Trypanosoma cruzi–Trypanosoma rangeli co-infection ameliorates negative effects of single trypanosome infections in experimentally infected *Rhodnius prolixus*

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SUMMARY

Trypanosoma cruzi, causative agent of Chagas disease, co-infects its triatomine vector with its sister species *Trypanosoma rangeli*, which shares 60% of its antigens with *T. cruzi*. Additionally, *T. rangeli* has been observed to be pathogenic in some of its vector species. Although *T. cruzi–T. rangeli* co-infections are common, their effect on the vector has rarely been investigated. Therefore, we measured the fitness (survival and reproduction) of triatomine species *Rhodnius prolixus* infected with just *T. cruzi*, just *T. rangeli*, or both *T. cruzi* and *T. rangeli*. We found that survival (as estimated by survival probability and hazard ratios) was significantly different between treatments, with the *T. cruzi* treatment group having lower survival than the co-infected treatment. Reproduction and total fitness estimates in the *T. cruzi* and *T. rangeli* treatments were significantly lower than in the co-infected and control groups. The *T. cruzi* and *T. rangeli* treatment group fitness estimates were not significantly different from each other. Additionally, co-infected insects appeared to tolerate higher doses of parasites than insects with single-species infections. Our results suggest that *T. cruzi–T. rangeli* co-infection could ameliorate negative effects of single infections of either parasite on *R. prolixus* and potentially help it to tolerate higher parasite doses.

Key words: *Trypanosoma cruzi*, *Trypanosoma rangeli*, *Rhodnius prolixus*, *T. cruzi–T. rangeli* co-infection, Chagas disease, infected-vector fitness.

INTRODUCTION

Upon infection of its insect vector, the parasite *Trypanosoma cruzi*, aetiological agent of Chagas disease, joins a diverse microbial community (Eichler and Schaub, 2002; Espino *et al*. 2009; Vallejo *et al*. 2009), consisting of up to eight species of bacteria (Vallejo *et al*. 2009), six genera of fungi (De Moraes *et al*. 2001, 2004; Luz *et al*. 2004), four other trypanosomatid species (Schaub, 1992) and at least one virus (Marti *et al*. 2015). These taxa can interact indirectly via resource competition, immune modulation, competition for immune-free space (Dobson, 1985; Cox, 2001; Pedersen and Fenton, 2007) and even sometimes directly through physical attack (Azambuja *et al*. 2004; Castro *et al*. 2007), all of which have potential consequences for the survival and reproduction of the insect.

One species of particular interest is *Trypanosoma rangeli*, a *T. cruzi* congeneric that infects several of the same mammal and triatomine species as *T. cruzi*. *Trypanosoma rangeli* is of interest in the study of Chagas disease because it shares at least 60% of its antigens with *T. cruzi* (Guhl and Marinkelle, 1982; Saldaña and Sousa, 1996; Guhl and Vallejo, 2003). These antigenic similarities can lead to cross-reactions in immunogenic diagnostic tests, which can result in erroneous Chagas disease diagnoses (Guhl *et al*. 1987) and in turn interfere with the ability to predict and describe Chagas disease distribution in Chagas-endemic regions. *Trypanosoma cruzi* and *T. rangeli* are often found co-infecting together in field-caught triatomine bugs of the genus *Rhodnius* (Fig. 1), some of which are considered key vectors of *T. cruzi* to humans (Gorla and Noireau, 2010).

Although not pathogenic in mammals (Herbig-Sandreuter, 1957), in triatomine bugs, *T. rangeli* has been observed to negatively affect the survival and development of the triatomine species *Rhodnius prolixus* when experimentally infected with the parasite (Grewal, 1957; Tobie, 1965; Gómez, 1967; Watkins, 1971; Añez, 1984; Añez *et al*. 1987). Until recently, *T. cruzi* was not believed to have negative consequences for its invertebrate hosts (Schaub, 1989*a*, 1992, 1994), although this has now been shown to be variable (Elliot *et al*. 2015; Peterson *et al*. 2015). Little is known about the consequences of *T. cruzi–T. rangeli* co-infection for the triatomine bug, and to our knowledge, has been investigated just once (Añez *et al*. 1992); that study reported delayed nymphal development and

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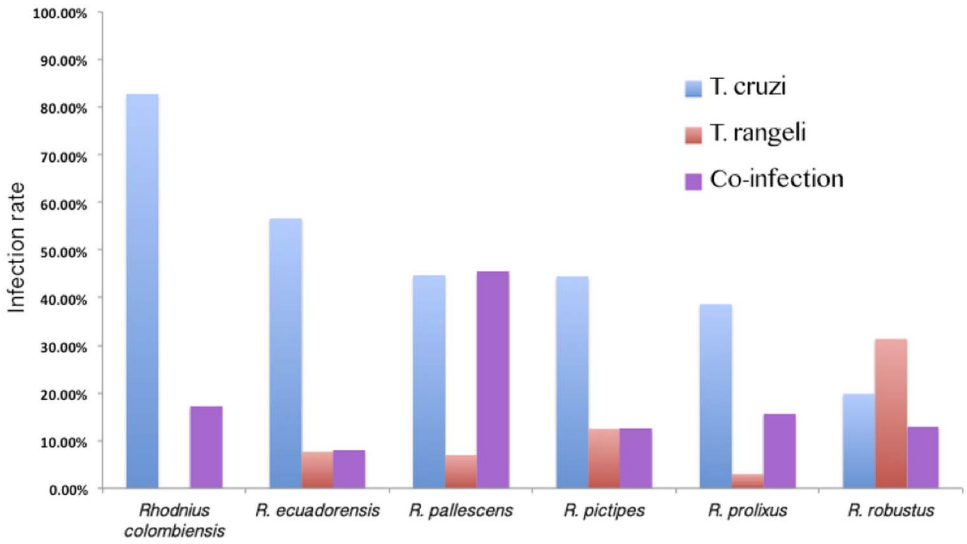

Fig. 1. Reported co-infection prevalence in field-caught *Rhodnius* triatomines (Carcavallo *et al*. 1975; Vallejo *et al*. 1988; Pavia *et al*. 2007; Pineda *et al*. 2008; Grijalva *et al*. 2012).

increased mortality in *R. prolixus* co-infected with *T. cruzi* and *T. rangeli* compared with singly-infected insects. However, the sustained effects of such co-infection on triatomines (e.g. on their reproduction or overall fitness) have never been investigated. In mammals, it was found that *T. rangeli* exposure in vertebrates prior to *T. cruzi* infection modulated the host immune response to *T. cruzi*, resulting in reduced disease severity in both acute and chronic *T. cruzi* infections (Basso *et al*. 1991, 2007, 2008, 2014; Marini *et al*. 2011; Basso, 2013). These studies suggest that *T. cruzi–T. rangeli* co-infection could affect triatomine fitness differently than single-species infections.

Here, we compared the fitness of triatomine bugs (*R. prolixus*) experimentally co-infected with *T. cruzi* and *T. rangeli* with the fitness of bugs with single-species infections of *T. cruzi* or *T. rangeli*. We defined fitness as the net contribution to future generations of each insect. We aimed to determine if there is a difference in fitness between bugs with different infection types, as we propose that the extent to which *T. cruzi–T. rangeli* co-infection alters the impact of each infection on individual vector fitness may in turn alter the transmission potential of the parasites. This, in turn, could have implications for vector control and Chagas disease prevention strategies.

MATERIALS AND METHODS

Experimental design

We infected 100 *R. prolixus* fifth instar females with just *T. cruzi*, just *T. rangeli*, or *T. cruzi* and *T. rangeli* (Table 1). A total of 33 additional uninfected insects were used as controls, for a total of 133 insects used in the experiment. After moulting into the adult stage, each female was mated with an uninfected male, and survival and reproduction were measured for up to 96–140 days. All experiments were carried out in the laboratory of the Grupo de la Biología y Control de Enfermedades Infecciosas [Biology and Control of Infectious Diseases Group (BCEI)], University of Antioquia, Medellín, Colombia.

Table 1. Treatment groups

Treatment group	
T. cruzi (Gal61 strain)	24
T. rangeli (Choachí strain)	33
T. cruzi–T. rangeli co-infection	43
Control	33

Triatomines

All *R. prolixus* used in the experiment were from laboratory colonies reared in the BCEI insectary, where triatomine colonies are kept under semi-controlled climate conditions (~27 ± 1 °C and 65 ± 15% RH) and a 12 h photoperiod, and given the opportunity to feed twice weekly on hens according to the animal ethics committee regulations of the Sede de Investigación Universitaria [University Investigation Headquarters (SIU)] of the University of Antioquia. Insects used in the experiment were fed on hens once per oviposition cycle, described below. Colonies were founded by *R. prolixus* eggs collected in Colombia between 2000 and 2009. All insects used in the experiment were 5th instar nymphs at the time of infection. Nymphs were collected manually from the colonies, and sex was subsequently determined (prior to infection)

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by examining the two concentric terminal segments around the anus on the insect's ventral side under a dissecting microscope, as described in Chiang *et al*. (2013) and Gillet (1935).

Parasites

We used the parasite strains 'Gal61' (*T. cruzi*) and 'Choachí' (*T. rangeli*). Gal61 was originally isolated from a mouse in Galeras, Colombia, and belongs to the *T. cruzi* discrete typing unit (DTU) group I (Rojas *et al*. 2007; Falla *et al*. 2009). Choachí was originally isolated from an *R. prolixus* individual collected in Cundinamarca, Colombia (Grisard *et al*. 1999; Vargas *et al*. 2000; Urrea *et al*. 2011), and belongs to the KP1(+) kDNA (kinetoplastid deoxyribonucelic acid) group (Vallejo *et al*. 2002), which is associated with the Prolixus complex of *Rhodnius* (Urrea *et al*. 2005).

Trypanosoma cruzi parasites were cultured and maintained as described in Peterson *et al*. (2015). Briefly, epimastigotes were cultured at 28 °C in a RPMI-1640 liquid medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS). Epimastigotes of the *T. rangeli* Choachí strain were supplied by Professor Gustavo Vallejo of the University of Tolima, where they were cultured at 28 °C in NNN medium and supplemented with 10% FBS. Infectivity was maintained by cyclic *R. prolixus*–mouse passages every 3 months.

Insect infection

We prepared the parasites (epimastigote stage) and infected the insects as described in Peterson *et al*. (2015). Briefly, parasites were counted in a Neubauer chamber, washed through centrifugation and resuspended in 1 mL of sterile phosphate-buffered saline. Insects were starved for about 2 weeks before the infection, upon which each bug was marked with a small dot of non-toxic water-based paint at the top of the pronotum, and then weighed before and after feeding to estimate the number of parasites ingested. Only females were fed infected blood, while the females from the control group and all males were fed uninfected blood. A total of 5–10 insects were grouped in small jars, which were then placed under a membrane feeder containing defibrinated, de-complemented human blood (heated to 37·5 °C) supplemented with inactivated FBS with an estimated concentration of $3{\cdot}3–3{\cdot}5 \times 10^6$ parasites/mL. This concentration falls within (a) the range of peak parasitaemias observed in mice and guinea pigs experimentally infected with *T. cruzi* (Bice and Zeledon, 1970; Urdaneta-Morales and Rueda, 1977; Perlowagora-Szumlewicz and Muller, 1982; Schaub and Losch, 1989*a*; Schaub *et al*. 1989; Kollien *et al*. 1998) and *T. rangeli* (Urdaneta-Morales and Tejero, 1986; Zuñiga *et al*. 1997*a*, *b*), and oral infectious doses used in prior published studies of *T. cruzi* and *T. rangeli* infection in triatomines (Garcia *et al*. 1994, 2004; Mello *et al*. 1996; Ratcliffe *et al*. 1996; Whitten *et al*. 2001; Borges *et al*. 2006; Araújo *et al*. 2007, 2014; Nogueira *et al*. 2007; Mejía-Jaramillo *et al*. 2009; Ferreira *et al*. 2010; Castro *et al*. 2012, 2014; Fellet *et al*. 2014). *Trypanosoma cruzi–T. rangeli* co-infections were carried out at a similar total parasite concentration, consisting of equal concentrations of each species (i.e. $1{\cdot}65 \times 10^6$ of each parasite species/mL of blood, for a total of $3{\cdot}8 \times 10^6$ parasites/mL of blood).

Insect reproduction

After moulting into the adult stage, we paired each female with a recently fed adult male (Buxton, 1930; Davey, 1965). Males were paired with females of just one treatment group throughout the experiment to avoid cross-contamination. Copulation was determined 1 day after insects were paired from the presence of the spermatophore casing in the jar, ejected by the female (Ruegg and Davey, 1979). If we did not find the spermatophore casing after the first night, then 2–3 additional males were placed in the jar with the female, and left for another night (G. Chiang, personal Communication, 2013). If copulation did not occur after three nights with several males, then we recorded the female as unmated for that oviposition cycle. Unmated individuals from the first oviposition cycle were given a second opportunity to mate for the second oviposition cycle. After mating, females were fed on hens (males were fed 3–4 days prior to copulation for sperm production). We marked each female with a small coloured dot of non-toxic, water-based paint on the pronotum (Mac Cord *et al*. 1983; Henriques *et al*. 2012), weighing it before and after feeding to calculate the volume of blood ingested. We recorded oviposition and eclosion 3–4 times per week until the second oviposition cycle, 31–38 days later.

We measured reproduction as fecundity (egg production) and the percentage of oviposited eggs that hatched. Fecundity in *R. prolixus* is correlated with the quantity of blood ingested and weight before feeding (Friend *et al*. 1965), and the standard index used when comparing fecundity in *R. prolixus* is the *E* value (Ruegg and Davey, 1979). The *E* value is calculated as the total number of eggs produced by a given individual divided by the product of the blood meal volume multiplied by its pre-feeding weight. This represents the efficiency with which the insect converts nutrition (blood) into food, while normalizing for blood and insect mass, allowing for comparison across feedings. The *E* value is independent of the timing of the oviposition cycle in an insect's lifetime. In analysing the *E* values,

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we did not include insects that died before an oviposition cycle began (i.e. resulting in an E value of 0), in order to compare E value independent of mortality rate. In addition to these measurements, time-dependent reproductive values were also generated for each individual in our fitness analyses, described below.

Infection confirmation

After insect death, we extracted total DNA from each insect using Qiagen DNeasy blood and tissue kit. Additionally, we extracted DNA from pooled males and pooled offspring to check for horizontal and vertical transfer of parasites. We amplified DNA in an RT–PCR (StepOnePlus Real-Time PCR System, Applied Biosystems), with the *T. cruzi* primer pair [TcZ1/2 (Cummings and Tarleton, 2003)] and *R. prolixus* reference gene primer (RP18S, Paim *et al*. 2012). To obtain a *T. rangeli*-specific primer of the optimal size [<150 base pairs (bp)] that did not cross-amplify *T. cruzi*, we designed a primer denoted as 'PEEL5' −F (5′-TGCTTTCGTAGTTGGCACTG-3′) and −R (5′-ACGCACCTCCTCCTCTCTCT-3′), which amplifies a 93 bp fragment of *T. rangeli* telomeric DNA. We designed this primer from the *T. rangeli* clone TrTel 10 telomeric sequence (GenBank ID: AF426020·1), using the Primer3 plus software (Untergasser *et al*. 2007).

Statistical analyses

We carried out all statistical analyses using the R statistical computing environment software version 3.03 (R Core Team, 2014) using non-parametric tests to avoid normality assumptions. We tested for differences between treatments in the amount of parasites or blood ingested per unit of insect mass using the Kruskal–Wallis rank sum tests. We tested for differences in the amount of parasites or blood ingested per unit of body weight using Wilcoxon Rank Sum tests. We applied the 'kruskalmc' function from the 'pgirmess' package (Giraudoux, 2013) to carry out multiple comparisons and control for family wise error when a difference was found in Kruskal–Wallis tests. This function implements comparisons between treatments, and one- and two-tailed comparisons *vs* control. We accepted P-values under 0·05 as statistically significant.

We analysed survival function for each treatment group using the Kaplan–Meier (K–M) method in the R 'survival' package (Therneau and Grambsch, 2000; Therneau, 2015). We compared survival function (the probability of total time until failure) between treatment groups using the 'survdiff ' function in the 'survival' package, a two-tailed test for censored data that implements the G–ρ family of tests (Harrington and Fleming, 1982), where deaths at various times are weighted by a factor of $S(t)^\rho$ (S = K–M estimate; t = time), and ρ is a scalar parameter that determines the type of test used. When set at 0, all deaths are weighted equally across time and a log-rank test is used. When set at 1, deaths at the beginning of the time period are more heavily weighted, and the Peto and Peto test (Peto and Peto, 1972) is employed. We set ρ at 1, to offset insect death events related to senescence. We carried out pairwise comparisons between K–M survival curves with Chi-squared (χ^2) distribution tests and adjusted P-values to control for the familywise error rate using the Holm–Bonferroni correction method (Holm, 1979).

We used Cox proportional hazards (PH) models (Cox, 1972) to examine the main effects and two-way interactions of parasite treatment, parasite dose and blood ingested on treatment hazard rates (the instantaneous rate of failure at any given time, given that the individual has survived up until that time). The PH assumption, (i.e. hazards were proportional over time) was tested with the Coxph function in the 'survival' package. We selected model covariates using Akaike's Information Criterion (AIC) with the stepAIC function in the 'MASS' package (Venables and Ripley, 2002), and manual one-variable-at-a-time reduction.

We log2 transformed parasite dose data, and centred them on the log2 transformation of $5{\cdot}0 \times 10^5$ parasites, the round number closest to the mean. We used the Predict function from the 'rms' package (Harrell, 2014) to estimate log relative hazards and their 95% confidence intervals based on 1000 simulations of the model.

We ran the Cox model with three variations. In the first variation, we investigated the interaction between treatment and blood:weight ratio, and compared the parasite treatment group hazards with the control hazard. In the second and third variations, we included only parasite treatment groups to investigate relative hazard. To control for a possible effect of absolute number of parasites *vs* relative number of each parasite species in the mixed parasite species dose, we ran the model with data for the absolute number of parasites ingested by the mixed group in the second variation. In the third variation, we ran data for the mixed group as the relative number of each parasite species ingested. This does not change the power of the model or the summary statistics; the change was reflected only in effect size. Cox PH model outputs are in Tables S1–S3 in the Supplementary Materials.

Fitness estimates

We used individual survival and reproduction data to construct an age-classified population projection matrix for each insect (McGraw and Caswell, 1996;

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Twombly *et al*. 1998). Each matrix was 3 × 3, with age-specific survival (P_i) on the sub-diagonal [always 0 or 1 in individual matrices (McGraw and Caswell, 1996)], and age-specific realized reproductive output (F_i) in the first row. All other matrix elements were zeros. Each time step (t_i) in the matrix represented one month (with t_0 being the day of insect infection). The model for each individual A was constructed as:

$$A = \begin{matrix} 0 & F_2 & F_3 \\ 1 & 0 & 0 \\ 0 & 1 & 0 \end{matrix}$$

The dominant eigenvalue (λ) of each matrix is a maximum-likelihood estimate of individual fitness, with values above one indicating population growth, and values below one indicating population shrinkage. The dominant left eigenvector of each matrix is an estimate of individual reproductive value v_i for each time step. We calculated dominant eigenvalues (λ) using the eigenfunction in the R base package, and reproduction values were calculated by hand based on these values, as in McGraw and Caswell (McGraw and Caswell, 1996; based on Fisher, 1930). The reproductive value for t_1 (v_1) is scaled to one, and other values are given relative to v_1. In an individual population projection model where F_1 is equal to 0, v_2 is equal to lambda. The model assumes a closed population with unlimited resources, no genetic structure, and does not account for effects of population density.

RESULTS

Parasites ingested

Insects ingested between 30·1 and 337·9 mg of blood (mean 214·8 mg), and an estimated 62 000–1 079 000 total parasites (mean 708 000). The ratio of the volume of blood ingested to insect pre-feeding weight ranged from 0·99 to 14·25 (mean 8·23), and the ratio of the estimated number of parasites ingested per mg of insect biomass ranged from 2000 to 48 000 parasites (mean 28 000). There were no differences between treatments in the absolute parasites dose, nor were there any linear relationships between the parasite dose and death day, E value, reproductive value or estimate of total fitness. There was a significant difference between treatments in the ratio of the volume of blood ingested per mg of insect biomass (Kruskal–Wallis, blood: $P = 1{\cdot}67 \times 10^{-4}$; parasites: $P = 0{\cdot}01$), with the mixed group ingesting significantly more blood than the *T. cruzi* or control groups (Fig. 2; KruskalMC, $P < 0{\cdot}05$ for comparisons).

Reproduction

87·8–97·6% of insects in each group laid eggs, and there was no significant difference between treatment groups in this respect. The E values were significantly different between treatments in both the first and second oviposition cycles (Kruskal–Wallis; cycle 1: $P = 8{\cdot}98 \times 10^{-8}$; cycle 2: $P = 3{\cdot}24 \times 10^{-4}$, Fig. 3A and B). In both cycles, E values for the *T. cruzi* or *T. rangeli* treatments were significantly lower than the co-infected treatment E values (Kruskalmc, $P < 0{\cdot}05$). The *T. cruzi* treatment had a significantly lower E value than the control group in cycle 1 only (Kruskalmc, $P < 0{\cdot}05$). The mean percentage of oviposited eggs that hatched ranged between 79·4 and 84·3% for cycle 1; 62·4–81·8% for cycle 2; and 77·6–83·7% overall. The percentage of eggs that hatched was not significantly different between treatments. Additionally, there was no association between E value and per cent of eggs hatched.

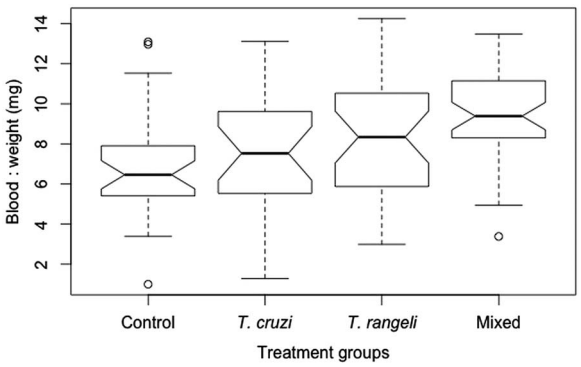

Fig. 2. The distribution of the ratio of the volume of blood consumed in the infective blood meal to mg of insect biomass, across treatments. The mixed group blood: weight ratio was significantly higher than that of the *T. cruzi* and control groups.

Survival function

K–M survival curves (representing survival function, i.e. the probability of total time until failure), were significantly different from each other ($\chi^2 = 8{\cdot}4$, 3 df, $P = 0{\cdot}03$, Fig. 4). The *T. cruzi* treatment group had a significantly shorter time to failure than the mixed treatment group (χ^2 distribution comparisons, $P < 0{\cdot}05$).

Hazards analysis

The Cox model variation investigating the interaction of treatment with blood:weight ratio was significant (Likelihood ratio test, 24·67, 7 df, $P = 8{\cdot}67 \times 10^{-4}$; Supplementary Materials Table S1), suggesting hazard (i.e. instantaneous risk of death) was not the same between treatment groups even when blood meal and body size were taken into account. Investigating the blood:weight ratio allowed us to control for differences in insect size by measuring the effect of the quantity of blood (and also therefore,

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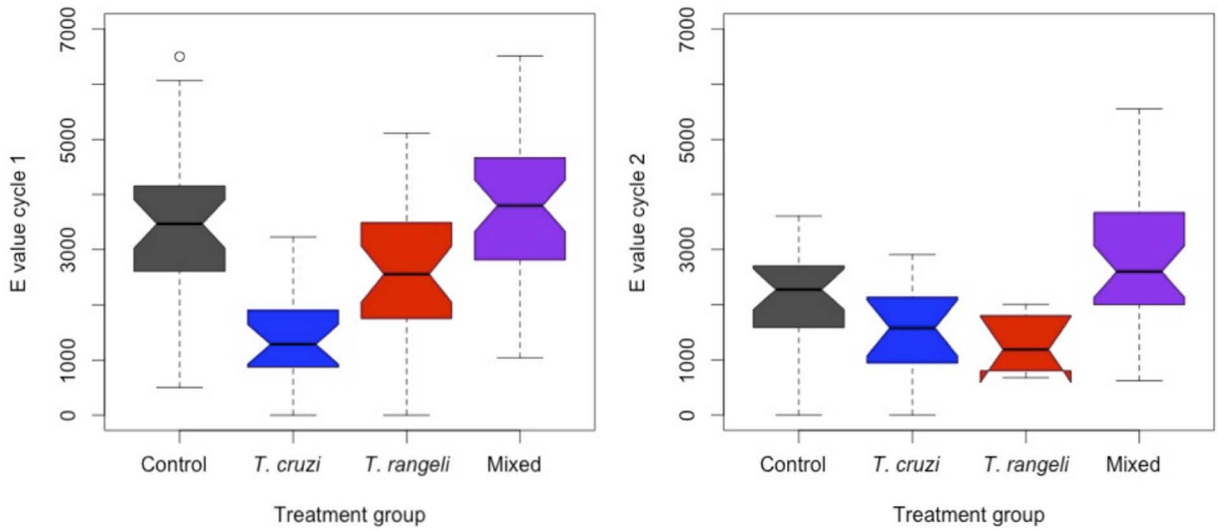

Fig. 3. E value distributions in each treatment group for oviposition cycle 1 (left) and oviposition cycle 2 (right). In both cycles, the mixed group *E* values were significantly higher than the *T. cruzi* and *T. rangeli* treatment group *E* values. The control group *E* values were significantly higher than the *T. cruzi* and *T. rangeli* treatments in cycle 1. In cycle 2, the control group is higher than just the *T. rangeli* treatment.

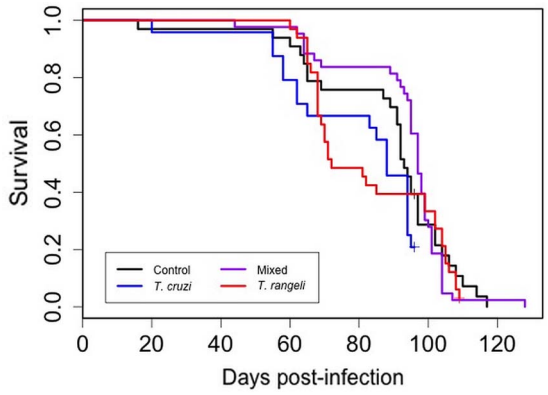

Fig. 4. K–M survival curves for each treatment group. The *T. cruzi* treatment survival function was significantly different than that of the mixed group.

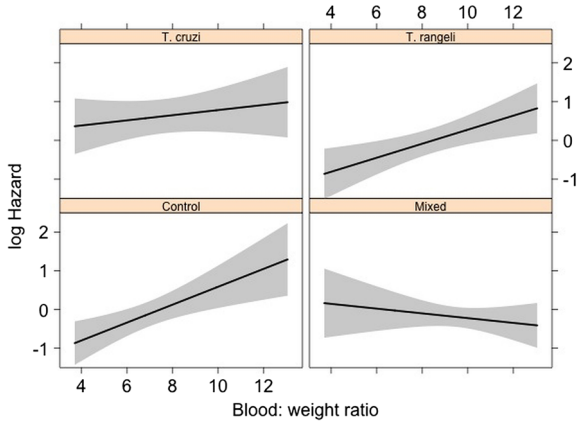

Fig. 5. Interaction of treatment with the blood:weight ratio of the infective blood meal. Hazards were predicted after 1000 simulations of the model. Figures are centred on the mean ratio, 8·23. Grey shading indicates 95% confidence intervals. Just the interactions in the bottom row (the control and mixed treatment groups) were significant.

number of parasites for infected groups) per unit of body mass. Quantity of blood was used in the calculation rather than parasite dose to be able to include the control group. The main effects of *T. cruzi* treatment were significant, with a hazard 2·17 times that of the control group ($e^{\beta} = 2{\cdot}17$, $P = 4{\cdot}33\text{–}04$). The control and mixed treatments interacted significantly with the blood:weight ratio, but in opposite directions; the control group hazard increased as the blood: weight ratio increased, while the mixed group hazard decreased with increases in the blood:weight ratio (control: $e^{\beta} = 1{\cdot}26$, $P = 1{\cdot}55 \times 10^{-3}$; mixed: $e^{\beta} = 0{\cdot}74$, $P = 5{\cdot}64\text{–}03$, Fig. 5).

The Cox model investigating the main and interaction effects of parasite dose was also significant (Likelihood ratio test, 29·63, 5 df, $P = 1{\cdot}74 \times 10^{-5}$). The patterns and significant effects were the same in both variants of the model (examining the effect of absolute *vs* relative parasite dose), with effects being slightly larger in the model investigating absolute parasite dose. In both model variations there were no differences in the main effects of treatment on hazard. Main effects of parasite dose were significant for *T. rangeli* and marginally significant for *T. cruzi*, with a 3-fold increase in hazard at a dose of 1 million parasites from the hazard at 500 000 parasites (*T. rangeli:* $e^{\beta} = 3{\cdot}27$, $P = 4{\cdot}33\text{–}04$; *T. cruzi:* $e^{\beta} = 3{\cdot}07$, $P = 6{\cdot}5 \times 10^{-2}$). Effects of the interaction between treatment and parasite dose were significant for the mixed group in both model variations (absolute and relative parasite doses of the mixed group). At 250 000 parasites, the mixed group hazard was

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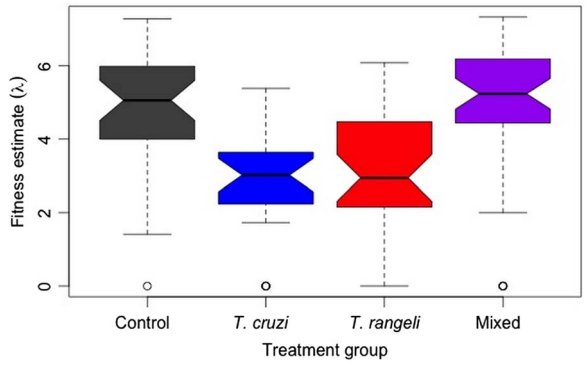

Fig. 6. Distribution of fitness estimates in each treatment group. The control and mixed groups had significantly higher fitness estimates than the *T. cruzi* and *T. rangeli* groups.

significantly higher than either single-species infection treatment, while at 1 million parasites the mixed group hazard was significantly lower (mixed *vs T. cruzi:* $P = 0{\cdot}025$; Mixed *vs T. rangeli,* $P = 0{\cdot}00006$; full summary in Supplementary Materials, Tables S2 and S3). Interaction effects were not significant when comparing the *T. cruzi* treatment with the *T. rangeli* treatment, suggesting their hazards were not significantly different from each other at any parasite dose.

Fitness

Fitness estimates (λ) and reproductive values v2 and v3 (corresponding to 60 and 90 days) were significantly different between treatments (Kruskal–Wallace; λ and v_2: $P = 1{\cdot}69 \times 10^{-7}$; v_3: $P = 1{\cdot}42 \times 10^{-2}$), with *T. cruzi* and *T. rangeli* treatment groups having significantly lower λ and v_2 values than the mixed and control groups (KruskalMC, $P < 0{\cdot}01$, Fig. 6). The reproductive value at 90 days (v_3) was significantly different between the *T. cruzi* and mixed group, with *T. cruzi* being lower (KruskalMC, $P < 0{\cdot}05$). The *T cruzi* and *T. rangeli* treatment group fitness estimates and reproductive values were not significantly different from each other at any time point.

Infection status at death

The difference between treatment groups in the proportion of samples that amplified in the qPCR was marginally non-significant (Fisher's Exact Test for Count Data, $P = 0{\cdot}09$), although there were no significant differences after performing individual comparisons between each treatment and adjusting the *P*-values for multiple comparisons. 90% of *T. cruzi* treatment group samples amplified; 76·92% of *T. rangeli* samples amplified; 61·53% of samples from the mixed treatment group amplified *T. cruzi*; and 84·61% amplified *T. rangeli*. There was no parasite DNA amplification for the pooled male and offspring groups.

DISCUSSION

Co-infection: advantageous for the host and parasite?

We observed that insects co-infected with *T. cruzi* and *T. rangeli* had higher survival, reproduction and overall fitness, suggesting that *T. Cruzi–T. rangeli* co-infection could reduce negative life history consequences of a single infection with *T. cruzi* or *T. rangeli* for *R. prolixus*. This could in turn, lead to increases in the transmission potential of *T. cruzi* and/or *T. rangeli*. Additionally, this could be a way that virulent strains persist, especially *T. rangeli*, which, as mentioned, is known to be pathogenic to *R. prolixus*. Reported prevalences of *T. cruzi–T. rangeli* co-infection in field-caught triatomines have been found to be higher than single infections of *T. rangeli* in *R. prolixus* (Groot, 1951; Vallejo *et al.* 1988), *R. pallescens* (Pineda *et al.* 2008; Calzada *et al.* 2010; Gottdenker *et al.* 2016); and *R. colombiensis* (Pavia *et al.* 2007), which would support the idea of a co-infection advantage for *T. rangeli* (Fig. 1). However, more data on fitness in trypanosome-infected field-caught triatomines are needed to support this result.

Additionally, we found a threshold parasite dose below which insects infected with a single species infection had a lower instantaneous hazard rate (i.e. risk of death) and above which co-infected insects had a lower risk. This might increase the transmission potential of the parasites if the parasite dose in the blood meal were associated with higher numbers of parasites transmitted by the bugs. However, *T. cruzi* infective dose does not correlate with the number of parasites excreted (Wood, 1954; Urdaneta-Morales and Rueda, 1977; Chowdury and Fistein, 1986; Azambuja *et al.* 2004, 2005), and the total trypanosome population size and composition (proportion of each form present) within a triatomine will fluctuate with feeding status; significant decreases in parasite numbers can occur within 4 h after feeding by as much as 50% in some parts of the bug (Schaub and Lösch, 1988; Schaub, 1989*b*; Kollien and Schaub, 1998*a*). Thus, it seems unlikely that the higher infective doses tolerated by co-infected insects increase the parasites' transmission potential, aside from increasing the transmission probability by keeping the insect alive longer.

Insect reproduction: quality vs quantity

While the efficiency of egg production seemed to be affected by parasite treatment, the per cent of oviposited eggs that hatched was not. It is known that the processes of egg growth and oviposition are

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controlled separately in *R. prolixus* (Mundall, 1978). Oviposition of badly formed eggs, which has been observed in *Cimex* species, is rare, even in cases of insect malnutrition (Buxton, 1930). This investment in egg quality over quantity could be a mechanism of insecticide resistance, which has been observed in *T. infestans* eggs (Toloza *et al*. 2008), and could be one factor that explains residual populations in human homes after insecticidal spraying.

T. cruzi *vs* T. rangeli *virulence*

As mentioned, *T. rangeli* is considered to be pathogenic to triatomines of the genus *Rhodnius*, while *T. cruzi* has been described in several publications as 'subpathogenic' (Schaub, 1989*a*, 1990, 1992; Schaub and Losch, 1989*a*), i.e. pathogenic only in the presence of external stress. In this light, it is surprising that the fitness of the treatment group infected with *T. cruzi* was not significantly higher than the fitness of the *T. rangeli* treatment group. However, the majority of studies supporting the subpathogenic theory of *T. cruzi* in triatomines have been carried out in the species *T. infestans* (Schaub, 1988*a*, *b*; Schaub and Lösch, 1988; Schaub and Losch, 1989*a*, *b*; Kollien and Schaub, 1998*a*, *b*; Kollien *et al*. 1998). Most studies investigating effect of *T. cruzi* on *R. prolixus* life history have found a mild effect (D'Alessandro and Mandel, 1969; Neves and Peres, 1975; Fellet *et al*. 2014), and effects have also been observed in *Panstrongylus megistus* (Lima *et al*. 1992) and *Mepraia spinolai* (Botto-Mahan, 2009). Additionally, Añez *et al*. (1992) also found no significant difference in development or mortality between insects infected with *T. cruzi* and insects infected with *T. rangeli*. Moreover, recent studies have found that *T. cruzi* can negatively affect *R. prolixus* life history outcomes, depending on temperature (Fellet *et al*. 2014; Elliot *et al*. 2015) and parasite strain (Peterson *et al*. 2015). This could be due to increased parasite replication rates at higher temperatures (Wood, 1954; Asin and Catalá, 1995). However, the insects in this study were reared under climate conditions similar to those found in *R. prolixus*-endemic areas of Colombia (Hoyos *et al*. 2007; Gutierrez *et al*. 2013), thus, if temperature were an underlying factor in *T. cruzi* virulence, it would suggest that *T. cruzi* may also be virulent to free-living Colombian *R. prolixus*.

Our survival results are not in agreement with the other published study of *R. prolixus* survival when co-infected with *T. cruzi* and *T. rangeli*, which found that insects with mixed infections had higher mortality (Añez *et al*. 1992). This could be due to differences in temperature between the studies (ours was carried out at higher temperatures), insect stage and/or parasite strains. Considering the high degree of polymorphism found within both the *T. cruzi* and *T. rangeli* species, it seems possible that the outcome of triatomine infection with either or both trypanosomes could lie in a wide range of outcomes from mildly virulent to positive. In our work, we have observed a wide range of survival in insects infected with different *T. cruzi* DTU I strains (Peterson *et al*. 2015).

Concluding remarks

Due to the inherent limitations of laboratory experiments, the extrapolation of effects observed in the laboratory to their meaning in the natural system must be carried out cautiously. That said, our findings suggest that some *T. Rangeli–T. cruzi* co-infections could ameliorate the negative effects of single-species infections, allowing more virulent strains to persist and potentially increasing the transmission potential of both parasites. Further research into *T. cruzi–T. rangeli* co-infections in other triatomine systems and in field-caught bugs will provide more insight into this topic.

SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found at http://dx.doi.org/10.1017/S0031182016000615.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

J.K.P. conceived and designed the study, carried out all assays, performed the statistical analysis, and drafted the manuscript. A.L.G. participated in the study design, helped with statistical analysis and critically revised the manuscript. R.J.E. helped with insect and molecular assays and helped with the analyses of the results; A.P.D. participated in

the study design and revised the manuscript. O.T.C. participated in the design of the study, helped to coordinate the experiments, and critically revised the manuscript.

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