration defined by a brown central area that did not reach the scutal posterior edge, as in the description for *A. maculatum* s. s. (morphotype II) (Figure 1, g).

Hybrid males possessed comma-shaped spiracular plates as described for *A. maculatum* s. l. (morphotype III) (Figure 1, i and l). The tibial armatures for male and female hybrids were consistently of unequal thickness, though there was a range of thicknesses of the smaller spur, similar to those identified for both the Arizona *A. maculatum* s. l. (morphotype III) and *A. triste* s. s. (morphotype I) (Figure 1, h and k).

Discussion

In the 175 years since Koch first described *Amblyomma maculatum*, there has been no consensus reached on how to easily define ixodid tick species (Dantas-Torres 2018, Goddard et al. 2019). While morphology is arguably the most practical tool in separating specimens, the characters that distinguish valid tick species are often subjective and difficult to decipher for even trained entomologists. This can become nearly impossible when trying to identify immature stages; nymphs and larvae of *A. maculatum* s. s. and *A. triste* s. s. are rarely collected and are not readily distinguished using contemporary morphological keys (Mertins et al. 2010, Mukherjee et al. 2014, Cohen et al. 2015). Molecular analysis can be an effective tool to separate valid species and identify potentially cryptic species, however a biological approach is perhaps the most robust method of elucidating species relationships in Ixodidae (Goddard et al. 2019).

The most basic principle of the biological species concept is reproductive isolation, or an accumulation of reproductive isolating mechanisms sufficient to prevent successful gene flow between two populations of closely related organisms (Mayr 1970). This principle has been used to verify species identities within ixodid ticks through crossbreeding experiments many times before. Interspecific studies between *Dermacentor variabilis* (Say), *D. occidentalis* Marx, and *D. andersoni* Stiles (Oliver et al. 1972), *Rhipicephalus (Boophilus) microplus* (Canestrini) and *Rh. (Boophilus) decoloratus* (Koch) (Spickett and Malan 1978), and *D. marginatus* Sulzer and *D. reticulatus* (Fabricius) (Zahler and Gothe 1997) produced no viable F1 hybrids. Similar interspecific crossbreeding analyses between the *Ixodes ricinus* (Linnaeus) – *I. persulcatus* Shulze complex (Balashov et al. 1998) and *Rh. (Boophilus) annulatus* (Say) and *Rh. (Boophilus) microplus* (Canestrini) (Graham et al. 1972) produced infertile F1 hybrids. In both cases, these studies support the validity of these taxa as separate species.

Intraspecific analyses have also been conducted to test conspecificity, such as between the tropical and temperate lineages of *Rh. sanguineus* (Latreille) (Levin et al. 2012) and geographically distinct populations of *Rh. (Boophilus) microplus* (Labruna et al. 2009), *Amblyomma cajennense* (Fabricius) (Labruna et al. 2011), temperate *Rh. sanguineus* s. l. (Dantas-Torres et al. 2018), and *Amblyomma parvum* Aragão (Nava et al. 2016). With the exception of *A. parvum* and temperate *Rh. sanguineus* s. l., all of these intraspecific analyses resulted in reproductive incompatibility, to suggest that the taxa represent multiple biological species.

In the present study, *A. maculatum* s. s. from Georgia and *A. maculatum* s. l. from Arizona effectively hybridized when placed together on animals in a laboratory setting. However, these F1 hybrids did not produce fertile progeny and were unable to establish an F2 generation. Though the F1 hybrids readily recognized each other as mates, they exhibited diminished fitness, most notably seen in the GA ♀/AZ ♂ cohort's apparent inability to feed to repletion across multiple attempts. It is thus likely that one or both of the F1 hybrid sexes is infertile.

Hybrid sterility serves as a postzygotic reproductive isolating mechanism to prevent the exchange of genes between populations, thus preserving the genetic integrity of species. Almost invariably, hybrid sterility follows Haldane's rule of scarcity, preferential impairment, or infertility of the heterogametic sex (Haldane 1922). In *Amblyomma* species, as in most ixodid ticks, males are heterogametic (Oliver 1989) and would be expected to be rare or infertile if Haldane's rule holds true. Such was the case in the attempted crosses of *Amblyomma americanum* (Linnaeus) with *A. maculatum* s. s. and *Amblyomma variegatum* (Fabricius) with *Amblyomma hebraeum* Koch performed by Gladney and Dawkins in 1973 and Clarke and Pretorius in 2005, respectively. In the first experiment, only the cross of *A. maculatum* s. s. males and *A. americanum* females produced F1 offspring, all of which were female (Gladney and Dawkins 1973). The second study showed similar results, with the cross of male *A. variegatum* and female *A. hebraeum* producing F1 progeny that were entirely female (Clarke and Pretorius 2005). In the work shown here, 5/6 of the individual F1 hybrid lineages produced more females than males at ratios from 1.05 – 1.96, with the highest female-to-male ratio in the AZ ♀/GA ♂ hybrid cohort. This cohort is significantly different from the F1 homologous cohorts, although the homologous cohorts also exhibit female-to-male ratios over 1 (Table 2). Nonetheless, we did not cross F1 hybrid males with F1 homologous fertile females, so it remains uncertain if Haldane's rule of sterility applies. Similarly, without performing the reciprocal cross of the F1 hybrid females with fertile F1 homologous males, we cannot know if the hybrid females are potentially the sterile sex.

The two allopatric populations of *A. maculatum* group ticks presented here lack the genetic divergence necessary to be classified as separate species (Lado et al. 2018). The adults can be separated morphologically, but the characters to do so are slight and somewhat subjective. There is inevitably a gradient of morphological variation within populations of individual species, and although the few characters that separate *A. maculatum* s. s. and *A. maculatum* s. l. might otherwise be attributed to this gradient, our observation of reproductive isolation past the F1 generation precludes consideration of these disjunct populations as a single biological species (Mayr 1970). However, while the two populations of ticks in this study

from southern Arizona and Georgia appear to represent distinct biological species, more work is required to accurately determine the taxonomic status of *A. maculatum* s. l. ticks identified in the American southwest, including crossbreeding experiments with *A. triste* s. s. from South America. Investigations into variable markers such as microsatellites could further provide clarity to the species relationships between these and other populations within the *Amblyomma maculatum* group (Fagerberg et al. 2001). As more cases of *R. parkeri* rickettsiosis are identified (Yaglom et al. 2020) and the recognized range of this pathogen's *Amblyomma* vector extends in the southwestern United States (Hecht et al. 2020, Paddock et al. 2020), it is vital to determine whether the ticks in this region represent a previously described or a unique species.

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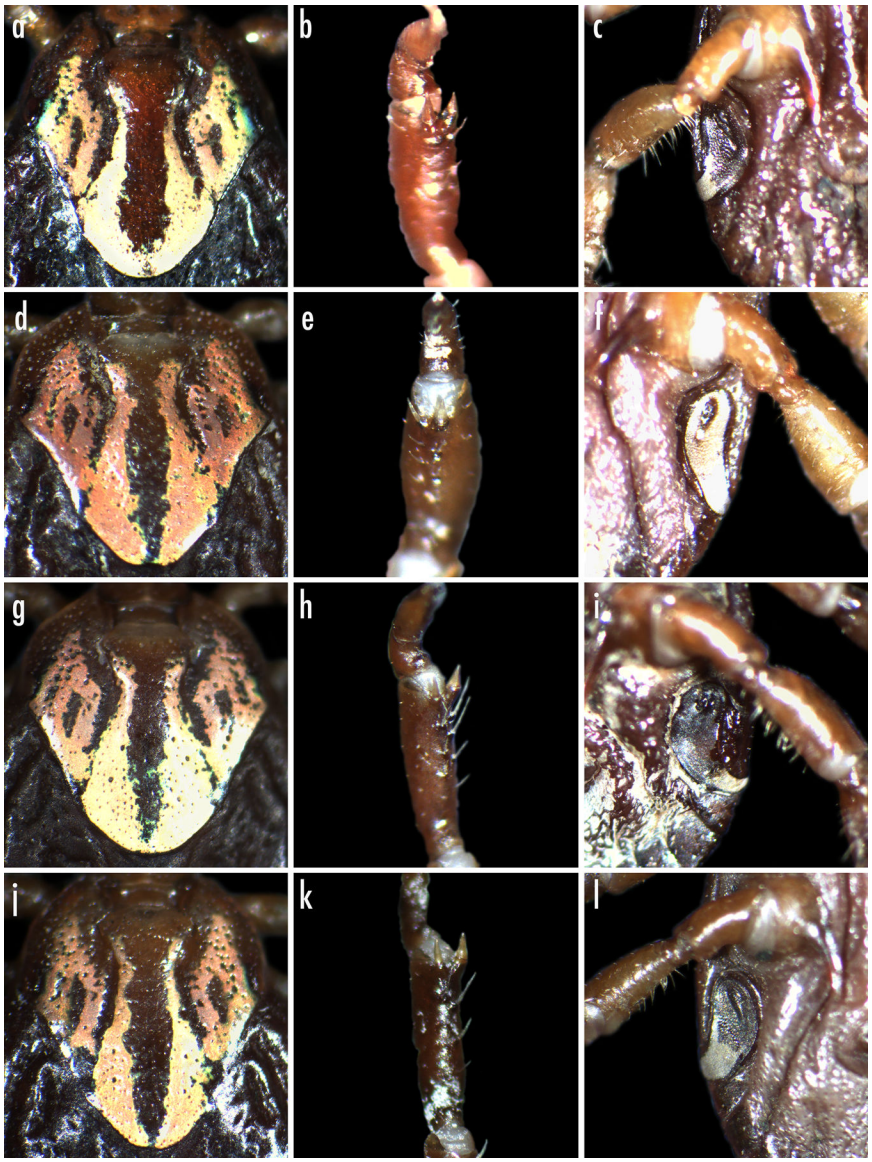

Fig. 1.

Images of the morphology of the scutum, armatures on tibiae II – IV, and male spiracular plates for the GA ♀/GA ♂ (a – c), AZ ♀/AZ ♂ (d – f), GA ♀/AZ ♂ (g – i), and AZ ♀/GA ♂ (j – l) F1 adults

Table 1.

Summary of fertility and fecundity parameters for the adult F0 and F1 ticks.

	F0 → F1				F1 → F2			
	Homologous Cohorts		Heterologous Cohorts		Homologous Cohorts		Heterologous Cohorts	
	GA ♀ / GA ♂	AZ ♀ / AZ ♂	GA ♀ / AZ ♂	AZ ♀ / GA ♂	GA ♀ / GA ♂	AZ ♀ / AZ ♂	GA ♀ / AZ ♂	AZ ♀ / GA ♂
	n = 24	n = 24	n = 24	n = 24	n = 20	n = 20	n = 20	n = 20
Female feeding success (%) ***	95.8 (79.8, 99.8)^{a,b}	95.8 (79.8, 99.8)^{a,b}	95.8 (79.8, 99.8)^{a,b}	100 (86.2, 100)^{a}	100 (83.9, 100)^{a}	100 (83.9, 100)^{a}	10.0 (2.8, 30.1)^{c}	70.0 (48.1, 85.5)^{b}
	n = 23	n = 23	n = 23	n = 24	n = 20	n = 19	n = 2	n = 14
Female feeding duration (days) **	10.5 (10.1, 10.9)^{a}	8.17 (7.77, 8.58)^{b}	10.6 (10.2, 11.0)^{a}	8.29 (7.76, 8.83)^{b}	10.4, (9.89, 10.9)^{a}	10.1 (9.51, 10.7)^{a}	12.5 (6.15, 18.9) *	12.1 (11.2, 13.1)^{c}
Engorgement weight (g) **	0.93 (0.84, 1.03)^{a}	0.87 (0.81, 0.93)^{a}	0.94 (0.82, 1.05)^{a}	0.84 (0.74, 0.94)^{a}	0.99 (0.87, 1.11)^{a}	0.87 (0.79, 0.95)^{a}	0.32 (0.25, 0.38) *	0.94 (0.74, 1.14)^{a}
Replete females ovipositing (%) ***	100 (85.7, 100)^{a}	100 (85.7, 100)^{a}	100 (85.7, 100)^{a}	100 (86.2, 100)^{a}	100 (83.9, 100)^{a}	95.0 (76.4, 99.7)^{a}	100 (34.2, 100) *	100 (78.5, 100)^{a}
Preoviposition period (days) **	5.21 (4.48, 5.60)^{a}	4.96 (4.58, 5.34)^{a}	7.05 (5.99, 8.10)^{b}	4.62 (4.26, 4.99)^{a}	6.75 (6.45, 7.05)^{b}	5.21 (4.87, 5.55)^{a}	5.5 (0, 11.9) *	5.43 (4.84, 6.02)^{a}
Egg mass weight (g) **	0.55 (0.50, 0.61)^{a}	0.51 (0.47, 0.55)^{a}	0.54 (0.45, 0.63)^{a,b}	0.50 (0.43, 0.57)^{a}	0.57 (0.47, 0.66)^{a,b}	0.55 (0.48, 0.63)^{a,b}	------	0.71 (0.61, 0.82)^{b}
Blood meal conversion index **	57.5 (55.0, 60.0)^{a}	58.0 (55.7, 60.3)^{a}	55.7 (51.2, 60.2)^{a}	58.7 (54.7, 62.8)^{a}	56.7 (52.6, 60.9)^{a}	63.7 (59.6, 67.9)^{a}	------	60.8 (54.9 66.6)^{a}
Minimum incubation period to eclosion (days) **	36.0, (35.1, 36.9)^{a,b}	36.0 (35.3, 36.7)^{a,b}	34.3 (33.4, 35.3)^{a}	36.5 (35.9, 37.1)^{b}	33.3 (32.8, 34.3)^{a}	34.6 (33.6, 35.5)^{a,b}	------	------
Hatching success (%) ***	100 (85.7, 100.0)^{a}	100 (85.7, 100.0)^{a}	100 (85.1, 100.0)^{a}	100 (86.2, 100.0)^{a}	100 (83.9, 100.0)^{a}	100 (83.9, 100.0)^{a}	0 (0, 65.8) *	0 (0, 21.5)^{b}

All cells show means with 95% confidence intervals for each cohort. Superscripts that differ indicate significant statistical difference between crosses.

* This generation-cross group was not included in pairwise comparison analysis due to small sample size.

** Linear regression and t tests were used to test differences in means between cohorts.

*** Logistic regression and t tests were used to test differences in log-odds between cohorts.

Table 2.

Summary of the F1 nymphs and larvae, including nymphal feeding success, larval and nymphal molting success, and female-to-male ratio. Superscripts that differ indicate significant statistical difference between crosses.

	Homologous Cohorts		Heterologous Cohorts	
	GA ♀ / GA ♂	AZ ♀ / AZ ♂	GA ♀ / AZ ♂	AZ ♀ / GA ♂
	n = 2765	n = 4997	n = 1410	n = 3700
Larvae molting success (%)	93.5 (92.5, 94.4)[a]	97.3 (96.8, 97.7)[b]	93.7 (92.3, 94.8)[a,b]	98.0 (97.4, 98.4)[a,b]
	n = 1050	n = 1050	n = 1050	n = 1050
Nymph feeding success (%)	56.3 (53.3, 59.3)[a]	88.7 (86.6, 90.4)[b]	83.7 (81.4, 85.8)[b]	87.7 (85.6, 89.6)[b]
Nymph molting success (%)	94.9 (92.8, 96.4)[b]	98.0 (96.9, 98.7)[a,b]	95.4 (93.9, 96.6)[a,b]	99.0 (98.2, 99.5)[a]
Female-to-male ratio	1.20 (1.02, 1.42)[a,b]	1.19 (1.04, 1.36)[a]	1.20 (1.05, 1.38)[a,b]	1.71 (1.50, 1.96)[b]