Relationships between altitude, triatomine (Triatoma dimidiata) immune response and virulence of Trypanosoma cruzi, the causal agent of Chagas’ disease


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Abstract. Little is known about how the virulence of a human pathogen varies in the environment it shares with its vector. This study focused on whether the virulence of Trypanosoma cruzi (Trypanosomatida: Trypanosomatidae), the causal agent of Chagas’ disease, is related to altitude. Accordingly, Triatoma dimidiata (Hemiptera: Reduviidae) specimens were collected at three different altitudes (300, 700 and 1400 m a.s.l.) in Chiapas, Mexico. The parasite was then isolated to infect uninfected T. dimidiata from the same altitudes, as well as female CD-1 mice. The response variables were phenoloxidase (PO) activity, a key insect immune response, parasitaemia in mice, and amastigote numbers in the heart, oesophagus, gastrocnemius and brain of the rodents. The highest levels of PO activity, parasitaemia and amastigotes were found for Tryp. cruzi isolates sourced from 700 m a.s.l., particularly in the mouse brain. A polymerase chain reaction-based analysis indicated that all Tryp. cruzi isolates belonged to a Tryp. cruzi I lineage. Thus, Tryp. cruzi from 700 m a.s.l. may be more dangerous than sources at other altitudes. At this altitude, T. dimidiata is more common, apparently because the conditions are more beneficial to its development. Control strategies should focus activity at altitudes around 700 m a.s.l., at least in relation to the region of the present study sites.

Key words. Triatoma dimidiata, Trypanosoma cruzi, altitude, amastigotes, Chagas’ disease, parasitaemia, phenoloxidase.
Introduction

Parasitic diseases transmitted by arthropods represent significant causes of death around the world (Buarque et al., 2013), and include dengue, malaria, leishmaniasis and Chagas’ disease [World Health Organization (WHO), 2014]. The last of these is categorized as an acute public health problem in Latin America as a result of its broad distribution and high prevalence, with an estimated nine million people infected (Schofield et al., 2006; Noireau et al., 2009).

Chagas’ disease is caused by the protozoan Trypanosoma cruzi (Parker & Sethi, 2011), a parasite that circulates in the blood of over 150 species of mammal and is transmitted by haematophagous hemipterans of the subfamily Triatominae (Hemiptera: Reduviidae), commonly known as assassin bugs or kissing bugs (Noireau et al., 2009). Since its first parasitological, clinical and epidemiological descriptions, the occurrence of Chagas’ disease has been related to environmental factors that include temperature, precipitation, vegetation, humidity, latitude and altitude (Cruz-Reyes & Pickering-López, 2006; Mischler et al., 2012). In fact, some studies have tried to find links between these factors and the biology of triatome vectors (Rocha et al., 2001; Heger et al., 2006; Martínez-Ibarra et al., 2008; Mendes-Pereira et al., 2013). For example, it has been reported that differences in altitude cause variations in the distribution of triatomines (Noireau et al., 2005), in the colonization and infection of the insect vectors (Ramsey et al., 2000), and in some clinical manifestations of Chagas’ disease (Pereira-Lopes et al., 2013). Of course, these abiotic factors are unlikely to be the sole factors explaining triatomine biology and distribution as they may interact with biotic factors. It is known that Tryp. cruzi may negatively affect the fitness of triatomines through changes in fecundity (Fellet et al., 2014), development and survival (Schaub, 1989; reviewed by Flores-Villegas et al., 2015). It is highly likely that such negative effects are also modulated by abiotic variables. For example, Tryp. cruzi development within the triatome Triatoma infestans became faster as the ambient temperatures experienced by the insect increased (Asin & Catalá, 1995). By contrast, the parasite induced higher mortality in the triatome Rhodnius prolixus (Hemiptera: Reduviidae) at ambient temperatures of 24–27 °C in comparison with more extreme temperatures of 21 and 30 °C (Elliot et al., 2015). Because triatomines are ectotherms, they can be expected to demonstrate a close temperature-dependent physiological response and thus it can be hypothesized that, in field conditions, interactions between abiotic and biotic factors will influence the effects of Tryp. cruzi infection in triatomines and, ultimately, hosts.

Given the relationships of abiotic and biotic factors with both Tryp. cruzi development and infection patterns, and triatome distribution, one very general hypothesis is that varying geographic factors associated with Tryp. cruzi distribution may drive triatome immune responses and infectivity in mammal hosts. An exploration of these possible relationships may deepen understanding of the occurrence of Chagas’ disease. For example, if such relationships were identified, further studies might elucidate why infectivity is dependent on specific abiotic factors and clarify the associated risk to human populations. Additionally, these insights would help to explain the ecological context of the disease, which is a subject that has received little attention (Abad-Franch et al., 2009; Telleria & Tibayrenc, 2010; Nouvellet et al., 2015).

The insect immune system consists of cellular and humoral components. Whereas the cellular component is carried out by haemocytes (phagocytes, encapsulation and nodulation), in the humoral component different factors are secreted in the circulatory system (Eleftherianos & Revenis, 2011). Factors of particular importance include reactive oxygen species (ROS), reactive nitrogen species (RNS), antimicrobial peptides and the phenoloxidase (PO) cascade. This last response is initiated by the recognition of foreign elements and the activation of the PO enzyme, which converts phenols to quinones. By a non-enzymatic pathway, quinones continue their transformation until they become melanin, which can encapsulate foreign agents (González-Santoyo & Córdoba-Aguilar, 2012). However, the PO pathway can also give rise to other key immunological mechanisms (González-Santoyo & Córdoba-Aguilar, 2012). For this reason, PO has been suggested as a general indicator of immunocompetence (Mucklow et al., 2004; González-Santoyo & Córdoba-Aguilar, 2012).

The present study analyses the relationships among an altitudinal gradient (as a broad-scale indicator of geographic variation) in a Mexican community (Chiapas State), the immune response of Try. dimidiata measured as the activity of PO, and the virulence of Tryp. cruzi in a mammal host. For this purpose, uninfected triatomines were collected at different altitudes and infected with Tryp. cruzi from corresponding altitudes. Health services personnel from Chiapas State had previously indicated that at these sites Try. dimidiata is present and cases of Chagas’ infection exist. These personnel had also provided verbal (e.g. non-measured) indications that suggested altitudinal variation in Tryp. cruzi infectivity. Assuming that this altitudinal variation represented one explaining factor, three steps were carried out. Firstly, PO activity was measured in the triatomines to determine whether there were immunological differences according to altitude that might also explain Tryp. cruzi infectivity. Secondly, the virulence of the parasite was investigated in experimentally infected mice to compare the number of resulting parasites and the number of amastigotes lodged in different mouse organs according to the altitude of Tryp. cruzi origin. Thirdly, the issue of whether Tryp. cruzi isolates from different altitudes correspond to distinct lineages was investigated as, if this were the case, the effects of Tryp. cruzi might also differ (reviewed by Manoel-Caetano & Silva, 2007). These different steps were then merged to support a discussion of the relevance of altitude and associated factors in the triatome immune elicited and Tryp. cruzi infectivity.

Materials and methods

Insects

Triatoma dimidiata adults were collected from three sites at distinct altitudes in Chiapas, Mexico: (a) at the town of Benito Juárez in the municipality of Berriozábal (300 m a.s.l.; 16°58'00" N, 93°20'30" W); (b) at El Paraíso in the municipality of Copainalá (700 m a.s.l.; 17°53'33" N, 93°00'21" W), and
(c) at Independencia in the municipality of San Juan Cancuc (1400 m a.s.l.; 17°08′24″ N, 92°22′15″ W) (Figure S1). Local health services personnel had already provided information on the presence of both T. dimidiata and Chagas’ disease infection. Mean temperature and relative humidity (RH) at these sites are, respectively: 30 ± 2 °C and 50% at Benito Juárez; 26 ± 1 °C and 70% at El Paraíso, and 18 ± 2 °C and 85% at Independencia. After 1 day of collection, all samples were taken to the Laboratory for Parasite Biology on the Mexico City campus of the Universidad Nacional Autónoma de México, for analysis. Parasite presence was determined through the analysis of fresh faeces of T. dimidiata using optical microscopy (magnification ×40). Samples of faeces were obtained by exerting pressure on the insect abdomen at 24 h after capture, which represented the amount of time required to transport the collections to the laboratory. After this analysis, the insects were maintained alive by providing access to mouse blood.

Trypanosoma cruzi isolates

Trypanosoma cruzi parasites were isolated from the rectal blister caused in female CD-1 mice by naturally infected triatomines. Mice were supplied by the vivarium of the Faculty of Medicine of the Universidad Nacional Autónoma de México. Faeces were gathered from three infected triatomines collected at a given altitude, and a pool of these faeces was mixed with phosphate-buffered saline (PBS) at pH 7.2. Each parasite isolate was denominated according to the altitude of the region from which it was sourced (TC300, TC700 and TC1400) and inoculated intraperitoneally in five mice (Instituto Nacional de Salud, 2005).

Assays of PO activity in T. dimidiata infected with Tryp. cruzi

Fifteen uninfected insects from each altitude (a total of 45) were infected with the Tryp. cruzi isolate from the same altitude; these groups represented the experimental groups. For this, previously infected mice were placed in immobilizers and offered to the triatomines for feeding until satiation. After 5 days, the insect haemolymph was extracted with a 1-mL syringe by puncturing the membrane region that separates the coxa and trochanter (Espinoza-de-Aquino, 2012). The haemolymph was immediately collected in Eppendorf tubes and mixed with PBS of NaCl (pH 7.2) at a ratio of 1:2. During extraction, haemolymph was maintained on dry ice. As controls, 15 uninfected insects from each altitude were handled and maintained in the same way as the experimental insects except that these were not infected. Control groups were confirmed to be uninfected by examining their fresh faeces for Tryp. cruzi using optical microscopy (×40); only those with faeces from which Tryp. cruzi was absent were used.

Quantification of proteins

As recommended for sample standardization (Moreno-García et al., 2013), proteins were quantified using the commercial Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Inc., Rockford, IL, U.S.A.). In a 96-well plate (Costar 96; Sigma-Aldrich Corp., St Louis, MO, U.S.A.), 10 μL of haemolymph (from each altitude and from infected and uninfected triatomines) and 40 μL of PBS were placed in each well, with each isolate assayed in duplicate. To each well, 150 μL of the reactive provided by the kit was added and then incubated at 37 °C for 30 min with an aluminium cover. Afterwards, plate absorbance was measured using an enzyme-linked immunosorbent assay (ELISA) reader (ELX 800; BioTek Instruments, Inc., Winooski, VT, U.S.A.) at 562 nm. Using the absorbance data from the samples and a standard concentration curve constructed by utilizing albumin, the total protein concentration in the sample was calculated using regression analyses (Moreno-García et al., 2013). Thirty micrograms of protein were used per sample.

Phenoloxidase activity

For each of the groups representing the three altitudes (n = 15 for each), PO activity was measured spectrophotometrically by adding 100 μL of t-DOPA substrate (dihydroxyphenylalanine, 4 mg/mL; Sigma-Aldrich Corp.) to 30 μg of protein from each sample in a 96-well plate. The plate was covered and incubated at 37 °C for 20 min. Afterwards, readings were taken every 15 min for 2 h with an ELISA reader at 492 nm. As a blank, 50 μL of PBS and 50 μL of t-DOPA were used. To show PO activity, the absorbance data [= average rate of change; for example, Cornet et al. (2009)] during the 2 h of the assay were used to construct the slope of the curve for each group. The experiment was carried out in duplicate to give a total of 30 samples for each altitude.

Trypanosoma cruzi virulence

The virulence of each Tryp. cruzi altitudinal isolate was measured using infected female CD-1 mice (weighing 18–20 g). At 20 days post-infection, a count was made in a Neubauer chamber of the parasites per mL of peripheral blood of mice used to isolate the parasite. Once this estimate was obtained, 15 mice were infected per isolate with 1 × 106 of blood trypomastigotes through intraperitoneal inoculations. Response variables, established by Barreto (1964) and the WHO (1986), were registered during a period of 37 days after infection (with only one replica). During the prepatent period, a daily check was made by direct microscopic examination (at magnification ×4) of the peripheral blood of mice. For this, samples were obtained by making a small cut in the distal part of the mouse tail. During the period of parasitaemia, parasites per mL (par/mL) were quantified by counting them in a Neubauer chamber using 10 μL of peripheral blood at a dilution of 1 : 10 with sodium citrate (3.8%). To evaluate mortality, daily records were kept until the mice were killed at 37 days. To determine cellular tropism, tissues from the heart, brain, gastrocnemius and oesophagus of mice were assessed. For this purpose, the organs were fixed in 10% formaldehyde until use. Afterwards, 10 slices (each 10 μm thick) per organ were obtained and dyed with haematoxylin and eosin (H&E) stain. For each slice, 100 fields were viewed under microscopy (×100).
Typing of Tryp. cruzi isolates

To determine whether the lineage of the Tryp. cruzi isolates from the three altitudes were different or similar, part of the intergenic region of Tryp. cruzi mini-exon genes was used (Souto et al., 1996). For this, DNA of the isolates was extracted using a modified protocol phenol-chloroform isoamyl alcohol (Espinoza & Garcia, 2003). A pool of three primers (IDT) 5'-GTGTCCGCCACCTCTCTCGGCC (TCI, group 1-specific), 5'-CCTGACGGCAGCAGTGTGTTG (TCII, group 2-specific), and 5'-CCCCCTCCCCAGGCCACTG (TC, common to groups 1 and 2) was used for amplification of the mini-exon genes. Amplification reactions were performed in a final volume of 25 μL containing 12 μL of Go Taq Green Master Mix 2X, 10 μL of nuclease-free water, 0.4 μM of each primer, and 20 ng of Trypanosoma DNA. Cycle amplification was performed using a MyGene MG96G thermal cycler (Hangzhou LongGene Scientific Instruments Co. Ltd, Hangzhou, China) under the following conditions: 5 min at 94°C, followed by 27 cycles of 40 s at 94°C, 40 s at 61°C, and 1 min at 72°C, and a final elongation of 5 min at 72°C. Polymerase chain reaction (PCR) products (300 bp for TCII and 350 bp for TCI) were analysed in a 2% agarose gel.

Statistical analysis

Differences in PO activity between the experimental and control groups for each altitude were analysed using two-way analysis of variance (ANOVA). Data for PO activity were previously transformed using the Box–Cox procedure. This transformation indicated that the normality (Shapiro–Wilk test, \( W = 0.982, P = 0.089 \)), as well as the homogeneity of variance (Fligner–Killeen test, \( \chi^2 = 10.177, \) d.f. = 5, \( P = 0.0703 \)) of the PO data complied with the assumptions for parametric tests. For ANOVA, the predictor variables were altitude (300, 700 and 1400 m a.s.l.), group (experimental and control groups), and the interaction between these two variables. Post hoc Tukey tests were then used to determine significant differences between group combinations. The virulence of the parasite was assessed using an ANOVA with altitude as the predictor variable and the number of parasites on day 29 as the response variable. Given that the three groups differed in peak, the best logistical solution was to use the nearest day to the peak for the three groups, which was day 29. Previous to analysis, data were normalized using a cubic root transformation (Shapiro–Wilk test, \( W = 0.975, P = 0.09 \)) and homogeneity of variance (Fligner–Killeen test, \( \chi^2 = 3.933, \) d.f. = 2, \( P = 0.139 \)) assumptions. As there were very few amastigotes in the brain, comparisons between amastigote levels for the three altitudes were made for the three remaining organs (heart, oesophagus and gastrocnemius) using a two-way ANOVA. Amastigote data were normalized using a square root transformation to fulfill parametric test assumptions (Shapiro–Wilk test, \( W = 0.988, P = 0.348 \)) and homogeneity of variance (Fligner–Killeen test, \( \chi^2 = 9.953, \) d.f. = 8, \( P = 0.268 \)). For this analysis, organ and isolate were entered as predictor variables, and amastigotes as the response variable. Tukey’s test was used to determine which groups showed significant differences. All analyses were carried out using R Version 3.2.3 (R Core Team, 2015).

Results

Phenoloxidase activity in T. dimidiata in relation to altitude and group

Significant differences were found in PO activity as a function of altitude (\( F_{2,129} = 12.316, P = 0.001 \)), experimental group (\( F_{1,129} = 1813.839, P = 0.001 \)), and the interaction of these two variables (\( F_{2,129} = 64.838, P = 0.001 \)). The post hoc comparisons and visual inspection of the values of these combinations indicated that for each of the three altitudes, infected groups had higher values for PO activity than their corresponding control groups (Fig. 1). Within the experimental groups, the highest values for PO activity were those for 700 m a.s.l., followed by those for 300 m a.s.l. and finally those for 1400 m a.s.l. (Fig. 1). Within the control groups, the lowest values for this parameter were those for 300 m a.s.l., and there was no difference between the groups representing the other two altitudes (Fig. 1).

Virulence of Tryp. cruzi

The periodic observations of the peripheral blood of experimental animals allowed the determination of the prepatent periods of the three isolates, which oscillated between 13 and 15 days. Based on circulating trypanosomes, there was a continuous increase in parasitaemia for each of the three isolates. With an inoculum of 1 × 10⁶ parasites, the isolate sourced from 700 m a.s.l. showed a peak of 22.57 × 10⁶ par/mL at 33 days post-inoculation (p. i.). Isolate TC300 reached a peak of 15.66 × 10⁶ par/mL at day 27 p. i., whereas isolate TC1400 reached a peak of 11.17 × 10⁶ par/mL at day 27 p. i. (Fig. 2). The analysis at day 29 p. i. indicated significant differences

Fig. 1. Changes in phenoloxidase (PO) activity in relation to the altitude of Triatoma dimidiata collection and the different experiment and control groups. Values are expressed as the mean ± standard error. Distinct letters indicate significant differences (Tukey’s test, \( P < 0.05 \)).
Effects of altitude on *Trypanosoma cruzi*

Fig. 2. Time curves of parasitaemia in female CD-1 mice infected with three isolates of *Trypanosoma cruzi* sourced from different altitudes (TC300, 300 m a.s.l.; TC700, 700 m a.s.l.; TC1400, 1400 m a.s.l.). Values are expressed as the mean ± standard error. Numbers in parenthesis indicate the quantity of mice per group that died during the experiment.

Fig. 3. Parasitaemia in female CD-1 mice inoculated with *Trypanosoma cruzi* from different isolates sourced from different altitudes (TC300, 300 m a.s.l.; TC700, 700 m a.s.l.; TC1400, 1400 m a.s.l.). Values are expressed as the mean ± standard error. Distinct letters indicate significant differences (Tukey’s test, \( P < 0.05 \)).

between groups (\( F_{2,35} = 264.61, P < 0.0001 \)), whereby isolate TC700 showed the greatest level of parasitaemia, followed by isolate TC300 and finally isolate TC1400 (Fig. 3). The mortality recorded during the 37 days of the experiment was low: in the 90 mice infected, only three deaths were observed during the entire experiment, including two from isolate TC700 and one from isolate TC1400.

Nests of amastigotes were found through dissection of the four different organs tested. (Examples from TC700 are shown in Figure S2) It is noteworthy that only isolate TC700 was able to invade the brain (in four insects). Indeed, this isolate produced the greatest number of amastigotes (\( F_{2,126} = 897.56, P = 0.001 \)) (Fig. 4). Additionally, all three isolates resulted in cellular tropism with greater frequency in the cardiac muscle than in the other organs (Fig. 4). Nests of amastigotes were observed to a lesser degree in the oesophagus, followed by the gastrocnemius and brain (\( F_{2,126} = 1640.94, P = 0.001 \)). The interaction between isolates (by altitude) and organs was significant (\( F_{4,126} = 24.24, P = 0.001 \)). The interaction indicated that, for each organ, the level of amastigotes was highest for the isolate sourced from 700 m a.s.l. (Fig. 4).

which can reduce but not eliminate the parasite (Araujo et al., 2015) is related to an efficient insect immune reaction against the parasite is that of nitric oxide and superoxide (Whitten et al., 2001). However, it is difficult to establish a link between the production of PO and that of nitric oxide or superoxide because they are involved in distinct cascades (Rivero, 2006; González-Santoyo & Córdoba-Aguilar, 2012). Studies related to any defensive effect against Tryp. cruzi by triatomines are sorely needed.

One novel aspect of the present study with regard to PO and the parasite is the environmental context (limited to the altitudinal gradient). Phenoloxidase activity was highest in insects collected at 700 m a.s.l. In this zone in the present study, the average temperature is 26 ± 1 °C and RH is 70% (Instituto Nacional de Estadística y Geografía, 2016). These ranges are appropriate for the optimum physiological development of T. dimidiata (Reyes & Angulo, 2009; Reyes-Novelo et al., 2011). Such appropriateness can be linked to the effects of stressful conditions on the immune response in these animals. The immune response is known to be energetically costly to produce and hence its maintenance must be balanced with regard to other costly functions (Sadd & Schmid-Hempel, 2009). Environments that are less favourable may cause an energetic imbalance that negatively affects the immune response (e.g. Zhivotovsky et al., 1996). For example, suboptimal environments can lead to immune downregulation as a result of a lack of resources for the host (e.g. Bowden, 2008). One such example refers to a cold season or environment, which, together with low availability of food, can negatively affect immune response and survival (e.g. Demas & Nelson, 1998). For T. dimidiata, it is possible that the environment at 700 m a.s.l. is better for achieving a more robust immune response than that at 1400 m a.s.l. This may refer to local adaptation, given that T. dimidiata at the altitude of 700 m a.s.l. may be able to strengthen its immune capacity more than T. dimidiata at other altitudes (e.g. Karl et al., 2010). A second explanation is that, at 700 m a.s.l., the supply of pathogens presents a greater challenge to which T. dimidiata responds more intensely. This supposes that at other altitudes the pressure exerted by pathogens is less intense [for a similar idea, see Moller & Rózsa (2005)]. A third explanation is that Tryp. cruzi at 700 m a.s.l. promotes a more intense immune response as a form of manipulation of the host (Damian, 1997). Each of these explanations warrants further investigation. Furthermore, although the only ‘controlled’ variable in the present study was altitude, there are certainly other abiotic and biotic factors involved, some of the most important of which, based on other insect studies (Schmid-Hempel, 2005), may be temperature and food.

With regard to virulence and infectivity, the isolate from 700 m a.s.l. showed different patterns in comparison with the other two isolates, although all Tryp. cruzi collections from the different locations were of the same strain (TCI). However, judging by other studies (Higo et al., 2004), it would not be surprising to find genetic variation in geographical regions that are relatively close to one another, as were the present study sites. In fact, even the same Tryp. cruzi strain can give rise to different symptoms as a result of large genetic differences (e.g. Del Puerto et al., 2010). Thus, it is likely that genetic differences may underlie the differences in isolates sampled from the different altitudes, as evidenced by: (a) different organ tropism, with TC300 more likely to invade brain tissues in comparison with isolates sourced

### Discussion

The overall results demonstrate that the highest values for triatomine PO activity, and for the number of parasites and organs invaded, were found with Tryp. cruzi from 700 m a.s.l. In addition, the isolates from the three altitudes indicated that all Tryp. cruzi populations belong to the same strain I. The activation of PO in triatomines infected with Tryp. cruzi is not a novel event (e.g. Mello et al., 1995; Ursic-Bedoya & Lowenberg, 2007; Castro et al., 2012; Espinosa-de-Aquino, 2012). For example, PO activity increases after infection of the triatomine R. prolixus with Tryp. cruzi (Castro et al., 2012) in a similar way to that found in the present study. It is not clear whether PO acts directly against the parasite or is part of a systemic response of R. prolixus faced with an invasion. In fact, rather than the increase in PO leading to a reduction of the parasite in the insect, the exact opposite occurs (Castro et al., 2012). It has even been proposed that both Tryp. cruzi and the related parasite Trypanosoma rangeli are capable of dealing successfully with an increase in PO levels (Flores-Villegas et al., 2015). If this is true, then it is likely that the insect does indeed detect the presence of the parasite, but is incapable of eliminating it. Another possibility is that the rise in PO is a systemic effect that facilitates other immune pathways to eliminate the parasite. For example, the activation of PO leads to the activation of highly toxic compounds (e.g. 5,6-dihydroxyindole) against viruses and parasitoid wasps (Zhao et al., 2011).

It is still unclear whether the fitness cost of Tryp. cruzi infection to triatomines (Fellet et al., 2014; Flores-Villegas et al., 2015) is related to an efficient insect immune reaction against the parasite. Infection by the parasite is known to lead to the production of other immune components such as defensin Def1, which can reduce but not eliminate the parasite (Araujo et al., 2006). Perhaps the best evidence of an efficient action against the parasite is that of nitric oxide and superoxide (Whitten et al., 2001). However, it is difficult to establish a link between the production of PO and that of nitric oxide or superoxide because they are involved in distinct cascades (Rivero, 2006; González-Santoyo & Córdoba-Aguilar, 2012). Studies related to any defensive effect against Tryp. cruzi by triatomines are sorely needed.

### Lineage of Tryp. cruzi isolates

Amplification of the mini-exon genes with the three isolates of Tryp. cruzi (TC300, TC700 and TC1400) resulted in a PCR product of 350 bp (Fig. 5). This confirms that the three populations belong to the same TCI lineage.

### Fig. 5. Amplification of polymerase chain reaction products from the analysis of mini-exon genes according to the altitude at which Trypanosoma cruzi isolates were sourced (TC300, 300 m a.s.l.; TC700, 700 m a.s.l.; TC1400, 1400 m a.s.l.). C1 indicates TCI lineage control (Qro strain); C2 indicates TCII lineage control (Y strain). L indicates the 50-bp ladder. Note that both control strains have been typified previously (Espinoza et al., 2010).

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from the other altitudes; (b) a prepatent period of 13–15 days unlike the 8–12 days reported for the H4 and H5 strains of Tryp. cruzi in Yucatán (Barrera-Pérez et al., 2001), a state near Chiapas, and, to a lesser extent, (c) mortality, whereby the TC700 isolate led to two of the three mouse deaths.

By contrast, the level of parasitaemia has been related to the invasion of Tryp. cruzi in tissues (Tay et al., 1973; Salazar-Schettino et al., 1978; Sánchez-Guillén et al., 2006). This relationship is in accordance with the present results, given that the isolate that showed the greatest parasitaemia (that from 700 m a.s.l.) also demonstrated the greatest amount of amastigote nests in the organs tested. The preference for amastigote nests in cardiac muscle among the three isolates coincides with the majority of strains evaluated in Mexico (Marie-France et al., 2002), which have been related to the lineage Tryp. cruzi I, which predominates mainly in the central and northern parts of the American continent (Noireau et al., 2009). Indeed, this variability has hindered efforts to establish adequate parameters for classification and taxonomy, which are key to effective parasite study and control (Guzmán-Marín et al., 1999).

In conclusion, the present study sheds light on the role of one abiotic factor (i.e. altitude) related to the origin of Tryp. cruzi in the pathogen–host interaction under natural conditions. Understanding of which factors are masked by such altitudinal differences should certainly support the development of strategies for the prevention of Chagas’ disease. In fact, studies on the collection and prediction of niche ecology in the state of Chiapas indicate greater densities of T. dimidiata in zones at 800 m a.s.l. (Benítez-Alba et al., 2012). Given that the present results show the isolate from 700 m a.s.l. to be the most infective, altitudes of 700 and 800 m a.s.l. seem to represent a greater risk to correpsonding human populations. However, the most common cases of seropositivity in Chiapas come from altitudes of around 1340 or <300 m a.s.l. (Mazariego-Arana et al., 2001). This suggests the existence of other factors that may explain why there are not necessarily more cases of infection with Tryp. cruzi at 700 m a.s.l., although the virulence of this parasite is greater at that altitude.

**Supporting Information**

Additional Supporting Information may be found in the online version of this article under the DOI reference: DOI: 10.1111/mve.12198

**Figure S1.** Locations of collection of three different groups of Triatoma dimidiata and isolates of Trypanosoma cruzi.

**Figure S2.** Amastigote nests in the (A) heart, (B) oesophagus, (C) gastrocnemius and (D) brain of female CD-1 mice inoculated with Trypanosoma cruzi (isolate TC700). Original magnification ×100.

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