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Chiggers (Acariformes: Trombiculoidea) do not increase rates of infection by *Batrachochytrium dendrobatidis* fungus in the endemic Dwarf Mexican Treefrog *Tlalocohyla smithii* (Anura: Hylidae)

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ABSTRACT

Amphibian populations are globally declining at an alarming rate, and infectious diseases are among the main causes of their decline. Two micro-parasites, the fungus *Batrachochytrium dendrobatidis* (Bd) and the virus *Ranavirus* (RV) have caused mass mortality of amphibians and population declines. Other, less understood epizootics are caused by macro-parasites, such as Trombiculoidea chiggers. Infection with chiggers can affect frog behavior and survival. Furthermore, synergistic effects of co-infection with both macro and micro-parasites may lead to higher morbidity. To better understand these potential synergies, we investigated the presence and co-infection by chiggers, Bd and RV in the endemic frog *Tlalocohyla smithii* (*T. smithii*). Co-infection of Bd, RV, and/or chiggers is expected in habitats that are suitable for their co-occurrence; and if infection with one parasite facilitates infection with the others. On the other hand, co-infection could decrease if these parasites were to differ in their micro-environmental requirements (i.e. niche apportionment). A total of 116 frogs of *T. smithii* were studied during 2014 and 2016 in three streams within the Chamela-Cuixmala Biosphere Reserve in Jalisco, Mexico. Our results show that 31% of the frogs were infected with Trombiculoidea chiggers (*Hannemania* sp. and *Eutrombicula alfreddugesi*); *Hannemania* prevalence increased with air temperature and decreased in sites with high canopies and with water pH values above 8.5 and below 6.7. Bd prevalence was 2.6%, RV prevalence was 0%, and none of the frogs infected with chiggers were co-infected with Bd. Together, this study suggests that chiggers do not facilitate infection with Bd, as these are apportioned in different micro-habitats. Nevertheless, the statistical power to assure this is low. We recommend further epidemiological monitoring of multiple parasites in different geographical locations in order to provide insight on the true hazards, risks and conservation options for amphibian populations.

1. Introduction

Currently, amphibian populations are globally declining at an alarming rate, and emerging diseases are one important factor in their mortality (Wake and Vredenburg, 2008). *Batrachochytrium dendrobatidis* (Bd) affects at least 500 amphibian species worldwide, while *Ranavirus* (RV) affects at least 105 amphibian species and both parasites have the capacity to produce mass mortality (Scheele et al., 2019; Duffus et al., 2015). The wide distribution of these microparasites suggests that amphibians are facing a pandemic of Bd and RV infection; therefore, it is of utmost importance to know about their effects on rare and micro-endemic species.

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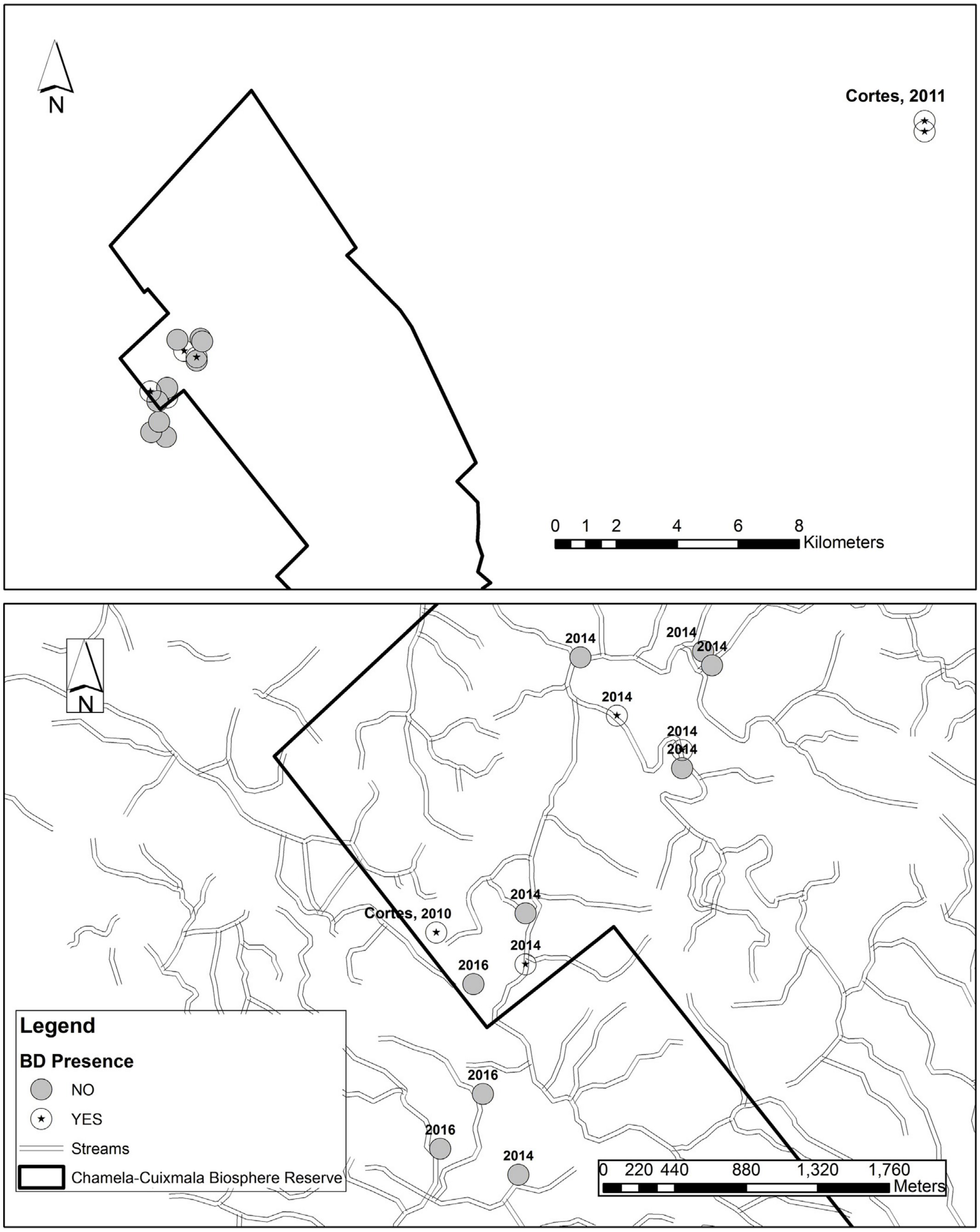

Fig. 1. Study area, showing November 2014 and 2016 sampling sites. Gray and white circles show presence or absence of *Batrachochytrium dendrobatidis*. Sampling sites in 2010 and 2011 show fungus presence, reported by Cortes in 2014.

The result of a host-parasite interaction is context-dependent, as many factors in the host, parasite and environment, (the disease triangle), interact to determine the presence and severity of the disease. Biotic and abiotic factors can either decrease or facilitate the infection of amphibians with pathogens such as Bd and RV (Brunner et al., 2015). The probability of acquiring a Bd infection is related to factors such as water temperature, air humidity, latitude, elevation, habitat preference, and seasonality. Several authors report that prevalence of Bd is higher in moist environments, with a wide range of temperatures and elevations in different habitats (Murrieta-Galindo et al., 2014; Mutnale et al., 2018; Kriger and Hero, 2007; Ruggeri et al., 2018; Cohen et al., 2019). Other authors mention that Bd presence is higher near developed centers (urban zones) with high variability in rainfall regimes (Bacigalupe et al., 2017). Likewise, several studies suggest that human-modified habitats, such as artificial ponds used for cattle, and warm water increase the prevalence of RV in the hosts (Hoverman et al., 2012; Rojas et al., 2005; Brand et al., 2016). Furthermore, RV infection may increase if the host's immune system is depressed, regardless of water temperature (Ariel et al., 2009; Grant et al., 2003; Rojas et al., 2005; Speare and Smith, 1992).

Synergies among Bd, RV and other parasites may increase the risk of host mortality (Stutz et al., 2018; Ezenwa and Jolles, 2011). Presence of different types of parasites including viruses, fungi, and chiggers, among others, and the relationships between them must be accounted for to understand the risk to amphibians (Koprivnikar et al., 2012). Often in nature, pathogens can simultaneously be found in the same hosts, and environmental changes can drive their ecological interaction, resulting in additive, antagonistic or synergistic effects within the hosts

(Romansic et al., 2011). Parasites other than Bd and RV affecting amphibian populations include chiggers belonging to family Leeuwenhoekiidae (Trombiculoidea), such as *Hannemania* sp. chiggers. Experimental studies have proven that such chiggers produce deformities and the loss of chemosensory function, as well as decreasing foraging capacity and the reproduction of host amphibians (Anthony et al., 1994; Duszynski and Jones, 1973; Maksimowich and Mathis, 2000). Current knowledge on infections by chiggers and information related to the ecology of these amphibian parasites is scarce and no previous studies concerning co-infection with Bd and RV are known (Alvarado-Rybak et al., 2018; Costa-Silveira et al., 2019; Paredes-León, 2019; Hoffmann, 1970; Hyland, 1961). To our knowledge, the possibility that chiggers may impair the protection of frogs from pathogens and facilitate co-infection with Bd and RV has never been investigated.

High levels of infestation by chiggers have been linked to environments suitable for Bd and RV with high humidity or sites close to water bodies; hence, the presence of chiggers may facilitate infection with Bd and RV (Hatano et al., 2007; Espino del Castillo et al., 2011; Attademo et al., 2012; Kpan et al., 2019 Warne et al., 2016; Whitfield et al., 2013; Stutz et al., 2018). Alternatively, a lack of co-infection between these parasites could exist if the parasites differ in their micro-environmental requirements, and hosts infected with one parasite species (e.g. chiggers) live in microhabitats that are not suitable for the other parasites, i.e. niche apportionment (Mouillot et al., 2003; Olori et al., 2018).

In this study, we tested for the above predictions by analyzing the presence and co-infection of chiggers, Bd and RV in populations of *Tlalocohyla smithii* (*T. smithii*), commonly known as the Dwarf Mexican Treefrog (Boulenger, 1902). This frog species is distributed from central and southern Sinaloa (Mexico) along the lowlands of the Pacific, to the south of Oaxaca and towards the Balsas river within the Tepalcatepec basin in the states of Morelos and Puebla (Santos-Barrera et al., 2010). *T. smithii* is a species of frog in the Hylidae family and it is endemic to Mexico. Its natural habitats include dry tropical or subtropical forests, intermittent rivers and intermittent freshwater marshes. It is threatened with extinction due to the destruction of its natural habitat, and some studies have shown that members of this family have one of the highest risk of being attacked by RV. Despite of this, no studies have been carried out on the RV prevalence in *T. smithii,* and its associations with other parasites like chiggers or Bd, and other biotic and abiotic variables.

2. Materials and methods

2.1. Study site

The study was performed in the Chamela-Cuixmala Biosphere Reserve in Jalisco, Mexico, located on the Pacific coast, within a region marked by mountains and alluvial flat-lands. The climate is warm and humid with an annual temperature of 24.9 °C and vegetation is dominated by tropical deciduous forest (Ceballos and Garcia, 1995).

Frogs were sampled nocturnally during November of 2014 and 2016, based on previous prospective sampling and unpublished studies that revealed a higher abundance of *T. smithii* during November (Fig. 1). In 2014, specimens of *T. smithii* were collected along three seasonal streams: Zarco, Colorado and Hornitos, located in the Reserve (Ceballos and Garcia, 1995).

Precipitation was low during 2016, so the Colorado and Hornitos streams were dry; hence, we only found and collected frogs along the Zarco stream, at four sites in 300-m intervals minimum.

Sampling sites were selected based on previous observations where amphibian presence was recorded in the three different streams. The distance between sites was greater than 300 m, to ensure the independence of observations between sites. Previous studies show that species of Hylidae, move 5 m per night on average with a maximum distance of 50 m in one night (Lemckert and Slatyer, 2002). At each sampling site, along each stream, amphibian specimens were collected using disposable vinyl gloves to manipulate each specimen, in a transect 30 m up and 30 m down. Sampling effort was 4–6 h per day by 3 workers on 2 days. All specimens studied were manually collected under the FAUT-0250 scientific collection permit issued by the Mexican Secretary of the Environment and Natural Resources (SEMARNAT).

2.2. Abiotic variables

Physicochemical water parameters were registered twice in each sample site, within a 24-h period, using Multiparametric Hanna Instruments® model HI 9828/04–1. Water parameters registered were: Water temperature, Stream pH (pH), Stream pH in millivolts (pH mV), Oxidation-reduction potential (ORP), Dissolved oxygen percentage (OD %), Water conductivity (uS), Water resistivity (Mohm), Total dissolved solids (TDS) and Salinity. Environmental temperatures (an average of the maximum and minimum temperature and humidity) were registered within a 24-h period from capture, using a model HTC-1 thermometer and hygrometer.

Stream width and depth at each sampling site were obtained using a measuring tape. Stream width was measured at the widest point within each 2 m radius (buffer). Depth measurements were taken at five positions within each buffer: one in the center and four along the circumference. The average depth per site was based on these depth measurements.

2.3. Biotic variables

A 40-square grid densiometer was employed to measure canopy cover at each sampling site, from five positions: one in the center and four around a 2m radius. These canopy cover measurements were summed by site, to give the grand total measurements used in these analyses.

Amphibian species richness and diversity were recorded within a 25-m radius around each sampling site.

2.4. Chigger extraction and identification

A stereoscopic microscope was used to search for chiggers on the skin of each captured frog. For taxonomic identification, chiggers were extracted from the frogs' skin using needles and tweezers, and then preserved in 70% and 98% ethanol, chiggers were cleared with lactophenol and mounted individually on semi-permanent microscope slides in Hoyer's medium (Brennan and Goff, 2006; Hoffmann, 1990). All specimens were deposited in the National Mite Collection (CNAC) at the Biology Institute of the National Autonomous University of México, with access number: *Hannemania* sp. (CNAC011431-011453) and *Eutrombicula alfreddugesi* (*E. alfreddugesi*) (CNAC011454-011458).

2.5. Bd extraction and amplification

The frog's skin was swabbed following Hyatt et al. (2007), and DNA was extracted from the swabs using the PrepMan Ultra kit. A total of 40 μl of PrepMan Ultra were added to each swab and 40 mg of zirconia/silica pearls 0.5 mm in diameter (Biospec) were added to a tube. The mix was homogenized twice for 1 min and centrifuged for 30 s at 13 X 103 g. The obtained elements were placed in a water bath and boiled for 10 min. They were then cooled at room temperature for 2 min and centrifuged at 13,000 g for 3 min. A total of 20–25 μl was recovered. DNA was quantified in a nanodrop and then stored at −80 °C, a conventional PCR and the primers to detect internal transcribed spacer 1 and 5.8S ribosomal RNA gene were performed as previously reported in the literature (Boyle et al., 2004). The PCR product (146 bp) was visualized in 1% agarose gel and stained with ethidium bromide.

A positive control provided by the University of Utrecht (Saucedo et al., 2018) was used to determine the presence of Bd. The thermocycler conditions for the PCR were set at 94 °C for 5 min; 30 cycles at

94 °C for 45 s; 50 °C for 45 s; 72 °C for 45 s, and a final extension at 72 °C for 5 min.

2.6. RV extraction and amplification

DNA was extracted from 100 mg of tissue samples from the heart, liver, kidney, spleen and skin. We used a Purelink Genomic DNA kit following the provider's instructions; except that a disintegration step using sterile sand was added to the sample.

In order to confirm that the extraction was performed correctly, an external extraction control was applied, and an internal amplification control was used for the RV PCR.

RV amplification was performed based on the FV3 genome (AY548484) using the following primers: Forward 5′GACTTGGCCACT TATGAC-3′and Reverse 5′GTCTCTGGAGAAGAAGAA-3, allowing an amplification of a 531 bp product. These primers bind to a conserved region of the Major Capsid Protein gene (MCP) that is present in the Iridoviridae family (Boyle et al., 2004). Each individual DNA sample was analyzed. A total of 2 μl of IAC were added to the reaction sample at a 0.1 ng/μl concentration; the primers' concentration was 20 nM. We used a Master Mix (2X) PCR kit. PCR conditions were: an initial denaturation step at 94 °C for 5 min; 30 cycles at 94 °C for 1 min; alignment at 52 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min (Campos, 2014). The PCR product obtained was visualized in 1% agarose gel stained with ethidium bromide.

2.7. Statistical analysis

Parasite loads for each parasite type were estimated following Bush et al. (1997), considering the prevalence as the number of hosts infected with a particular parasite species divided by the number of hosts examined for that parasite species. Abundance is the number of individuals of a parasite in/on a single host regardless of whether the host is infected divided by the number of hosts examined for that parasite species. Mean intensity or the average intensity, it is the total number of parasites of a species found in a sample divided by the number of hosts infected with that parasite.

Correlations between the presence of each parasite and each biotic and abiotic variable were tested by using generalized linear mixed-effects models, GLMMs (Bolker et al., 2009). We analyzed the presence of each parasite found in a frog (response variable) with the predictors at each sampling site: biotic (canopy % and amphibian diversity) and abiotic variables (air temperature, humidity and water quality). The biotic variables recorded at each sampled site in 2014 and 2016 were Canopy cover (Canopy) and Amphibian richness and diversity (Shannon and Simpson indexes). The abiotic variables recorded in both years were: Average maximum temperature (Tmax), Average minimum temperature (Tmin), Average maximum humidity (Hmax), Average minimum humidity (Hmin), Width stream, Depth stream (Depth), Water temperature (Water t °C) and Stream pH (pH). The following water variables were only recorded one year (2014) due to the availability of equipment: Stream pH in millivolts (pH mV), Oxidation-reduction potential (ORP), Dissolved oxygen percentage (OD%), Conductivity Water (uS), Resistivity Water (Mohm), Total dissolved solids (TDS), and Salinity (see Table 1).

Sampling sites were grouped by stream Colorado 1, Colorado 2 and Colorado 3 as Colorado and analogously for Zarco and Hornitos streams. Number of observations (all amphibians collected) at each stream were: Zarco (79), Colorado (33) and Hornitos (4). For analysis, sites included were the ones where at least one amphibian was collected; eight sites in total, in the three streams (Zarco, Colorado and Hornitos).

Before our analysis, correlations between continuous variables were tested by using the Pearson correlation coefficient (ρ), to assess multicollinearity. The critical correlation value was defined as $|\rho| \geq 0.7$, as an indicator of interaction among predictors with significant

Table 1
Biotic and abiotic variables registered in November 2014 and 2016. Variables registered during 2014 and 2016 were: Average maximum temperature (Tmax), Average minimum temperature (Tmin), Average maximum humidity (Hmax), Average minimum humidity (Hmin), Stream width in meters (Stream width), Stream depth in centimeters (Depth), Water temperature, Stream pH (pH), Stream pH in millivolts (pH mV), Oxidation-reduction potential (ORP), Dissolved oxygen percentage (OD%), Water conductivity (uS), Water resistivity (Mohm), Total dissolved solids (TDS), Salinity, Indexes (Shannon and Simpson), Amphibian richness (Richness) and Not registered (NR). Sites: Z1 = Zarco 1, Z2 = Zarco 2, Z3 = Zarco 3, Z4 = Zarco 4, C1 = Colorado 1, C2 = Colorado 2, C3 = Colorado 3, H1 = Hornitos 1, H2 = Hornitos 2, H3 = Hornitos 3.

Year	Site	Tmax °C	Tmin °C	Hmax %	Hmin %	Canopy %	Stream width m	Depth cm	Water t °C	pH	pH mV	ORP	OD %	uS	Mohm	TDS ppm	atm	Salinity	Shannon	Simpson	Richness
2014	Z1	27.9	20.4	97	51	66.6	5.3	38.4	24.5	9.34	−131	−258	82.8	139	0.01	70.0	1	0.06	1.8	0.8	8
	Z2	27.9	20.4	97	51	75.4	4.1	32.1	24.5	8.01	−64.8	−179	84.1	130	0.01	65.0	1	0.06	1.5	0.7	9
	Z3	27.3	20.1	99	47	92.7	4.9	27.0	24.0	7.61	−45.3	−173	78.1	135	0.01	68.0	1	0.06	0.5	0.3	3
	C1	30.1	21.1	99	47	62.3	7.9	39.7	24.3	7.82	−55.2	44.3	−197	124	0.01	32.0	1	62	1.9	0.8	9
	C2	27.2	20.8	95	77	61.9	8.6	24.0	24.4	8.51	−89.4	47.8	−233	123	0.01	61.0	1	61	2	0.8	11
	C3	27.9	20.2	99	67	58.7	8.0	29.2	24.6	7.98	−63.4	54	−231	118	0.01	58.5	1	58.5	0.8	0.4	5
	H1	35.05	19.7	98	52.5	33.7	6.4	22.8	24.3	8.64	−96.6	48.65	48.65	219	109.5	109.5	1	0.1	1.1	0.6	4
	H2	39.8	19.5	99	35	81.7	6.7	29.4	24.1	9.37	−133	53.25	53.25	218	109.0	109.0	1	0.1	1.7	0.7	8
	H3	28.2	19.4	99	93	82.2	7.9	26.8	24.1	7.5	−41.6	53.9	53.9	214	106.7	106.7	1	0.1	1.6	0.7	6
2016	Z1	33.5	22.4	99	42	0.0	5.2	27.0	27.0	7.2	NR	NR	NR	NR	NR	NR	NR	NR	1.4	0.6	9
	Z2	37.1	19.1	98.5	39	0.0	2.0	25.0	25.0	7.4	NR	NR	NR	NR	NR	NR	NR	NR	1.4	0.6	6
	Z3	35.5	19.5	99	52	0.0	5.1	25.5	25.5	6.9	NR	NR	NR	NR	NR	NR	NR	NR	0	0	1
	Z4	39.8	19	52	10	0.0	2.55	25.5	25.5	6.7	NR	NR	NR	NR	NR	NR	NR	NR	1.4	0.6	3

collinearity.

First, bivariate analysis were performed, using the mixed effects logistic regression methodology (Ten Have et al., 2000; Johnson et al., 2015). The chigger variable (i.e. presence of *Hannemania* sp.) was considered the "response variable", the three streams (Zarco, Colorado and Hornitos) were considered random effects, and the environmental variables that remained after carrying out the correlation (collinearity) analysis were considered "explanatory variables". We used a GLMM of the binomial family (logistic regression) to be specific on the nature of the binary response variable, and mixed (random) effects were used to include the variance within streams to the estimation of each parameter (Zuur and Ieno, 2016). GLMMs were nested, hence the fit of the models was evaluated with the Akaike's information criteria (AIC), and the estimated parameter beta was considered as a measure of weight and importance of each explanatory variable on the chigger presence. Statistical significance of beta was evaluated with a Z test, comparing the fit of the model with the null hypothesis, assuming that the mean of beta = 0.

Finally, co-occurrence among parasites infecting frogs in the same stream (but not necessarily infecting the same frog) was tested using Fisher's exact test. Co-infection of parasites in the same frog was zero, thus no further test was performed.

We conducted all statistical analyses in R using the "nlme", "lme4" and "arm" packages (R Core Team, 2019; Pinheiro et al., 2019; Bates et al., 2014; Gelman and Hill, 2007).

3. Results

A total of 116 individuals of *T. smithii* were collected over two sampling years (Table 2).

3.1. Chiggers, Bd and RV

All samples tested negative for RV infection. As for the detection of the Bd fungus, in 2014 there was a 4.3% prevalence while in 2016 Bd was not recorded, yielding an overall prevalence of 2.3% when considering both years. All chiggers collected in the skin of the frogs belonged to the superfamily Trombiculoidea, of the genera *Hannemania* sp. Oudemans (1911) (Leeuwenhoekiidae) (n = 24) and *Eutrombicula alfreddugesi* Oudemans (1910) (Trombiculidae) (n = 5). In total, we collected 29 chiggers over both sampling seasons.

The prevalence, abundance and mean intensity of chiggers and Bd in *T. smithii* found in 2014 are shown in Table 3.

The 2014 prevalence levels of *Hannemania* sp. chiggers were as follows: the highest at Z3 and C3 (20%) and the lowest at C2 (8.33%). Bd was found at Z2, C1 and C3 with 9.09%. of prevalence.

The highest co-occurrence of *Hannemania* sp. chiggers and Bd during 2014 was recorded at C3 with a prevalence of 20% for chiggers and 9.09% for Bd.

Prevalence, abundance, and average intensity of Trombiculoidea chiggers (*Hannemania* sp. and *E. alfreddugesi*) in *T. smithii* during 2016 are presented in Table 3. Chiggers were found at all streams. However, *Hannemania* sp. was present at Z1, Z3 and Z4 and *E. alfreddugesi* only at Z2.

The highest 2016 *Hannemania* sp. prevalence levels were recorded at Z4 (100%), while the lowest levels of infestation (24%) were recorded at Z1.

Table 2
Tlalocohyla smithii individuals, sampled November 2014 and 2016. N (total number of captured anurans), UD (sex undetermined) and weight.

Month	Year	N	Females	Males	UD	Weight Average (range)
November	2014	69	1	68	0	0.75 (0.6–1.1)
November	2016	47	1	26	20	0.73 (0.5–1)

Despite finding co-occurrence of chiggers and Bd, no frog was found having co-infection with two parasites.

Fig. 2 shows *Hannemania* sp. chiggers embeds and encapsulates themselves in the dermis of *T. smithii*. Chiggers infected mainly the ventral zone of *T. smithii,* possibly it is due to proximity with the soil where the chiggers would be present.

3.2. Biotic and abiotic factors linked to Bd and chiggers

Analysis of the environmental variables associated with the presence of Bd (n = 3) or *E. alfreddugesi* chiggers (n = 5) was not possible, due to the small number of observations obtained.

For the *Hannemania* sp. chiggers, however, we discovered a positive and significant association with maximum environmental temperature (Tmax) and a negative association with canopy and pH (Table 4). No significant link between Trombiculoidea chiggers and Bd (Fisher's exact test: $F_1 = 1$, $p > 0.05$).

4. Discussion

Dwarf Mexican Treefrog was found parasitized with Trombiculoidea chiggers. We present the first report of *Hannemania* sp. and *E. alfreddugesi* chiggers in *Tlalocohyla smithii*.

In the two sampling seasons studied, *Hannemania* sp. prevalence (2.3%) was significantly narrower than in previous studies, for example in *Hannemania hylae*, Jung et al. (2001) reported 67% in *Eleutherodactylus guttilatus* and 81% prevalence in *Dryophites arenicolor* (formerly *Hyla renicolor*) from the USA; whereas in Mexico, Goldberg and Wrenn (2002) reported 70% prevalence in *Incilius mazatlanensis* (formerly *Bufo mazatlanensis*) from Sonora; Espino del Castillo et al. (2011) 23.4% prevalence in *Eleutherodactylus longipes* from Queretaro and Jacinto-Maldonado et al. (2013) reported 5% prevalence in *Leptodactylus melanonotus* from Jalisco. Possibly, the differences among the prevalence can be attributed to the larger sample size, in our study we sampled 116 animals whereas in the others 3, 37, 20, 47 and 20 animals were studied respectively.

Another possibility is that these differences in prevalences are due to differences in the biology of each amphibian specie as well as the environmental conditions of each sampling site. Statistical analyses show that presence of *Hannemania* sp. increases with the maximum air temperature recorded (Table 4); an important factor for the development of chiggers. Some studies have shown that *Hannemania hegeneri* needs temperatures from 10 to 30 °C to survive and reproduce (Hoffmann, 1990; Hyland, 1961). Canopy is negatively associated with the presence of chiggers, since greater vegetation cover creates a microenvironment (temperature, humidity, etc.) that may result in a cooler, more humid micro-habitat than sites with less vegetation cover (canopy %) (Jacinto-Maldonado et al., 2016). On the contrary, sites that are more exposed to sunlight may present slightly higher temperatures, and therefore, higher chigger abundance. Both lower humidity and higher temperatures are key for chiggers to complete their life cycle (Hoffmann, 1990).

We found that presence of *Hannemania* chiggers was negatively associated to water pH in the streams, which seems to indicate sensitivity to the pH of the environment. However, there are no studies of *Hannemania* sp. and its relationship with water pH.

Eutrombicula alfreddugesi was only present at one site (Zarco 2) and in one year (2016). However biotic and abiotic factors could not be statistically analyzed due to the small sample size (n = 5).

Although *E. alfreddugesi* is a generalist parasitic mite, it is not usually reported in amphibian species. Mertins et al. (2011) hypothesize that *E. alfreddugesi* larvae may be preadapted to occasionally feed on amphibians.

Eutrombicula species have been previously reported in amphibians. *E. alfreddugesi* has been found on: *Lithobates* sp. in Mexico (Hoffmann, 1990; Paredes-León et al., 2008); *Spea bombifrons* and *Spea multiplicata*

Table 3
Prevalence, abundance and average of intensity for Trombiculoidea chiggers and Bd fungus in *Tlalocohyla smithii*. Chiggers and fungus registered at sample sites along three streams, November 2014 and 2016 (N: number of individuals captured).

Stream	Point	Coordinates	N	***Hannemania sp.*** Prevalence % (Abundance ± SD) (Average intensity ± SD)	***Batrachochytrium dendrobatidis*** Prevalence % (Abundance ± SD) (Average intensity ± SD)	***Eutrombicula*** *alfreddugesi* Prevalence % (Abundance ± SD) (Average intensity ± SD)
Year 2014						
Zarco	1	19° 29′ 4.8″ N, 105°02′ 22.5″ W	11	0	0	0
Zarco	2	19° 29′ 47″ N, 105°02′ 21″ W	11	18.18% (0.18 ± 0.4) (2 ± 0)	9.09% (0.09 ± 0.03) (1 ± 0)	0
Zarco	3	19° 29′57.2″ N, 105°02′ 17.7″ W	10	20% (0.02 ± 0.42) (1 ± 0)	0	0
Colorado	1	19° 30′ 36.8″ N, 105°02′ 01.7″ W	11	9.09% (0.09 ± 0.03) (1 ± 0)	9.09% (0.09 ± 0.03) (1 ± 0)	0
Colorado	2	19° 30′ 26.3″ N, 105°01′ 54.6″ W	12	8.33% (0.08 ± 0.28) (1 ± 0)	0	0
Colorado	3	19° 30′ 29.9″ N, 105°01′ 47.9″ W	10	20% (0.3 ± 0.67) (1.5 ± 0.7)	9.09% (0.09 ± 0.03) (1 ± 0)	0
Hornitos	1	19° 30′ 48.5″ N, 105°02′ 09.4″ W	4	0	0	0
Hornitos	2	19° 30′ 49.7″ N, 105°01′ 43.5″ W	0	0	0	0
Hornitos	3	19° 30′ 46.9″ N, 105°01′ 41.6″ W	0	0	0	0
Year 2016						
Zarco	1	19° 29′ 10″ N 105° 02′ 39″ W	25	24% (0.4 ± 0.87) (1.67 ± 1.03)	0	0
Zarco	2	19° 29′ 21″ N 105° 02′ 36″ W	9	0	0	44.44% (0.89 ± 1.27) (2 ± 1.15)
Zarco	3	19° 29′ 46″ N 105° 02′ 30″ W	12	25% (0.67 ± 1.5) (2.67 ± 2.08)	0	0
Zarco	4	19° 29′ 43″ N 105° 02′ 32″ W	1	100% (1 ± 0) (1 ± 0)	0	0

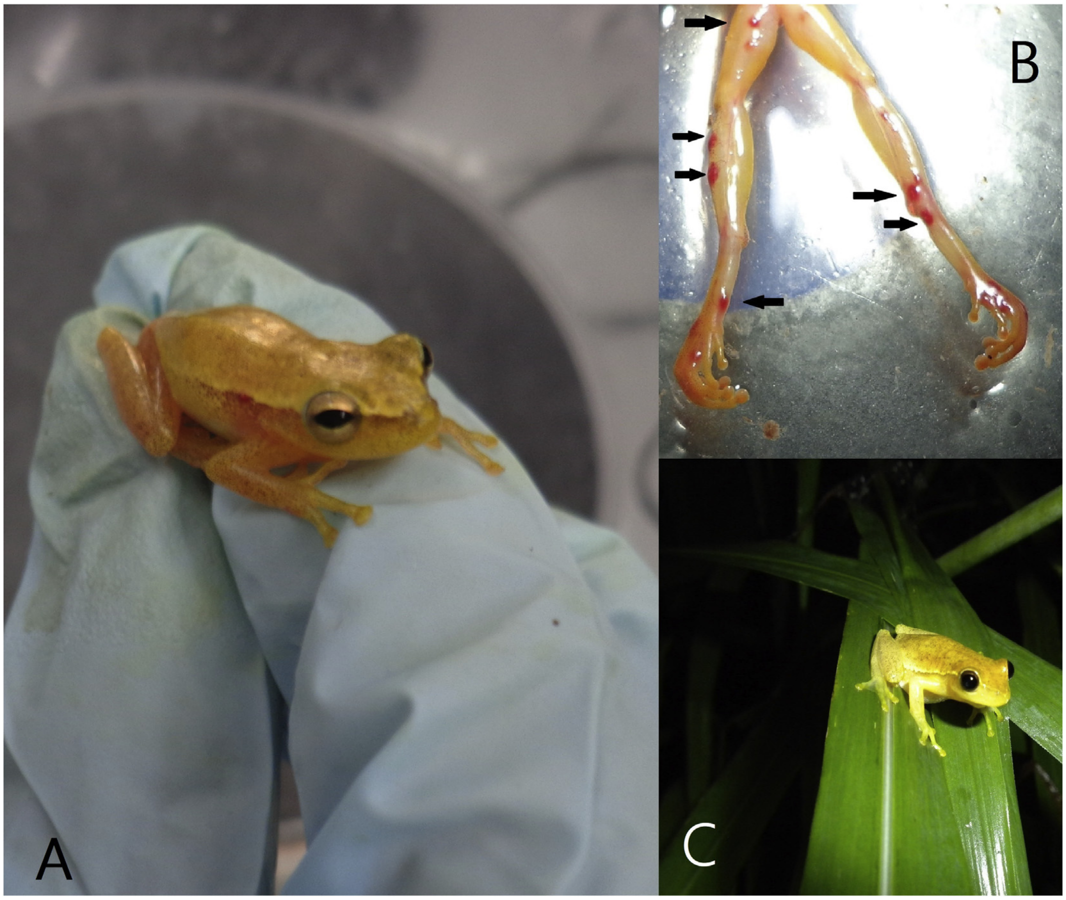

Fig. 2. Photographs. A: restrained *T. smithii* specimen; B: anatomical areas of host (*T.smithii*) parasitized by Trombiculoidea chiggers; C: another specimen of *T smithii* in its natural habitat.

Table 4
Generalized linear mixed models on the bivariate relationships between Chigger presence (response) and the biotic and abiotic variables: maximum and minimum values of temperature T (°C) and humidity H (%); canopy % at each sampling site; stream water variables: width, depth, water temperature and pH; and Shannon's index of amphibian diversity. All models are nested and have 113 degrees of freedom. AIC: AKAIKE Criterion, deviance, Std.Error: Standard error of beta parameter, Z different from 0 at 95% of confidence (*).

Model predictors	AIC	Residual deviance	B1	Std. Error	Z	P-value
T max	113.1	107.1	1.12	0.4844	2.319	0.02 *
T min	118.2	112.2	−0.32	0.4911	−0.671	0.5
H max	116.9	110.9	−0.53	0.4518	−1.182	0.23
H min	114.2	108.2	−1.15	0.5957	−1.932	0.05
Canopy	114.4	108.4	−1	0.4984	−2.019	0.04 *
Stream width	115.3	109.3	−0.91	0.5128	−1.775	0.07
Stream depth	117.5	111.5	−0.51	0.4795	−1.084	0.27
Water temperature	116.9	110.9	0.61	0.4484	1.368	0.17
pH	110.2	104.2	−1.68	0.6541	−2.572	0.01 *
Shannon index	116.9	110.9	−0.62	0.4539	−1.369	0.17

in USA (Mertins et al., 2011; Torrence, 2007); *Acris gryllus*, *Anaxyrus* sp., *Anaxyrus americanus*, *Anaxyrus woodhousii*, *Lithobates palustris*, *Lithobates pipiens* and *Duttaphrynus melanostictus*, have been reported respectively in the USA, Mexico and Bangladesh (Jenkins, 1949; Wolfenbarger, 1952; Wharton and Fuller, 1952; Hoffmann, 1990; Asmat, 1995). In the USA, Jenkins (1948) recorded *Eutrombicula splendens* feeding on *Dryophytes squirellus* (formely *Hyla squirella*) and Loomis (1956) found *Eutrombicula lipovskyana* on *Acris gryllus* and *Anaxyrus woodhousii* (formely *Bufo woodhousii*).

This study reports that chiggers and Bd co-occur in the same streams. Both were infesting *T. smithii* at the same site, although not in the same host. Results of the Fisher test showed no connection between chiggers and Bd. When chiggers were present in a host, the Bd fungus was absent. This may be due to the different micro-environmental conditions that Bd and chiggers need to survive; and the specific requirements may be the cause of the non-frequent interaction between them (Hoffmann, 1990; Hyland, 1961; Mendoza-Almeralla et al., 2015; Woodhams et al., 2008; Johnson et al., 2003). Nevertheless, it may be that prevalence of Bd is very low at this stage to find any co-infections of both parasites in the same frog, thus further monitoring is needed to falsify this observation, and discard the possibility of co-infection.

In regards of chiggers, this study suggest that high environmental temperatures favor its presence, as it has been found by other authors. A specific case is *Hannemania hegeneri,* it needs temperatures from 10 to 30 °C to survive and reproduce (Hyland, 1961).

Concerning Bd, high humidity is considered necessary for its survival (Mendoza-Almeralla et al., 2015), as well as the ambient temperature that influences the progression of a Bd (Woodhams et al., 2008).

Bd has exhibited a maximum growth rate in culture within a range of 17–25 °C (Piotrowski et al., 2004). Mendoza-Almeralla et al. (2015) mention that a rise in temperature produces an imbalance in the parasite-host relationship, promoting higher Bd virulence and/or higher susceptibility to infection in frogs. Johnson et al. (2003) found that Bd perishes at higher temperatures under experimental conditions (4 h at 37 °C, 30 min at 47 °C and 5 min at 60 °C). Our results support these observations, since chiggers were found in sampling sites at higher temperatures (31.45 ± 3.8) and Bd in lower temperatures (20.33 ± 0.09).

One possibility related to Bd absence in one of the streams under study is that the sampling months may not have coincided with the periods of highest Bd prevalence (Daversa et al., 2018) or that such prevalence may have been linked to amphibian species susceptibility (Kärvemo et al., 2018). For example, Talbott et al. (2018) found that *Lithobates sylvaticus* had the highest prevalence compared to the other amphibian species under study (*Pseudacris maculata* and *Hyla versicolor*).

4.1. Bd prevalence

Studies reporting the presence of Bd in Mexico exist (Martínez et al., 2019; Bolom-Huet et al., 2019; Hernández-Martínez et al., 2019; Hale et al., 2005; Frías-Alvarez et al., 2008; Muñoz-Alonso, 2010; Cheng et al., 2011; Van Rooij et al., 2011; Luja et al., 2012; Luría-Manzano et al., 2011; Murrieta-Galindo et al., 2014; Mendoza-Almeralla et al., 2016b; Cabrera-Hernández, 2012; García-Feria et al., 2017; Peralta-García et al., 2018; Familiar-López, 2010; Gómez, 2013; Cortes, 2014; López-Velázquez, 2014; Mendoza-Almeralla, 2016a; Ortiz-Millán, 2016; Solís-Sotelo, 2017).

There have been four previous works on the Bd prevalence-environmental factor relationship in Mexico. The first study, by Cortes in 2014, reports Bd prevalence at two sites in Jalisco state - one at the Chamela Biological Station and another in the Cuixmala river basin. Bd prevalence found was 0.08% and 0.22% respectively, compared to the 2.6% prevalence we found in our research. Despite the similarity of diagnostic techniques and sample size between that study and ours, the increase in prevalence that we report can be explained with the sampling seasons. We performed our study in November 2014 and 2016; in contrast, Cortés's study was carried out in July and September 2010, and in May, June and December 2011. Special host susceptibility, seasonality and environmental stochasticity are all known to strongly influence the prevalence of Bd (Ruggeri et al., 2018; Lenker et al., 2014; Kinney et al., 2011; Longo et al., 2010; Guayasamin et al., 2014; Searle et al., 2011; Lamirande and Nichols, 2002; Berger et al., 2004; Daszak et al., 2004). Monitoring infectious diseases over seasonal fluctuations can help us to predict spillover to amphibian populations at sites of high biodiversity or where endemic and endangered species occur.

The second study, performed by Muñoz-Alonso (2010), analyzes the relationship between Bd prevalence and macroecological factors for wildlife in the Isthmus of Tehuantepec. Muñoz-Alonso analyzed 77 anuran species from six amphibian families, finding that Hylidae was the family with the highest tendency of infection. In our research, the Bd prevalence that we found (prevalence = 2.6%), compared to that of Muñoz-Alonso (prevalence 21%), may be explained by difference in sample sizes (n = 1106, versus our study with n = 116 respectively) and the nature of the sampled sites. Our study was executed at preserved sites, while Muñoz-Alonso's samples were obtained in disturbed areas. Another possible explanation for the difference between these findings is environmental conditions, such as vegetation and altitude. Our study was performed under the tropical conditions of deciduous forests with an altitude range of 10–580 m asl, whereas the Muñoz-Alonso study was undertaken in 17 different types of vegetation with an altitude range of 4–100 masl.

The third study related to the topic at hand analyzes Bd prevalence and its connection to environmental factors (Murrieta-Galindo et al., 2014). The difference in Bd prevalence between their research (range 0–38.8%) and ours (2.6%) may be attributed to the detection of Bd in 12 different amphibian species versus the single-species approach in our study. Another difference could be the type of vegetation at sample sites. While the vegetation in our study area was tropical deciduous forest, that found at Murieta-Galindo's site was cloud forest, where humidity is higher and favors Bd. Even though temperature and tree density were similarly recorded in both studies, we were not able to statistically analyze the environmental variables associated with the presence of Bd due to the small number of observations obtained.

The fourth study, García-Feria et al. (2019), analyzes 13 Mexican amphibian species for the presence of Bd associated to abiotic as well as biotic factors in seven types of vegetation during dry and rainy seasons. They reported prevalences for adults and tadpoles of 47.78% and 72.53% respectively and found that host species and precipitation were the most important factors linked to Bd presence. The difference between their prevalence findings and ours may be attributed to the host species, since they mention 13 species in their study while we only analyzed one. Another difference between studies was the vegetation type; they did not collect amphibians in tropical deciduous forest, while in our study it was the sole vegetation type under analysis. Sampling seasons posed another difference. Garcia-Feria and collaborators sampled in both the dry and rainy seasons whereas our samples were only taken in the latter. However, it is interesting to note that in 2014 our study registered higher precipitation than 2016 and Bd was present in 2014.

4.2. RV prevalence

RV has been witnessed on all continents. There are reports of this infection all over the Americas, except El Salvador, Belize and Guatemala (Duffus et al., 2015). In Mexico, it was recently reported in Sinaloa in captive American bullfrogs (*Lithobates catesbeianus*) (Saucedo et al., 2019), but has not yet been reported in wildlife. Thanks to contact between native species and exotic species through international trade, the disease is also likely to spread to wildlife. We have not found cases of RV, this can be explained because our study only aimed at a single species in a preserved area, compared to other studies covering multiple species (Hoverman et al., 2011; Schock et al., 2008). Therefore, to better understand the potential prevalence of RV in Mexican wildlife, we recommend conducting further studies considering both preserved and disturbed areas, in different amphibian species, and at different stages of development. Research should also be conducted in distinct provinces at local and regional scales, using a variety of diagnostic techniques.

This study shows the importance of analyzing biotic and abiotic factors at sampling sites, since differences between environmental factors can be key to the presence or absence of a wide variety of parasites. For example, co-infection with two parasites may not be possible because certain parasites are unable to survive within a microhabitat. Furthermore, changes in environmental conditions have the power to modify interactions between parasite species and produce higher levels of co-infection and co-occurrence.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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