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DINÁMICA POBLACIONAL DE LAS ESPECIES DE GARRAPATAS EN ESTADO NO PARASÍTICO (ACARI: IXODIDA) EN EL PARQUE NACIONAL PALO VERDE, GUANACASTE, COSTA RICA Y DETERMINACIÓN DE *RICKETTSIA* SP. (RICKETTSIALES) EN LAS ESPECIES CAPTURADAS.

Tesis sometida a la consideración de la Comisión del Programa de Estudios de Posgrado en Biología para optar al grado y título de Maestría Académica en Biología

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Dedico esta tesis a mi lindo Cucho. Gracias por tu apoyo y tu amor brindado durante este tiempo tan corto que estuviste a mi lado.

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RESUMEN

La distribución y dinámica poblacional de las garrapatas es afectada por factores climáticos y ambientales. La temperatura, el fotoperiodo, la humedad relativa, así como la estructura y composición de la vegetación, afectan de forma diferente a los diversos estadios de las garrapatas. La importancia médica de estos ácaros como transmisores de bacterias y virus ha sido reportada múltiples veces en la literatura. En los últimos diez años se han descrito nuevas interacciones entre garrapatas y varias especies de *Rickettsia* patogénicas; además, en las garrapatas se han aislado especies de *Rickettsia* con patología no comprobada. Lo anterior pone de manifiesto la importancia de las garrapatas como vectores de *Rickettsia*.

En Costa Rica varios estudios reportan la presencia de *Rickettsia* spp. en garrapatas en la vertiente Caribe; sin embargo, no hay estudios sobre estos organismos en el bosque seco tropical costarricense. Este estudio tiene dos metas principales: la primera parte, se enfoca en determinar el efecto del tipo de hábitat (estructura vegetal) y las condiciones climáticas (temperatura y precipitación) sobre la fluctuación en la abundancia de larvas, ninfas y adultos de garrapatas (*Amblyomma* spp.) en el bosque seco tropical. En general, la abundancia de garrapatas presenta cambios drásticos temporales y entre hábitats, según el estadio. Las larvas presentan dos picos máximos de abundancia durante el año: época seca que va de diciembre a mayo y el veranillo de San Juan entre julio y agosto, seguido por picos de abundancia de ninfas. Por otra parte, los adultos sólo presentan un pico de abundancia durante la época seca del año. Las larvas fueron abundantes en todos los sitios, pero las ninfas y los adultos fueron abundantes solamente en los sitios boscosos.

Para la segunda parte de este estudio, se determinó la presencia de *Rickettsia* spp. en muestras aleatorias de garrapatas de cada estadio mediante la técnica de Reacción en Cadena de la Polimerasa convencional (PCR) para dos genes, *gltA* y *ompA*, presentes en las rickettsias del grupo de las fiebres manchadas. Un alto porcentaje de garrapatas resultaron infectadas con *Rickettsia*, pero la incidencia fue mayor en larvas y ninfas. Todas las secuencias obtenidas para el gen *ompA* en este estudio, a excepción de una, fueron identificadas como *R.amblyommatis*. La secuencia restante fue similar a *R. colombianensi*. El efecto patogénico sobre humanos de *R. amblyommatis* no ha sido comprobado, razón por la cual no se puede descartar por completo. La detección de *Rickettsia* en varias muestras de larvas sugiere la existencia de transmisión transovarial de esta bacteria. Este es el primer reporte de *R. amblyommatis* en el bosque tropical seco costarricense y el primer reporte de *R. colombianensi* para Costa Rica.

ABSTRACT

Population distribution and dynamics of ticks is affected by climatic and environmental factors. The temperature, photoperiod, relative humidity, as well as the structure and composition of the plants, affects differentially the three ticks stages. The medical importance of ticks as transmitters of bacteria and viruses has been reported several times in the literature. In the last ten years, new interactions between ticks and several pathogenic *Rickettsia* species have been described. In addition, several *Rickettsia* species have been isolated in ticks with unknown pathology. This indicates the importance of ticks as vectors of *Rickettsia.*

In Costa Rica, several studies have reported the presence of *Rickettsia* spp. in ticks on the Caribbean slope; however, there are no studies on these organisms in the tropical dry forest. *This* study has two main goals: the first part focuses on determining the effect of habitat type (plant structure) and climatic conditions (temperature and precipitation) on the fluctuation in abundance of larvae, nymphs and adults of ticks (*Amblyomma* spp.) in the tropical dry forest. In general, the abundance of ticks has temporal drastic changes and between habitats, according to the stage. Larvae present two peaks of abundance during the year: dry season from December to May and between July and August (Veranillo de San Juan), followed by peaks of abundance of nymphs. On the other hand, adults only show a unique peak of abundance during the long dry season of the year. Larvae were abundant in all sites, but nymphs and adults were more abundant in forest sites.

For the second part of this study, the presence of *Rickettsia* spp. was determined in random samples of ticks from each stage by the technique of polymerase chain reaction (PCR) for two genes, *gltA* y *ompA*, present in the rickettsias of spotted fever group. A high percentage of ticks were infected with *Rickettsia*, but the incidence was higher in larvae and nymphs. All the sequences for the *ompA* gene in this study, with exception of one, were identified as *R. amblyommatis*. The remaining sequence was similar to *R. colombianensi*. The pathogenic effect on humans of *R. amblyommatis* has not been proven, so that it cannot be ruled out completely. The detection of *Rickettsia* in several larval samples suggests the existence of transovarial transmission of this bacterium. This is the first report of *R. amblyommatis* in the Costa Rican dry tropical forest and the first report of *R. colombianensi* for Costa Rica

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Effect of habitat and climatic fluctuation on abundance of off-host *Amblyomma* ticks (Acari; Ixodidae) in the Palo Verde National Park Costa Rica

Resumen

El tipo de hábitat (estructura vegetal) y las fluctuaciones en las condiciones climáticas afectan la abundancia de las poblaciones de garrapatas. No obstante, el efecto de estos cambios influye de manera diferente según el estadio del ciclo de vida de las garrapatas. El objetivo de este estudio es determinar el efecto del tipo de hábitat (estructura vegetal) y las condiciones climáticas (temperatura y precipitación) en la fluctuación en la abundancia de larvas, ninfas y adultos (*Amblyomma* spp.) en el bosque seco tropical. En general, la abundancia de garrapatas presenta cambios drásticos temporales y entre hábitats. La abundancia de larvas presenta picos máximos durante dos períodos del año: el época seca y veranillo de San Juan, seguido por picos de abundancia de ninfas, mientras que los adultos sólo presentan picos de abundancia durante la época seca del año. Las larvas fueron abundantes en todos los sitios, pero las ninfas y los adultos fueron abundantes solamente en los sitios boscosos. La precipitación y la temperatura mínima están negativamente correlacionadas con la abundancia de adultos y ninfas. Los sitios boscosos proveen mejores condiciones (humedad relativa) para la sobrevivencia de los tres estadios de garrapatasy una mayor abundancia de huéspedes potenciales.

Abstract

Vegetation types and fluctuation in environmental conditions often strongly influence fluctuations of animal populations, and the effect of such changes may affect differently each stage of their life cycle. Here we evaluate the effect of different vegetation types and environmental conditions (e.g., temperature and precipitation) on the fluctuation of abundance of larvae, nymphs, and adults of ticks (*Amblyomma* spp.) in a northwestern Costa Rican tropical dry forest (Palo Verde National Park). Abundance changes drastically, both temporally and across vegetation types. In general, abundance of larvae reaches a peak during the dry periods of the year, followed by peaks of abundance of nymphs. The peak of

abundance of adults occurs during the dry season, but the three stages were found every month indicating a year round reproduction. Larvae were abundant in all sites, but nymphs and adults were more abundant in forested sites. The rainfall and minimum temperature correlated negatively with the abundance of nymphs and adults. Forested sites likely provide more suitable conditions (e.g., relative humidity) for survivorship of the three off-host tick stages (larvae, nymphs and adults) and a larger abundance of potential hosts.

Introduction

Individuals within a population are adapted to a range of different environmental conditions, and this range varies widely across different species (Cloudsley-Thompson 1962, Andrewartha and Birch 1982, Levings 1983). Part of this range of conditions is considered optimal because survivorship and reproduction of individuals are higher (Andrewartha and Birch 1982, Levings and Widsor 1982). Therefore, spatial and temporal changes in ecological and environmental factors are expected to affect abundance of animals' populations (Kühnelt 1963). In terrestrial arthropods these changes could differently affect the different stages, possibly more than in other groups of animals (e.g., birds and mammals). The life cycle of most terrestrial arthropods includes several stages, each with quite different ecological requirements. For instance, most tick species include four different stages to complete their life cycle (i.e., egg, larva, nymph, and adult), each of these stages with different ecological requirements (Randolph 2014), and in some tick species the need of a different host for larvae, nymphs, and adults (Oliver 1989).

Tick prevalence is known to vary across habitats and seasons often experiencing a peak in abundance during a certain period of the year. This temporal dynamic in ticks commonly correlates with climatic and microclimatic conditions, which are often determined by the structure of the vegetation (Milne 1950, Perret et al. 2004, Labruna et al. 2009). Ticks spend most of their life cycle off-host, living free in their habitat. Hence it is important to consider the possible effect of microclimate on survival and development of these arthropods. Previous studies have shown that abundance of different tick stages is

influenced by temperature, as well as humidity, rainfall, vegetation type (or habitat structure), soil water content and host availability to which the population is exposed (Szabó et al. 2007, Tack et al. 2012)

The type of vegetation that a habitat includes could provide microclimates where ticks may persist, or where conditions may be less favourable and ticks may not survive or may not live long enough to access a host. It is known that ticks, especially eggs and larvae, are highly vulnerable to dehydration in hot and drier climates, while extremely rainy conditions are also associated with high mortality (Milne 1950, Tack et al. 2012). Vegetation could provide refuges with moderate and more stable conditions where ticks can stay and survive longer or on the contrary could result in sites with extreme conditions whichmay become critical for persistence of the tick population. For example, more exposed sites, with scattered or no vegetation, have conditions with high ambient temperatures that may accelerate the molting process, but may also increase the chance of tick dehydration (Needham and Teel 1991). These conditions affect tick activity, their questing behavior and their chance of attaching to a host, and thus the seasonal patterns of tick abundance (Kaiser et al. 1988,Perret et al. 2004, Randolph 2014). For this reason, evaluating the effect of different types of vegetation and temporal fluctuation in climatic conditions on abundance of different tick species is fundamental to understand the spatial and temporal fluctuation in their populations.

Considering the possible effect of climate and vegetation type on tick abundance, and the scarcity information available about the seasonal dynamics of ticks in tropical dry forests, we aim to determine the effect of both vegetation structure and climatic factors (temperature, humidity and rainfall patterns) on the seasonal dynamic of different off-host tick stages in Palo Verde National Park, Guanacaste, Costa Rica. This park is characterized by a long dry season which lasts between four to six months, followed by an intense rainy season (Mata and Echeverría 2004), and the park has a mosaic of different vegetation types that makes it an ideal location to study de effect of vegetation, and climatic conditions on the prevalence and abundance of non-parasitic ticks.

Materials and Methods

Study site

We conducted this study from February 2012 to February 2013, in Palo Verde National Park, Guanacaste province (10° 21' N, 85° 21' W, 50-250 m elevation) Costa Rica. Palo Verde has an extension of 19800 ha that includes part of the southernmost fragments of the Mesoamerican tropical dry forest, from which only 0.1% of its original extension remains (Janzen 1988, Quesada and Stoner 2004), with a mean annual precipitation of 1700mm and temperatures that oscillate between 22 °C and 32 °C. The precipitation in the region occurs from June through November with a long dry season from December through May, with a short dry period between July and August.

The park includes different types of vegetation: deciduous dry forest, evergreen forest growing along rivers and permanent and seasonal streams, thorn scrub-lands, grasslands, mangroves, and disturbed areas caused by anthropogenic practices developed in the area (mainly livestock) before being declared a National Park in 1978. It also includes seasonal wetlands, intermittent streams, swamps and several waterholes that are used as major water sources by the wildlife in the area, especially during the dry season (Slud 1980, Hartshorn 1983). This diversity of habitats has conferred to the park a wide species richness of flora and fauna, and has become an area of vital importance for breeding and feeding of a large number of species of mammals, migratory birds and resident waterfowl species (Bartantes and Sánchez 2004).

Collection and identification of ticks

We collected free living ticks monthly (from February 2012 to February 2013) by dragging a piece of cloth (a blanket fabric of 1m x 1.5 m) on the litter and herbaceous layer (0-50 cm

above the ground) in seven different sites. We selected this method over CO_2 traps, because the strong windy condition during the entire dry limit the tick-attracting efficacy of these traps. Each site is characterized by a different vegetation structure based on Vaughan et al. (1982) and Hartshorn (1983) (Table 1). Every 2.5 meters we carefully examined (visually) the cloth and manually collected all ticks adhering or walking on it and preserved them in tubes containing 70% ethanol. In the laboratory, adult, nymphs and larvae were counted individually. Only adults were identified to species level using the taxonomic keys of Onofrio et al. (2006). We used keys from Keirans and Durden (1998) and Vargas (2006) and to identify larvae and nymphs to genus level. We deposited voucher specimens in the Museo de Zoología, Escuela de Biología, Universidad de Costa Rica.

Statistical analyses

We tested the effect of site (e.g., structure of vegetation), climatic factors, and area sampled at each site on the abundance of larvae, nymphs and adult ticks. Climatic data were obtained from the climatic station located in the Palo Verde National park, from OTS (http://www.tropicalstudies.org). We first selected monthly minimum and maximum temperature, monthly rainfall, rainfall from each week prior to each sampling, and relative humidity, as the climatic variables that could affect abundance of different tick stages. We then correlated these variables and excluded those that were highly correlated. Maximum temperature was highly correlated with minimum temperature ($r = 1$), and monthly rainfall with relative humidity ($r = 0.92$), so that we ended up with minimum temperature, monthly rainfall, and rainfall of the week prior to each sampling as the climatic predictor variables included in the analyses. The area sampled at each site had no effect of tick abundance, so this variable was not included in the final models.

The data on tick abundance consisted of counts that included a large number of zeros. Considering the characteristics of these data, we compared the abundance of each stage across sites using Zero Inflated Models with negative binomial distribution (library

glmmADMB). These models are appropriate when the response variable (e.g., abundance of larvae) consisted of counts and the number of zeros in the data exceed the amount expected by a Poisson or a negative binomial distribution (Zuur et al. 2009, 2012) as is the case in this study. Specifically, the Zero Inflated Models included site and climatic variables as predictor variables, and sampling month as random factor. We initially included all three climatic factors and excluded those that had no effect on predicting abundance of ticks from the final model using the Akaike Information Criterion (AIC) and analyses of residuals. We used the R statistical Language (version 3.02: R Core Team 2013) for all statistical and graphical analyses.

Results

Species richness and abundance

We collected 12 505 off-host ticks in seven sites of the Palo Verde National Park. From this total, 87% corresponded to larvae, 9.5% to nymphs, and 3.5 % to adults (Table 2). We identified all larvae and nymphs only to genus level as *Amblyomma* spp. and four different species of adult ticks: *Amblyomma mixtum*, *A. oblongoguttatum*, *A. parvum*, and *A. dissimile*. *A. mixtum* was the most common species, followed by *A. oblongoguttatum*, and both were present in all seven sites (Table 3), while *A. parvum* was collected in all but the two most disturbed sites, La Cantera and Laguna. We collected only one female *A. dissimile* from one site (Ojo de agua).

The total number of ticks collected varied widely among sites. Laguna had the highest number of ticks while LosMangos site had the lowest number. However, the abundance of each of the different stages followed a different pattern. Larvae were more abundant in the Laguna site; nymphs and males in Ojo de Agua, and females in Sendero Guayacán (Table 2).

The abundance of all three stages fluctuated widely over time. Abundance of larvae peaked in February, June, and December (Fig. 1), though given the short period of time separating between the peaks in December and February, these may correspond to a single peak. Nymphs had two defined peaks in March and August, just following the peaks of larvae. Adults had a poorly defined peak of abundance in August-September, and a clearly defined peak in November-December.

Abundance by site

The abundance of ticks varied widely among stages and sites (Tables 4-6). Sendero Guayacán site had a significantly larger abundance of larvae than La Cantera and Ojo de Agua, and Laguna than La Cantera and Ojo de Agua (Fig.2; Table 4). The abundance of larvae was similar among all other sites. The abundance of nymphs had a different pattern. Ojo de Agua had significantly more nymphs than all the other sites, followed bySendero Guayacán which had more nymphs than the Camino, La Cantera, and Laguna sites (Fig. 3; Table 5). The site with fewest nymphs was La Cantera, followed by Laguna and Camino. The two sites covered by mature forests, Sendero Guayacán and Ojo de Agua, had the highest abundance of adult ticks when compared with all other sites, whereas La Cantera and Laguna sites had the lowest abundance of adult ticks (Fig. 4, Table 6). The mean monthly rainfall and the mean minimum temperature correlated negatively with the abundance of nymphs ($Z = -3.54$, $p = 0.00039$) and adults ($Z = -3.05$, $p = 0.00231$) respectively, but none of the environmental variables correlated with larval abundance. Both of these variables strongly correlated with relative humidity ($r = -0.79$, $p < 0.0001$ with rainfall; $r = -0.84$, $p < 0.0001$ with minimum temperature), which is known to affect the molting success and survivorship of arthropods (Kahl and Knülle 1988).

Sex ratio

In general, adult males and females were present year round, but adults increased in abundance from August through December, and drastically decreased from February through July (Figs. 1 and 4). Sex ratio of each of the three species with a sufficient sample

size to conduct a statistical analysis did not differ from a 1:1 proportion. We collected153 males and 138 females of *A. mixtum* with a sex ratio that did not differ statistically from a 1:1 proportion. Within each of the seven sites the sex ratio of *A. mixtum* was also 1:1, but with a large monthly variation (Table 7). We also collected 47 males and 35 females of*A. oblongoguttatum*, and 29 males and 18 females of*A. parvum*. Sex ratio did not differ from a 1:1 proportion for the total of females and males for both species, nor within each of the sites, but similar to *A. mixtum*, these species showed a large monthly variation (Table 7).

Discussion

In the Costa Rican dry forest the abundance of ticks changes drastically over time. The increase in larval abundance correlates with the periods of lower precipitation; the driest period of the year occurs from December to May, with a second, short dry period in July-August (Herrera, 1985). Heavy precipitations likely flushes away and kills larvae (Rawlins, 1979), so that the peaks of abundance observed in this stage could be either determined by a lower mortality of larvae due to lower precipitation, or by females adjust their reproductive events (laying eggs) to the dry periods. At the population level the peaks of nymphal abundance just follow the peaks of larvae. That the peak of individuals of one stage follows the peak of the previous stage is common in population dynamics, since reproduction could be relatively synchronic even in tropical species (Barrantes and Weng 2007, Labruna et al. 2009). Adults drastically increase in abundance during the driest period, with a small peak during August-September. This indicates that the periods of drought in the dry forest are important for the population dynamics of ticks, since the peaks of abundance of non-parasitic ticks, particularly larvae and nymphs, match the occurrence of these periods.

The peaks of abundance of the three stages in each particular site approximate the peaks detected for all sites combined (Figs. 1-4); however, some sites varied in a few respects. Abundance of larvae reaches a peak during the driest period of the year (December-April) in nearly all sites, but only in four sites did the abundance of larvae increases during the

mid-year dry period (Fig. 2). Abundance of nymphs also showed a well defined peak of abundance, in most cases during the second half of the dry season, often, but not always, following the larval peak (e.g., Camino, Cantera, and Laguna sites) (Fig. 3). Adults had a single peak of abundance during the driest period of the year in five of the seven sites (Fig. 4). The overlap that occurs between the peaks of abundance of the three stages supports the argument of continuous annual reproduction, with some synchrony among sites, but is far from being strict. In addition, this pattern also indicates that the environmental conditions prevalent during the drought periods have a strong influence on the abundance of all three non-parasitic tick stages.

The structure of the vegetation and some environmental variables had a strong impact on the abundance of different stages of ticks in the tropical dry forest. The structure of the vegetation plays an important role in determining the presence of different assemblages of arthropods, because groups of species converge in adapting to particular environmental conditions and biotic interactions associated to particular types of vegetation (Levings and Windsor 1982, 1984). Most tick species require more than one host and this condition allow the evaluation of the effect of the vegetation structure and climatic conditions on the different free-living stages: larvae, nymphs, and adults. The forested sites (Forest and Ojo de Agua) apparently have the most suitable conditions for molting (larvae and nymphs) and survivorship of the three stages of free-living ticks. Trees in both of these sites retain the leaves during the driest period of the year (Hartshorn 1983), so that the crown of the trees drastically reduce the radiation and temperature at ground level, also maintaining a higher relative humidity at this level (Borchert 1994).

Another factor associated with the apparent success of ticks in the forested sites is the presence and abundance of potential hosts. Both sites include small water springs that remain flowing during the entire dry season, or nearly so. The presence of water, benign environmental conditions, and occurrence of some fleshy-fruit trees (Frankie et al. 1974, Opler et al. 1980) that produce their crops during the dry season made these two sites very attractive for a large number of potential mammal hosts (e.g., white tailed deer, peccaries,

raccoons, coatis; Vaughan and Weis 1999) . We have collected *Amblyomma mixtum* from some of these mammals in this dry forest (unpubl. data) and these mammals have been reported elsewhere as primary hosts of the same tick species we found in this dry forest (Fairchild et al. 1966, Guglielmone and Nava 2006). Considering that most of these medium-size and large mammals move back and forth between forest patches and between forest patches and altered areas, it is likely that they serve as dispersal agents between forested areas, and between forested and altered areas. This needs to be confirmed, but the presence of larvae, and the nearly absence of other stages, in some sites circumstantially supports this hypothesis.

There is some consensus among researchers that tick species have a single annual breeding period (Oorebeek and Kleindorfer 2008, Szabo et al. 2007). In particularly, Labruna et al. (2009) state that *A. cajennense*, the most abundant species in their study, has one generation per year, based on behavioral diapause of larvae. In the Costa Rican dry forest, based on peak abundance of larvae, there are at least two well defined annual reproductive events. And if we assume that most larvae belong to the two most common adult species, at least *A. mixtum* (a close related species to *A. cajennense*; Nava et al. 2014) and A. *oblongoguttatum* would have two reproductive peaks during the year. However, the reproduction of ticks in this forest likely occurs year round, since we collected larvae, nymphs, and adults of both sexes each month during the study period (Fig.1).

In conclusion, vegetation structure plays an important role in determining fluctuation in abundance of the three free-living stages of *Amblyomma* spp. ticks in a Costa Rican dry forest. Abundance of the three stages is higher in the two forested sites, indicating that these sites offer more suitable conditions for molting and survivorship of ticks. In addition, these two sites concentrate a rich and abundant mammal fauna (Janzen and Wilson 1983, Stoner and Timm 2004), with many of the mammal species serving as primary hosts for the tick species found in this dry forest (Fairchild et al. 1966, Guglielmone and Nava 2006). The abundance of tick larvae indicates the occurrence of at least two annual peaks of reproduction, but the presence of larvae, nymphs, and adults during the entire study period

suggests that ticks in this Costa Rican dry forest have a continuous reproduction, with two well defined peaks.

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Table 1. Environmental characterization of sites sampled in Palo Verde National Park, Guanacaste.

Sites sampled in Palo Verde	Description
Sendero Guayacán	Evergreen forest dominated by large trees of *Brosimun alicastrum* growing on limestone soil surrounding a small seasonal water spring on a slope of about 20 degrees. The undergrowth is not very dense where *Garcia nutans* and *Saprantus palanga* are the most common shrubs. Herbaceous layer is dominated by some bambusoids and some Acantaceae herbs. Area sampled 492 m^2
Ojo de agua	Permanent water spring surrounding by old second ground forest. Herbaceous layer is very dense, dominated by Acantacea and grasses. The canopy is dominated by trees of the species *Calycophylumcandidisimun, Ura crepitans, Manilkara zapota* y *Cocoloba umbifera*.Area sampled 855 m^2
Sendero Mapache	Rocky limestone soil, with little dense undergrowth, with some shrubs of *Vachellia* and large limestone rocks that cover much of the area. Trees of *Sterculia apetala, Calycophylum candidisimun* y *Brosimun allicastrum* are abundant.Area sampled 928 m^2
Los Mangos	Altered area with the ground covered by a thick layer of litter and grasses. This site includes a camping area for visitors and 11 large mango trees *Mangifera indica*.Area sampled 921 m^2
Camino Principal (border)	It corresponds to the rocky soil and herbaceous edge of the main road of the park, located after camping area (Los Mangos). Area sampled 382 m^2

La Cantera	Flat trail with a herbaceous layer mainly composed of grasses and shrub layer dominated by *Vachellia* (= *Acacia*) and deciduous trees (e.g., *Guazuma ulimifolia*).Area sampled 276 m^2
Laguna y pista de aterrizaje	Edge of a seasonal lagoon where some abundant tree species such as *Pithecellobim lanceolatum* y *Parkinsonia aculeata* trees. Herbaceous layer is dominated by Acantacea and grasses. The lagoon is used by migratory and resident water birds to feed and breed throughout the year or part of it.Area sampled 922 m^2

Table 2. Total number of ticks collected in sites sampled en Palo Verde, Guanacaste

Site/ Instar	Guayacán	Ojo de agua	Mapache	Mangos	Camino	Cantera	Laguna
Larvae	1480	1872	1830	360	1032	1655	2671
Nymphs	272	324	269	171	37	41	69
Males	57	65	39	21	35	8	4
Females	64	38	32	25	26	6	2
Total	1873	2299	2170	577	1130	1710	2746

Table 3. Tick species collected in sites sampled in Palo Verde, Guanacaste.

Specie/ site	*Amblyomma mixtum*		*Amblyomma oblongoguttatum*		*Amblyomma parvum*	
	Male	Female	Male	Female	Male	Female
Guayacán	39	51	15	11	3	2
Ojo de agua	50	25	8	5	7	6
Mapache	22	16	11	13	6	3
Mangos	11	16	1	2	9	7
Camino	25	25	6	1	4	0
Cantera	5	3	3	3	0	0
Laguna	1	2	3	0	0	0
Total	153	138	47	35	29	18

Table 4. Results of Zero Inflated Models with negative binomial distribution for larvae. The habitat type and monthly rainfall were included as predictor variables and sampling date as random factor.

Sites	Estimated	SE	Z value	P
Guayacán-Camino	-2.03	1.185	-1.71	0.087
Guayacán - Cantera	-3.17	1.441	-2.20	0.028
Guayacán - Laguna	-0.08	0.961	-0.08	0.935
Guayacán - Mangos	-1.68	0.947	-1.77	0.077
Guayacán - Mapache	-1.02	0.942	-1.08	0.280
Guayacán - Ojo	-2.45	1.146	-2.13	0.033
Camino-Cantera	-1.14	1.163	-0.98	0.320
Camino-Laguna	1.95	1.097	1.77	0.076
Camino-Mango	0.35	1.228	0.29	0.776
Camino-Mapache	1.01	1.220	0.83	0.409
Camino-Ojo	-0.42	1.177	-0.36	0.722
Cantera-Laguna	3.09	1.367	2.26	0.024
Cantera-Mango	1.49	1.515	0.99	0.324
Cantera-Mapache	2.15	1.437	1.50	0.134
Cantera-Ojo	0.72	1.393	0.52	0.603
Laguna- Mango	-1.60	1.039	-1.54	0.125
Laguna-Mapache	-0.94	0.995	-0.94	0.346
Laguna- Ojo	-2.37	1.119	-2.11	0.034
Mango-Mapache	0.66	1.137	0.58	0.563
Mango- Ojo	-0.77	1.291	-0.60	0.551
Mapache-Ojo	-1.43	1.096	-1.30	0.190

Table 5. Results of Zero Inflated Models with negative binomial distribution for nymphs. The habitat type and monthly rainfall were included as predictor variables and sampling date as random factor.

Sites	Estimated	SE	Z value	P
Guayacán -Camino	-1.92	0.379	-5.07	<0.00001
Guayacán - Cantera	-1.90	0.383	-4.96	<0.00001
Guayacán - Laguna	-1.20	0.358	-3.37	0.00076
Guayacán - Mangos	0.02	0.349	0.07	0.94808
Guayacán - Mapache	0.41	0.333	1.23	0.21967
Guayacán - Ojo	0.65	0.331	1.98	0.04805
Camino-Cantera	0.022	0.427	0.05	0.95896
Camino-Laguna	0.72	0.402	1.78	0.07436
Camino-Mango	1.94	0.398	4.88	<0.00001
Camino-Mapache	2.33	0.384	6.07	<0.00001
Camino-Ojo	2.58	0.385	6.70	<0.00001
Cantera-Laguna	0.69	0.403	1.72	0.08486
Cantera-Mango	1.92	0.400	4.81	<0.00001
Cantera-Mapache	2.31	0.384	6.01	<0.00001
Cantera-Ojo	2.55	0.390	6.55	<0.00001
Laguna- Mango	1.23	0.370	3.32	0.00091
Laguna-Mapache	1.61	0.355	4.54	<0.00001
Laguna- Ojo	1.86	0.357	5.21	<0.00001
Mango-Mapache	0.39	0.336	1.15	0.24988
Mango- Ojo	0.63	0.340	1.86	0.06318
Mapache-Ojo	0.25	0.323	0.76	0.44535
Monthly rain	-0.01	0.003	-3.54	0.00039

Table 6. Results of Zero Inflated Models with negative binomial distribuition for adult ticks. The habitat type and monthly rainfall were included as predictor variables and sampling date as random factor.

Sites	Estimated	SE	Z value	P
Guayacán -Camino	-1.11	0.3202	-3.47	0.00052
Guayacán - Cantera	-2.39	0.4101	-5.83	<0.00001
Guayacán - Laguna	-3.25	0.5450	-5.96	<0.00001
Guayacán - Mangos	-1.36	0.3296	-4.14	<0.00001
Guayacán - Mapache	-0.73	0.3031	-2.41	0.01602
Guayacán - Ojo	-0.04	0.2936	-0.12	0.90263
Camino-Cantera	-1.28	0.415	-3.08	0.00208
Camino-Laguna	-2.14	0.558	-3.83	0.00013
Camino-Mango	-0.25	0.346	-0.73	0.46325
Camino-Mapache	0.38	0.325	1.17	0.24187
Camino-Ojo	1.07	0.336	3.20	0.00137
Cantera-Laguna	-0.86	0.614	-1.40	0.1613
Cantera-Mango	1.02	0.431	2.38	0.0173
Cantera-Mapache	1.66	0.415	4.00	<0.00001
Cantera-Ojo	2.35	0.421	5.59	<0.00001
Laguna- Mango	1.88	0.566	3.33	0.00087
Laguna-Mapache	2.52	0.552	4.56	<0.00001
Laguna- Ojo	3.21	0.550	5.84	<0.00001
Mango-Mapache	0.63	0.340	1.86	0.06229
Mango- Ojo	1.33	0.343	3.87	0.00011
Mapache-Ojo	0.69	0.313	2.22	0.02640
Minimum temp.	-0.59	0.192	-3.05	0.00231

Table 7. Sex proportion of Amblyomma spp.ticks collected in sites sampled in Palo Verde, Guanacaste. (n= numbers of months in which a particular species was collected).

Species	Site	n	Females Mean (Desv.std)	Males Mean (Desv.std)
A. mixtum	Guayacán	10	0.54 (0.29)	0.46 (0.29)
	Ojo de agua	11	0.54 (0.42)	0.46 (0.42)
	Mangos	7	0.45 (0.45)	0.55 (0.45)
	Mapache	9	0.47 (0.36)	0.53 (0.36)
	Camino	6	0.48 (0.32)	0.52 (0.32)
	Cantera	4	0.58 (0.50)	0.42 (0.50)
	Laguna	3	0.67 (0.58)	0.33 (0.58)
A. oblongoguttatum	Guayacán	4	0.42 (0.17)	0.58 (0.17)
	Ojo de agua	4	0.38 (0.48)	0.63 (0.48)

	Mapache	5	0.49 (0.13)	**0.51 (0.13)**
	Mangos	2	0.75 (0.35)	**0.25 (0.35)**
	Camino	2	0.17 (0.24)	0.83 (0.24)
	Cantera	1	0.50 (NA)	0.50 (NA)
	Laguna	1	0.00 (NA)	1.00 (NA)
	Guayacán	4	0.38 (0.48)	0.63 (0.48)
	Ojo de agua	6	0.46 (0.45)	**0.54 (0.45)**
	Mapache	4	0.35 (0.47)	**0.65 (0.47)**
A. parvum	Mangos	4	0.21 (0.28)	**0.79 (0.28)**
	Camino	3	**0.00 (NA)**	**1.00 (NA)**

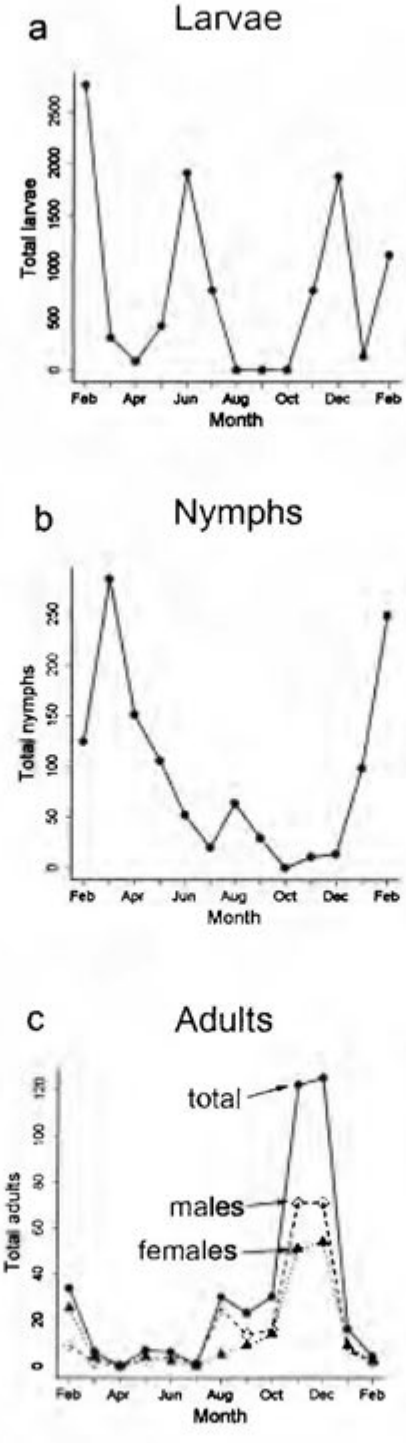

Figure 1. Monthly variation of abundance of larvae (a), nymphs (b) and adults in Palo Verde National Park.

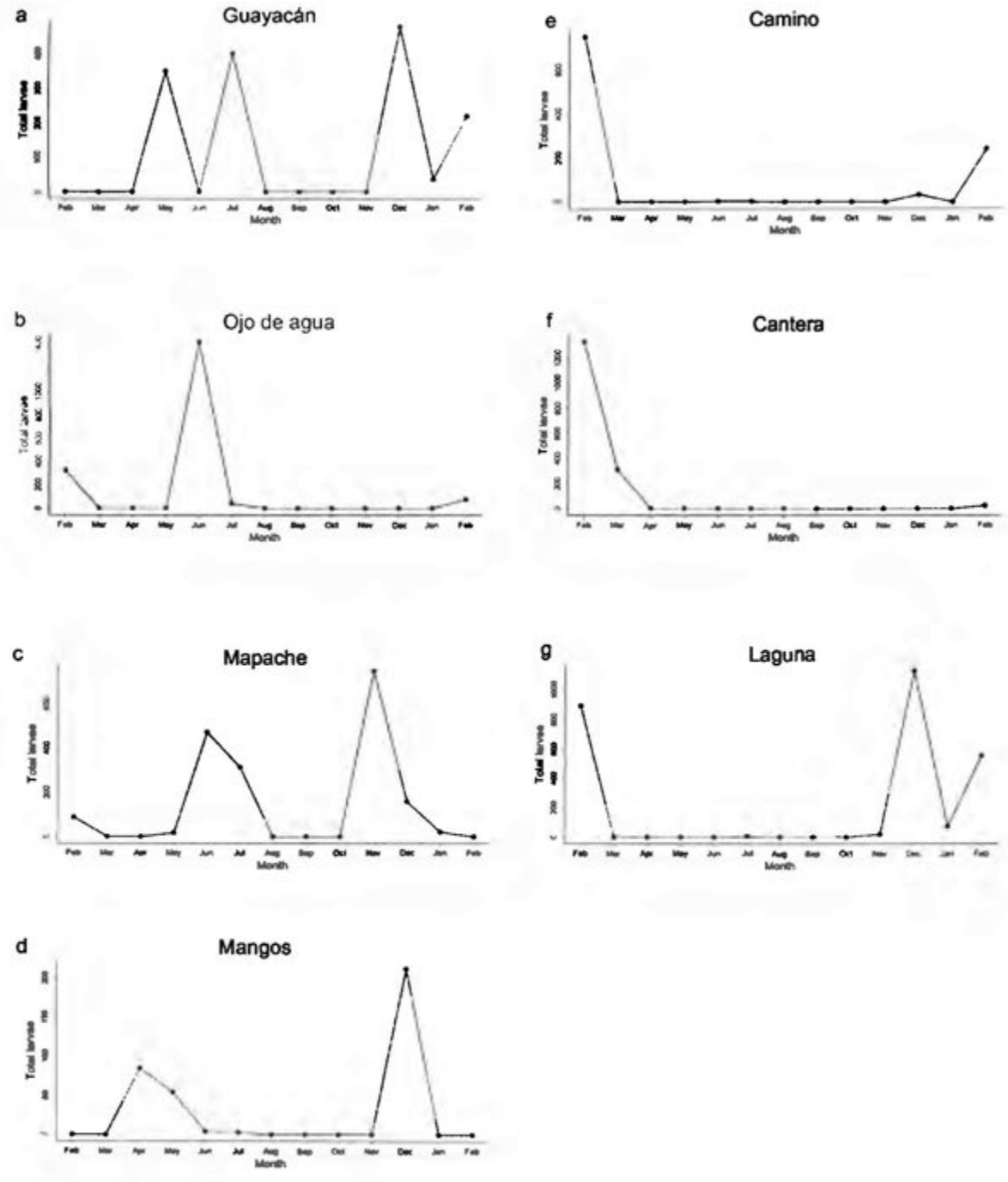

Figure 2. Monthly variation of larvae abundance in seven sites. The sites are ranked from the most forested (a) to the most altered site (g: grassy terrain).

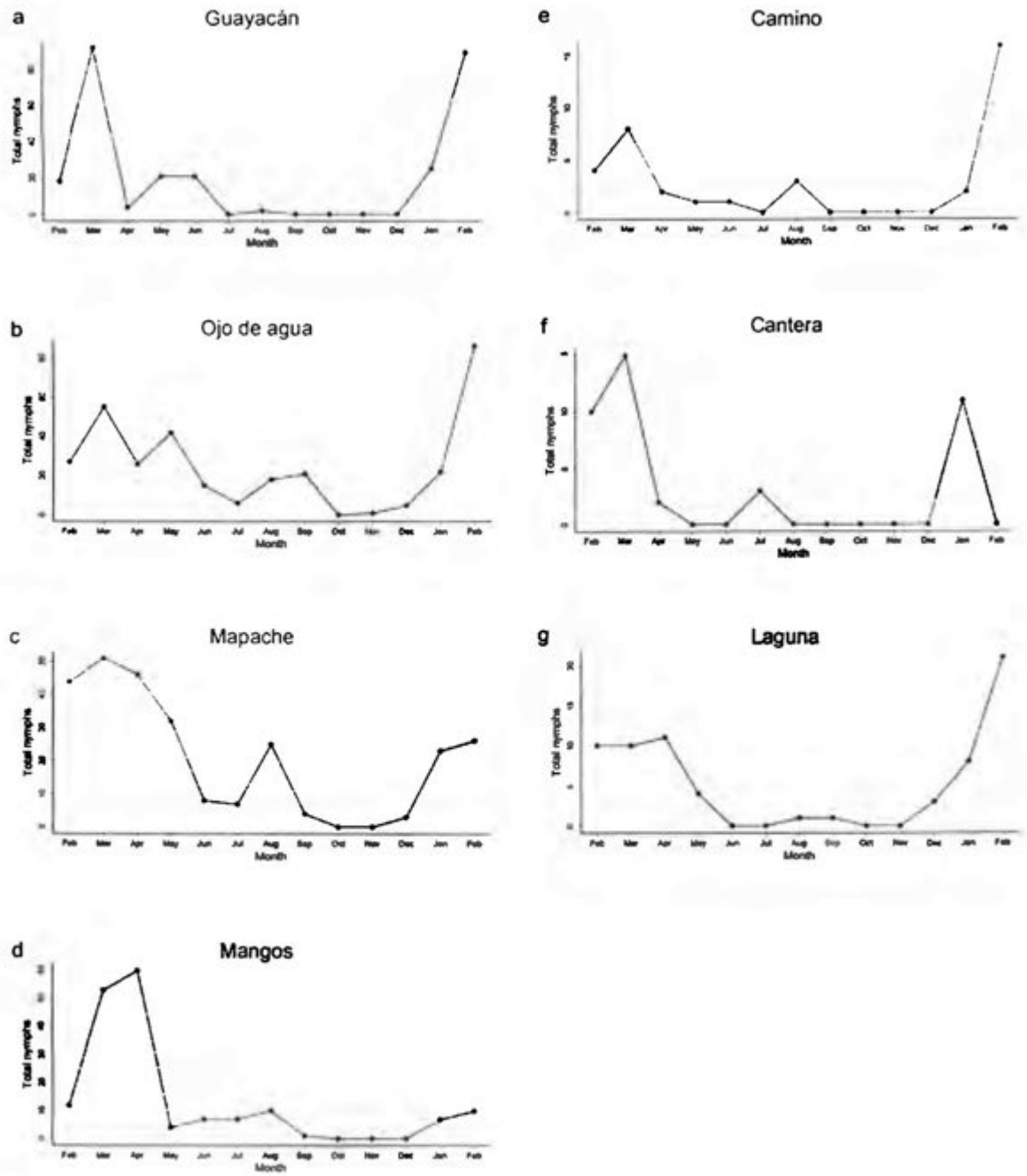

Figure 3. Montlhy variation of nymphs abundance in seven sites. The sites are ranked from the most forested (a) to the most altered site (g: grassy terrain).

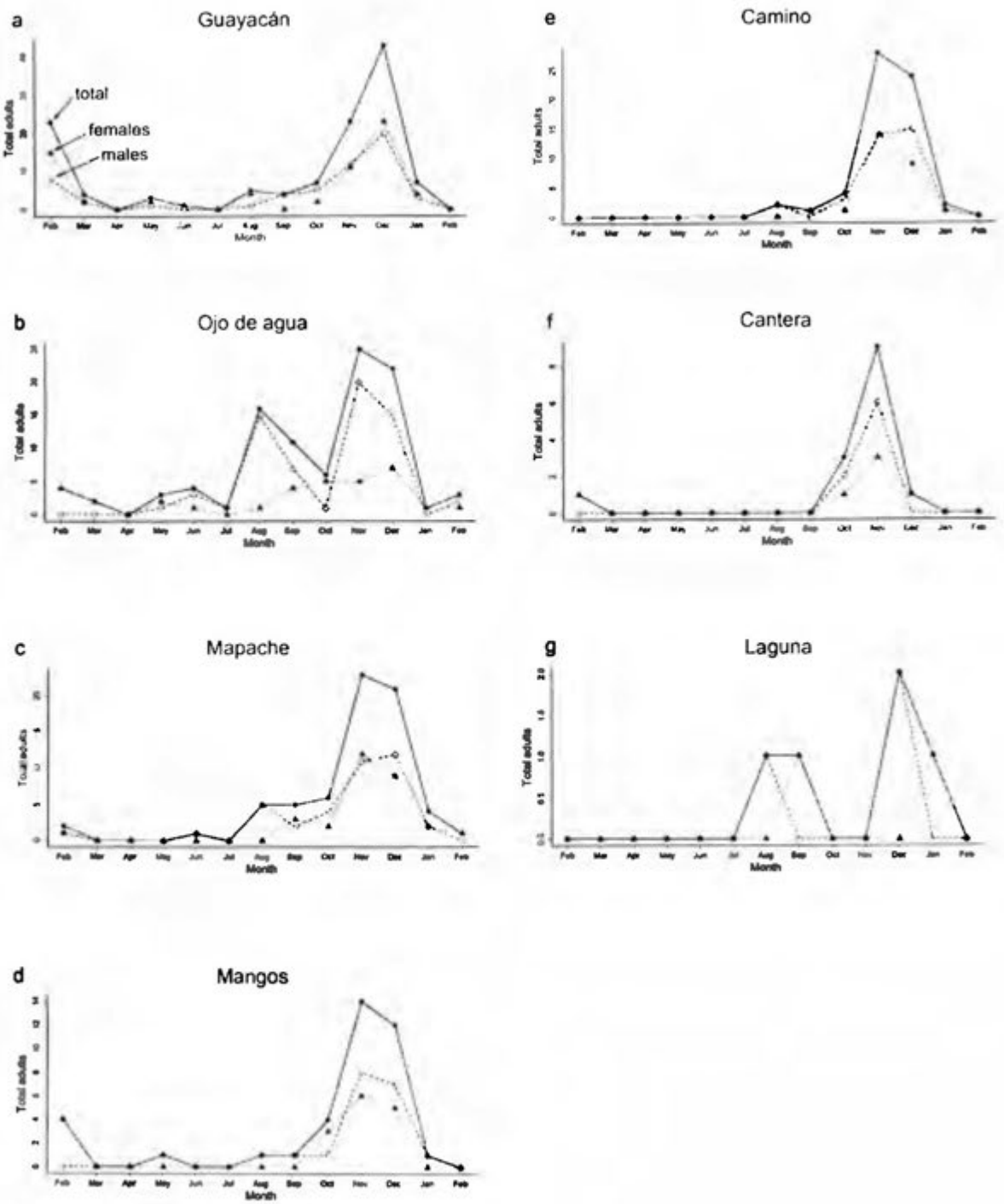

Figure 4. Monthly variation of adults abundance in seven sites. The sites are ranked from the most forested (a) to the most altered site (g: grassy terrain).

Molecular detection of *Rickettsia amblyommatis* in off-host *Amblyomma* ticks in the Palo Verde National Park, Costa Rican and the first record of *Rickettsia* sp. strain *colombianensi*

Resumen

En el bosque tropical seco costarricense no se han reportado, previamente, especies de *Rickettsia* infectando garrapatas. Un alto porcentaje de garrapatas de vida libre (larvas, ninfas y adultos de *Amblyomma*) resultaron infectadas con *Rickettsia*, pero la incidencia fue mayor en larvas y ninfas. Todas las secuencias obtenidas para el gen *omp A* en este estudio, a excepción de una, fueron identificadas como *R.amblyommatis*. La secuencia restante fue similar a *R.* sp. strain*colombianensi*. *R. amblyommatis* es muy abundante en diferentes regiones geográficas y climáticas en América, pero su efecto patogénico en humanos no ha sido comprobado, razón por la cual no se puede descartar por completo. La detección de *Rickettsia* en varias muestras de larvas sugiere la existencia de transmisión transovarial de esta bacteria. Este es el primer reporte de *R. amblyommatis*en el bosque tropical seco costarricense y el primer reporte de *R.* sp. strain*colombianensi*para Costa Rica.

Abstract

Species of *Rickettsia* parasitizing ticks have not previously been reported in Costa Rican dry forests. A large percentage of off-host larvae, nymphs, and adults of *Amblyomma* ticks were infected with *Rickettsia* spp., and the incidence was higher in larvae and nymphs than in adults. All but one sequence of the gene *ompA* identify the rickettsias found in this study as *R. amblyommatis*. The other sequence was nearly identical to *Rickettsia* strain *colombianensi*. *R. amblyommatis* is very abundant in different geographic and climatic regions in the America's, but its pathogenic effect on humans has not been proven, though it cannot be discarded entirely. The detection of *Rickettsia* in several larval samples, suggests

a transovarial transmission of this bacteria in dry forest species of *Amblyomma*. This is the first report of *R. amblyommatis* in the Costa Rican northwestern tropical dry forest and the first report of *R. colombianensi* for Costa Rica.

Introduction

Rickettsia species are bacterial endoparasites widespread in different groups of arthropods (Parola et al. 2005a, Merhej et al. 2014). At least 16 species are human pathogens transmitted by ectoparasites such as fleas, lice, mites, and ticks (Parola & Raoult 2006, Zavala-Velázquez et al. 2006, Summer et al. 2007, Nicholson et al. 2009, Hun et al. 2011, Labruna et al. 2011a, Troyo 2012, Parola et al. 2013). In the past, identification of *Rickettsia* species was difficult and imprecise, but new sensitive molecular techniques have proven to be a valuable tool for a rapid identification of *Rickettsia* species, and now new species are frequently identified from ticks and other arthropods (Zavala-Velázquez et al. 2006, Walker 2007, Paddock et al. 2010, Labruna et al. 2011a, Troyo et al. 2012, 2014, Miranda & Mattar 2014). Several rickettsias, previously identified as a different species, have been re-identified or in some cases re-assigned to a different or even new species (Paddok et al. 2004, Raoult et al. 2005, Hechemy et al. 2006). This new information has shed some light on the evolution and relationship among different species of *Rickettsia* (Parola et al. 2005b, Weinert et al. 2009, Venzal & Nava 2011, Merhej et al. 2014).

The pathogenic effect of some of the new species is still unknown. However, this is a topic that deserves special attention because some species that have previously been considered innocuous, have resulted pathogenic to humans (Paddock et al. 2004, Parola et al. 2005b, Parola & Raoult 2006, Walker 2007, Parola et al. 2009, Labruna et al. 2011a, Parola et al. 2013).

Most of the recognized pathogenic rickettsias are classified into the spotted fever group (SFG). This group includes the species responsible for the spotted fever rickettsiosis which

are transmitted by ticks to humans in different parts of the world, including tropical regions (Parola & Raoult 2006, Labruna et al. 2011a, Parola et al. 2013). In Costa Rica, the first lethal human case of rickettsiosis, attributed to *Rickettsia rickettsii*, occurred in 1975. After that time, 16 new cases of this infection have been reported. Most of these cases have occurred in lowlands along the Caribbean coast, but lately (2010-2011) some additional cases have occurred in urban areas (Fuentes 1979, Fuentes et al. 1985, Fuentes 1986, Hun et al. 1991, Hun-Opfer 2008, Argüello et al. 2012, Troyo et al. 2012). However, other regions in the country remain unexplored.

In Costa Rica, there is little information on the presence and distribution of the species of *Rickettsia* along the entire Pacific lowlands, particularly the dry forest in the northwestern region, which is a common destination for thousands of tourists and students year-round (Instituto Costarricense de Turismo, 2016). Ticks are extremely abundant in the tropical dry forest of Costa Rica (Sánchez-Quirós and Barrantes unpubl. data), and this makes it a good target area for studying the presence of *Rickettsia* species and their pathogenic potential. Thus, the objective of the present study is to identify the species of *Rickettsia* of free living ticks collected in different areas of the Palo Verde National Park in the northwestern Costa Rica.

Materials & Methods

Study site

We conducted this study from February 2012 to February 2013, in Palo Verde National Park, located in the northwest of Costa Rica, Guanacaste province (10° 21' N, 85° 21' W, 50-250 m elevation). Palo Verde has an extension of 19800 ha that includes part of the remaining Mesoamerican dry forest (Janzen 1988), with a mean annual precipitation of 1700 mm and temperatures that oscillate between 22° C and 32° C. The precipitation in the region occurs from June through November with a long dry season from December through May.

The park includes different habitats (Vaughan et al. 1982): deciduous dry forest, evergreen forest growing along rivers, permanent and seasonal streams, thorn scrub-lands, grasslands, mangroves, and disturbed areas caused by anthropogenic practices (mainly livestock), developed in the area before being declared a National Park in 1978. It also includes seasonal wetlands, intermittent streams, swamps and several waterholes, that are used as major sources of water by the wildlife in the area, especially during the dry season (Slud 1980, Hartshorn 1983).

Collection and identification of ticks

We collected free living ticks monthly by dragging a piece of fabric (flannel, 1m x 1.5 m) on the litter and herbaceous layer (0-50 cm above the ground) in seven different sites. Each site was characterized by a different vegetation structure based on Vaughan et al. (1982) and Hartshorn (1983). Every 2.5 meters the fabric was examined and all ticks were collected and preserved in tubes containing 70% ethanol. In the laboratory, adult, nymphs and larvae were counted individually. Only adults were identified to species level following the taxonomic keys of Onofrio et al. (2006); and usingthe taxonomic criteria proposed by Nava et al. (2014) for the designation of *Amblyomma mixtum* within the *Amblyomma cajennense* group. We used keys from Keirans & Durden (1998) and Vargas (2006), to identify larvae and nymphs to genus level. For the molecular analyses, we included samples of the three stages from all seven sites (Table 1).

Detection of Rickettsia

We extracted DNA from tick samples using the Nucleospin® Tissue kit (Macherey-Nagel) following the manufacturer's instructions for animal tissue. Adults were processed individually (58 females and 67 males) and nymphs and larvae were processed in pools using routine PCR assay with the primers CS 78F and CS 323R, designed to amplify a 401-bp fragment of the enzyme citrate synthase (*gltA*) of *Rickettsia* spp. (Roux et al. 1997,

Labruna et al. 2004a). PCR assays were performed with a final volume of 25 µL per reaction, which included five microliters of DNA extract, following the procedure described by Labruna et al.(2004a). Positive and negative controls were included in each reaction. We then processed the positive PCR samples for the presence of *Rickettsia* spp. with the primers Rr190.70p and Rr190.701n, which target a 631-bp fragment of the rickettsial 190-kDa outer membrane protein gene (*ompA*) (Regnery et al. 1991, Roux et al.1996). This gene identifies those species of *Rickettsia* that belong to the spotted fever group (SFG). PCR products of the expected sizes were sequenced in a ABI 3730XL Analyzer (Bioneer Sequencing Service, South Korea). We used the BioEditprogram (Hall, 1999) to determine the consensus sequences and then BLAST to determine the genetic similarity to other *Rickettsia* species (Altschul et al. 1990).

Statistical Analysis

We tested whether the proportion of samples with presence of *Rickettsia* vary among sites with Chi-squared analyses. We used the R statistical language, version 3.0.1 (R Development Core Team 2014) for all analyses.

Results

We collected 12 505 off-host ticks from seven sites in Palo Verde National Park, 87% larvae, 9.5% nymphs, and 3.5 % adults. From adult specimens, we identified four *Amblyomma* species (*Amblyomma mixtum, A. oblongoguttatum*, *A. parvum*, and *A. dissimile*). *Amblyomma mixtum* was the most common species (69.3%) and both *A. mixtum* and *A. oblongoguttatum* (19.5%) were found in all seven sampled sites; *A. parvum* (11.2%) was absent in the two most disturbed sites; and only one female of *A. dissimile* was found.

We detected *Rickettsia* in 38.0% (n = 80) of the total samples analyzed, based on *gltA*. From the total of larvae samples (n = 37), we detected *Rickettsia* in 54%; for nymphs (n =

64) in 51%; and for adults (n = 26) in 23% (Table 2). The proportion of positive samples did not differ across sites for the three stages: larvae- $X^2 = 2.57$, df = 6, p = 0.861; nymphs- $X^2 = 10.28$, $df = 6$, p = 0.113; adults- $X^2 = 6.06$, df = 6, p = 0.416.

From the positive samples for the presence of *Rickettsia* (based on *gltA*) we sequenced the gene *ompA* from 43% of them to identify the species of *Rickettsia*. All but one of the sequences obtained matched the sequences of *Rickettsia amblyommatis* (previously considered as *R.amblyommii*; Karpathy et al. 2016) 97.7% (SD =2.69; range = 87% – 100%) deposited in the Gene Bank. The other sequence obtained from a larval pool, was nearly identical (99%) to *Rickettsia* sp. strain *colombianensi*; thus, we considered this *Rickettsia* as *R.* sp. strain *colombianensi*.

Discussion

The incidence of *Rickettsia* spp. (38.0%) is similar to that found in North America (Moncayo et al. 2010), in South America (Labruna et al. 2004b), and in Panamá (Bermúdez et al. 2009, 2016), and it was higher for larvae (53.1%) and nymphs (54.0%) than for adults (24.3%). In addition, we found that females and males of the three *Amblyomma* species analyzed (*A. mixtum*, A. *oblongoguttatum*, *A. parvum*) were infected in similar proportion with *Rickettsia*; these tick species that had previously been reported in Costa Rica (Calderón-Álvarez et al. 2005). The higher percentage of infection in adults compared to larvae and nymphs could be produced by differential mortality caused by *Rickettsia*, as has been experimentally shown by Nielbylski et al. (1999) for *R. rickettsii* in *Dermacentor variabilis*. It could also be an artifact of using a pool instead of individuals in testing for the presence of *Rickettsia* in samples of larvae and nymphs; if in a pool one or a few larvae or nymphs are infected, the sample would be registered as positive, and this would inflate the positive samples for immature stages.

All, but one *Rickettsia* detected in this study were identified as *R. amblyommatis* following the criteria of Karpathy et al. (2016) who formally described this species, as candidatus *Rickettsia amblyommii* was not validly described. We also found a large proportion of ticks - the two immature stages and adults of *Amblyomma mixtum*, *A. oblongoguttatum*, *A. parvum* - infected with *Rickettsia amblyommatis* in this Costa Rican dry forest. These and other results (Labruna et al. 2004b, Bemúdez et al. 2009, Moncayo et al. 2010) indicate that this *Rickettsia* species has a high incidence in populations of different species of *Amblyomma* across several geographic regions. In addition, the high density of mammals that likely serve as primary and secondary hosts of ticks (Fairchild et al. 1966, Guglielmone and Nava 2006), and the movement of mammals between different habitats in the study site (Vaughan and Weis 1999) may also increase the probability of ticks infected with *Rickettsia*.

Finding *R. amblyommatis* in several larval samples strongly suggests that transovarial transmission may be occurring naturally in these *Amblyomma* species; this transmission has been confirmed for other tick and *Rickettsia* species (Horta et al. 2006, 2009, Parola et al. 2005b, Labruna et al. 2008, 2011b, Saraiva et al. 2013). Transovarial transmission could explain, at least partially, the high incidence of *Rickettsia* in these *Amblyomma* ticks, as it might increase the probability of *R. amblyommatis* infection in non-infected hosts, and thus, maintain a high population in the dry forest. The high incidence of *R. amblyommatis* in ticks of this dry forest could occur through two different mechanisms: first, uninfected ticks parasitizing an infected host, and second, uninfected hosts being parasitized by infected ticks, whose probability of becoming infected increases if transovarial transmission occurs in these *Amblyomma* species.

Our study is the first to report *R. amblyommatis* in the Costa Rican tropical dry forest, though Hun et al. (2011) reported the same *Rickettsia* in *A. cajennense* (= *A. mixtum*) collected from horses in the wet lowland areas of the Caribbean slope. Bemúdez et al. (2016) also reported *R. amblyommatis* from different tick species collected from domestic animals in different climatic regions in Panama. This suggests that *R. amblyommatis* is

capable to infest a wide range of tick species from several genera across a wide range of climatic conditions (Bemúdez et al. 2016). It cannot be discarded that, at least in part, the ample distribution of *R. amblyommatis* could be attributed to the movement of domestic animals across different geographic regions (within or between countries), but this hypothesis needs to be tested.

The pathogenic effect of *R. amblyommatis* in humans is unclear. This *Rickettsia* specie belongs to the spotted fever group and is closely related to *R. rickettsii* which is the main cause of human infection of the most severe of all tick-borne rickettsiosis– the Rocky Mountain spotted
fever (Moncayo et al. 2010). However, the cases of RMSF that have been attributed to *R. amblyommatis* have not been confirmed (Moncayo et al. 2010, Parola et al. 2013). Hence, Moncayo et al. (2010) consider *R. amblyommatis* as non-pathogenic or only mildly pathogenic, but this *Rickettsia* species cannot be ruled out as a potential pathogenic agent of human diseases (Rivas et al. 2015).

One *Rickettsia* sequence was similar to *R.* sp. strain *colombianensi*, and this is the first record of this *Rickettsia* in Costa Rica. In Colombia *R.* sp. strain *colombianensi* was obtained from adults and nymphs of *A. dissimile* parasitizing *Iguana iguana* and from free-living larvae of *Amblyomma* spp. and larvae of *Rhipicephalus microplus* collected from vegetation in cattle pastures and agriculture lands (Miranda et al. 2012). They suggest that this *Rickettsia* also infect livestock. Palo Verde National Park contains large populations of *Iguana iguana* and *Ctenosaura similis* (Savage 2002), and livestock is also abundant in the park, used for vegetation management. Thus, there is an abundance of hosts for *A. dissimile* in the study site. The pathogenic human effect of this new *Rickettsia* is still unknown and no evidence of transmission to humans has been demonstrated. However, its pathogenic effectcannot be discarded since it is closely related to *R. tamurae* and *R. monacensis* which both have been found infecting humans (Jado et al. 2007, Imaoka et al. 2011)

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Table 1 Sample size of larvae, nymphs and adult (females and males) ticks, from the seven sampled sites, included in molecular analyses. The number of amplicons of *gltA* and *ompA* are included for each stage and site.

	Guayacán	Ojo de agua	Mapache (%)	Mangos	Camino	Cantera	Laguna
Larvae	7	4	8	3	5	3	7
Positive	4	2	5	1	2	1	5
Negative	3	2	3	2	3	2	2
gltA seq.	2	1	5	0	2	1	5
ompA seq.	2	1	2	0	0	0	2
Nymphs	9	13	12	10	7	5	8
Positive	7	5	7	8	2	1	4
Negative	2	8	5	2	5	4	4
gltA seq.	6	4	5	5	1	1	3
ompA seq.	4	4	4	1	1	1	3
Females	12	12	8	7	10	2	2
Positive	3	3	1	1	2	0	1
Negative	9	9	7	6	8	2	1
gltA seq.	3	3	0	1	2	0	1
ompA seq.	1	2	0	0	0	0	0
Males	14	12	11	8	11	3	3
Positive	5	1	1	1	5	2	0
Negative	9	11	10	7	6	1	3
gltA seq.	4	0	0	1	3	1	0
ompA seq.	2	0	0	1	2	1	0

Table 2. Prevalence and identity of *Rickettsia* from the three stages of free-living *Amblyomma* ticks. a- number of samples analyzed for the gene *gltA*. b- and c- the number of amplicons of *gltA* and *ompA* respectively. In parentheses, the percentage of the amplicons for which sequences were obtained.

	Total samples tested	No. samples positive for *gltA*DNA[a]	No. samples of *gltA*sequenced[b]	No. samples of *ompA*sequenced[c]
Larvae	37	20 (54)	16 (80)	7 (44)
Nymphs	64	34 (53)	25 (73)	18 (72)
Females	53	11 (20)	10 (91)	3 (30)
A.mixtum	46	9 (19)	8 (89)	3
A.oblongoguttatum	5	1 (20)	1 (100)	0
A.parvum	2	1 (50)	1 (100)	0
Males	62	15 (24)	9 (60)	6 (67)
A.mixtum	45	13 (29)	8 (61)	6 (75)
A.oblongoguttatum	12	1 (8)	0	0
A.parvum	5	1 (20)	1 (100)	0

How many larvae or nymphs of *Amblyomma* spp. ticks are needed to detect the presence of *Rickettsia*?

Resumen

Las rickettsiosis transmitidas por garrapatas son enfermedades emergentes en muchos países tropicales, por lo que la rápida detección de estas enfermedades podría reducir el impacto en la salud pública. Para la detección de especies de *Rickettsia,* se procesan en grupos las larvas y ninfas; mientras que los adultos se procesan de forma individual, pero el número de larvas y ninfas en los grupos probablemente podría afectar la probabilidad de detección de *Rickettsia* spp. Probamos si la cantidad de larvas y ninfas incluidas en el grupo afectaba la probabilidad de detección de *Rickettsia*. Encontramos que el número de larvas en cada grupo no afecta la probabilidad de detectar *Rickettsia* spp., pero con el número de ninfas la probabilidad de detección aumenta rápidamente. Para este estudio, 20 ninfas son suficientes para acercarse a una probabilidad de 1 de detectar *Rickettsia.*

Abstract

Tick-borne rickettsiosis is becoming a common emergent disease in many tropical countries, and the rapid detection of this disease could reduce the impact on public health. Pools of larvae and nymphs, and individual adult ticks are used for detection of *Rickettsia* species, but the number of larvae and nymphs in the pools likely affect the probability of detection of *Rickettsia* spp. We tested whether the number of larvae and nymphs included in the pool affected the probability of detection. We found that the number of larvae in each pool did not affect the probability of detecting *Rickettsia* spp., but the probability of detection increased rapidly with the number of nymphs. For this study, 20 nymphs are enough to approach a probability of 1 of detecting *Rickettsia.*

Introduction

The rapid detection and identification of the *Rickettsia* causing human infections, is a priority for two reasons: first, to determine the identity of the pathogenic *Rickettsia*, and second, to determine the vector responsible for causing the infection (Parola, et al. 2013; Santibáñez, et al. 2013). To increase the probability of detecting *Rickettsia* in tick samples, several larvae and nymphs are included in single pools that vary widely in number of individuals (Saraiva, et al. 2013; Alves, et al. 2014; Troyo, et al. 2016). To our knowledge, there have been no tests to determine whether the number of individuals included in the pools affects the probability of detecting *Rickettsia* spp.

We used off-host immature ticks to test if the probability of detecting *Rickettsia* spp. is affected by the stage (larvae and nymphs), and by the number of larvae or nymphs included in the pools. We expect that the probability of detecting *Rickettsia* spp. is not affected by the number of larvae included in the pools, but this probability should increase with the number of nymphs. This because if the tick female is infected and transovarial transmission occurs in ticks, all larvae that emerge from this female's eggs will also be infected. Similarly, if the female is not infected all larvae emerged from the eggs of this female neither would be infected. Larvae in each pool are likely to come from the same female as they emerged from eggs and stay close together forming dense groups in the field. On the contrary, nymphs in a pool are likely coming from different females that may or may not be infected (Oliver, 1989; Nicholson, et al. 2009). Because, most off-host nymphs collected in the wild come from larvae that had released from their hosts, and then molted to this stage.

Materials and Methods

Collection of ticks

We collected off-host larvae and nymphs monthly in Palo Verde National Park, northwestern Costa Rica, Guanacaste province (10° 21' N, 85° 21' W, 50-250 m elevation)

from February 2012 to February 2013. We collected ticks monthly by dragging a piece of fabric (a flannel cloth of 1m x 1.5 m) on the litter and herbaceous layer (0-50 cm above the ground) in seven sites with different vegetation structure (Hartshorn, 1983). Every 2.5 meters we examined the fabric, collected all immature ticks and preserved them in tubes containing 70% ethanol. We used the key from Vargas (2006) to identify larvae and nymphs to genus level.

Polymerase chain reactions and sequence of DNA form ticks

We extracted DNA from 37 larval pools and 64 nymphal pools using the Nucleospin® Tissue kit (Macherey-Nagel) following the manufacturer's instructions for animal tissue. To test the effect of number of larvae and nymphs on detection of *Rickettsia* we made pools with different numbers of larvae (from 3 to 1427) and nymphs (from 3 to 51). We individually processed all tick samples (pools) using routine PCR assay with primers CS 78F and CS 323R, designed to amplify a 401-bp fragment of the enzyme citrate synthase (*gltA*) of *Rickettsia* spp. which allows identification of all rickettsiae species (Roux, et al. 1997; Labruna, et al. 2004). PCR assays were performed with a final volume of 25 µL per reaction, which included five microliters of DNA extract, following the procedure described by Labruna et al. (2004). Positive and negative controls were included for each reaction. PCR products of the expected sizes were sequenced in a ABI 3730XL Analyzer (Bioneer Sequencing Service, South Korea). We used the Bio Edit program (Hall, 1999) to determine the consensus sequences which were subsequently analyzed by BLAST to confirm the genetic similarity to *Rickettsia* species. The sequences obtained were aligned for *gltA* with the corresponding sequences of other *Rickettsia* species available in GenBank, using the BLASTn searching.

Statistical analyses

To test the effect of the number of larvae or nymphs included in pools on the probability of detecting *Rickettsia*, we used General Linear Model with a binomial distribution. We run two separate analyses for larvae, the first included pools from 3 to 55 larvae, and the second from 3 to 1427. Thus, the first group of larvae pools is similar in range to the pools of nymphs. We used the R statistical language, version 3.0.1 (R Development Core Team, 2014) for analyses.

Results

The number of larvae included in the pool samples for both groups did not affect the probability for detection of *Rickettsia* (group 1: $z = 0.09$, $p = 0.926$; group 2: $z = -0.73$, $p = 0.462$, Table 1, Fig. 1a). However, the probability of detection of *Rickettsia* in nymphs increased with the number on individuals included in the pool ($z = 2.35$, $p = 0.019$, Fig. 1b).

Discussion

Our results are congruent with differences in the biology of larvae and nymphs. Engorged adult females of most tick species free themselves from their hosts to lay the eggs on the litter. All larvae remain together a few days after emerging and then climb the vegetation where they quest for potential hosts (Nicholson, et al. 2009). Hence, when these free-living larvae are collected, they are usually collected in large groups, from which the pools are selected; thus, the larvae in a pool are likely from the same female. On the contrary, nymphs collected in the litter or herbaceous layer are not found in dense groups, they are often dispersed and probably from different tick hosts (Oliver, 1989).

Based on our data, the number of larvae placed in a pool does not affect the probability of detecting *Rickettsia*. Thus, a few individuals in each pool would be enough to detect the presence of *Rickettsia*. However, if the interest is in detecting *Rickettsia* from nymphs, pools with 10 nymphs yield a probability of detection of about 0.50, and with 20 nymphs in the pools the probability of detection is close to 1. In this case, more than 20 nymphs do not improve the probability of detection.

It is important to consider that the probability of detection may change with many different factors, e.g., type of habitat, abundance of ticks, and host abundance (Oorebeek and Kleindorfer, 2008). However, considering that larvae and nymphs of a large number of ticks have a free-living period, these findings could be useful for ticks collected in different habitats and geographic regions.

Ethics statement

The authors declare no conflicts of interest. DNA was only extracted from free-living ticks.

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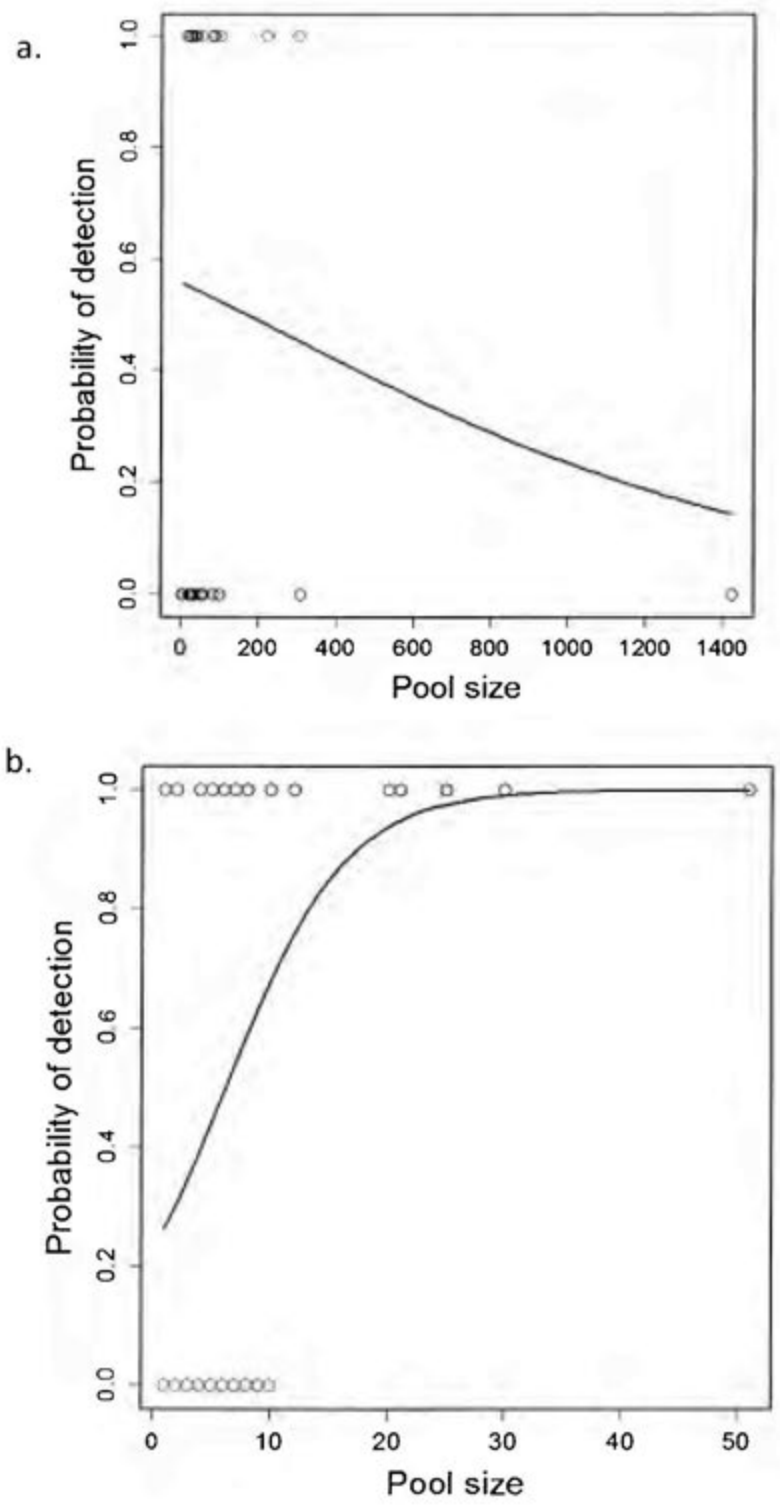

Figure 1. Probability of detection of *Rickettsia* as the number of larvae (a) and nymphs (b) in the pools increases.

Table 1. Effect of the number of larvae and nymphs included in the pools on the probability of detecting *Ricketssia* spp. The analyses were conducted for two groups of larvae that differed in the range of larvae included in the pools.

	Range of pool size for larvae: 3-55			
Factor	Coefficient	SE	Z-value	P
Intercept	-0.14	0.85	-0.17	0.865
Pool size	0.00	0.02	0.09	0.926
	Range of pool size for larvae: 3-1427			
Factor	Coefficient	SE	Z-value	P
Intercept	0.23	0.36	0.65	0.517
Pool size	-0.00	0.00	-0.73	0.462
	Range of pool size for nymphs: 3-51			
Factor	Coefficient	SE	Z-value	P
Intercept	-1.23	0.58	-2.14	0.0325
Pool size	0.20	0.08	2.35	0.0190