Published in final edited form as:

Ticks Tick Borne Dis. 2015 June ; 6(4): 463–472. doi:10.1016/j.ttbdis.2015.03.014.

Vector Potential and Population Dynamics for *Amblyomma inornatum*

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Abstract

We studied the natural life cycle of *Amblyomma inornatum* and its vector potential in South Texas. This tick is distributed throughout South Texas and most of Central America. *A. inornatum* represented 1.91% of the ticks collected by carbon dioxide traps during a study of free-living ticks in the Tamaulipan Biotic Province in South Texas. The life cycle of *A. inornatum* in South Texas showed a clear seasonal pattern consistent with one generation per year. Nymphs emerged in the spring with a peak in February through May. Adults emerged in the summer with a peak in July through September. Detection of *A. inornatum* larvae was negatively correlated with saturation deficit and positively correlated with rain in the previous few months. Adult activity was positively correlated with temperature and rain in the previous five weeks. Using PCR we detected the presence of species related to *Candidatus* *Borrelia lonestari*, *Borrelia burgdorferi*, *Rickettsia* species (*Candidatus* *Rickettsia amblyommii*), *Ehrlichia chaffeensis*, and another *Ehrlichia* related to *Ehrlichia ewingii*. Finally we sequenced the mitochondrial 16S rRNA genes and found that *A. inornatum* is most closely related to *Amblyomma parvum*. This is the first report of the life cycle, vector potential and phylogeny of *A. inornatum*.

Keywords

Amblyomma inornatum; ecology; life cycle; *Borrelia*

Introduction

Amblyomma inornatum (Banks, 1909) is found throughout South Texas (Eads & Borom 1975, Gladney et al. 1977), Mexico (Guzmán-Cornejo et al. 2011) and Central America. The species has been reported as far north as 30°N (Presidio and Bexar County, TX) (Brennan 1945, Eads 1951) and as far south as Costa Rica (Alvarez et al 2000). *A. inornatum* has been found on a wide range of hosts: occasionally on large mammals (Cooley and Kohls 1944, Guzmán-Cornejo et al 2011) including *Homo sapiens* Linnaeus, 1758, *Bos taurus* Linnaeus,

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1758, *Odocoileus virginianus* Zimmerman, 1780; but has been most commonly reported from a large variety of small mammals and birds (Eads 1951, Eads and Borom 1975, Gladney et al. 1977, Guzmán-Cornejo et al 2011, Samuel and Trainer 1970). Because *A. inornatum* feeds on humans (the most recent report from a traveler to the United States returning to Ontario, Canada - Nelder et al. 2014), a wide range of mammals, ground birds, and migratory birds (it has been collected far from its natural range off of migratory birds as far north as eastern Canada as reported in Ogden et al. 2008) it is important to understand its vector potential. It should be noted that Texas is a major flyway for migratory birds that could acquire tickborne diseases and/or infect ticks with tickborne diseases from other areas in the Americas. Other than occasional reports of the isolation of *A. inornatum* over the last century there is only a single report on its cytogenetics (Oliver and Osburn 1985) and a single report on its laboratory life cycle (Gladney et al. 1977). *Amblyomma auricularium* (Conil, 1878), *Amblyomma pseudoparvum* Guglielmone, Mangold and Keirans, 1990, *Amblyomma pseudoconcolor* Aragão, 1908, *Amblyomma parvum* Aragão, 1908 and *A. inornatum* are similar in their morphological characteristics (Cooley and Kohls 1939, Nava et al. 2008). Prior genetic analysis of this group has not included *A. inornatum*. In this study the population dynamics and phylogenetics of *A. inornatum* are reported for the first time. In addition, *A. inornatum* was tested for *Ehrlichia*, *Rickettsia* and *Borrelia* species which are known to be present in *Amblyomma americanum* (Linnaeus, 1758) and *Amblyomma mixtum* Koch, 1844. These ticks are also present in South Texas where *A. inornatum* is found (Billings et al. 1998, Estrada-Peña et al., 2004, Williamson et al. 2010). It should be noted that *A. mixtum* is the reinstated name of one of the “*Amblyomma cajennense* (Fabricius, 1787) sensu lato” strains from South Texas following observation of developmental features (Guglielmone et al. 1992), crossbreeding incompatibility (Labruna et al. 2011, Mastropaolo et al. 2011), and genetic analysis (Beati et al. 2013). These studies resulted in “*A. cajennense* sensu lato” being divided into six different species by Nava et al. 2014. Understanding tickborne disease in South Texas is critical as hundreds of thousands of people entering the United States illegally each year cross through this region on foot. Because of the circumstances of their migration they tend to avoid roads and pass through trails, made by both man and animal, that are potentially targeted by questing *A. inornatum* and *A. mixtum* ticks. As both of these species feed on humans, these individuals are extensively exposed to ticks and tickborne diseases during this time.

Materials and Methods

Study area, tick collection and meteorological analysis

The primary field site for this study was on the Texas A&M International University Campus in Webb County, Texas, USA (27°57'N 99°44'W) with an elevation of 155 m. The study site has been previously described (Beck et al. 2011). The study area is part of the South Texas Plains vegetation region (Gould 1975) and Tamaulipan Biotic Province (Dice 1943). The region is relatively dry (52 cm of precipitation per year), with mild winters and hot summers (mean temperature from 12°C in the winter to 31°C in the summer) and slight peaks of rainfall in the spring and fall. Vegetation consisted mostly of thorny woodland and shrub land. Tick surveys were undertaken monthly from March 2005 to November 2008 as previously described (Beck et al. 2011). Ticks were surveyed using carbon dioxide traps.

Actively questing and responsive questing ticks were collected using this method. As described in Beck et al. (2011) ticks were identified to species using published keys (Brinton et al. 1965, Cooley 1938, Cooley 1946, Cooley and Kohls 1944, Jones et al. 1972, Keirans and Durden 1998, Kohls 1958, Yunker et al. 1986). It should be noted that only five species of *Amblyomma* are present in South Texas: *A. americanum*, *Amblyomma imitator* Kohls, 1958, *A. inornatum*, *Amblyomma maculatum* Koch, 1844, and *A. mixtum*. *A. inornatum* larvae are easily distinguished from other *Amblyomma* larvae in this region by their short palpi, but must be carefully distinguished from some *Dermacentor* larvae which also have short palpi. Of the *Amblyomma* larvae, only *A. inornatum* larvae were identified to the species level. All larvae were identified by two different researchers. Meteorological data (daily averages and daily total rainfall) was from Laredo International Airport which is less than 2 km from the study site. The saturation deficit was calculated using this equation: $SD = (1 - RH/100) 4.9463 e^{0.01611 T}$, where SD represents the saturation deficit in millimeters of mercury, RH is the daily average relative humidity in percent, and T is the daily average temperature in degrees Celsius (Randolph and Storey 1999). Statistical analysis was as previously described (Beck et al. 2011) using IBM SPSS for Windows, Version 22 (13 Aug, 2013, IBM, Chicago, IL). Three week means were determined by averaging the daily average temperature, humidity saturation deficit or wind speed for 21 days prior to the trap day. Total rainfall was determined by totaling the daily total rainfall for 21 days prior to the trap day, unless otherwise indicated. Spearman's coefficient of rank correlation was used to study the relationship between tick trap data and meteorological data. For nymphs and adults the density (number of ticks) per trap was determined. For larvae the density per trap is highly variable due to oviposition sites having large numbers of larvae, and other nearby sites having very few larvae. For larvae the percent of traps per trap day that had the presence of larvae on traps was used for statistical analysis. Multiple regression analysis was done with tick density of nymphs or adult per trap/percent of traps per trap day with larvae as the dependent variable and meteorological indicators as the independent variables. Pearson's rho was calculated to determine if the independent variables were correlated with each other.

Detection of *Borrelia*, *Ehrlichia* and *Rickettsia* in *A. inornatum*

Ticks were frozen within 1-2 hours of collection at -20°C. Except for the purpose of identification of ticks to species, were kept frozen until they were used for DNA purification. A total of seventy male and female adult *A. inornatum* ticks were used for PCR analysis. The entire tick was utilized for DNA extraction using E.Z.N.A. Mollusc DNA Isolation Kits (Omega Bio-Tek, Inc., Norcross, GA, USA). The protocol of Williamson et al. (2010) was modified as described. Each tick was washed in bleach five times followed by washing five times in deionized water. The tick was then chopped using a sterile razor blade and homogenized in 350 µL MLI buffer, using a microtube pestle. Each tick was ground for five minutes. After adding proteinase K, the samples were incubated at 60°C for 4 hours. Subsequent sample purification was performed according to the manufacturer's protocol.

The samples were then subjected to PCR for amplification of tick 16S rDNA, as well as *Borrelia* spp., *Ehrlichia* spp., and *Rickettsia* spp. genes as described by Williamson et al. (2010). To minimize risk of contamination, barrier tips were used. DNA isolation and PCR

were conducted in different locations and at different times. Positive controls were added using separate hoods and pipettors from templates. The primers used in the present study are listed in Table 1. The PCR mixture was a 25 µL reaction volume containing 1.25 units of GoTaq polymerase (Promega, Madison, WI), 1X GoTaq Buffer, 160 ng/µL bovine serum albumin, 1.0 mM MgCl₂, 200 µM of each dNTP, 2 pmol primers, and 5 µL of template (1 µL for nested reactions). Amplifications were performed on a Bio-Rad MyCycler thermal cycler (Bio-Rad, Carlsbad, CA) with an initial five minute denaturation at 95°C, followed by 46 cycles of 95°C for one minute, 55°C for one minute, and 72°C for one minute. A final extension step was performed at 72°C for seven minutes. For the detection of *Borrelia* spp. DNAs, nested amplifications were performed with 1 µL from the initial reaction as a template with an initial five minute denaturation at 95°C, followed by 36 cycles of 95°C for one minute, 55°C for one minute, and 72°C for one minute. A final extension step was performed at 72°C for seven minutes. For PCR of mitochondrial 16S rDNA, an initial 2 minute denaturation at 94°C was followed by seven cycles with an increased annealing temperature of 0.3°C per cycle, denaturation 94°C for 30 s, annealing 47 to 48.8°C, extension at 72°C, followed by 28 cycles of 94°C for 30s, 50°C for 30 s, and 72°C for 45s. A final extension step was performed at 72°C for seven minutes.

PCR products were electrophoresed on 1.6% agarose gels stained with ethidium bromide (EtBr) in a 1X TAE buffer with EtBr. Gels were examined under UV light and results were compared to a known positive control. PCR samples were purified using SpinPrep PCR Clean-UP Kits (Novagen, La Jolla CA, USA) according to the manufacturer's protocols. DNA sequencing was performed at Cornell University Core Laboratories Center, at Texas A&M International University and MCLab (San Francisco, CA). The sequences were compared to sequences in the NCBI GenBank using BLAST. Sequences greater than 200 nucleotides long were submitted to GenBank (KM458241-KM458271).

Phylogenetic analysis

For phylogenetic analysis, initial alignments for genes from *Amblyomma*, *Borrelia*, *Ehrlichia*, and *Rickettsia* were each performed with MUSCLE (Edgar 2004) as implemented by the European Bioinformatics Institute's MUSCLE server (<http://www.ebi.ac.uk/Tools/muscle/>) using the default settings, with subsequent manual adjustments if needed. Bayesian inference phylogenetic analyses were conducted using MrBayes v.3.1.2 (Ronquist and Huelsenbeck 2003) with two runs, each for 10,000,000 generations, with eight chains and a temperature of 0.2, and trees sampled every 8,000 generations. The appropriate model for each genus was determined via jModelTest 2 (Darriba et al. 2012): GTR + I + Γ for *Amblyomma* and *Borrelia*, HKY + I + Γ for *Ehrlichia* and HKY + Γ for *Rickettsia*. For all models, the gamma distribution included six categories. After analysis, the first 250 trees from each run were discarded as burnin, and the consensus trees were viewed in FigTree v1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>). The following sequences were included in the *Amblyomma* phylogenetic analysis (species followed by isolate ID and/or GenBank accession number): two *A. inornatum* from South Texas (A19C and A41C) KM458244 and KM458245, three *A. mixtum* from South Texas (B13M-F1, B16A-F2, B4O-M1) KM458241-KM458243 and reference sequences from GenBank – *A. americanum* L34313, *Amblyomma aureolatum* (Pallas, 1772) JN800433, *A.*

auricularium FJ627951, *Amblyomma boeroi* Nava, Mangold, Mastropaolo, Venzal, Oscherov and Guglielmone, 2009 JN828797, *Amblyomma brasiliense* Aragão, 1908 FJ424399, *Amblyomma coelebs* Neumann, 1899 FJ424408, *Amblyomma dubitatum* Neumann 1899 DQ858955, *Amblyomma hebraeum* Koch, 1844 L34316, *Amblyomma incisum* Neumann, 1906 FJ424405, *Amblyomma latum* Koch, 1844 L34319, *Amblyomma longirostre* (Koch, 1844) KF702345, *A. maculatum* AY498559, *Amblyomma multipunctum* Neumann, 1899 KJ584366, *Amblyomma naponense* (Packard, 1869) KC677674, *Amblyomma neumanni* Ribaga 1902 AY498560, *Amblyomma oblongoguttatum* Koch, 1844 FJ424407, *Amblyomma ovale* Koch, 1844 AF541255, *Amblyomma parvitarsum* Neumann, 1901 AY498561, *A. parvum* EU306137 & EU306159, *A. pseudoconcolor* AY628137, *A. pseudoparvum* FJ627952, *Amblyomma sabanerae* Stoll, 1890 KF702344, *Amblyomma sculptum* Neumann, 1899 FJ424404, JX573118, & KJ557135, *Amblyomma tigrinum* Koch, 1844 AY836005, *Amblyomma triste* Koch, 1844 AY498563, *Amblyomma tuberculatum* Marx, 1894 U95856, *Amblyomma variegatum* (Fabricius 1794) L34312, *Haemaphysalis bispinosa* Neumann 1897 KC853420, *Haemaphysalis doenitzi* Warburton and Nutall 1909 JF979402, *Haemaphysalis japonica* Warburton 1908 AB861936.

Results

Life cycle and meteorological indicators

As previously described in a study of *A. mixtum* from March 2005 to November 2008, there were 70,873 ticks collected by carbon dioxide traps in Webb County, Texas. Of these we identified 1.91% as *A. inornatum* (1007 larvae, 315 nymphs, 65 adult males, and 17 adult females) (Beck et al. 2011). Adults were present throughout the year with a peak in July through September in each of the trap years (Figure 1). Nymphs showed a clear peak in February through May (Figure 2). This trend was not observed in 2007 (individual monthly data not shown). However, in 2007 the mean temperature dropped to below 10°C in February for several weeks, which probably negatively impacted the emergence of nymphs and a second year of drought may have significantly affected tick and host activity. In 2007 half as many nymphs were noted per trap as observed in 2006 or 2008. Larvae were collected on a total of 19 traps and two tick walks in Webb County during the study period (Table 2). During September and November 2008 some traps were set in Jim Hogg County on which *A. inornatum* larvae were collected (Table 2). All traps with more than three *A. inornatum* larvae present were collected in August–November. One positive trap was collected in January, March, and June during the study period.

The presence of larvae on traps was negatively correlated with saturation deficit (Spearman's $\rho = -0.204$, $df = 98$, $p < 0.04$), and mean wind speed over a three week period (Spearman's $\rho = -0.386$, $df = 98$, $p < 0.001$) which would correlate with drying conditions associated with increased dehydration and decreased survival of larvae (Table 3). No larvae were detected on traps when the three week mean saturation deficit exceeded 12.2 mmHg (data not shown). In contrast, presence of larvae was positively correlated with mean humidity over a three week period (Spearman's $\rho = 0.337$, $df = 98$, $p < 0.001$) which would promote survival of larvae. Indeed no larvae were detected when the mean humidity over a three week period was below 58.9%, while 87.5% of the traps with larvae were found when humidity was

above 64%. Presence of larvae was compared with total rainfall in three-week windows. A positive correlation was found with rainfall and larvae from Week 3-17 (Table 3). A multiple regression model for the prediction of the presence of larvae on traps from total rainfall values from 5-7 weeks and total rainfall values from 13-15 weeks was run. These variables statistically significantly predicted the presence of larvae on traps; $F(2,95)=11.977$, $p<0.0005$, $R^2=0.201$. Both variables added statistical significance to the prediction, $p<0.001$. Increased precipitation would allow for more abundant vegetation, increased shade and lowered saturation deficit at the ground level promoting survival of larvae. Other factors such as host density were not measured in this study.

Nymphs were primarily observed in the spring. It was noted that almost all trap days with >0.25 mean nymphs per trap were on days that had between 11.5 hrs and 13.2 hrs of daylight (Figure 3). As shown in Figure 3, by the box plot at the top of the graph, 50% of the traps were set on days that had daylight from 11.5 hours to 13.4 hours, yet all trap days that had a large number of nymphs fell within (except for one) those hours-of-daylight values.

The density per trap of adults was positively correlated with the three week mean humidity values (adults $\rho=0.220$, $df=98$, $p<0.03$), total rainfall from the six week period prior to setting the trap (adults $\rho=0.207$, $df=98$, $p<0.04$), total rainfall from 9-11 weeks and total rainfall 11-13 weeks prior to setting the trap (adults $\rho=0.85$, $df=98$, $p<0.004$, $\rho=0.43$, $df=98$, $p<0.02$). A multiple regression model for the prediction of the density per trap of adults from total rainfall from 1-5 weeks values and three week mean temperature values prior to the trap day was run. These variables had a statistically significant effect on predicted density of adults on traps; $F(2,95)=6.817$, $p<0.002$, $R^2=0.126$. Both variables added statistical significance to the prediction, $p<0.03$. Other factors such as host density were not measured in this study.

Field observations

During field observations of trap sites it was noted that *A. inornatum* ticks were less likely to be present in trap sites dominated by dicots and tall buffelgrass where *A. mixtum* was often very abundant (data not shown and Beck et al. 2011). In traps sites characterized by a mixture of monocots and mostly smaller dicots (shrubs and forbs with less buffelgrass), both *A. inornatum* and *A. mixtum* were detected on the traps. *A. inornatum* was thus present in a wider variety of vegetation alliances than *A. mixtum* as much of the region is characterized by intermittent shrubs and forbs.

Detection of DNA of tick-borne bacteria

DNA was purified from a total of 70 ticks; 50 males and 20 females. Of these, 59 ticks were positive for DNA from the genera *Borrelia*, *Ehrlichia*, or *Rickettsia* (Table 4). As shown in Figure 4, we detected *Candidatus Rickettsia amblyommii* by sequencing; it is possible that other *Rickettsia* species were present as this amplicon is difficult to sequence (Phillip Williamson personal communication). The second most frequently detected genus was *Borrelia*. The primers for *Candidatus B. lonestari* often detect *B. burgdorferi* sequences, thus sequencing of the PCR products is essential for species identification. Sequencing revealed that DNA from *Candidatus Borrelia lonestari* was present in 6 of 70 ticks (8.6%).

The *Candidatus* B. lonestari-like sequences from our study clustered with previous reported *Candidatus* B. lonestari sequences (Figure 5). DNA from the *Borrelia burgdorferi* complex of species was detected in 26/70 (37%) of ticks. The sequences from *A. inornatum* were highly similar to known species in the *B. burgdorferi* complex (Figure 5). Both *Ehrlichia chaffeensis* DNA and DNA closely related to *Ehrlichia ewingii* were detected in *A. inornatum*. The *E. chaffeensis* like amplicons clustered with known sequences of *E. chaffeensis*. The *E. ewingii* amplicon clustered as a distinct clade from known *E. ewingii* sequences (Figure 6). Three co-infections of *Ehrlichia* and *B. burgdorferi* complex were detected.

The mitochondrial 16S rDNA genes from *A. inornatum* and *A. mixtum* ticks from South Texas were sequenced. A rooted Bayesian inference tree including these sequences and other published *Amblyomma* sequences is shown in Figure 7. *A. auricularium*, *A. inornatum*, *A. parvum*, and *A. pseudoconor* and *A. pseudoparvum* formed a clade with a posterior probability (PP) of 0.99. As for other *Amblyomma* species, there was high PP support for a clade of *A. trigrinum*, *A. maculatum* and *A. triste* (1.00 PP), a clade of *A. hebraeum* and *A. variegatum* (0.99 PP), and a clade of *A. ovale* and *A. aureolatum* (1.00 PP) which is consistent with previously published results (Nava et al 2008). *A. sculptum* and *A. mixtum* (both formerly *A. cajennense* sensu lato species) formed two separate clades with a PP support of 1.00 PP. This is consistent with the recently published reassessment of the taxonomy of *A. cajennense* (Beati et al. 2013, Nava et al. 2014).

Discussion

Life cycle

A. inornatum represented a small percent of the total populations of ticks (1.91% of ticks collected) throughout the study area as compared to *A. mixtum* (93.66% of ticks collected) during the study period (Beck et al. 2011). However, *A. inornatum* was present in a wider variety of vegetation alliances in the environment than was *A. mixtum*, which was primarily observed in areas of higher brush density (areas mostly populated by dicots). Our results are in contrast to a report by Samuel and Trainer 1970 that *A. inornatum* was collected more frequently from the fawns of white-tailed deer in more densely wooded areas than in other areas. However, our observation is consistent with reports that the immature stages of *A. inornatum* were most commonly observed on *Sigmodon hispidus* Say and Ord, 1825 (the Hispid cotton rat) in Cameron County, Texas (Eads and Borom 1975). This small rodent is known to be more common in areas of less dense vegetation with a mixture of monocots and dicot shrubs (Kincaid and Cameron 1985). *Sigmodon hispidus* and two closely related species, *Sigmodon toltecus* Saussure, 1860 and *Sigmodon hirsutus* Burmeister, 1854 are found throughout the known range of *A. inornatum* (Musser and Carelton, 2005). However, it should be noted that *A. inornatum* feeds on a wide variety of hosts.

Laboratory studies show that *A. inornatum* can complete its entire life cycle in as little as 120 days (Gladney et al. 1977). In the present field study, the data primarily support a single life cycle per year with larval presence peaking in August-November (Table 2), nymphs peaking in February through May (Figure 2), and adults peaking in July through September

(Figure 1). Whether this is the case further south in Mexico or Central America, which lack freezing temperatures, would require further research. Both nymphs and adults (and to a lesser extent larvae) are detected at low trap densities throughout the year. It is possible that some individuals of *A. inornatum* may complete more than one life cycle per year. The peak in adults is consistent with reports of adults collected from animals in South Texas (Cook et al. 1969, Eads 1951, Samuel and Trainer 1970). Eads and Borom (1975) reported that the immature stages of *A. inornatum* were found in February, May, and August through December without distinguishing between larvae and nymph activity. Likewise, consistent with our results Teel et al. (1988) reported larvae were detected in December and on Eastern and Western meadowlarks as well as nymphs were detected on meadowlarks peaking from March until June.

The small body size of the larvae makes them more sensitive to dehydration by wind. Questing ticks will often leave a questing position to return to the leaf litter to rehydrate which would lead to decreased survival and detection of ticks (Sonenshine, 1991). The presence of larvae on traps was positively correlated with mean humidity, and negatively correlated with mean wind speed and cloud cover on the trap day, with mean saturation deficit and mean wind speed over the three weeks prior to the trap day. It was noted that no larvae were detected on traps when the mean humidity of the preceding three weeks was below 58.9% RH and that most of the larvae were collected when the mean humidity was above 64% RH. This demonstrates that larvae were clearly sensitive to prolonged dehydrating conditions. A similar effect was observed with *A. mixtum* in South Texas (Beck et al. 2011). In studies in Brazil on *A. cajennense* sensu lato it was observed that larvae hatched at the same rate when placed in the field at different times. However, the larvae underwent a behavioral diapause and became active at the same time due to shorter days or colder temperatures (Cabrera and Labruna 2009, Labruna et al. 2003). Whether *A. inornatum* undergoes a similar behavioral diapause has not been investigated.

In the laboratory the nymphs are able to emerge 16-24 days after larva find a host (Gladney et al. 1977), suggesting that nymphs could begin emerging in November or December. However the nymphs emerge with a clear beginning and peak in February, strongly suggesting that some environmental event, such as temperature or photoperiod, is triggering their emergence. Interestingly, few nymphs were collected on trap days with a photoperiod of less than 11.5 hours (Figure 3). The density per trap of adult male *A. inornatum* ticks was positively correlated with the length of the day. *A. mixtum* adult activity increased significantly in February but peaked in June dropping to low trap densities again in October (Beck et al. 2011). In contrast, as with the nymphs, *A. inornatum* adults suddenly emerged in large numbers and reached peak density per trap in the same month (July). It is worth noting that the longest day of the year is in late June. This observation supports the hypothesis that *A. inornatum* adults are emerging in response to a decline in the photoperiod.

There are numerous factors that influence the establishment and persistence of tick populations in the natural environment. Long term multidisciplinary studies are needed to explain the patterns of tick density per trap that are observed in a study such as ours (Pfäffle et al 2013). Our concluding hypothesis is that the emergence of nymphs and adults is primarily dependent on photoperiod and/or temperature. Temperature and photoperiod are

highly correlated in the natural environment. Additional experiments would need to be conducted to test this hypothesis. Based on a photoperiod model the emergence of different life stages was observed when the day length drops below 12.5 hrs for larvae (about September 1), increases above 11.5 hrs for nymphs (about February 1) and begins to decline from the peak of 13.9 hrs for adults (after about June 20).

Implications for human risk

A. inornatum has also been repeatedly been shown to bite humans (Gibson and Carrillo 1959, Guzmán-Cornejo et al. 2011, Hoffman and López-Campos 2000, Nelder et al. 2014, Williamson et al. 2010). Sequences belonging to spotted fever group rickettsia were the most frequently detected (48/70, 69%) in *A. inornatum*. At the species level we identified *Candidatus R. amblyommii*. *Candidatus R. amblyommii* is of unknown pathogenicity and was recently successfully cultured (Labruna et al. 2004, Sayler et al. 2014, Zhang et al. 2012). It is frequently detected in *A. americanum* (Williamson et al. 2010) and other *Amblyomma* species (Soares et al. 2015). It has been implicated in infections of humans (Apperson et al. 2008, Bermúdez et al. 2013, Vaughn et al. 2014). It was also noted in a recent study of “naturally” acquired infections of dogs taken for walks in tick infested areas that the dogs had a much higher rate of seroconversion (confirmed by PCR) to *Candidatus R. amblyommii* than to *Rickettsia rickettsii* (Barrett et al. 2014). Thus, there is now strong evidence that infections previously identified as *R. rickettsii* included infections caused by *Candidatus R. amblyommii*.

We detected *Candidatus B. lonestari* in 6 of 70 (8.6%) of ticks by sequencing which is implicated as the causative agent of STARI – Southern Tick Associated Rash-like Illness, but thus far has not been successfully cultured for more than a short time. This disease is primarily seen after tick bites with *A. americanum* (Barbour et al 1996, Masters et al. 2008) and has also been detected in *A. cajennense* (Williamson et al 2010). A sequence related to the *B. burgdorferi* species complex was detected in 26/70 (37%) of the *A. inornatum* samples. *B. burgdorferi* is a species complex with a large number of closely related organisms (Jacquot et al. 2014, Margos et al. 2011). The clades within this complex often correspond to different host specificities (Brisson et al. 2008, Margos et al. 2011). Lyme disease has been occasionally diagnosed in Webb County (CDC). Whether the *Borrelia* detected in this study are able to cause disease is currently not known, but studies from Mexico reported that the seroprevalence to *B. burgdorferi* in Coahuila, Nuevo León and Tamaulipas, Mexico was 6%, 3.9%, and 14.1% respectively (Gordilla-Perez et al. 2003). These states in Mexico are south of Webb County, TX. In Tamaulipas and Nuevo León, “*B. burgdorferi* sensu stricto” was detected in 8.4% of ticks, including *A. cajennense*, *Ixodes scapularis* Say 1821, *Ixodes texanus* Banks, 1909, and *Dermacentor andersoni* Stiles, 1908 (Gordilla-Perez et al. 2009). *A. cajennense* sensu lato has also been suggested to have an unculturable spirochete-like bacteria which causes a Lyme disease-like syndrome was in Brazil in regions where no *Ixodes* ticks are present (Mantovani et al. 2007, da Costa et al. 2007).

E. chaffeensis was detected in three in *A. inornatum* ticks (4.3%), and a sequence closely related to *E. ewingii* was detected in one (1.4%). *E. chaffeensis* is frequently isolated from *A.*

americanum (Nair et al. 2014), and has also been detected in *A. mixtum* and *A. maculatum* (Williamson et al. 2010). The latter two ticks are also present in the study area. *E. chaffeensis* is the causative agent of human monocytic ehrlichiosis (Paddock and Childs 2003). *E. ewingii* causes similar disease symptoms but is more common in dogs.

A. inornatum larvae were present from August through November, this overlaps with adult *A. inornatum* and *A. mixtum* peak density per trap. *A. mixtum* larvae are also detected with a peak in August and September (Beck et al. 2011). When adult ticks co-feed on a host with larvae, there is a much higher risk of disease amplification (Randolph 2004). It is likely that both *A. inornatum* and *A. mixtum* adults and larvae are co-feeding on the same hosts as they both feed on a wide variety of mammals. Co-feeding of *A. inornatum* adults, *A. maculatum* adults, *A. americanum* adults and *Amblyomma* larvae has been observed in South Texas on a variety of hosts (Eads and Boron 1975, Samuel and Trainer 1970). This co-feeding would also help explain the high rate of infection of ticks with pathogens in this study.

Acknowledgements

We thank Josué Zavala, Eric O. Montalvo, J.P. Orozoco, M.A. Vela, K. Witt, F. Garcia, C.A. Bernal, L. Miller, M. Hernandez, J. Perez, J. Cortez, C. Gonzalez, Y. Alcaraz, L. Nunez, R.F. Palacios-Bruno, J. Serrato, M. Caballero, M. Kidd for their technical assistance. John Gunderson for review of the manuscript. David L. Beck was funded for this project by Tennessee Technological University and Texas A&M International University faculty development funds. Some undergraduates working on this project were part of the BUSCAR – Bridges to the Baccalaureate Program funded by NIH Grant# 5R25GM07856-03.

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Fig. 1.

Mean monthly collection of *A. inornatum* adults from March, 2005 to November, 2008 in the Tamaulipan Biotic Province (South Texas) with carbon dioxide traps. Each month represents the mean of three or four years. Error bars show the standard error of the mean.

Fig. 2.

Mean monthly collection of *A. inornatum* nymphs from March, 2005 to November, 2008 in the Tamaulipan Biotic Province (South Texas) with carbon dioxide traps. Each month represents the mean of three or four years. Error bars show the standard error of the mean.

Fig. 3.

Mean nymphs per trap compared to day light in hours. Each circle represent a different trap day. The Tukey box plot at the top of the graph shows that 50% of the trap days were between 11.5 hours and 13.4 hours. The Tukey box plot at the right of the graph shows that 50% of the trap days had less than 0.06 nymphs per trap. The whisker shows the highest data point within the 1.5 interquartile range. The box shows that the majority of the outliers (days with >0.4 nymphs per trap were on days with a day length from 11.5 hours and 13.4 hours).

Fig. 4.

Bayesian inference consensus tree inferred from *ompA* of *Rickettsia* species. Node support is indicated by the posterior probabilities before the node. The name of the species is followed by the GenBank accession number. C31R-F represents an amplicon for *A. inornatum*.

Fig. 5.

Baysian inference consensus tree inferred from *flaB* of *Borrelia* species. Node support is indicated by the posterior probabilities before the node. The name of the species is followed by the GenBank accession number. B38A-M, C30N-M1, C30N-M2, D19R-Ma, D19R-Mb, and H24O-F represent amplicons from *A. inornatum*.

Fig. 6.

Baysian inference consensus tree inferred from *dsb* of *Ehrlichia* species. Node support is indicated by the posterior probabilities before the node. The name of the species is followed by the GenBank accession number. D36N, D39H, and E24K represents amplicons from *A. inornatum*.

Fig. 7.

Bayesian inference consensus tree inferred from 16S rDNA. Node support is indicated by the posterior probabilities before the node.

Table 1

Primers used in this study

Primer Name	Gene	Primer Sequence (5' - 3')	Specificity	Screen	Reference
85F	<i>l2S</i>	TTAAGCTTTTCAGAGGAATTTGCTC	Not Reported	Initial	Williamson et al 2011
225R	<i>l2S</i>	TTTWWGCTGCACCTTGACTTAA	Not Reported	Initial	Williamson et al 2011
FlaLL	<i>flaB</i>	ACATATTCAGATGCAGACAGAGGT	<i>Borrelia</i> Genus	Initial	Barbour et al 1996
FlaRL	<i>flaB</i>	GCAATCATAGCCATTGCAGATTGT	<i>Borrelia</i> Genus	Initial	Barbour et al 1996
FlaLS	<i>flaB</i>	AACAGCTGAAGAGCTTGGAATG	<i>Borrelia</i> Genus	Nested	Barbour et al 1996
FlaRS	<i>flaB</i>	CTTTGATCACTTATCATTCTAATAGC	<i>Borrelia</i> Genus	Nested	Barbour et al 1996
BL-Fla 522F	<i>flaB</i>	GGTACATATTCAGATGCAGACAGAGGG	<i>Candidatus B. lonestari</i>	Initial	Williamson et al 2011
BL-Fla 1182R	<i>flaB</i>	GCACTTGATTTGCTTGTGCAATCATAGCC	<i>Candidatus B. lonestari</i>	Initial	Williamson et al 2011
BL-Fla 662F	<i>flaB</i>	AACTGCTGAAGAGCTTGGAATGC	<i>Candidatus B. lonestari</i>	Nested	This study
BL-Fla 860R	<i>flaB</i>	AGCTGGTTGAACCTTCCTGTTGT	<i>Candidatus B. lonestari</i>	Nested	This study
Rr. 190 70P	<i>rompA</i>	ATGGCGAATATTTCTCCAAAA	<i>Rickettsia</i> Genus	Initial	Regnery et al 1991
Rr. 190 602N	<i>rompA</i>	AGTGCAGCATTCGCTCCCCCT	<i>Rickettsia</i> Genus	Initial	Regnery et al 1991
Ehr DSB 330F	<i>dsb</i>	GATGATGTCTGAAGATATGAAACAAAT	<i>Ehrlichia</i> Genus	Initial	Doyle et al 2005
Ehr DSB 728R	<i>dsb</i>	CTGCTCGTCTATTTACTTCTTAAAGT	<i>Ehrlichia</i> Genus	Initial	Doyle et al 2005
MT-16S-F	<i>mito16S</i>	CCGGTCTGAACTCAGATCAAGT	Ixodidae	Phylogeny	Mangold et al 1998
MT-16S-R	<i>mito16S</i>	GCTCAATGATTTTTTAAATTGCTGT	Ixodidae	Phylogeny	Mangold et al 1998

Table 2

Detection of *A. inornatum* larvae on carbon dioxide traps, and tick walks in South Texas.

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
2005 ^a			1	0	0	0	0	0	0	0	2, 1 ^b	0
2006 ^a	1	0	0	0	0	0	0	0	0	25	0	0
2007 ^a	0	0	0	0	0	2	0	66, 1	4	10, 47, 1, 1 ^b	47, 1	0
2008 ^a	0	0			0	0	0	0	740	1, 1	5, 51, 1	
2008 ^c									121 ^c , 1820 ^c , 76 ^c		517 ^c , 559 ^c , 50 ^c , 1 ^c , 153 ^c	

^a Number of larvae shown for each positive trap in Webb County. Different numbers of traps were set each month, a minimum of 8 traps were set each month. If no traps were set no data is given for that month.

^b Number of larvae collected from tick walks. Tick walks were done every time traps were set.

^c Number of larvae collected from Jim Hogg County

Table 3
Correlation of selected meteorologic indicators and life stages of *A. inornatum* collected on carbon dioxide traps.

Meteorological Indicator ^a	Larvae ^b	Correlation ^d	Nymphs ^c	Correlation ^d	Males ^e	Correlation ^d	Females ^c	Correlation ^d	Adults ^c	Correlation ^d
Day Length	-0.217	p<0.03			0.234	p<0.02			0.191	p<0.06 ^g
Mean Temperature on Trap Day					0.280	p<0.005			0.255	p<0.01
3 Week Mean Temperature ^e			-0.185	p<0.07 ^g	0.231	p<0.02			0.202	p<0.05
3 Week Mean Humidity ^e	0.337	p<0.001			0.184	p<0.07 ^g	0.273	p<0.006	0.220	p<0.03
3 Week Mean Saturation Deficit ^e	-0.204	p<0.04								
Mean Wind Speed on Trap Day	-0.231	p<0.02								
3 Week Mean Wind Speed ^e	-0.386	p<0.001								
Cloud Cover	-0.326	p<0.001								
Total Rain from weeks 1-3			-0.178	p<0.08 ^g	0.180	p<0.08 ^g	0.193	p<0.06 ^g	0.178	p<0.08 ^g
Total Rain from weeks 1-4							0.195	p<0.06 ^g		
Total Rain from weeks 1-5					0.229	p<0.02	0.193	p<0.05	0.214	p<0.03
Total Rain from weeks 1-6	0.179	p<0.08 ^g	-0.182	p<0.07 ^g	0.238	p<0.02			0.207	p<0.04
Total Rain from weeks 2-4 ^f										
Total Rain from weeks 3-5 ^f	0.190	p<0.06 ^g								
Total Rain from weeks 4-6 ^f	0.275	p<0.006			0.268	p<0.008				
Total Rain from weeks 5-7 ^f	0.318	p<0.001								
Total Rain from weeks 6-8 ^f	0.371	p<0.001								
Total Rain from weeks 7-9 ^f	0.273	p<0.007								
Total Rain from weeks 9-11 ^f	0.366	p<0.001			0.302	p<0.002	0.190	p<0.06 ^g	0.285	p<0.004
Total Rain from weeks 11-13 ^f	0.185	p<0.07 ^g			0.225	p<0.03			0.243	p<0.02
Total Rain from weeks 13-15 ^f	0.197	p<0.05								
Total Rain from weeks 15-17 ^f	0.290	p<0.004			-0.260	p<0.01	-0.209	p<0.04	-0.274	p<0.006

^a Only meteorological indicators with significance p<0.08 or better are shown, df=98 for all correlations.

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- q For larva the mean number of traps that were positive for larva representing different larval hatch events was used.
- c For nymphs, males, females and total adults the mean number of ticks per trap was used.
- d Spearman's Coefficient of Rank Correlation was used.
- e The mean of the daily mean temperature for the previous three weeks (21 days). Similarly for humidity, saturation deficit, wind speed.
- f Rainfall was totaled for multiple three week (21 days) windows prior to the day of tick collection.
- g Not Significant

Table 4Detection of bacterial DNA in *A. inornatum* ticks.

	<i>Ehrlichia</i>	<i>B. lonestari</i>	<i>B. burgdorferi</i> Complex	<i>Rickettsia</i>
Adult Male	4% (2/50)	8% (4/50)	40% (20/50)	62% (31/50)
Adult Female	15% (3/20)	10% (2/20)	30% (6/20)	85% (17/20)
Total	7.1% (5/70)	8.6% (6/70)	37% (26/70)	69% (48/70)
2006	0/3	0/3	1/3	2/3
2007	0/16	2/16	2/16	7/16
2008	3/40	4/40	18/40	32/40
2009	1/9	0/9	4/9	6/9
2010	1/2	0/2	1/2	1/2
Total	5 of 70	6 of 70	26 of 70	48 of 70