# LIFE HISTORY CONSEQUENCES OF INFECTION WITH CHAGAS DISEASE AGENT *TRYPANOSOMA CRUZI* FOR ITS INVERTEBRATE HOST *RHODNIUS PROLIXUS*

Jennifer Kate Peterson

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Advisers: Andrea L Graham and Andrew P. Dobson

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**Abstract**

Every interaction between species occurs in a heterogeneous environment that presents countless contexts that shape the interaction over time and space. The consequences of these interactions can regulate populations, as they trickle down to influence the genes that an individual passes on to its offspring, and then, in turn, scale back up to influence the genetic and phenotypic composition of future populations. In this work, I sought to uncover how these principles play out in the interactions between an invertebrate vector of human disease and the disease agent it carries. Disease vectors are often considered in a context that is faithful to the word as it is used in physics, where the vector is viewed as public transportation that moves the pathogen between hosts, experiencing no consequences of parasite infection. However, vectors face the challenge of how to maximize individual fitness in a stochastic environment with limited resources just as all other species do, so why would they be exempt from the effects of being parasitized? As such, I investigated the triatomine bug species *Rhodnius prolixus* when infected with the parasite *Trypanosoma cruzi* (etiological agent of Chagas disease), and co-infected with *T. cruzi* and its sister species, *T. rangeli*. I asked, does *T. cruzi* affect *R. prolixus* fitness, and under what contexts does this effect vary? I found a large range of variation in *R. prolixus* fitness when infected with *T. cruzi*, with the outcome being influenced by parasite strain, co-infection with *T. rangeli*, parasite dose, and the timing and order of infection. These factors did not act alone, but seemed to be dependent on one another: it was better to have a co-infection at lower *T. rangeli* doses, but at high *T. rangeli* doses, it was better to be infected with *T. cruzi* first, suggesting an interaction between dose, order and timing. These results illustrate the interactions across

scales of both biological and spatio-temporal complexity that can be revealed when studying infectious disease through an ecological lens. Moreover, this work emphasizes the importance of taking into account the ecology of vector-borne neglected tropical diseases such as Chagas disease.

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## Introduction

*If:*

*A vector (mathematics) is a matrix with one row or one column,*

*and a vector (physics) is a quantity with size and direction,*

*and a vector (aeronautics) is the course of direction of an airplane.*

*Then:*

*Is a vector (disease) a quantity of parasites moving toward a host?*

Disease vectors are often considered in a context that is faithful to definitions of the word vector outside of disease ecology, where the vector is almost literally a vector. The host and vector are two different entities with different functions: the host is where the parasite carries out its life cycle, often to the detriment of the host, causing disease, while the vector functions just as parasite transportation to the next host. However, from an ecological and evolutionary standpoint, vectors face the same challenges as hosts: to maximize individual fitness in a stochastic environment with limited resources. As such, in this thesis, I investigate the insect vector as an invertebrate host that experiences context-based consequences from parasite infection, just as does its vertebrate host. I ask, how does a human disease agent affect its invertebrate host, and under what contexts is variation found?

To answer this question, I focus on the fitness of the triatomine bug (Reduviidae: Hemiptera), when infected with the protozoan parasite *Trypanosoma cruzi* (etiological agent of Chagas disease) and co-infected with *T. cruzi* and a congeneric parasite, *T. rangeli*. I

begin by investigating if *T cruzi* has the potential to regulate *R. prolixus* populations by reducing the fitness of infected individuals, analogous to "top-down" regulation by predators[1,2]. Although predation and parasitism can differ in the rate at which the prey is killed, the mobility of the 'predator' and the size difference between the two species, both involve one species benefitting at the expense of another. In the second part of this work, I examine the potential of *T. cruzi* -*T. rangeli* co-infection to interfere with *T. cruzi* regulation of *R. prolixus* populations; if the co-infection has a different net effect on *R. prolixus* fitness than a single *T. cruzi* infection, it could either release or tighten *T. cruzi* regulation of host populations [3,4].

By asking questions based in classic processes of population ecology, predation and competition, I aim to (i) deepen the current understanding of vector-borne Chagas disease, an important neglected tropical disease in Latin America, and (ii) to refine the current characterization of the triatomine-trypanosome relationship to include context dependent variability, namely parasite polymorphism and co-infection. This work will add more specificity to the body of knowledge that is drawn upon for the control of Chagas disease and other vector-borne diseases, aiding the development of more effective strategies that are rooted in a detailed biological understanding of the system.

*Research questions and hypotheses*

Current opinion on the effect of *T. cruzi* on its vector assumes no change in vector fitness relative to uninfected vectors. I hypothesize that *T. cruzi* can change vector fitness, as detailed below chapter by chapter. Along with these hypotheses, I have included a simple

graphical representation illustrating how change in vector fitness due to *T. cruzi* infection (i.e., vector population regulation by *T. cruzi)* could potentially impact the proportion of hosts infected with Chagas disease. This image shows the zero growth isoclines for the proportion of infected vectors (y) and the proportion of infected hosts (x), represented by dx/dt = 0 and dy/dt = 0, respectively. The equations for each isocline are expressed for the infected host population as the population density of triatomines (T) multiplied by the vector-host contact rate (a) and infection success rate (b) divided by the density of infected hosts (N), written as (abT/N). This value is then multiplied by the proportion of infected vectors (y) and then multiplied by one minus the proportion of infected hosts (x). Finally, the product of the host recovery rate (r) times the proportion infected hosts (x) is subtracted. The full equation is expressed as:

$$dx/dt = (abT/N)\, y(1-x) - rx$$

The zero growth infected vector isocline is expressed as vector-host the contact rate (a) multiplied by the proportion of infected hosts (x), and then multiplied by one minus the proportion of infected vectors (1-y). The product of the triatomine death rate (μ) and the proportion of infected vectors (y) is then subtracted, for a final expression of:

$$dy/dt = ax(1-y)-\mu y$$

The $R_0$ for the system would then be the square-root of product of the slope of each isocline[5,6], expressed as:

$$\sqrt{(a^2bM/\mu r)}$$

My work will focus on the parameters for the triatomine death rate μ, which in combination with reproduction affects the triatomine population density T. If enough *T. cruzi*-infected vectors experience a decrease in fitness, this would increase their mortality

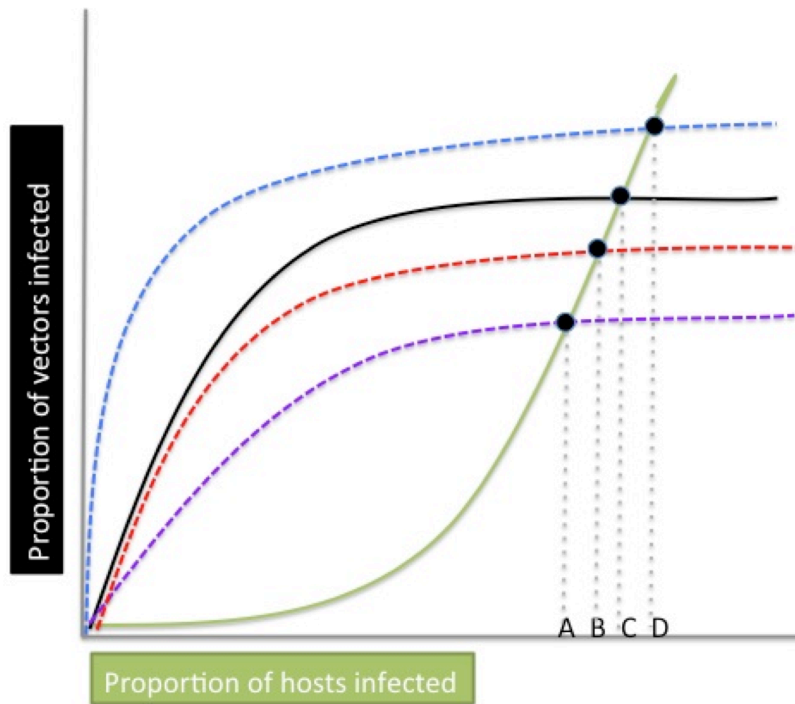


**Figure A. Graphical representation of the potential effects of changes in the proportion of infected vectors on the proportion of infected hosts** [7]. **Each line is a zero growth isocline; green is the proportion of *T.* cruzi-infected hosts, black is *T. cruzi*-infected vectors. The dotted red and purple lines represent a scenario where infected vectors have decreased fitness and the dotted blue line represents increased fitness of infected vectors. Each intersection is where the fitness of *T. cruzi* is at equilibrium.**

μ, leading to a decrease in the proportion of vectors infected *y* and the overall triatomine population density T. This in turn, could lead to fewer infected hosts, and lower *T. cruzi* fitness (Points A and B in Figure A), and a decrease in $R_0$. The opposite could also occur if *T.* cruzi infection conferred a fitness advantage to the insect (Point D in Figure A). It must be kept in mind however, that this is a generalized idea, as many factors are at play in Chagas

disease transmission, including low host parasitemias, fecal parasite transmission, and the existence of chronically infected hosts with long lifespans.

In Chapter 1, I ask (1) **does *T. cruzi* infection affect the survival and molt success of *R. prolixus*?** and (2) if so, **is there a difference between *T. cruzi* strains in this effect?** Based on the variability observed in *T. cruzi* across scales, I predicted that yes, *T. cruzi* can affect *R. prolixus* survival and molt, and this effect would vary between strains from no effect to a negative effect (Points A-C in Figure A).

In Chapter 2, I asked **(1) does *T. cruzi* infection affect the survival, reproduction and/or total fitness of *R. prolixus*?** and **(2) if so, is there a difference between *T. cruzi* strains in this effect?** I hypothesized that yes, *T. cruzi* can affect *R. prolixus* survival, reproduction and/or total fitness, and that the effects will vary from no effect to a negative effect between strains (Points A-C in Figure A).

In Chapter 3, I switch gears to investigate *T. cruzi*-*T. rangeli* co-infection of *R. prolixus.* Similar to Chapter 2, I asked **(1) does *T. cruzi* -*T. rangeli* infection affect the survival, reproduction and/or total fitness of *R. prolixus*,** and **(2) does *T. cruzi*-*T. rangeli* co-infection have a different effect on survival, reproduction and/or total fitness than single infection with *T. cruzi* or *T. rangeli*?** I hypothesized that yes, *T. cruzi*-*T. rangeli* co-infection would have an effect on *R. prolixus* fitness and that the fitness of co-infected insects would be lower than the fitness of insects infected with just one of the parasites

(Points A and B, Figure A). Alternative hypotheses are that there is no effect on fitness (Point C, Figure A), or that co-infection confers a fitness advantage (Point D, Figure A).

In Chapter 4, I investigate some potential mechanisms of the effects observed in *R. prolixus* when co-infected with *T. cruzi* and *T. rangeli.* I asked, **in *T. rangeli-T. cruzi* co-infection of *R. prolixus,* does *T. rangeli* infective dose, co-infection timing (simultaneous or delayed), or co-infection order affect *R. prolixus* survival?** I hypothesized that (a) a higher *T. rangeli* dose will have a negative effect on survival (Points A and B, Figure A); (b) a simultaneous co-infection would have a more detrimental effect on survival than a delayed co-infection (Points A and B, Figure A); and (c) that infection with *T. cruzi* first would lessen the detrimental effects of a subsequent *T. rangeli* infection (Points B, C or D, Figure A).

Finally, in Chapter 5, I ask, **what are the "true" effects of *T. cruzi* and *T. rangeli* on triatomines?** I discuss this question in light of the results presented in Chapters 1-4, along with a review of the published studies on *T. rangeli* infection in triatomines.

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# Chapter 1

*R. prolixus* survival and molt outcomes when infected with different *T. cruzi* strains[1]

## 1.1 Background

*Chagas disease*

Chagas disease is a parasitic infection that can lead to heart failure and other related pathologies. Caused by the protozoan parasite *Trypanosoma cruzi,* Chagas disease is found throughout the new world, mainly in poor areas with settings conducive to human contact with its insect vector, the triatomine bug (Hemiptera: Reduviidae). An estimated 10 million people are currently infected and more than 10,000 deaths per annum are related to Chagas disease [1].

Due to the absence of a comprehensive treatment or vaccine, Chagas disease prevention is focused primarily on the interruption of *T. cruzi* transmission by domiciliated triatomine bugs. The triatomine species *Rhodnius prolixus* is considered to be the principal domestic vector of *T. cruzi* in Venezuela, Colombia and parts of Central America [2,3], and thus a comprehensive understanding of the effect of this parasite on its insect host is critical to improving vector surveillance and control strategies.

[1]A portion of the work presented in this chapter was presented at the International Conference on Parasitology (2014); the Federación Latinoamericana de Parasitología meeting (2013); the 62nd annual meeting of the American Society for Tropical Medicine and Hygiene (2013); and the Ecology and Evolution of Infectious disease meeting (2013).

T. cruzi *diversity*

*T. cruzi* is diverse across scales, from its molecular make up to the ecotopes it inhabits. As a generalist parasite, *T. cruzi* can infect hundreds of mammal species and all 142 triatomine bug species [2]. It is considered to be harmless to its triatomine bug hosts in the absence of external stressors, but pathogenic to mammals [3,4]. Its pathogenicity in mammals is highly variable, both within and between species and individuals, ranging from asymptomatic to severe pathology [2,5–9]. Geographically, *T. cruzi* is found from Patagonia to the Great Lakes of North America, following the range of the estimated 142 triatomine bug species found in the new world. It is not believed to infect the seven species found in Asia, although this has never been formally studied, and at least one vector in Asia, *Triatoma rubrofasciata,* is competent for the parasite. Interestingly, *Triatoma rubrofasciata* is the only triatomine species found both within and outside the new world (always in port cities; it is associated with ship rats), and it is believed that all 7 species found outside of the new world are derived from *T. rubrofasciata* [10].

Across its expansive range, *T. cruzi* has three main transmission cycles: domestic, peri-domestic and sylvatic. Additionally, the parasite circulates between humans and anthropophilic mammal populations such as opossums via triatomine bugs capable of peri-domestic and domestic invasion; this is thought to be one of the drivers of the high degree of genetic diversity found across the species [11]. *T. cruzi* shows such high levels of genetic diversity that it has been proposed as a complex rather than a species [12], and this has continued to be a contentious subject for the past decade. The currently accepted division of *T. cruzi* is six genetic classifications called discrete typing units (DTUs I-VI;[13]), which

are thought to be loosely associated with different geographic regions, mammal hosts, triatomine bug genera and clinical manifestations of Chagas disease[14–17].

*Research question and hypothesis*

Considering its broad host diversity, ample geographic range, and extensive genetic diversity, I asked if *T. cruzi* variability extends to survival and molt outcomes of one of its invertebrate hosts, the triatomine species *R. prolixus*, when infected with *T. cruzi.* I hypothesized that *T. cruzi* strains would have different effects on *R. prolixus* survival and molt.

## 1.2 Materials & Methods

*Experiment design*

I infected 168 *R. prolixus* 5$^{th}$ instar nymphs with one of five different *T. cruzi* (DTU I) strains (Table 1.1). Following infection, I recorded the survival and molt outcome (successful, with deformities, or death) of each bug for up to 65-127 days. I carried out all experiments in the laboratory of the Biology and Infectious Diseases group (BCEI) at the University of Antioquia in Medellín, Colombia lead by Professor Omar Triana.

| ***Treatment group*** | **N** |
|---|---|
| Cas15 | 29 |
| Cas20 | 27 |
| Gal61 | 29 |
| SO-8 | 27 |
| Sebas1 | 26 |
| Control | 30 |

**Table 1.1.** *T. cruzi* strain treatment groups

*Insects*

*Rhodnius prolixus* individuals came from five different colonies maintained by BCEI staff in the BCEI insectarium. Each colony was founded by *R. prolixus* eggs collected in Colombia. BCEI *R. prolixus* colonies are kept under semi-controlled climate conditions (~27+/-1°C and 65% +/- 15% RH) in a 12 hour light/dark cycle. Insects are given the opportunity to feed twice weekly on hens, according to animal ethics committee regulations of the Sede de Investigacion Universitaria (SIU), of the University of Antioquia.

*Parasites*

All *T. cruzi* strains used were DTU I (or "Tc1"), as Tc1 is believed to be the predominant DTU in Colombia [17–19]and is associated with the genus *Rhodnius* [13,14,20]. All parasite cultures were grown up by BCEI researchers. Epimastigote forms of *T. cruzi* strain Gal61 were cultured at 28 °C in RPMI- 1640 liquid medium (Sigma-Aldrich) supplemented with 10% Fetal Bovine Serum [21,22]. Parasite strain information is presented in Table 1.2.

| Strain name | Geographic origin* | Biological origin | Genetic grouping† | International ID | Ecotope |
|---|---|---|---|---|---|
| Cas15 | Villanueva, Casanare | *R. prolixus* | Id | TPRX/CO/2000/ CAS15 | Extradomestic |
| Cas20 | Villanueva, Casanare | *R. prolixus* | I** | TPRX/CO/2000/ CAS20 | Extradomestic |
| Gal61 | Galeras, Sucre | *Mus* spp. | Id | XXXX/CO/91/ Gal61 | Extradomestic |
| SO-8 | San Ofre, Sucre | *R. pallescens* | Ia | TPAC/CO/1995/ SO8 | Extradomestic |
| Sebas1 | San Sebastián, Magdalena | *R. pallescens* | Ib | I.RHO/CO/06/ SEBAS-1.MAG | Extradomestic |

**Table 1.2.** Background information for each *T. cruzi* strain used. *All Colombian locations, listed as municipality, state. †Refers to groupings based on intergenic regions of sliced leader (SL-IR) genes [19,23].

*Insect Infection: parasite preparation*

I collected epimastigote forms in their exponential growth phase for insect infection. Although epimastigotes are not the infective form of the parasite for the insect, this is the standard protocol used in the study of established trypanosome infections in triatomines, because it is easier and more practical to maintain epimastigotes in culture (Dr. Alessandra Guarneri, FIOCRUZ Institute, personal communication). Studies have shown that this does not affect the ability of the parasite to establish in triatomines [17,18], because trypomastigotes (the infective stage) transform into epimastigotes inside the insect within 24 hours of infection. The only case where trypomastigotes are necessary is when investigating the first 24 hours of infection. Of course, that said the most ideal scenario would be with trypomastigotes rather than epimastigotes, and in future experiments, I would prefer to include this methodology. However, in this experiment, it was not possible.

To calculate the parasite concentration in culture medium, I gently swished flasks containing the parasites in the medium to facilitate even parasite dispersion, and then I counted them in a neubauer cell counter under a compound light microscope [19–24]. After estimating parasite concentration, I centrifuged the parasites in 1.5ul eppendorf tubes for 10 minutes at 3000RPM to separate them from the medium. After the centrifugation, I poured off the medium and replaced it with sterile 0.15M NaCl, 0.01 M phosphate-buffer, pH 7.2 (PBS). I gently pipetted the solution up and down to re-suspend the parasites and then I repeated the process once more for a final solution of parasites re-suspended in 1ml of PBS.

*Insect infection: blood preparation*

After counting and washing the parasites, I gently pipetted the parasite solution into a given quantity of 37.5°C defibrinated, decomplemented human blood* supplemented with fetal bovine serum, for a final estimated concentration of 3.3 -3.5 x $10^6$ parasites per ml of blood. This parasite concentration is similar to that used in several other published studies of *T. cruzi* infection in triatomines [17,24–32], and blood meals taken by triatomines at this parasite concentration lead to infective doses that falls within the range of peak parasitemias observed in mice and guinea pigs experimentally infected with *T. cruzi* [33–38].

* Blood was obtained by BCEI staff under the Colciencias project number 111549326149, which was approved by the bioethics committee of the Sede de Investigación Universitaria (SIU), of the University of Antioquia.

*Insect infection: insect preparation and feeding*

Prior to the infective feeding, I weighed and marked each *R. prolixus* 5th instar nymph with a small dot of non-toxic, water-based paint at the top of the pronotum [39,40] for recognition after feeding, and placed it a small jar (6cm x 7cm, 200ml) with 5-10 other weighed and marked nymphs. Each *R. prolixus* 5th instar larva had not eaten for about 2 weeks [41,42]. I placed each jar of nymphs under an artificial membrane feeder containing the parasite-blood solution at 37.5°C for about 30 minutes ([43]; Image 1.1). Control insects consumed blood

**Image 1.1. 5th instar *R. prolixus* in various engorged states after the infective blood meal. Photo: JK Peterson**

without parasites. I weighed each nymph after feeding to estimate the number of parasites ingested, which was calculated by subtracting each insect's pre-feeding weight from its post feeding weight, dividing this amount by the average density of human blood (1.06mg/ml), and multiplying the result by 3.3-3.5 x $10^6$ flagellates/ml, depending on treatment. Insects that did not eat were not used in the study. After infection, each insect was kept in a small glass jar (4.5cm x 4.5cm, 60ml) with two pieces of folded carton inside and a mesh top [44], in still-air incubators (Hovabator model #1602N) with dimensions of 18" X 18" X 9½" to minimize climate variation. Climate conditions within the incubators ranged between 27.5 +/- 1.5 ºC and 68% +/- 8% RH, in accordance with the recommended climate for rearing triatomines [44]. Insects were fed tri-weekly on hens post-infection.

*Infection confirmation*

I confirmed insect infection in a subset of the insects by direct microscopic observation and PCR. Upon death, I macerated and centrifuged each insect, forming an insect-parasite tissue pellet as described in [30,45], and I extracted total DNA from each pellet with a Qiagen DNeasy blood and tissue kit. I followed the kit protocol for tissue extraction, but with an additional hour of lysis in ATL buffer at 56ºC, vortexing every 15 minutes. I amplified the DNA in a PCR with the primer pair TcZ1 (5′-CGAGCTCTTGCCCACACGGGTGCT-3′) and TcZ2 (5′-CCTCCAAGCAGCGGATAGTTCAGG-3′; [46]) that amplify a 188 base pair sequence of *T. cruzi* satellite DNA.

*Molt Analyses*

I evaluated four molt variables between treatment groups: molt occurrence (yes or no), molted but with deformities (yes or no), death during molt (yes or no) and molt day. 'Molted with but with deformities' refers to deformities resulting from the molting process that would prevent insect reproduction and/or dispersal, such as incomplete shedding of the exuviae or molting with a broken neck or crumpled wings (see Appendix 1 for pictures). Sex was determined after molting occurred, and in most cases was only possible in those individuals that molted without deformities. Therefore, differences between males and females were not tested for, as the majority of insects with their sex determined had successfully molted without any problems.

*Statistical Analyses*

I carried out all statistical analyses using the R statistical computing environment software version 3.03 [47]. I used only parametric tests to avoid normality assumptions. I 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. I tested for differences between binomial outcomes (such as death during molt) in the amount of parasites or blood ingested per unit of body weight using Wilcoxon Rank Sum tests. I evaluated differences between treatment groups in the variables 'molt occurrence', 'molted with deformities', and 'death during molt' with Fisher's Exact Test for Count Data. I evaluated molt day between treatments with the Kruskal-Wallis rank-sum test. I applied the 'kruskalmc' function from the 'pgirmess' package [48] 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 versus control. I accepted p-values under 0.05 as statistically significant.

*Survival Analysis*

I generated survival curves for each treatment group using the Kaplan-Meier (K-M) method in the R 'survival' package [49,50]. I compared survival between treatment groups using the 'survdiff' function in the 'survival' package, a two-tailed test for censored data that implements the G-rho family of tests [51], where deaths at various times are weighted by a factor of $S(t)^\rho$ ($S$ = K-M estimate; $t$ = time), where $\rho$ is a scalar parameter that determines what type of test is 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 [52] is employed. I set $\rho$ at 1, to offset death events related to senescence. I carried out pairwise comparisons between K-M survival curves were with Chi-Squared Distribution tests and I adjusted p-values to control for the family-wise error rate using the Holm-Bonferroni correction method [53].

I used Cox Proportional Hazards (PH) models [54] to examine the main effects and two-way interactions of parasite treatment, parasite dose and blood ingested on hazard rates (the instantaneous rate of failure at any given time, given that the individual has survived up until that time). The proportional hazards (PH) assumption, (i.e., hazards were proportional over time) was tested with the Coxph function in the 'survival' package. Data violated the (PH) assumption, so I ran them in an extended Cox model, with data split at 28 days, which was the final molt day for the majority of the insects, and within the time when

hazard curves became disproportionate (between 21-30 days post-infection). I created dummy variables representing 2 episodes, up to 28 days post-infection ("early") and after 28 days post infection ("late"), to examine the hazard ratios in each episode.

I selected model covariates using Akaike's Information Criterion (AIC) with the stepAIC function in the 'MASS' package [55], and manual one-variable-at-a-time reduction. Final covariates included in the model were the main effects of parasite treatment and main effects of the ratio of blood ingested in the infective blood meal to the insect weight prior to feeding (referred to from here on "blood:weight"), which can also be seen as a proxy for estimated number of parasites ingested per mg of body mass. I used the blood:weight ratio to normalize for variation in insect size. I included the interaction effects of the dummy variables representing the treatment before 28 days with blood:weight. I centered blood:weight data on the mean (6.05:1), as no insect in the study weighed nothing or consumed a volume of blood equal to 0. Blood:mean data were log2 transformed after being centered. Thus, main effects of blood:weight can be interpreted as the effects of a blood meal 6 times that of a given insect's weight. Blood:weight-treatment interaction effects represent the effect of a blood meal of a specific treatment that is 3 and 12 times the insects' body weight, depending on whether the beta value given by the model is subtracted or added from the beta value given for treatment main effects.

I excluded the interaction of blood:weight with treatment after 28 days, after the insects took several more blood meals in this period of time. The change in an insect's parasite load after the ingestion of uninfected blood meals is discussed extensively in Chapter 3, but

briefly, parasite loads in triatomines are dramatically reduced upon ingestion of an uninfected blood meal [56–58].

I excluded sex, as it was determined after insect molt, meaning that after a majority of deaths of insects of unknown sex had already occurred. Full Cox model outputs and information on interpreting the results is provided in Appendix 1.

### 1.3 Results

#### *I. Comparison of effects of T. cruzi strains on vector survival and molting*

*Volume of blood and estimated number of parasites ingested*

Insects ingested between 20.0-277.0 mg of blood (mean 172.0 mg), and an estimated 68,000 – 920,000 of *T. cruzi* parasites (mean 559,000). The ratio of the volume of blood ingested to insect pre-feeding weight ranged from 0.74 to 16.71 (mean 6.05), and the ratio of the estimated number of parasites ingested per mg of insect biomass ranged from 2,000 to 55,000 parasites (mean 22,000). There was no significant difference in the amount of blood or the estimated number of parasites ingested between treatment

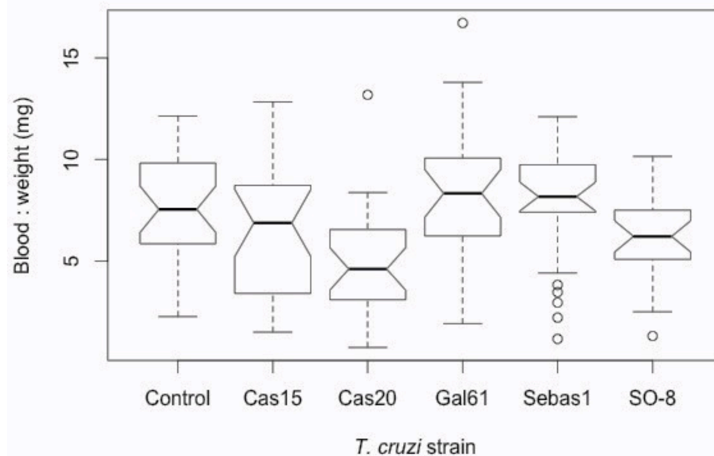


**Figure 1.1. The distribution of the ratios of the volume of blood consumed in the infective blood meal to insect biomass (mg), across treatments. The Cas20 group blood:weight ratio was significantly lower than that of the Gal61, Sebas1 and control groups.**

groups (Kruskal Wallis, blood: $p = 0.71$; parasites: $p = 0.66$). There was a significant difference between treatments in the ratio of the volume of blood ingested and estimated number of parasites ingested to pre-feeding weight (Kruskal-Wallis, blood: $p = 1.24e-04$; parasites: $p = 2.85e-04$, Figure 1.1), with both ratios significantly lower in the Cas20 treatment than that of the Gal61, Sebas1 and the blood:weight ratio of Cas20 lower than the control group (Kruskalmc, $p < 0.01$).

*Infection confirmation*

Infection was confirmed in ~50% of all insects (Table 1.3). There was no difference in the number of infections confirmed between treatments (Fisher's Exact test, $p = 0.63$).

| Treatment | # Confirmed | (%) |
|---|---|---|
| Cas15 | 16/29 | 55.2 |
| Cas20 | 14/29 | 48.3 |
| Gal61 | 15/27 | 55.6 |
| S08 | 14/26 | 53.8 |
| Sebas1 | 10/27 | 37.0 |

**Table 1.3. Number and proportion of insects in each treatment group with a *T. cruzi* infection confirmed by DMO and/or PCR.**

*Survival*

Kaplan-Meier survival curves were not the same between treatment groups (Chi Square = 25.2, 5 df, $p = 1.19e-04$, Figure 1.2). Pairwise comparisons revealed the difference to be between the Cas15 treatment group and the control and Sebas1 treatments (Chi-Squared Distribution comparisons, corrected $p < 0.05$ for comparisons of Cas15 with Sebas1 and with the Control group).

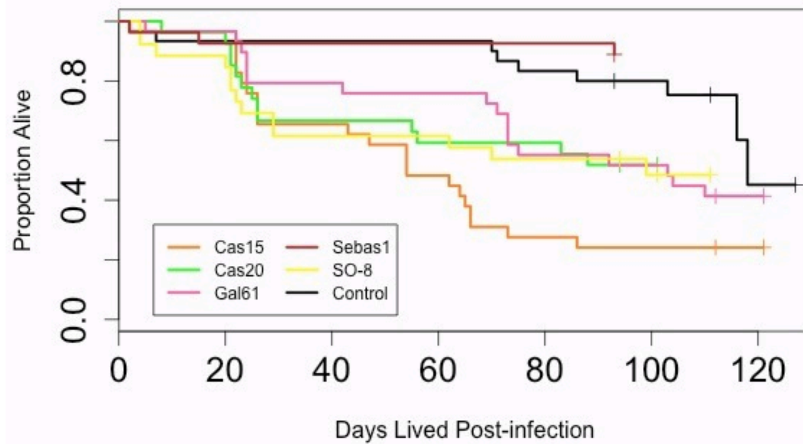


**Figure 1.2. Kaplan-Meier curves representing the survival of each treatment group over time. Crosses represent right-censored data.**

Cox model summary results suggested that covariates did not influence survival (Likelihood ratio test, 62.86 on 16 df, p = 1.71e-07). Insects infected with strains Cas15 and Cas20 had statistically significant hazard ratios, with hazard being over 6 times higher than that of the control group in the time period up to 28 days post-infection (Cas15: $e^{\beta}$ = 6.20, p = 0.04; Cas20: $e^{\beta}$ = 6.81, p = 0.03, Figure 1.3). After 28 days, Cas15 was the only treatment group with a statistically significant hazard ratio, with a hazard that was 5.6 times that of the control group ($e^{\beta}$ = 5.63, p = 4.05e-04). Although not significant, there was a consistent pattern of decrease in the hazard ratio of each treatment from before 28 days to after 28 days. Main effects of blood:weight significantly decreased hazard by 86.52% ($e^{\beta}$ = 0.13, p = 1.70e-03). Cas20 significantly interacted with the blood:weight ratio, with hazard increasing with increasing volumes of blood (and/or parasites) per body mass ($e^{\beta}$ = 7.66, p = 7.06e-03). This effect was also marginally significant for the Gal61 and Cas15 groups (Gal61: $e^{\beta}$ = 4.70, p = 5.52e-02; Cas15: $e^{\beta}$ = 4.02, p = 7.67e-02)

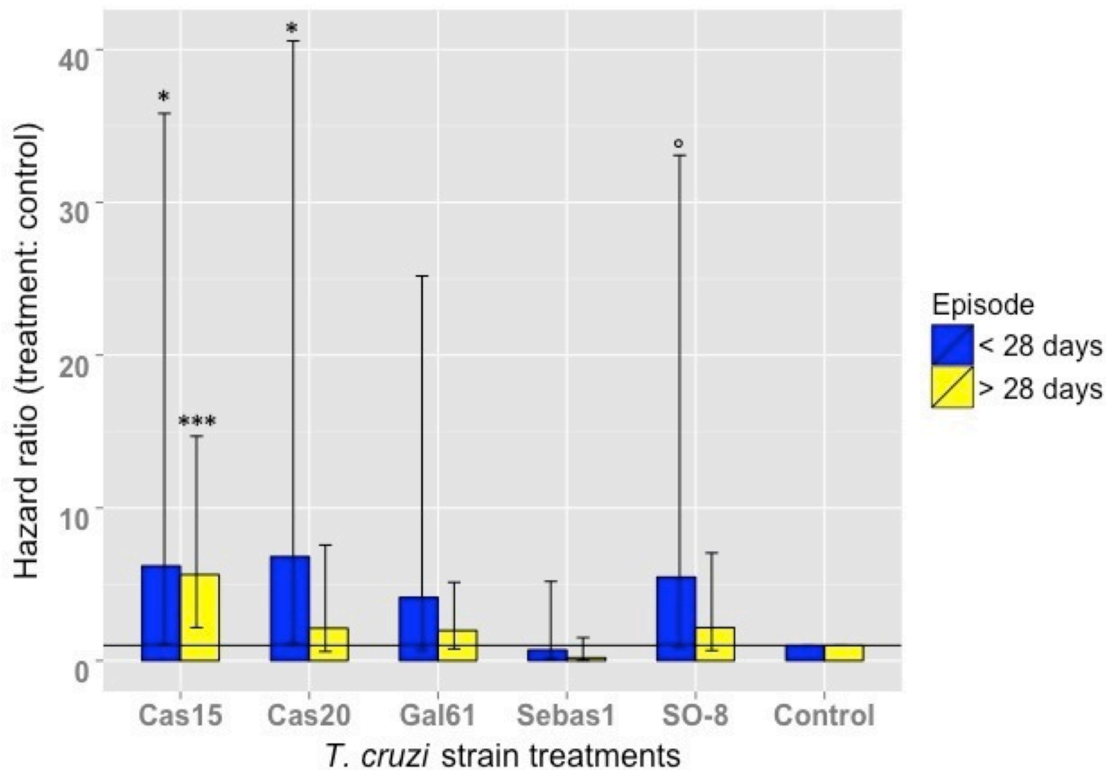


**Figure 1.3. Instantaneous hazard ratios ($e^{\beta}$) for *T. cruzi* strain treatment group main effects before and after 28 days post-infection. Confidence intervals of the β value indicated by vertical lines. Horizontal line crosses y axis at 1 to indicate where a hazard ratio of 1 lies. *p < 0.05; ***p < 0.001; °p <0.10**

*Molt*

There were no insects in the control or Sebas1 groups that died during molt or molted with deformities. The proportions of insects that died during molt and that molted with deformities were globally different between treatments (Fisher's Exact Test, died: p = 0.03, Figure 1.4; deformities: p = 8.19e-03), however there were no significant differences between treatment groups after pairwise comparisons were carried out between each treatment and the p-values were adjusted for multiple comparisons. There was no difference in the amount of blood ingested or parasites ingested per unit of body mass

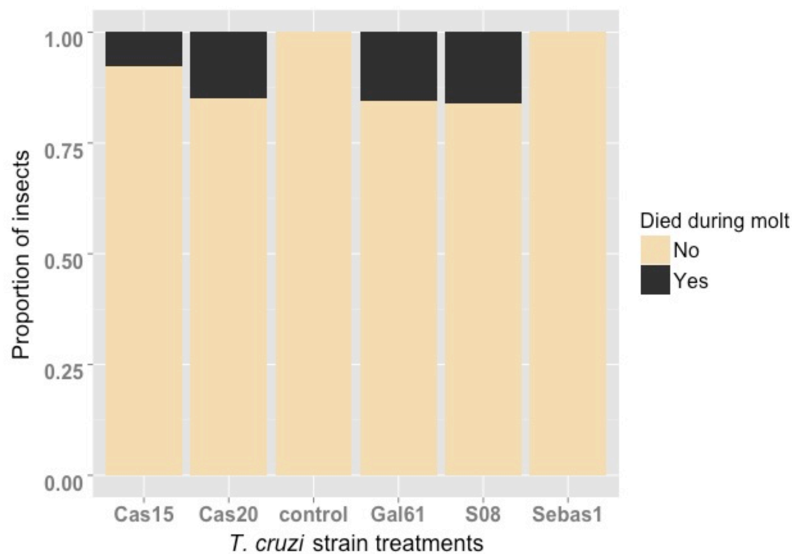


**Figure 1.4. Proportion of each treatment group that died during molt.**

between insects that died during molt and those that did not, nor between insects that molted with deformities and those that did not. The proportion of insects that did and did not molt was not significantly different between treatments nor was the day that molting occurred.

## 1.4 Discussion

Based on the variable effect of *T. cruzi* infection on its vertebrate hosts, I asked (a) if *T. cruzi* has an effect on its invertebrate host as well, and (b) if this effect is variable between *T. cruzi* strains. I hypothesized that yes, there would be an effect and it would vary. To test my hypotheses, I investigated survival and molt of the Chagas disease vector *R. prolixus* when infected with different strains of *T. cruzi* (DTU I). Taking into account time post-infection and the volume of blood/parasites ingested per unit of body mass, I found considerable variability in the effects of *T. cruzi* on *R. prolixus* survival and molting.

*T. cruzi strain variation reflected in invertebrate host survival outcomes*

I analyzed survival using two measurements, the Kaplan-Meier (K-M) survival function, (the probability that an individual survives longer than a given time point, adjusted for right-censored data), and the hazard function (the instantaneous rate of failure at any given time, given that the individual has survived up until that time). There was a global difference between K-M survival curves of each treatment group, with marked differences between survival in the Cas15 and Sebas1 treatments, suggesting that *T. cruzi* strains can have highly variable effects on *R. prolixus* longevity.

These differences in survival were confirmed in the hazards analysis, which is often considered more informative than survival function about underlying causes of death [59], as it allows for the inclusion of the main and interaction effects of the covariates in the analysis, which in this case were time and volume of blood/parasites ingested. The period between 0 and 28 days post-infection was more hazardous than the subsequent time period, with insects infected with strains Cas15 and Cas20 having a hazard rate over 6 times that of the control group. This time period is critical for both the insects and the parasite, as it includes the insect molt period, which in this study occurred between 16-28 days for the majority of the insects, and the parasite prepatent period which usually occurs up to 15 days post-infection [60]. The increased hazard could be attributed to parasite establishment and replication along with the physiological demands of molting within this relatively short period of time. The imaginal molt, (the molt from 5$^{th}$ instar to adult) is indeed very demanding, as the insect must undergo several morphological changes that do not occur in the previous four molts, including the development of wings and reproductive

organs. This was reflected in the molt outcomes, with the two treatment groups with the highest survival and lowest hazards, Sebas1 and control, having no insects that died during molt or molted with deformities. After 28 days, the Cas15 treatment continued to have the highest hazard at 4.5 times higher than the control group, suggesting that negative effects of some *T. cruzi* strains can continue after the molt period and the initial establishment of *T. cruzi* infection.

The blood:weight covariate interacted differently between the control and Cas20 groups. Higher volumes of blood:weight ratios significantly decreased hazard for the control group (expressed as blood:weight main effects), but significantly increased hazard for the Cas20 group. This pattern was also marginally significant in the Gal61 and Cas15 groups, suggesting that the addition of *T. cruzi* to the blood can override the nutritional benefits of a larger blood meal, if it translates to a higher infective dose.

T. cruzi *polymorphism*: *evidence and explanations*

*T. cruzi* has highly variable effects on its vertebrate hosts. In humans infected with *T. cruzi*, 60-70% never develop symptoms of infection, while the other 30-40% will develop symptoms that manifest themselves in one of at least three general ways (cardiac, digestive or both; [8,9]). *T. cruzi* parasitemia in mice and opossums has been found to vary with *T. cruzi* strain and clone [2,61–64], tissue lesions caused by *T. cruzi* have been found to be different between sylvatic and domestic animals [9], and differential tissue distribution was observed between different clones of *T. cruzi* when infecting mice [65]. The length of acute parasitemia is highly variable among *T. cruzi*-infected vertebrate hosts, with some

hosts exhibiting long-lasting high parasitemias and others moving from an acute stage to a chronic stage with low parasitemia in a matter of weeks [8,63,64]. Despite all of this attention to the varied effects of *T. cruzi* on vertebrate hosts, until now, there has been relatively little attention to variation in its effect on invertebrates.

Interestingly, in a prior study in the BCEI group, they found that Cas15, the most pathogenic strain in this experiment, was highly virulent in mice as well (unpublished undergraduate thesis, Juan Fernando Rios). Further comparison of the pathogenicity of a given *T. cruzi* strain in both of its obligate hosts, the vertebrate and invertebrate, could yield insights into the triatomine bug's potential for "ecological interference" in the transmission of more virulent strains [66], by filtering out virulent strains through insect death before it can infect a mammal host. This was tested in *Anopheles stephensi* mosquitoes infected with different *Plasmodium chabaudi* clones, but they found no correlation in parasite virulence between the mouse and in the vector [67]. However, they did find that the effect of *P. chabaudi* on mosquitoes was highly variable, dependent on the *P. chabaudi* clone infecting the mosquito, which has also been observed in *T. brucei*-infected tsetse flies [68].

This variation in parasite virulence toward their insect vectors could be attributed to differences between parasites strains driven by the selective pressures undergone by the parasite every time it infects a new host, vertebrate or invertebrate [63]. If different parasite genotypes are favored by different environments and/or host genotypes, then polymorphism will be maintained [69]. In parasites such as *Plasmodium* species,

reproduction occurs sexually, but in a limited number of host species; its polymorphism is probably maintained in a genotype by genotype scenario, where different parasite genotypes have higher fitness in different host genotypes that exist in host species populations. Environment will also play a role, both biotic and abiotic, as defined in [69], but not as strongly as for a clonally reproducing parasite such as *T. cruzi* [16] that infects 100s if not 1000s of different host species across multiple environments and varying spatio-temporal scales (due to differences in host size and lifespan). Since *T. cruzi* does not usually reproduce sexually, its potential for adaptation (genetic variation) is dependent on the presence of a high degree of clonal polymorphism within the species. Therefore, a heterogeneous environment across which the selection pressures are highly variable, allows for selection to differentially favor the *T. cruzi* clones that are able to adapt to specific local environments, thus maintaining polymorphism across its range and in the species. Each local environment presents a unique *T. cruzi* transmission scenario, and thus a unique challenge for controlling Chagas disease. Thus, it is critical to understand the local adaptations of *T. cruzi* to know how to best prevent and treat the disease. For example, the southern cone of South America is the only region where *T. cruzi* causes serious gastro-intestinal problems; in the northern parts of South America, Chagas disease manifests itself only in the heart tissue [70]. These disease variations have been linked to the different *T. cruzi* genotypes circulating in these areas [13,16].

In this light, variation in the effect of *T. cruzi* on its invertebrate hosts would seem to be guaranteed, but it must be kept in mind that each insect-parasite strain combination bears a unique combination of factors belonging to both the parasite and the host. Therefore, the

survival outcome of each infected host will also be influenced by its own biochemical and biological variables, such as its stage, size, immune system, and nutritional state [60,63], and how they interact with and respond to *T. cruzi* infection.

*The significance of* T. cruzi *DTU I*

Here I present a final note on the logic behind the *T. cruzi* strain selection in this experiment, and the significance of studying *T. cruzi* DTU I in Colombia. DTU I is the predominant DTU found in Colombia [17], and a significant amount of genetic variation and parasite-host co-adaptation has been reported within the genotype. This has resulted in the division of the group into the subgroups DTUs Ia-Ie [17,19,23,71], which are sometimes referred to as TC1 haplotypes. Although it was not possible in my experiment to include a sufficient number of strains to test for statistical differences associated with biological or geographical origins or TC I haplotypes, the strains I used were selected with these factors in mind, to get a crude indication of any potential underlying patterns.

In regard to the biological origin of the strain, I selected two strains isolated from extradomiciliary *R. prolixus* (Cas15, Cas20); two strains from extradomiciliary *R. pallescens* (Sebas1, SO-8); and one strain isolated from a vertebrate host (Gal 61). I chose these biological origins to examine their effect on *R. prolixus*, but I did not find any associations. The strains with the highest hazard in the first 28 days, Cas15, Cas20, and SO-8 were from both *R. prolixus* (Cas15 and Cas20) and *R. pallescens* (SO-8). Sebas1, the strain with the lowest hazard, was also isolated from *R. pallescens*. Additionally, Cas15 was the only strain

that significantly increased hazard after 28 days. Therefore, I cannot make any hypotheses linking strain biological origin and insect survival.

In terms of strain geographic origin, all strains were isolated in Colombia. Colombia presents within a relatively small geographic area, a wide variety of landscapes, including Caribbean and Pacific coastlines, Andes Mountains and dry plains regions, leaving the possibility of strain differences associated with the reproductive isolation of vectors, as gene flow in vector-borne parasites is believed to be primarily influenced by vector behavior and host preferences [72]. Furthermore, there are an estimated 700,000-1,200,000 people in Colombia infected with Chagas disease and 8,000,000 at risk, and *R. prolixus* is the key vector of *T. cruzi* in the country [73,74]. In the eastern plains region of Colombia, where strains Cas15 and Cas20 originated, the human seroprevalence for antibodies against Chagas disease is between 16.5-31.5% [75]. Additionally, in the Caribbean basin region, where Gal61, and SO-8 and Sebas1 were isolated, there are indigenous populations with a 40% human seroprevalence for antibodies to *T. cruzi* [76]. Therefore, the selection of Colombian *T. cruzi* DTU I infections of *Rhodnius prolixus* of Colombian origin allowed me the opportunity to study the interaction of a biologically appropriate parasite-host combination in a country with high endemicity for Chagas disease. More details on parasite-host combinations in other published studies are presented in Chapter 5.

### 1.5 Summary

In this experiment, I found that *T. cruzi* does affect *R. prolixus* survival and development, and that this effect differs significantly between *T. cruzi* DTU I strains, depending on time post-infection and the volume of blood or quantity of parasites ingested in the infective blood meal. My results suggest that *T. cruzi* can be pathogenic to the triatomine species *R. prolixus* without apparent external stressors, suggesting that the variability in the effect of *T. cruzi* on its vertebrate host extends to its invertebrate host as well.

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# Chapter 2

Effects of *T. cruzi* strains on *R. prolixus* fitness[2]

## 2.1 Introduction

The trypanosome-triatomine relationship is recognized as important for understanding vector competence and the spread of Chagas disease parasites. There are several published studies on this relationship from the perspective of *T. cruzi* that focus on the effect of different triatomine species on the development and infectivity of different *T. cruzi* strains and clones [1,2]. These works found that *T. cruzi* development (replication and transformation into its various forms) and infectivity (ability to establish and maintain infection in a new host) differs between triatomine species [3–7] and *T. cruzi* strain, clone and infectious form (blood stream form vs. metacyclic trypomastigote, [8–13]). Additionally, some triatomine species -*T. cruzi* clone/strain pairings consistently resulted in higher insect infection rates than others [12,14]. Other studies of the triatomine-*T. cruzi* relationship at the molecular level found that insect immune factors and other pathogens present in the insect gut affect *T. cruzi* development, which also varied by triatomine species, parasite strain and/or clone [15–23].

The effects of *T. cruzi* on its insect vectors are less well characterized. A few studies exist of the effect of *T. cruzi* infection on triatomine life history and fitness, but most are limited to a one *T. cruzi* strain-one triatomine species system [24–32]. The findings in these works are variable, with some results suggesting that *T. cruzi* reduces triatomine survival and or

[2]A portion of the work presented in this chapter was presented in an oral presentation at the 63rd annual meeting of the American Society for Tropical Medicine and Hygiene in November, 2014.

reproduction [24–26,28,29], and others finding little to no effect [27,30–32]. While some of these discrepancies may be due to differences in experimental conditions or design, it is also possible that the inherent variability of *T. cruzi* itself is a driver of the variable fitness outcomes in infected insects.

After observing different survival outcomes between *R. prolixus* infected with different strains of *T. cruzi*, I carried out a second experiment to test for an effect of different *T. cruzi* strains on insect reproduction in addition to survival, to estimate total fitness. I use the definition of fitness as described by McGraw & Caswell [33], i.e., the propensity to survive and reproduce. Thus, while survival is a component of fitness, there is still the possibility that insects with decreased survival could compensate with increased reproduction [34]. Therefore, to get a true estimate of the effect of different *T. cruzi* strains on *R. prolixus* fitness, and the potential for *T. cruzi* to regulate *R. prolixus* populations, it is necessary to measure reproduction in addition to survival.

*Research questions and hypotheses*

Considering its broad host diversity, ample geographic range, and extensive genetic diversity, I asked if *T. cruzi* variability extends to its effect on the survival, molt and reproductive output when it infects the triatomine species *R. prolixus.* I hypothesized that indeed *T. cruzi* strains would have different effects on *R. prolixus* survival, molt, and reproduction.

## 2.2 Materials & Methods

*Experimental design*

I infected 82 *R. prolixus* 5th instar females with one of three different *T. cruzi* (DTU I) strains; 33 additional uninfected 5th instar females were used as controls, for a total of 115 insects used in the experiment (Table 2.1). Following infection, I recorded the survival and reproduction of each bug for up to 96-148 days. I carried out all experiments in the laboratory of the Biology and Infectious Diseases group (BCEI) at the University of Antioquia in Medellín, Colombia. I carried out this experiment simultaneously with the co-infection reproduction experiment (see Chapter 3). The Gal61 and control groups in this experiment are also part of the analysis presented in Chapter 3. This allowed me to address the two branches of my thesis while taking advantage of limited numbers of insects. To ensure a clear and thorough presentation of each experiment and the subsequent analysis and interpretation, I considered it appropriate to divide them into two chapters.

| ***Treatment group*** | **N** |
|---|---|
| Cas15 | 33 |
| Gal61 | 24 |
| Sebas1 | 25 |
| Control | 33 |

**Table 2.1.** *T. cruzi* strain treatment groups

*Triatomines*

All *Rhodnius prolixus* used in the experiment were from laboratory colonies reared in the BCEI insectary, as described in Chapter 1. I determined the sex of each insect in its 4th or 5th instar by examining the two concentric terminal segments around the anus on the insect's ventral side under a dissecting microscope, as described in [35,36]. After determining insect sex, I separated the males and the females into separate jars of about 20 nymphs each.

*Parasites*

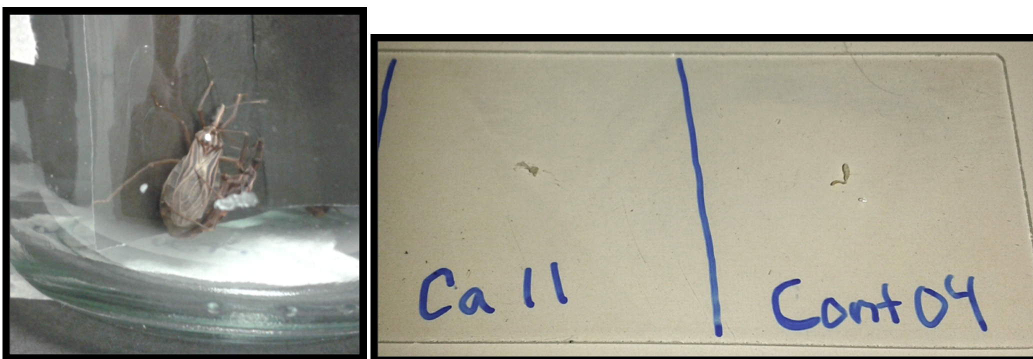
I selected the parasite strains Sebas1, Cas15 and Gal61, which were associated with the highest, lowest and mid-range insect survival, respectively, in Chapter 1. Information on these strains is presented in Table 1.2. Parasites were cultured and prepared for infection as described in Chapter 1.

*Insect infection*

Insect infections were carried out as described in Chapter 1. Only females were fed infected blood, while males to be used in the experiment were fed on the same uninfected blood as the control group.

*Insect Reproduction*

After molting into the adult stage, I paired each female with a recently-fed, uninfected adult male (Image 2.1a; [37,38]). I separated uninfected males into groups, and members of each




**Images 2.1a & b. Insect copulation (2.1a) and the resulting spermatophore casing (2.1b).**

group were paired with females of just one treatment group throughout the experiment to avoid cross-contamination. I left each pair together overnight [39] and confirmed

copulation the following day from the presence of the spermatophore casing (Image 2.1b), ejected by the female [40]. If I did not find the spermatophore casing after the first night, 2-3 additional males were placed in the jar with the female, and left for another night, as per advice I received from insect physiologist and *Rhodnius* specialist Professor Gary Chiang of Redeemer College. If copulation did not occur after three nights with several males, I recorded the female as unmated for that oviposition cycle. Unmated individuals from the first oviposition cycle were given a second opportunity to mate prior to the second oviposition cycle. After mating, each insect took a blood meal from a live chicken. I marked each insect as described in Chapter 1, weighing it before and after feeding to calculate the volume of blood ingested. I recorded oviposition and eclosion 3-4 times per week until the second oviposition cycle, 31-38 days later.

I measured reproduction as fecundity (egg production) and fertility (eggs that hatched). Fecundity in *Rhodnius prolixus* is correlated with the quantity of blood ingested and weight before feeding [41], and the standard index used when comparing fecundity in in *R. prolixus* is the E value [40]. 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 and its pre-feeding weight, and it represents the efficiency with which the insect converts nutrition (blood) into food while normalizing for blood and insect mass, which allows for comparison between feedings. The E value is independent of the timing of the oviposition cycle in an insect's lifetime. I did not calculate E values for insects that died in order to compare E value independent of mortality rates. In addition to these measurements, time-dependent

reproductive values were also generated for each individual in the analysis of fitness, described below.

*Infection confirmation*

Upon insect death, I stored each insect in a 1.5ul tube in 95% ethanol for later processing. At the end of the experiment, I air-dried each insect for 1-2 hours before maceration, centrifugation and extraction of total DNA, as described in Chapter 1. I then amplified the DNA in an RT-PCR (StepOnePlus Real-Time PCR System, Applied Biosystems) with the *T. cruzi*-specific primer pair TcZ1/2 [42] and the *R. prolixus*-specific primer RP18S [43], to confirm successful DNA extraction. Attempts to optimize a second *R. prolixus* reference gene primer to include in the qPCR were unsuccessful.

*Statistical Analyses*

I carried out all statistical analyses using the R statistical computing environment software version 3.03 [44]. I tested differences between treatments in the volume of blood ingested in the infective meal, estimated parasites dose, E values, fertility, and fitness estimates using Kruskal-Wallis rank sum tests with the 'kruskalmc' function from the 'pgirmess' package [45] as a post-hoc test to carry out multiple comparisons and control for family wise error. I tested for differences between treatments in the proportion of insects that did and did not lay eggs with a Fisher's exact test for count data. I carried out correlation tests with Spearman's correlation and linear regressions with the baseline stats package in R. I accepted p-values under 0.05 as statistically significant.

I carried out survival analyses as in Chapter one, using Kaplan-Meier (KM) estimates to compare the probabilities of total time to failure, and Cox models to compare instantaneous hazard rates. The data were run in two Cox models: one that included the controls to evaluate the difference in hazard between the parasite treatment groups and the control group; and a second without the control group to investigate main effects of parasite dose and the interaction of parasite dose with treatment.

I used individual survival and reproduction data to construct an age-classified population projection matrix for each insect [33,46]. Each matrix was 3 x 3, with age-specific survival ($P_i$) on the sub-diagonal (always 0 or 1 in individual matrices [33]), 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:

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

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. I calculated dominant eigenvalues (λ) using the eigen function in the R base package, and reproduction values were calculated by hand based on these values, as in McGraw and Caswell ([33]; based on Fisher [47]). The reproductive value ($v_i$) for $t_1$ 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.

*Genetic profile of* T. cruzi *strains across experiments*

In collaboration with Dr. Ana Mejia Jaramillo and Andres Felipe Diez of the BCEI group, a low-stringency single-primer polymerase chain reaction (LSSP-PCR; [9]) was carried out to characterize the kDNA profile of the strains Cas15, Gal61 and Sebas1. These strains had been cryopreserved before the experiment presented in Chapter 1, and then kept in culture until I carried out the experiment presented in this chapter (~11 months). Therefore, the LSSP-PCR was performed to check for a change in the genetic profile of the strains between experiments possibly due to time in culture.

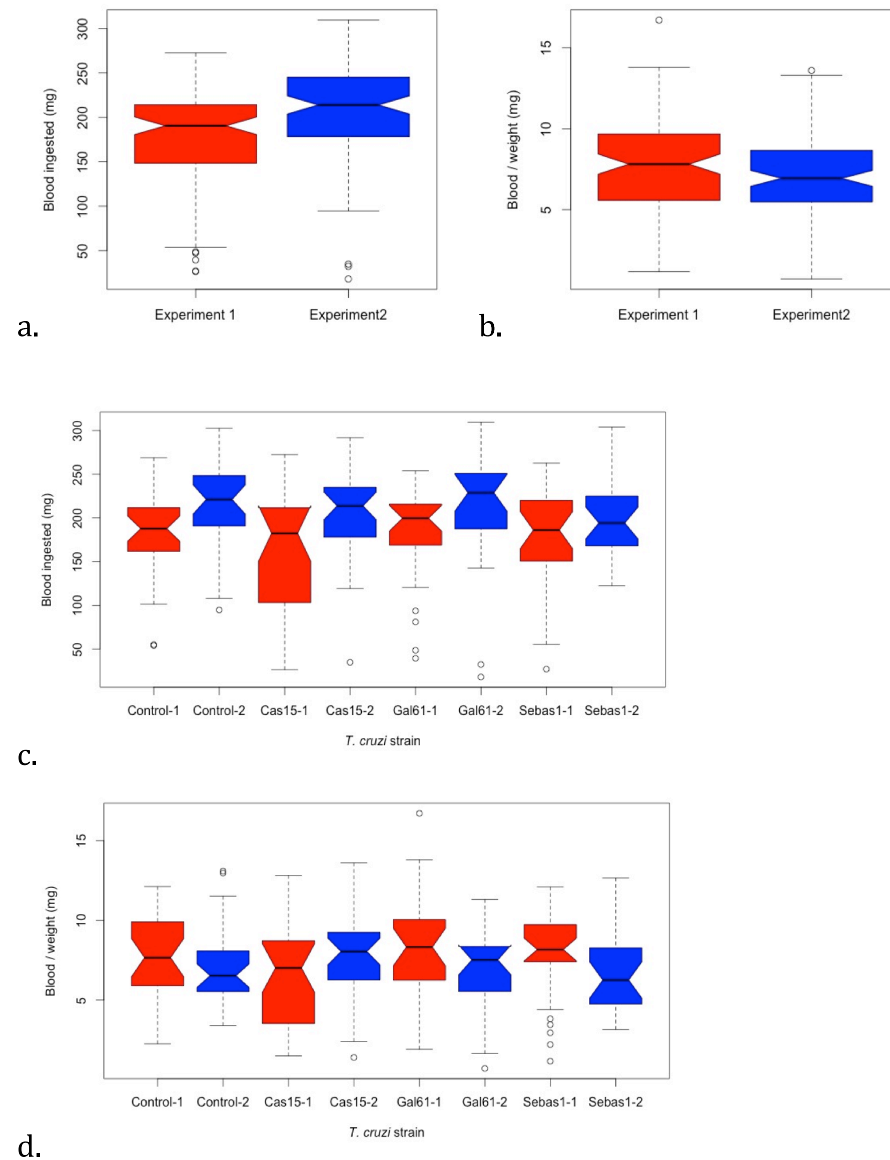
Briefly, LSSP-PCR is a PCR-based technique that uses extreme PCR conditions to the effect that the primer hybridizes to multiple regions of a DNA fragment, creating a set of reaction products that constitute a DNA profile for each sample. The technique is sensitive to single and multiple mutations in a fragment.

LSSP-PCR was carried out as described in [49,50], and complete methods description is in Appendix 2. Briefly, *T. cruzi* kDNA from the Cas15, Sebas1 and Gal61, extracted from insects infected in the experiment presented in Chapter 1 and cultures from strains used in Chapter 2 ,was amplified with the primer pair 121/122 [51], and the PCR product was run on an agarose gel. A 330 base pair *T. cruzi* kDNA fragment was cut out of the gel, diluted,

and used as the template for the LSSP-PCR. Amplification products from the LSSP-PCR were stained with ethidium bromide and visualized with silver staining.

## 2.3 Results

*Blood and Parasites ingested*




**Figures 2.1a-d. The distribution of the volume of blood ingested in the infective blood meal (2.1a,c) and the blood:weight ratios (2.1b,d) for insects in the experiments described in Chapter 1 (red) and Chapter 2 (blue). The groups in 2.1a are significantly different ($p < 0.001$).**

There was no difference between treatments in the volume of blood ingested or the estimated number of parasites ingested (Kruskal-Wallis, blood: $p = 0.41$; parasites: 0.19). There was a global difference in the absolute volume of blood ingested between insects in this experiment and the experiment presented in Chapter 1, (Wilcoxon, $p = 2.34e-04$, Figure 2.1a), but no significant differences were revealed in pairwise comparisons between treatments (Figure 2.1c). There was no difference between years or treatments in the blood ingested per mg of insect body mass, (Figures 2.1b and 2.1d).

*Molt outcomes*

Just two insects did not molt, one from the Gal61 treatment group and one from the control group. There were no visible signs of death due to molt in the insects that died nymphs. Eight insects molted with deformities, 7 with damaged wings and one with a broken neck. The deformities occurred across all treatments, with two insects per group molting with deformities in the control and Gal61 groups, and 3 deformed insects per group in the Cas15 and Sebas1 groups.

*Reproduction*

87.5-96.9% of insects in each group laid eggs, with no difference between treatments in this respect. E-values were significantly different between treatment groups in the first oviposition cycle Kruskal-Wallis, $p = 2.58e-05$, Figure 2.2), but not in the second cycle. In the first cycle, the E-value of the Gal61 treatment was significantly lower than all other treatments (KruskalMC, corrected for $p < 0.05$).

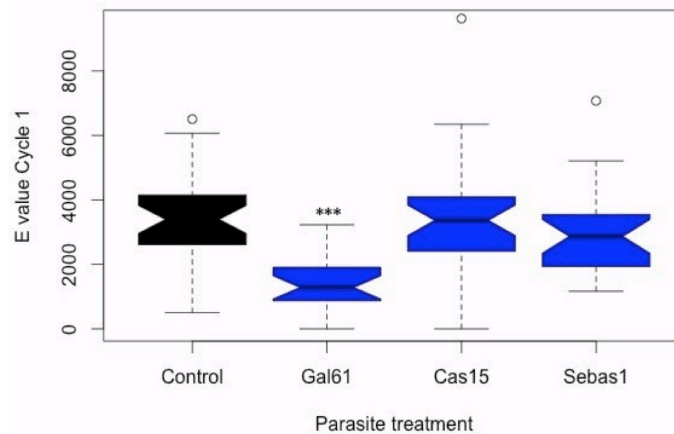



**Figure 2.2. The E value distribution for each treatment group. The Gal 61 E values were significantly lower than the other groups. *** p < 0.001.**

Mean egg fertility ranged between 71.57%-82.2% and was not different between treatment groups. Additionally, reproductive values produced by the matrix model for the time period encompassing the second oviposition cycle ($v_3$) were not different between treatments.

*Survival function*

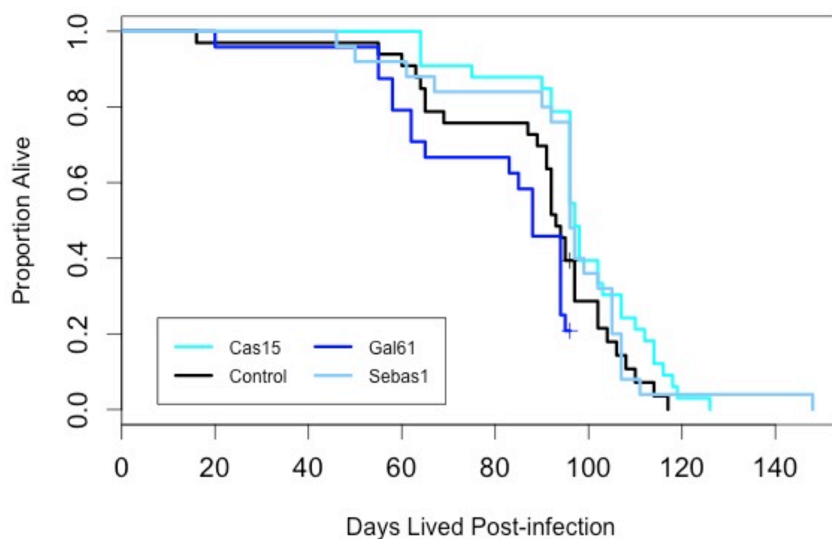



**Figure 2.3. Kaplan-Meier curves representing the survival of each treatment group over time. Crosses represent right-censored data.**

Kaplan-Meier survival function estimates were significantly different, (Chi Square = 16.6, 3 df, p = 8.34e-04, Figure 2.3), with the Gal61 treatment group having a shorter time to failure than the

Cas15 and Sebas1 treatment groups (Chi-Squared Distribution comparisons, $p < 0.02$).

*Hazards analysis*

In the Cox model comparing *T. cruzi* treatments with the control group, hazard was not the same (Likelihood ratio test, 12.96 on 3 df, $p = 4.72e\text{-}03$). The Cas15 treatment hazard was significantly lower than the control group hazard ($e^{\beta} = 0.60$, $p = 4.54e\text{-}02$, Figure 2.4, Appendix 2), while Gal61 hazard was significantly higher than the control group ($e^{\beta} = 1.84$, $p = 4.55e\text{-}02$, Figure 2.4). In the second model, there were no significant main effects or interaction effects of the number of parasites ingested.

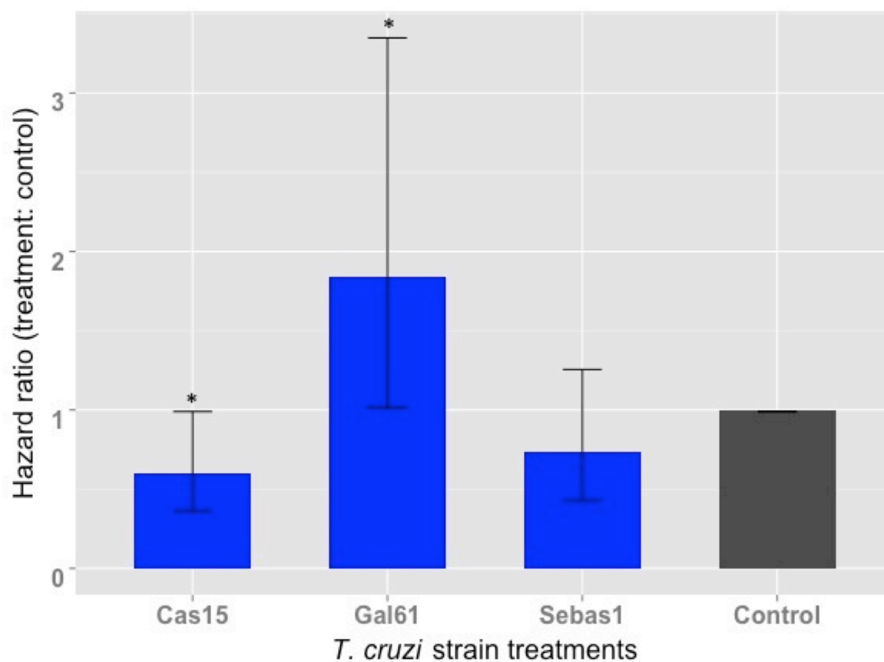



**Figure 2.4. Instantaneous hazard ratios ($e^{\beta}$) for *T. cruzi* strain treatment group main effects. Confidence intervals of the β value are indicated by the vertical lines. *p < 0.05**

*Fitness estimates*

Total fitness estimates were not the same between treatment groups (Kruskal-Wallis, p =

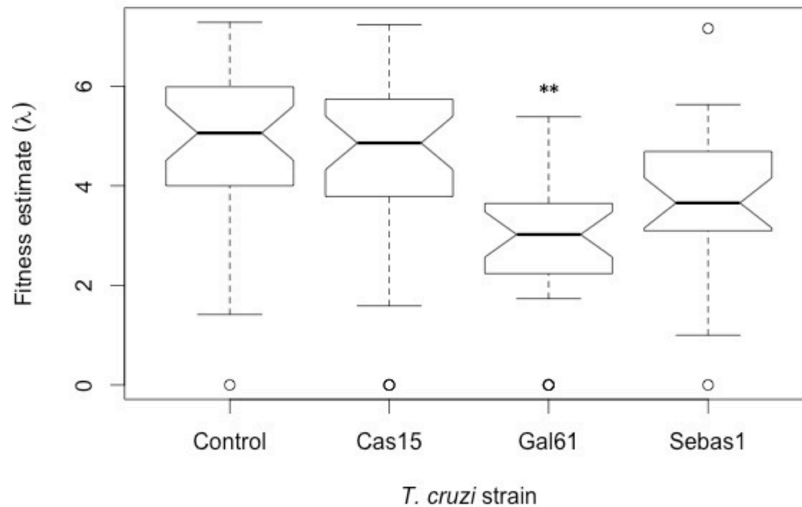



**Figure 2.5. Distribution of the total fitness estimates in each treatment group. Notches represent the 95% confidence intervals of the median. **p < 0.01.**

9.88e-05, Figure 2.5), with Gal61 having lower fitness than Cas15 and the control group (KruskalMC, p <0.01).

*Infection confirmation*

Successful DNA extraction was confirmed in 83.3% - 88.0% of samples from each treatment, (confirmed by the amplification of the *R. prolixus* reference gene primer). Out of these samples, 100% of Sebas1 (22/22) amplified, as well as 90.0% of Gal61 (18/20), and 75.0% of Cas15 (21/28).

*Genetic profile of* T. cruzi *strains across experiments*

Shown in Figure 2.6 are the genetic profiles of the *T. cruzi* strains I used in the experiments described in Chapter 1 (denoted with a "T"), next to the profile of the same strain after spending about 11 months in culture (denoted with a "C") The "C" strains were used in the

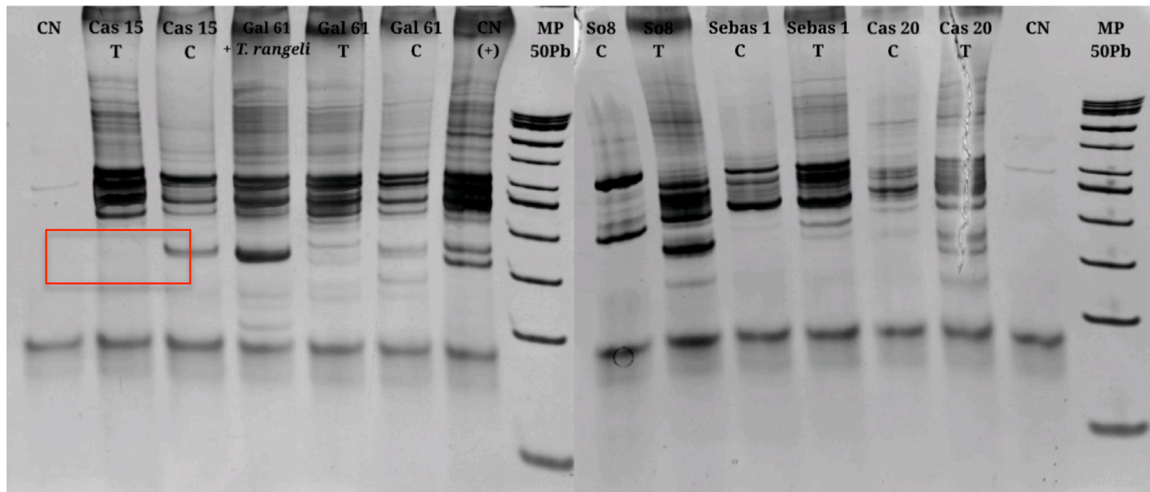



**Figure 2.6 Genetic profiles for each strain used in the experiment visualized in a polyacrimide gel. Strains marked with a "T" were used in the experiment described in Chapter 1. Strains marked with a "C" were those used in this chapter. Columns marked "CN" are negative controls and those marked "MP" are 50 base pair ladders. The pattern of the bands in each column should be similar between the 'T" and "C" version of each strain. However, Cas15 C (the third column in from the left) has a band that Cas15 'T" does not have, indicated by the red box.**

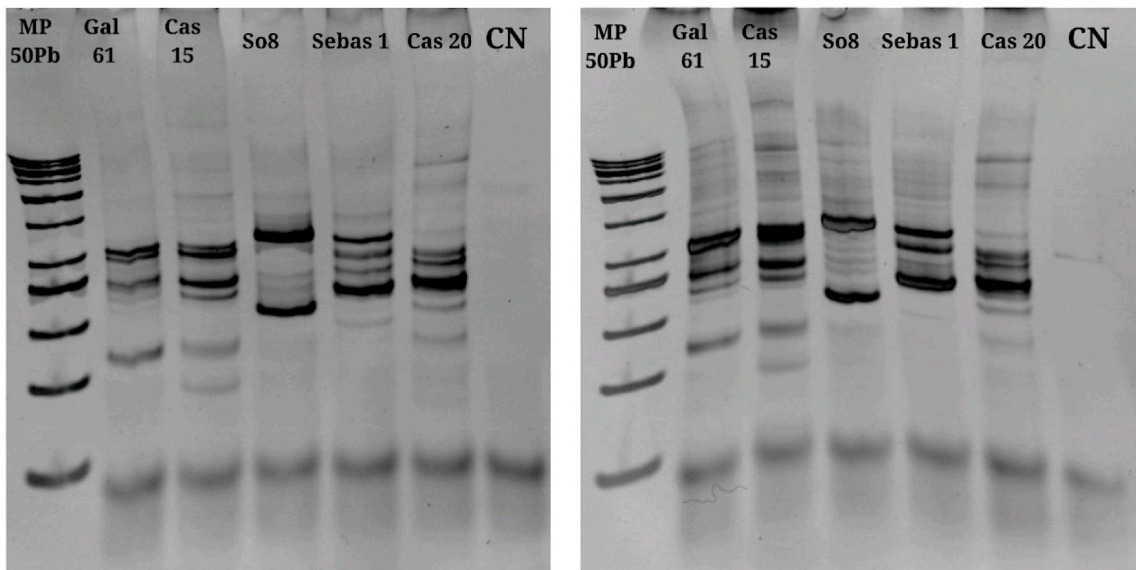



**Figure 2.7. Two replicates of the genetic profiles of the strains used in 2013. I included this photo to demonstrate the variability between gels.**

experiment presented in this chapter. The strain Cas15 “C” has a band at ~180 base pairs that is not present in the genetic profile of Cas15 “T,” indicated by the red box in Figure 2.6. There do not appear to be any other changes between the two samples from the other strains of interest, Gal61 and Sebas1. Although there is slight variation between the ‘C’ and ‘T’ samples of each of these strains, it does not seem to be more than the common variation found between gels. Shown below in Figure 2.7 are two replicates of the genetic profiles of the LSSP-PCR run with the samples that had been in culture. I include these photos to demonstrate the variability between the gels.

## 2.4 Discussion

As in Chapter 1, the effect of different *T. cruzi* strains on *R. prolixus* was not the same. In addition to survival, I found significant variation between treatment groups in reproduction and total fitness. That said, the pattern of the variation I observed in this experiment differed in some respects from that described in Chapter 1. From hereon, the experiment presented in Chapter 1 will be referred to as ‘Experiment 1’ and the experiment from this chapter will be referred to as ‘Experiment 2.’

*Molting*

In Chapter 1, hazard was significantly higher before 28 days, which was mostly due to insect death during molt. In this experiment, there were no apparent deaths due to molt, few insects that molted with deformities, and just 2 deaths before 28 days. One potential explanation for this is the volume of blood ingested in the blood meal prior to molting from the 5th instar into the adult stage, which in these experiments is also the infective blood

meal. When comparing the absolute volume of blood ingested between years, there was a global difference between the two experiments (Figure 2.1a) but no significant differences were revealed in pairwise comparisons between treatments (Figure 2.1c). However, when comparing the blood ingested per mg of insect body mass, there is no difference between years or treatments (Figures 2.1b and 2.1d), suggesting that insects in experiment 1 ingested less blood because they were smaller.

While the smaller size could be an indication of an inferior nutritional state in the insects used in experiment one, it could also be related to the population density of the colony and the size of the blood meal taken in the prior instar [52–55]. Whatever the reason, the insects infected with all *T. cruzi* strains except for Sebas1 experienced a higher hazard due to molting in Experiment 1, while insects in Experiment 2 did not. Moreover, although the control group insects in Experiment 1 were also small, they did not experience an increased death rate due to molting, suggesting that the molt problems were related to *T. cruzi* infection.

*Cas15 loss of virulence: possible attenuation?*

In Experiment 1, the Cas15 treatment group had significantly shorter time to failure and significantly higher hazard than the control and the Sebas1 treatment groups, across the entire experiment; it was the only treatment group with negative effects associated with parasite treatment continuing beyond the molt period. In Chapter 2, Cas15 seemed to lose its virulence. There was no difference in survival function or hazard between the Cas15 group and any other group, and in fact, the Cas15 treatment fared slightly better than all

other groups. One explanation could be that the insects used in Chapter 2 possessed a superior background nutritional state, but then again, the only change in virulence (expressed as insect survival relative to the control group) between chapters occurred in the Cas15 strain.

This led me to investigate the possibility of the attenuation of the strain Cas15 across experiments, by comparing the kDNA profiles of the strains across experiments. The results of the LSSP-PCR revealed a 180bp band in the Cas15 strain used in Experiment 2 (from hereon called “Cas15-2” for Experiment 2 and “Cas15-1 for Experiment 1) that is not in the Cas15-1 profile. As mentioned earlier, Cas15-1 had been cryopreserved before I used it, while Cas15-2 spent ~11 months in vitro before use. Therefore, it is possible that this extra band in the Cas15-2 profile could be evidence of a selection event of a subset of the clones of Cas15-1, leading to the attenuation of the strain into its Cas15-2 form, which was reflected in the difference in survival between insects infected with Cas15-1 and Cas15-2.

These types of selection events are not uncommon in *T. cruzi* strains that spent extended periods of time in vitro [56–58]. The *T. cruzi* population structure is heterogeneous and multiclonal [59,60], and in different environments, different subsets of clones can be selected for and become predominant [61], as touched upon in Chapter 1. In the case of parasites in culture medium, the environment is, in general, much more stable than that of a living host, as the parasites are released from resource limitation and host immune pressure. *T. cruzi* attenuation has been associated with decreased virulence in its vertebrate hosts [62–64], although there are also studies where no attenuation was

observed after upwards of 18 years in culture [63]. Therefore, it likely depends on intrinsic properties of the strain, which could explain why I did not observe a significant difference between the survival of insects infected with Gal61 and Sebas1, as compared to the control groups in each experiment.

An alternative hypothesis is that the clonality of Cas15 changed when infecting the insect. In a study of *Triatoma infestans* experimentally infected with mixed *T. cruzi* clones, it was found that insects had 73-93% of the clonal mixtures they begun with after passage through the insects [8], suggesting that the clonality can change within the insect. However, Cas15 spent far less time in the insect than in culture (3 months vs. 11 months), and passing *T. cruzi* through a host geneally results in an increase in virulence as opposed to a decrease[56]. Further research is needed to confidently predict if the change in virulence I observed was associated with in vitro clonal selection. This could be done in vitro by challenging different cultures of the Cas15 strain with triatomine immune molecules such as gut hemolytic factors [65] or lysozymes [66]; or in vivo, with an experiment similar to mine comparing the fitness of insects infected with strains maintained in vitro and in vivo.

*Reproduction changes survival*

There are other differences between Experiments 1 and 2 that merit consideration, namely that the insects were mated and reproductively active in Experiment 2 only. It has been observed that virgin females consume 12-22% less blood than mated females [67], which could lead to a suboptimal nutritional status. Moreover, an association between longevity and matedness has been observed in several insect species including the *Panstrongylus*

*megistus* (subfamily Triatominae;[68]), soldier bugs [69] and stink bugs [70], (both of the order Hempitera), and *Aedes aegypti* [71], *Anopheles gambiae* [72] (both of the class insecta and also vectors of human disease). I observed that insect death seemed to be timed to the oviposition cycles; rarely did an insect die before all its eggs had hatched. Therefore, survival across the two experiments should be compared only in the time period preceding reproductive activity, which coincides with 1-36 days post-infection. However, this does not explain the difference between Experiments 1 and 2 in the survival of the insects infected with Cas15, and the most viable explanation still appears to be strain attenuation.

*Effects on total fitness*

The Gal61 strain was the only treatment with significantly lower total fitness estimates, with a mean fitness estimate (λ) of about half that of the control group. Whether this translates to a reduction in fitness that would impact *R. prolixus* population dynamics is yet to be seen, because while fitness was reduced, it remained above one, indicating that the population would still grow. Sisterson [73] modeled vector population dynamics with infected vector death rates higher than uninfected vector death rates, and found that vector density and proportion of infected hosts was reduced. However, if the effect of *T. cruzi* on *R. prolixus* fitness is highly variable, as observed in Experiment one, then the effects on insect population dynamics would depend on the frequency of insects infected with virulent *T. cruzi* strains. At present, there are no published studies investigating variation in virulence among *T. cruzi* strains infecting triatomines in natural settings, presenting an interesting area of future research.

**2.5 Summary**

Considering the polymorphism found in *T. cruzi* across scales, from its genetic variation to its host and vector species richness, geographical range, and variable effect on its vertebrate hosts, I investigated the effect of *T. cruzi* on the life history of its triatomine bug host. Using the triatomine species *R. prolixus,* I posed two questions: (1) can *T. cruzi* affect *R. prolixus* fitness; and (2) if *T. cruzi* does affect *R. prolixus* fitness, is there variation between *T. cruzi* strains in the observed effect?

I found that *T. cruzi* does affect *R. prolixus* fitness, reflected in the significant variability I observed between treatment groups in several aspects of insect survival and reproduction. To the best of my knowledge this is the first study to (a) examine the effect of *T. cruzi* on triatomine fitness where survival and reproduction were measured in the same experiment; (b) to compare the effect of different *T. cruzi* strains on triatomine survival, reproduction and fitness; and (c) to propose a connection between a *T. cruzi* clonal selection event in culture and a significant change in individual host fitness and [projected] host population growth.

The attenuation of the strain Cas15 between experiments demonstrates the potential of *T. cruzi* to change over a short period of time to the extent that it manifests across scales at a population level. This is important to consider in the search for novel vector control methods, for example the current use of *Wolbachia* bacteria that is currently being employed to control the spread of the dengue virus, as the virus cannot transmit when it co-infects *Aedes aegypti* with certain *Wolbachia* strains [74,75]. A similar method in

triatomines may not be successful due to the ability of *T. cruzi* to adapt rapidly to changes in its environment. On the other hand, perhaps a *T. cruzi-Wolbachia* co-infection would increase the burden put on the invertebrate host by *T. cruzi* to the point of a significant reduction in fitness. The effects of co-infection can be powerful and surprising, as I will discuss in the subsequent chapters.

In sum, the results presented in Chapters 1 and 2 suggest that *T. cruzi* can influence *R. prolixus* individual fitness, although, like *T. cruzi* itself, the effect is highly variable. More studies on the effect of different *T. cruzi* strains on triatomine fitness will allow us to understand how the variation observed in this controlled experiment plays out in *T. cruzi* transmission settings, and, ultimately, whether or not *T. cruzi* reduces triatomine fitness at an intensity and frequency that is high enough for the parasite to be considered a top down regulator of triatomine populations.

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## Chapter 3

*Rhodnius prolixus* fitness outcomes when co-infected *T. cruzi* and *T. rangeli*[3]

### 3.1 Introduction

In Chapters 1 and 2, I suggested that the insect vector of Chagas disease agent *T. cruzi* experiences variable outcomes from *T. cruzi* infection, just as *T. cruzi*-parasitized vertebrate hosts do. I investigated the fitness consequences of *T. cruzi* infection for the triatomine species *Rhodnius prolixus,* and the effects of *T. cruzi* strain variation. I presented the idea that *T. cruzi* has the potential to regulate triatomine populations from the top down, analogous to predator regulation of prey populations.

In the next two chapters, I continue investigating triatomine bugs as parasitized hosts, but in the context of another phenomenon known to regulate populations, resource competition. Here, I investigate how *T. cruzi* competition with another parasite, *Trypanosoma rangeli,* changes the fitness consequences of *T. cruzi* infection for the insect, with the aim of characterizing the potential interplay between top down regulation and parasite competition in *T.* cruzi regulation of *R. prolixus* populations. Although these two parasites colonize different parts of the triatomine (detailed below and in Figure 3.1), *T. cruzi* and *T. rangeli* indirectly compete for control over the source of all resources, their shared host. First, each parasite competes to modulate the host immune response in order to facilitate their establishment [1–3]. Thereafter, the two parasites compete to maximize their replication but at the same keep their shared host alive in spite of the fact that each parasite co-opts host tissues, uses host micronutrients and metabolites[4], and requires

[3]A portion of the work presented in this chapter was presented in an oral presentation at the 63rd annual meeting of the American Society for Tropical Medicine and Hygiene in November, 2014.

that the host repurpose its energy toward immune responses and reparation of damage caused by each parasite[5].

## 3.2 Background

Upon infection of its invertebrate host, *T. cruzi* is faced with a diverse suite of gut microbiota [6–8], many of which are symbionts or commensals [4, 5], and at least one of which directly attacks *T. cruzi* [11,12]. At present, there are at least 8 species of bacteria identified to infect triatomines [6] along with at least 6 genera of fungi [13–15], 4 trypanosomatid species [16], and one virus [17]. All of these pathogens will interact directly or indirectly via resource competition, immune modulation and competition for immune-free space [18–20].

I focus on *T. cruzi* co-infection with a very similar parasite, its congeneric *T. rangeli. T. rangeli* is a euglenozoan parasite that circulates between mammals and triatomine bugs, most commonly in the triatomine genus *Rhodnius*. *T. rangeli* can infect sympatrically with *T. cruzi* in both vertebrate and invertebrate hosts. *T. rangeli* is of interest in Chagas disease epidemiology because it shares at least 60% of its antigens with *T. cruzi* [21–23], and is being used to develop a vaccine against Chagas disease in mice, guinea pigs and dogs [24–26]. These antigenic similarities lead to cross reactions in immunogenic diagnostic tests, which can lead to erroneous Chagas disease diagnoses [27], interfering with our ability to predict and describe Chagas disease distribution in Chagas-endemic regions. The true impact of Chagas disease diagnoses in relation to *T. rangeli* infection in hosts and vectors is still not known.

*T. rangeli* and *T. cruzi* differ in their vector-borne transmission routes (anterior vs. posterior, respectively, Figure 3.1). *T. rangeli* must invade the salivary glands of its triatomine host to be transmitted, and it is believed that *T. rangeli* usually invades the salivary glands just of members of the triatomine genus *Rhodnius* [16,28]. In contrast, it is thought that all triatomine genera have the capacity to transmit *T. cruzi* [29].

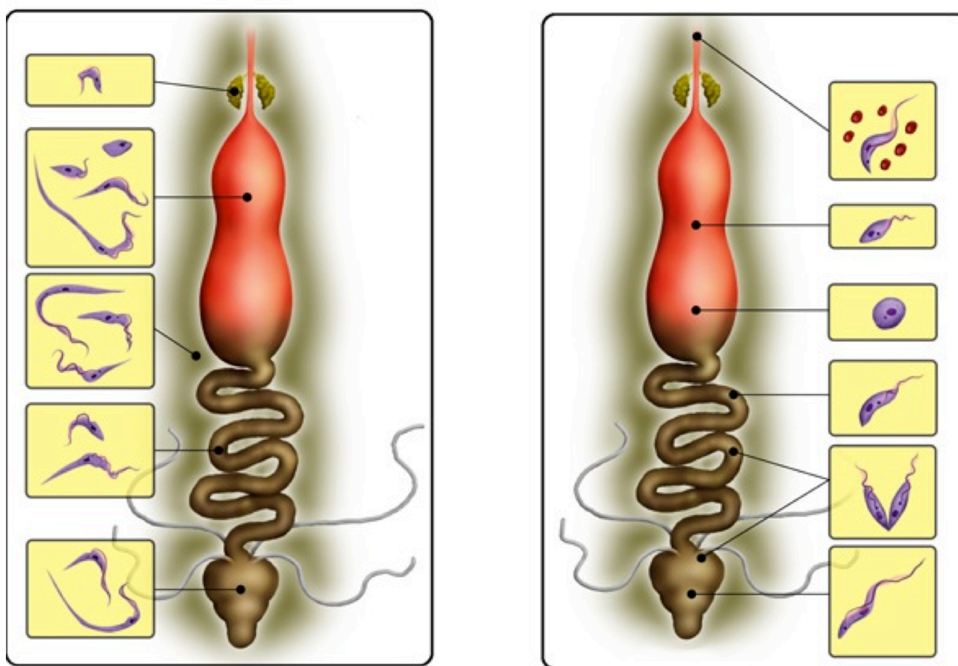


**Figure 3.1. The life cycle of *T. rangeli* (left) and *T. cruzi* (right) in the triatomine bug. Both parasites enter the insect with a blood meal. Afterward, *T. rangeli* passes through the gut wall into the hemolymph and invades the salivary glands; *T. cruzi* continues on through the digestive system, eventually exiting with insect excrement. Illustration by Bruno Eschenazi, used with his permission.**

The most important reported difference between *T. cruzi* and *T. rangeli* is in their pathogenicity: *T. rangeli* is not pathogenic to mammals [30], but has been observed to negatively affect molt, survival, movement, and excretion in *R. prolixus* [31–37]. *T. cruzi*, by contrast, can have very negative consequences for its mammal hosts, Chagas disease being

**Figure 3.2. Geographical range of *T. rangeli.* Image from Grisard, *et al.*** [114].

the prime example, but until recently, was not believed to have negative consequences for its invertebrate hosts [16,38,39]. In this chapter, I use the term 'pathogenicity' as a qualitative term that refers to the presence or absence of disease [40], and 'virulence' as a quantifiable decrease in host fitness after infection, as suggested by Read [41].

Like *T. cruzi, T. rangeli* is very polymorphic. It infects a wide range of mammals and follows the geographic range of its *Rhodnius* vectors (Figure 3.2), extending from central Argentina to northern Mexico. *T. rangeli* is divided into two lineages based on the presence or absence of a kinetoplastid DNA (kDNA) minicircle, and these lineages are referred to as KP1(+) and KP1(-) [42]. The KP1(+) and KP1(-) lineages have co-evolved separately with different *Rhodnius* species and each Rhodnius species is capable of transmitting just one of the two lineages [43], although *T. rangeli* can sustain infections in the hemolymph of the other species. These *Rhodnius* species that transmit the KP1(+) lineage are classified as the "Prolixus group" (as *R. prolixus* is the most well-studied member), and those that transmit KP1(-) are the "Pallescens group," [42,44,45].

Reductions of *T. cruzi* virulence in *T. cruzi* clone co-infections have been observed both in vivo [46] and in vitro (as measured by replication rates; [47]), and *T.* rangeli exposure in vertebrates prior to *T.* cruzi infection has been found to modulate the host immune response to *T. cruzi*, resulting in reduced disease severity in both acute and chronic *T. cruzi* infections [24–26,48–50]. These studies suggest that *T. cruzi* virulence can be affected by co-infection, and more specifically co-infection with *T. rangeli*.

In spite of the observations of decreased *T. cruzi* virulence in co-infections, experimental *T. cruzi*-*T. rangeli* co-infection has never been studied in its invertebrate host. Perhaps this is because *T. cruzi* is not believed to be pathogenic to its invertebrate hosts, while *T. rangeli* is considered pathogenic in invertebrates. Therefore, considering the public health importance of *T. cruzi*-infected triatomines and what is known about *T. cruzi* virulence in co-infections, *T. cruzi*-*T. rangeli* co-infection of invertebrates merits investigation. *T. cruzi* and *T. rangeli* are often found co-infecting together in field-caught triatomine bugs of the genus *Rhodnius*, some of which are considered key vectors of *T. cruzi* to humans [51]. Therefore, an understanding of the extent to which competition between *T. cruzi* and *T. rangeli* can change individual vector fitness (and thus the transmission potential of either parasite), regulate populations, and/or negate population regulation by *T. cruzi* could have implications for vector control and Chagas disease prevention strategies.

*Research questions and hypotheses*

I investigated the effect of *T. cruzi-T. rangeli* co-infection on the fitness of their triatomine vector *Rhodnius prolixus*. I asked if *R. prolixus* co-infected with *T. cruzi* and *T. rangeli* have different survival, reproductive success, and/or overall fitness than uninfected insects and/or insects infected with just *T. cruzi* or *T. rangeli.* Due to pathogenicity of *T. rangeli*, and the negative effects of *T. cruzi* infection I observed in Chapters 1 and 2, I hypothesized that fitness of co-infected insects would be lower than all other treatment groups.

## 3.3 Materials & Methods

*Experimental design*

I infected 101 *R. prolixus* 5th instar females with *T. cruzi*, *T. rangeli*, or *T. cruzi* and *T. rangeli* (Table 1). 33 additional uninfected insects were used as controls, for a total of 134 insects used in the experiment. After molting into the adult stage, each female was mated with an uninfected male, and survival and reproduction were measured for up to 96-140 days.

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

**Table 3.1. Treatment groups.**

*Triatomines*

All *Rhodnius prolixus* used in the experiment were from laboratory colonies reared in the BCEI insectary, as described in Chapter 1.

*Parasites*

I used the parasite strains "Gal61" (*T. cruzi;* strain information presented in Table 2.2), and Choachí (*T. rangeli*). The Choachí strain was originally isolated from an *R. prolixus* individual collected in Cundinamarca, Colombia [52–54], and belongs to the KP1(+) kDNA group [42], which, as stated earlier, is associated with the Prolixus complex of *Rhodnius* [43].

*T. cruzi* parasites were cultured and maintained as described in Chapter 1. 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% inactivated fetal bovine serum. Infectivity was maintained by cyclic *R. prolixus*-mouse passages every 3 months.

*Insect infection*

**Image 3.1. 5th instar *R. prolixus* in various engorged states after the infective blood meal.**

I prepared the parasites and infected the insects as described in Chapter 1 (Image 3.1). All insects were provided de-fibrinated, de-complemented human blood supplemented with inactivated fetal bovine serum blood with an estimated concentration of 3.3-3.5 x $10^6$ parasites/ml. (As reported in Chapter 1, this concentration falls within (a) the range of peak parasitemias observed in mice and guinea pigs experimentally infected with *T. cruzi*

[55–60], but here I add that this is also appropriate for *T. rangeli* in relation to peak parasitemias [61–63], and oral infectious doses used in prior published studies of *T. rangeli* infection in triatomines [64–68]). *T. cruzi-T. rangeli* co-infections were carried out at the same total parasite concentration, albeit with half the parasite concentration (i.e. 1.65 x $10^6$ parasite species/ml of blood for a total of 3.8 x $10^6$ parasites/ ml of blood).

*Insect Reproduction*

I mated experimentally infected nymphs that successfully molted into the adult stage, and measured reproduction, as described in Chapter 2. Briefly, I measured fecundity (egg production) and fertility (eggs that hatched) with the E value and the proportion of oviposited eggs that hatched, respectively.

*Infection confirmation*

Upon insect death, I processed all insects from each treatment group and extracted DNA as described in Chapter 2. Additionally I extracted DNA from pooled males and offspring to check for horizontal and vertical transfer of parasites. I amplified DNA in an RT-PCR as described in Chapter 2, with the same *T. cruzi* primer pair (TcZ1/2, [69]) and *R. prolixus* reference gene primer (RP18S, [48]). To obtain a *T. rangeli*-specific primer of the optimal size that did not also amplify *T. cruzi,* I designed a primer that I denoted as "PEEL5" -F (5' -TGCTTTCGTAGTTGGCACTG-3') and -R (5'-ACGCACCTCCTCCTCTCTCT-3´), which amplifies a 93 base pair fragment of *T. rangeli* telomeric DNA. I designed this primer from the *T. rangeli* clone TrTel 10 telomeric sequence (Genbank ID: AF426020.1), using the Primer3 plus software [71].

*Statistical Analyses*

Statistical analyses were carried out as in Chapters 1 and 2. I analyzed survival function and hazard rates using Kaplan-Meier estimates and Cox PH models, as described in Chapter 1. For the CoxPH model, I log2 transformed parasite doses, and centered them on the log2 transformation of $5.0e^5$ parasites, the round number closest to the mean. I used the Predict function from the 'rms' package [72] to estimate log relative hazards and their 95% confidence intervals based on 1000 simulations of the model.

I ran the Cox model with three variations. In the first variation, I investigated the interaction between treatment and blood:weight ratio, and I compared the parasite treatment group hazards with the control hazard. In the second and third variations, I included only parasite treatment groups to investigate relative hazard. To control for a possible effect of absolute number of parasites versus relative number of each parasite species in the mixed parasite species dose, I ran the model with data for the absolute number of parasites ingested by the mixed group in the second variation, and in the third variation, I 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 only reflected in the effect size. Cox PH model outputs are in Tables 3.2-3.4 in Appendix 3.

*Fitness estimates*

Fitness estimates were calculated as described in Chapter 2.

## 3.4 *Results*

*Parasites ingested*

Insects ingested between 30.1-337.9 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 prefeeding 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 2,000 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 and number of parasites ingested per mg of insect biomass (Kruskal-Wallis, blood: p = 1.67e-04; parasites: p = 0.01), with the mixed group ingesting significantly more blood than *T. cruzi* or control groups (Figure 3.3) and more parasites than the control

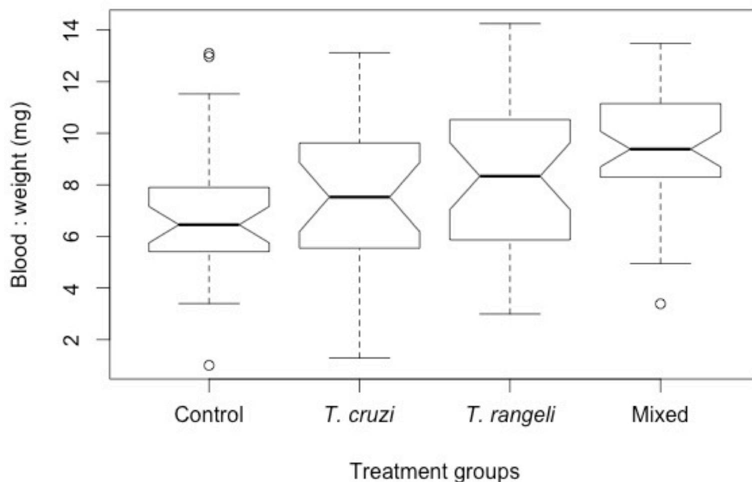
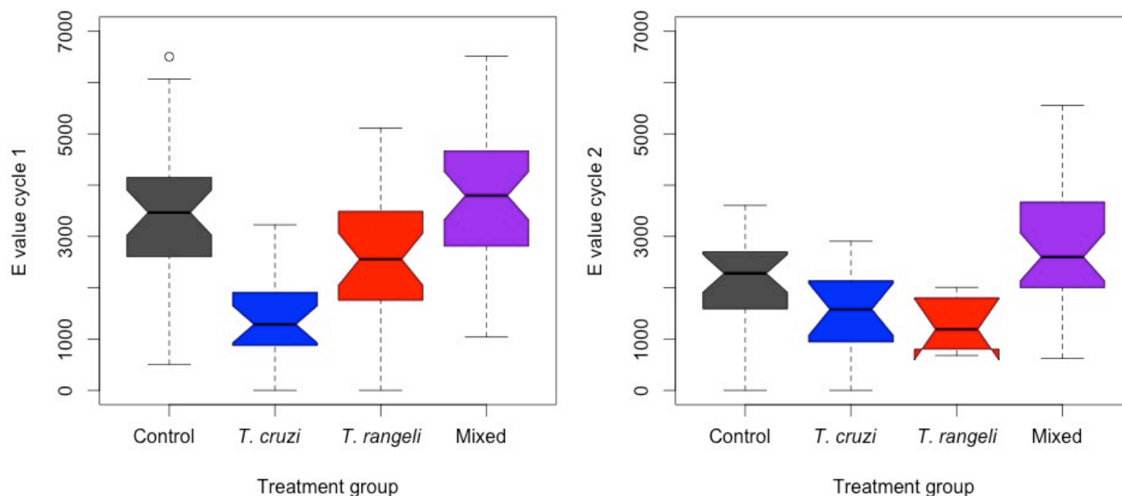



**Figure 3.3. The distribution of the ratio of the volume of blood consumed in the infective blood meal to mg of insect biomass across treatments. The mixed group blood:weight ratio was significantly higher than that of the *T. cruzi* and control groups.**

group (KruskalMC, $p < 0.05$ for both 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 = 8.98e-08; cycle 2: p = 3.24e-04, Figures 3.4a and 3.4b). In both cycles, E values for the *T. cruzi* or *T. rangeli* treatments were significantly lower than the co-infected treatment E value (Kruskalmc, $p < 0.05$). The *T. cruzi* treatment




**Figures 3.4a & 3.4b. 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 groups. 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.**

had a significantly lower E value than the control group in cycle 1 only, (Kruskalmc, $p < 0.05$). Mean egg fertility ranged between 79.4-84.3% for cycle 1; 62.4-81.8% for cycle 2; and 77.6-83.7% overall. Fertility was not significantly different between treatments. There was no association between E value and fertility.

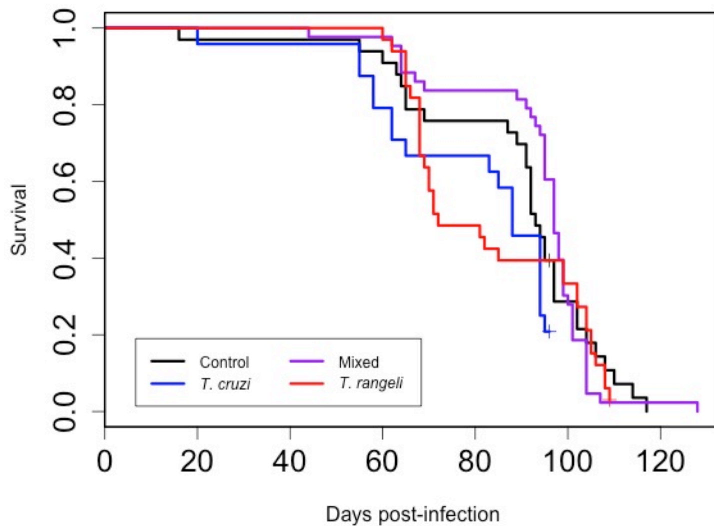



**Figure 3.5. Kaplan-Meier survival curves for each treatment group. The *T. cruzi* treatment survival function was significantly different than that of the mixed group.**

*Survival function*

Kaplan-Meier survival curves were significantly different, (Chi Square = 8.4, 3 df, p = 0.03, Figure 3.5), with the *T. cruzi* treatment group having a shorter time to failure than the mixed treatment group (Chi Squared Distribution comparisons, $p < 0.05$).

*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 = 8.67e-04), suggesting hazard was not the same between treatment groups. The main effects of *T. cruzi* treatment were significant, with a hazard about twice that of the control group ($e^{\beta}$ = 2.17, p = 4.33-04). The control and mixed treatments interacted significantly with the blood:weight ratio, but in opposite direction; the control group hazard increased with the blood:weight ratio increase, while the mixed group hazard decreased with increases in the blood:weight ratio, (control: $e^{\beta}$ = 1.26, p = 1.55e-03; mixed: $e^{\beta}$ = 0.74, p = 5.64-03, Figure 3.6).

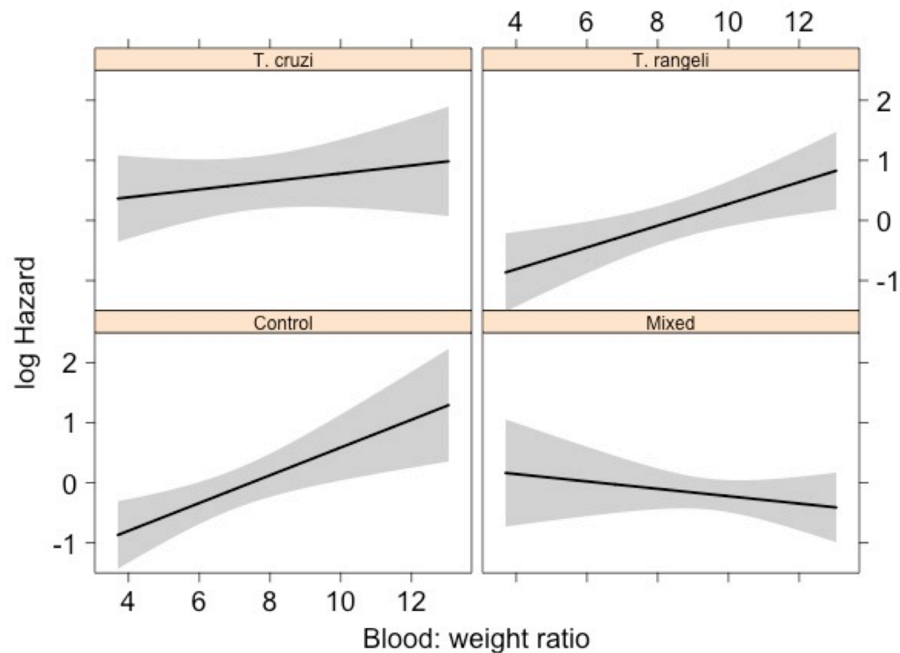
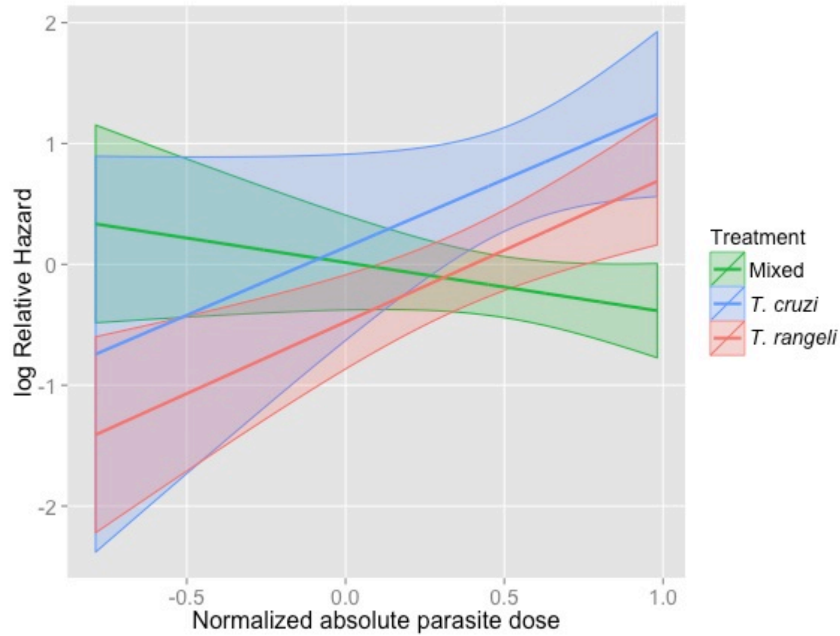
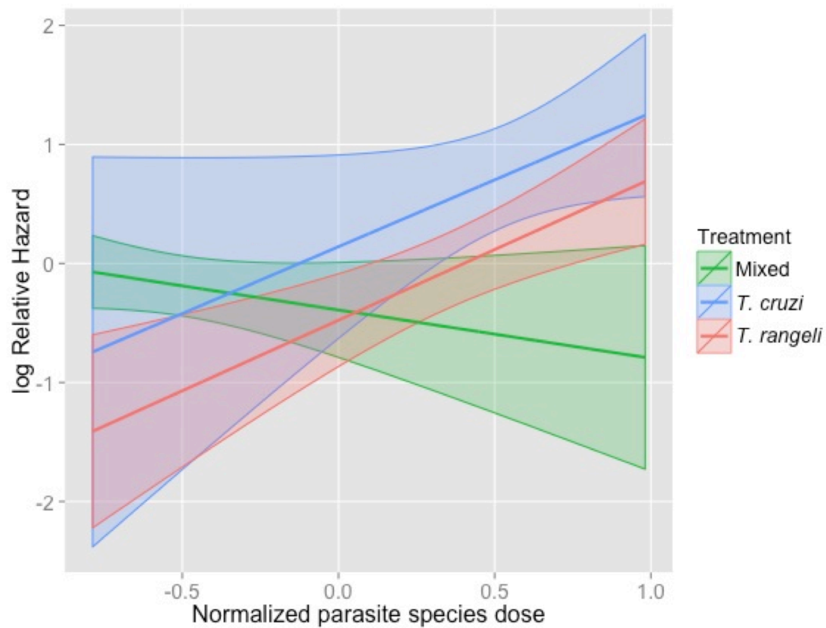



**Figure 3.6. Interaction of treatment with the blood:weight ratio of the infective blood meal. Hazards were predicted after 1000 simulations of the model. Figures are centered on a ratio of 8.32. Grey shading indicates 95% confidence intervals. Just the interactions in the bottom row (the control and mixed treatment groups) were significant.**

The Cox model investigating the main and interaction effects of parasite dose was also significant (Likelihood ratio test, 29.63, 5 df, p = 1.74e-05). The patterns and significant effects were the same in both variants of the model (examining the effect of absolute versus 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 threefold increase in hazard at a dose of one million parasites from the hazard at 500,000 parasites (*T. rangeli*: $e^{\beta}$ = 3.27, p = 4.33-04; *T. cruzi:* $e^{\beta}$ = 3.07, p = 6.5e-02).




**Figures 3.7a (above) and 3.7b (below). Predicted log relative hazard values and confidence intervals from 1000 simulations of the two variations of the Cox PH model investigating parasite dose. Figure 3.7a shows the interaction of treatment with the absolute number of parasites ingested. Figure 3.7b shows the hazard when the model was run with the number of parasites ingested by the mixed group run as the total number of each species ingested. Parasite dose is log2 transformed and centered on 500,000 parasites.**

Effects of the interaction between treatment and parasite dose were significant for the mixed group in the both of the model variations (absolute and relative parasite doses of the mixed group). At 250,000 parasites, the mixed group hazard was significantly higher than either single infection treatment, while at 1 million parasites the hazard was significantly lower, (Mixed vs. *T. cruzi*: p = 0.025; Mixed vs. *T. rangeli*, p = 0.00006; Figures 3.7a and Figure 3.7b, full summary in Appendix 3, Tables 3.3 and 3.4). Interaction effects were not significant when comparing *T. cruzi* and *T. rangeli* with each other, suggesting their hazards were not significantly different at any parasite dose.

*Fitness*

Fitness estimates (λ) and reproductive values $v_2$ and $v_3$ (corresponding to 60 and 90 days) were significantly different between treatments, (Kruskal Wallace; λ and $v_2$: $p = 1.69e^{-7}$; $v_3$: $p = 1.42e^{-2}$), with *T. cruzi* and *T. rangeli* treatment groups having significantly lower λ and $v_3$ values than the mixed and control groups (KruskalMC, $p < 0.01$, Figure 3.8).

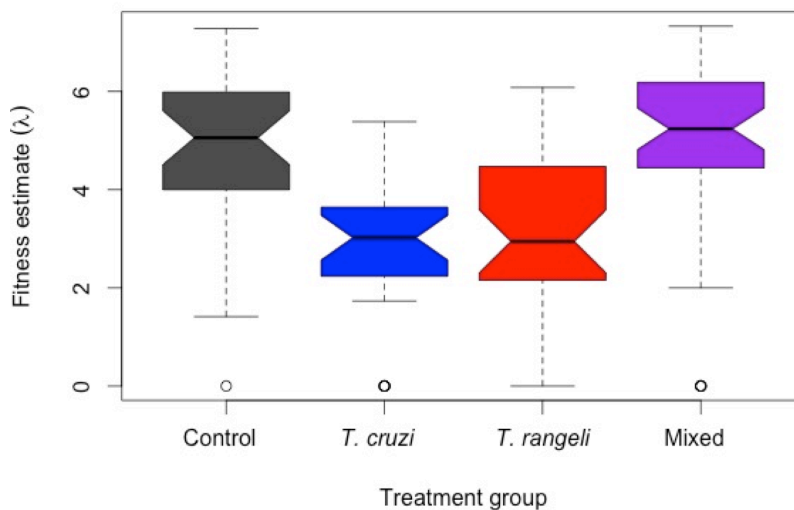



**Figure 3.8. Distribution of fitness estimates in each treatment group. The control and mixed groups had significantly higher fitness estimates than the *T. cruzi* and *T. rangeli* groups.**

The reproductive value at 90 days ($v_3$) was

significantly different between the *T. cruzi* and the mixed group, with *T. cruzi* being lower (KruskalMC, p <0.05). 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 significant (Fisher's Exact Test for Count Data, p = 0.09), 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* samples amplified (18/20); 76.92% of *T. rangeli* samples amplified (20/26); 61.53% of samples from the mixed treatment group amplified *T. cruzi* (16/26); and 84.61% amplified *T. rangeli* (22/26).

### 3.5 Discussion

Co-infections of parasites that differ in virulence are predicted to change the demands put on a host's immune system and available resources [18], with the possible outcomes being: (1) no change: overall virulence corresponds to that of one of the parasites when it infects without the presence of the other parasite; (2) increased virulence: virulence is higher than the more virulent parasite (3) intermediate virulence: virulence falls somewhere in between the virulence of the parasites when infecting alone; and (4) reduction in overall virulence; virulence is reduced to levels lower than when either parasite infects alone. Naturally, these outcomes will be sensitive to variation across different environments, host and parasite genotypes, and parasite infective doses. I hypothesized that *T. cruzi-T. rangeli*

co-infection in *R. prolixus* would lead to option 2, increased virulence, reflected in lower insect fitness outcomes. However, what I observed seemed to be option 4, a reduction in overall virulence. Below, I discuss the possible explanations for these results, and their implications for *T. cruzi* transmission.

*Co-infection: a potential protective effect?*

Cox model results predicted that the treatment groups interacted differently with parasite dose: at lower doses, hazard increased in co-infected insects and decreased in mono-infected insects; at higher doses, the opposite pattern was predicted, and this did not change when controlling for absolute and relative parasites species dose. These results suggest the existence of a threshold parasite dose, below which it is optimal to have a single infection and above which the optimal infection is a co-infection. The very low hazard for the co-infected group at high levels of parasites suggests that higher doses of each species when infecting together may modulate the insect immune system in such a way that it more effectively resists infection by one or both of the parasites [73]. Alternatively, a co-infection may be better tolerated by the immune system than a mono-infection, with the immune system not necessarily fighting the parasites better, but rather being more able to repair harm caused by one or both of the parasites in the presence of the other. Thus, while more data on fitness at different parasite doses are needed to confirm this pattern, I cautiously put forward the idea that co-infected insects could have a higher threshold dose of each parasite species before hazard increases. 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* [74,75], *R. pallescens* [76–78]; and *R. colombiensis* [79], which would support the idea of a co-infection advantage (Figure 3.9).

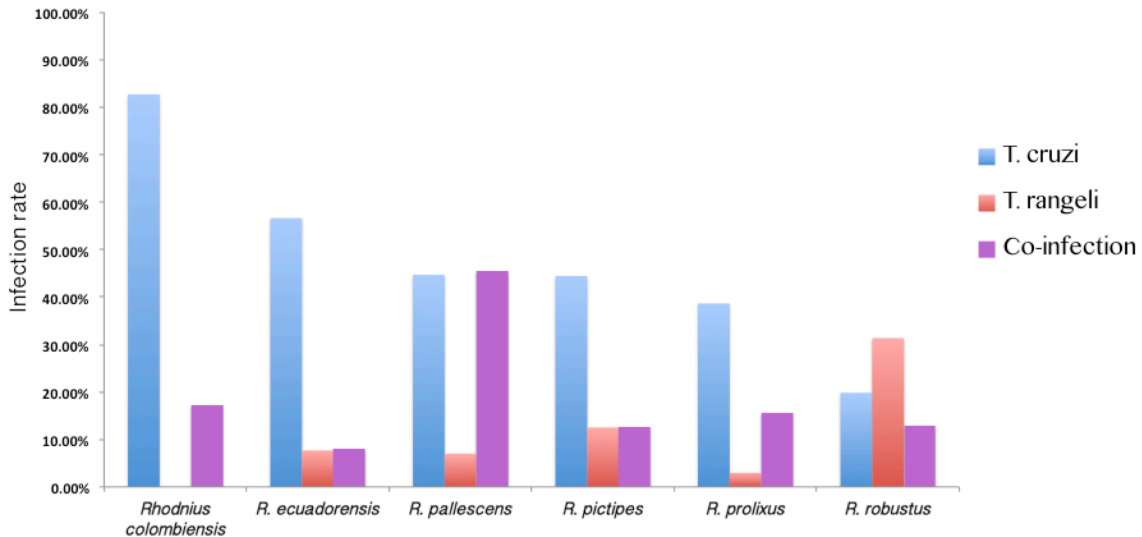



**Figure 3.9. Reported co-infection prevalences in field-caught *Rhodnius* triatomines** [75,76,79,115,116].

*Egg fertility: quality over quantity*

It is noteworthy that while the efficiency of egg production seemed to be affected by parasite treatment, egg fertility was not. It is known that the processes of egg growth and oviposition are controlled separately in *R. prolixus* [80], and that oviposition of badly formed eggs, as observed in *Cimex* species, is rare, even in cases of insect malnutrition[81]. This investment in egg quality over quantity could be a mechanism of insecticide resistance, which has been observed in *T. infestans* eggs [82], and could be one factor that explains residual populations in human homes after insecticidal spraying, if some eggs are not permeated by insecticide and are not eliminated during spraying.

*How much does infective dose matter?*

The dose used in this experiment was within the range of peak parasitemias observed in lab mice and guinea pigs [55–60,83–86], and is a dose commonly used in studies of *T. cruzi*-*T. rangeli*-triatomine interactions [2,47,56,64–68,83,87–94]. There is no publication that provides a rationale for this parasitemia/dose, and it is seems to be far above reported parasitemias of wild animals reported in the literature [30,95], which would presumably be a selective pressure on triatomines in the wild. (Triatomines that eat the most will reproduce optimally, as blood meals are linked with reproductive output, both in terms of energy [81,96] and the hormonal response triggered by a blood meal that cues egg production [97]. As such, bugs that could not tolerate parasites in their blood meals would eat less, and therefore reproduce less; on the other hand, the maintenance of an ability to tolerate unrealistic infective dose sizes could also divert energy from reproduction.) However, studies have shown that (1) the number of parasites in an infective blood meal decreases dramatically upon ingestion due to digestive enzymes, temperature changes and the gut microbiota community[12,98]; (2) the total trypanosome population size and composition (proportion of each form present) within a triatomine fluctuates according to feeding status; significant decreases in parasite numbers can occur within four hours after feeding, by as much as 50% in some parts of the bug [83,86,99]; and (3) *T. cruzi* infective dose does not correlate with the number of parasites excreted [58,100,101]. These studies suggest that the number of parasites ingested is fewer than the number that the insect will face, and that, while it may lead to temporary effect, this should change by the time the insect excretes parasites and takes its next blood meal. This is reflected in our study in the fact that there were no linear relationships between parasite dose and any other numeric

parameter measured (days lived, E value, fertility). Therefore it seems likely that if indeed dose was associated with decreased fitness, it was probably a temporary effect that didn't last throughout the entire experiment, as insects took several uninfected blood meals after their infective meal.

*Why were effects of* T. cruzi *and* T. rangeli *similar?*

*T. rangeli* is considered as fully pathogenic to triatomines, while *T. cruzi* is described as "subpathogenic" [16,38,56,102], meaning it makes the insects more vulnerable to external stress. Considering this orthodoxy, it was unexpected that the fitness of the treatment group infected with *T. cruzi* was not significantly higher than the fitness of the *T. rangeli* treatment group. Although my results from Chapters 1 and 2 suggested that *T. cruzi* pathogenicity is more variable than previously believed, I still expected *T. rangeli*-infected insects to have lower fitness than *T. cruz*-infected bugs. One possible explanation is that insect stage at infection influenced the fitness outcomes, as stage-dependent pathogenicity of *T cruzi* infection has been observed in the triatomine species *Mepraia spinolai* [93] and *Triatoma infestans* (Christine Merks, unpublished, reported in Schaub [38]). In Merks' study *T. cruzi*-infected insects had decreased egg production only if they were infected as $5^{th}$ instars (the stage used in my experiment as well), while insects infected at other stages showed no change.  However, this explanation does not quite hold up when considering the variable results I observed in Chapters 1 and 2; all insects were infected with *T cruzi* as $5^{th}$ instars, but not all treatments experienced a reduction in fitness.

Interestingly, Schaub does not propose infective dose as a potential mechanism behind the decreased egg production observed by Merks, even though they used the same high doses as I did. He proposes just insect stage at infection, as discussed above, and then additionally, the source of the blood used in the infective blood meal, which in his study was sheep blood. In my study, however, insects consumed human blood for the infective meal and chicken blood thereafter, both of which are considered to be acceptable blood meal sources for *R. prolixus* [55,94,103–105].

It has also been observed that *T. cruzi* replication rates with triatomines are positively associated with temperature [101,106], and Fellet *et al.* [88] observed decreased E values in *T. cruzi*-infected *R. prolixus* that were reared at 30°C and then transferred to 25°C after molting into adults compared to control and *T.* cruzi-infected insects kept at 25°C for the entire experiment. However, it is difficult to discern if this difference is related to the temperature itself or the change in temperature that occurred in the middle of the experiment. My insects were reared and maintained in climate conditions within the range recommended by Azambuja and Garcia [107], which also coincide with the climate conditions in areas of Colombia to which the insects are endemic [108,109]. Therefore, if temperature were an underlying factor in *T. cruzi* virulence, it would suggest that *T. cruzi* virulence is the norm and not the exception.

The last possibility, and the one that I believe to be the most likely, is that a mild decrease in fitness lies within the range of possible effects of *T. cruzi* on *R prolixus.* (I refer to the decrease as mild, because, while fitness estimates were lower than the control group, they

still projected population growth.) Considering the high degree of polymorphism found within the *T. cruzi* species*,* it seems possible that the outcome of *T. cruzi* infection in any triatomine species could range from no effect to low virulence, without the presence of an external stressor. (Although it must be mentioned that in free-living populations, 'external stressors' are likely quite frequent.) Moreover, the majority of studies supporting the subpathogenic theory of *T. cruzi* in tratomines have been carried out in the species *T. infestans* [56,60,83–86,94,110]*.* Most studies investigating effect of *T. cruzi* on *R. prolixus* life history have found a mild effect [88,111,112], and effects have also been observed in *Panstrongylus megistus* [113] and *Mepraia spinolai* [93]*.* Thus, it seems possible that the *T. cruzi* virulence I observed could be attributed to factors such as stage at infection, temperature, dose size or triatomine species. However, it seems equally likely that it could also have been a commonplace outcome of the infection of a heterogeneous parasite interacting with the intrinsic properties and processes of its host.

**3.6 Summary**

Virulence is a variable phenomenon affected by parasite diversity and the within host process of each infection, given the condition and type of individual host. This is an inherent limitation of any experiment of this kind, yet also captures inherent complexity of natural host-parasite systems. As such, extrapolation of effects observed in the laboratory to their meaning in the natural system must be carried out cautiously. That said, I observed a threshold dose size below which a single species infection is optimal and above which a co-infection is optimal to maximize insect fitness. This suggests that *T. rangeli*-*T. cruzi* co-infection potentially ameliorates the negative effects of single infections. More research is

needed on this theme, but my observations of *T. cruzi-T. rangeli* co-infection in its invertebrate host suggest that co-infection could increase the transmission potential of both parasites, transforming what I had called competition into an interaction more closely resembling symbiosis. In the next chapter, I will take a closer look at this question by isolating components of the co-infection process (dose, timing, and order) in order to characterize the host fitness outcomes associated with each infection component.

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# Chapter 4

Infection event characteristics that influence *R. prolixus* survival

## 4.1 Introduction

Every interaction between species occurs in a noisy, heterogeneous and ever changing environment that presents countless contexts that shape the interaction over time and space[1]. For *T. cruzi*-host interactions there is an additional component, because contact must first be made between the new host and an already infected host. In vector-borne Chagas disease, this is the contact made between the invertebrate and vertebrate hosts, (although *T. cruzi* is also spread through direct and indirect vertebrate-vertebrate contact, as in congenital, oral and tranfusional Chagas disease[2,3]). As such, *T. cruzi*-host interactions are influenced not just by intrinsic properties of the parasite strain and its current host, but also by the composition and number of infected hosts in the environment, and the characteristics of each individual infection. Variation in host infections at a population level, will affect the likelihood of the occurrence of the new host-parasite interaction, while variations in individual infections will determine the infective dose, and the parasite species composition of that infective dose.

Therefore, all *T. cruzi* infections of invertebrate hosts are not created equal; they begin with varying infective doses and co-infection statuses. In effect, each new infection event is characterized by the "baggage" of the prior host infection. However, does this matter? In this chapter I investigate this question in *Rhodnius prolixus* infected with *T. cruzi* and *T. rangeli.* I investigate 3 variations in infection events that were controlled for in my other

experiments: *T. rangeli* infective dose, co-infection order, and co-infection timing. My aim was to identify potential characteristics of the infection event that influence vector survival in order to better understand the contexts that influence the outcomes of *T. cruzi* and *T. rangeli* infection on *R. prolixus* at an individual and population level.

*Research questions and hypotheses*

I investigated the survival and development of *R. prolixus* mono- and co-infected with *T. cruzi* and *T. rangeli*. Insect treatment groups were set up to investigate the effect of the following variations in the infection event: (1) *T. rangeli* dose size; (2) the timing of *T. cruzi*-*T. rangeli* co-infection (staggered or simultaneous); and (3) the order of the *T. cruzi*-*T. rangeli* co-infection (*T. cruzi* first or *T. rangeli* first). I hypothesized that (a) a higher *T. rangeli* dose will have a negative effect on survival; (b) a simultaneous co-infection would have a more detrimental effect on survival than a staggered co-infection; and (c) that infection with *T. cruzi* first would lessen the detrimental effects of a subsequent *T. rangeli* infection.

## 4.2 Materials & Methods

*Experimental design*

I infected 185 *R. prolixus* 5$^{th}$ instar nymphs with one of eight different randomly assigned parasite treatments (Table 4.1) designed to investigate the effect of *T. rangeli* dose, co-infection timing (simultaneous vs. delayed, i.e., 32-34 days between each infection), and co-infection order when delayed (*T. cruzi* first vs. *T. rangeli* first). 23 additional insects were fed uninfected blood and used as controls, for a total of 208 insects used in the experiment.

Following infection, I recorded the survival of each insect for up to 89 days.

*Parasites and insects*

I prepared parasites and insects as described in chapters 1-3. Parasite strains used were the "Gal61" *T. cruzi* strain and the "444" *T. rangeli* strain. 444 was originally isolated from an *R. prolixus* collected in the Boyaca department of Colombia, and it belongs to the KP1(+) lineage, which is associated with the prolixus complex of *Rhodnius*[4]. This strain was provided by Professor Gustavo Vallejo of the University of Tolima, where the strain was maintained as described in Chapter 3.

| Treatment name | Parasites | *T. rangeli dose* | Co-infection timing | N |
|---|---|---|---|---|
| *T. cruzi* | *T. cruzi* | N/A | N/A | 22 |
| *T. rangeli1* | *T. rangeli* | low | N/A | 20 |
| *T. rangeli2* | *T. rangeli* | normal | N/A | 20 |
| Mixed1 | *T. cruzi + T. rangeli* | low | simultaneous | 26 |
| Mixed2 | *T. cruzi + T. rangeli* | normal | simultaneous | 31 |
| CrRa | *T. cruzi + T. rangeli* | normal | delayed | 22 |
| Ra1Cr | *T. rangeli + T. cruzi* | normal | delayed | 22 |
| Ra2Cr | *T. rangeli + T. cruzi* | low | delayed | 22 |
| Control | Controls | N/A | N/A | 23 |

**Table 4.1. Parasite treatment groups. "Treatment name" is the abbreviation used for the groups throughout the chapter and in figure legends.**

*Insect infection*

I infected the *T. cruzi*, *T. rangeli2* and Mixed2 treatment groups as described in chapters 1-3, with infective blood meals containing a concentration of parasites between 3.3-3.5 x $10^6$ per ml, which I will refer to as the "normal" dose.

*T. rangeli low dose*

Insects infected with a low dose of *T. rangeli* (*T. rangeli1,* Mixed1 and Ra1Cr*)* consumed blood with a concentration of *T. rangeli* parasites about ¼ that of the normal parasite dose. In the *T. rangeli1* mono-infection and the Ra1Cr infection, this concentration was an estimated 833,000 *T. rangeli* parasites per ml of blood. In the simultaneous co-infection (Mixed1), the parasite concentration was an estimated 1,667,000 *T. cruzi* parasites, and 417,000 *T. rangeli* parasites per ml of blood.

*Timing and order of infection*

Insects in the CrRa treatment group were infected with *T. cruzi* at the same time as insects mono-infected with *T. cruzi*, and 32 days later were infected with *T. rangeli* (at the normal dose). Insects in the Ra1Cr and Ra2Cr treatments were infected with *T. rangeli* with the *T. rangeli1* and *T. rangeli2* groups respectively, and then infected with *T. cruzi* 32 and 34 days later, respectively.

*Infection confirmation*

I confirmed infections in a subset of the insects by direct microscopic observations of insect biomaterials. I extracted DNA from the insects as described in chapters 1-3. Due to time limitations, PCRs were run with a small subset of the samples with *T. cruzi*-specific primers using the primer pair TcZ1/2.

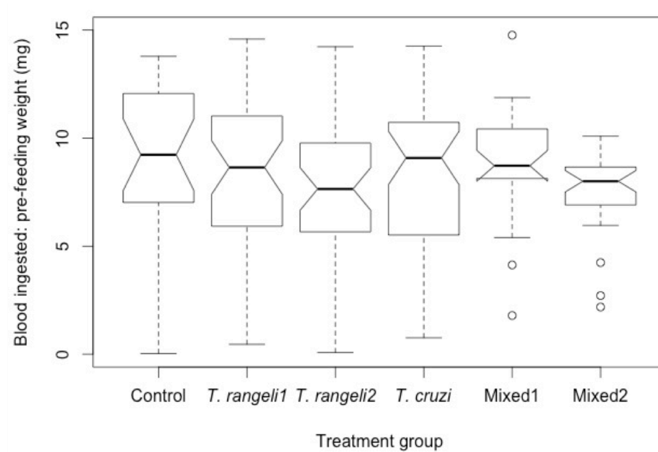
*Statistical Analyses*

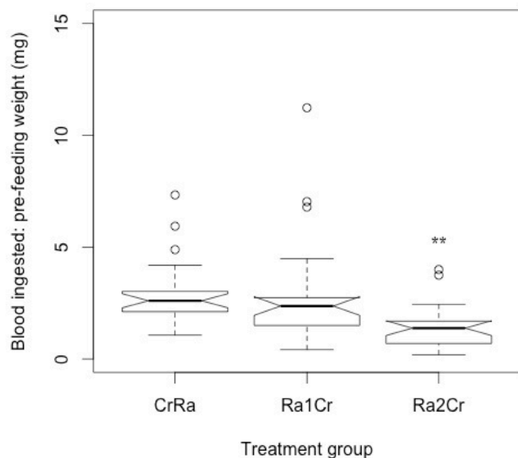
I carried out statistical tests and survival analyses out as described in chapters 1-3. Survival data did not meet the Cox proportional hazards assumption, so I created dummy "early" and "late" groups for each treatment, with early corresponding to before 32-34 days and "late" corresponding to afterward, and ran them in an extended Cox model for right-censored, left-truncated data. 32-34 days corresponded to the date of the second infection of the delayed mixed infections. As mentioned above, CrRa and Ra1Cr were infected with the second infection 32 days after the first infection, and therefore were split at 32 days in the model; Ra2Cr was infected with the second infection 34 days after the first infection, and thus split at 34 days; all other treatments were split at 33 days in the model. The model was first run with the same variations as described in chapter 3: first, with the control group as the comparative group, and then with just the parasite treatments. However, the model with the control group did not fit the PH assumption, so my analysis focuses just on the models examining the main and interaction effects of parasite treatment and quantity of parasites ingested, with the Mixed1 group was used as the comparative group, as its survival was very similar to the control group. Finally, as in Chapter 3, the mixed groups were run twice, once with the absolute number of parasites ingested and once with the relative parasite species number ingested. However, the PH assumption was met only when the model was run with the relative number of parasites ingested. For the delayed group, this was the number of parasites ingested in the second infection only.

## 4.3 Results

*Blood and parasites ingested*

Due to the large number of treatment groups, here I will report the differences between treatments that are relevant to my research questions. A full list of significant differences related to the components of the infective blood meal, (volume of blood ingested, parasites ingested, and the volume of blood and parasites ingested per mg of insect biomass), are found in Appendix 4.




**Figures 4.1a and 4.1b. Distributions of the ratios of the volume of blood ingested to the pre-feeding weight in each treatment group. 4.1a (top) is the distribution of ratios in the first infective feeding. 4.1b (bottom) are the ratios for the second infective feeding, meaning the feeding where insects were infected with the second parasite. A point in the Mixed2 group of 29.06 is not shown.**

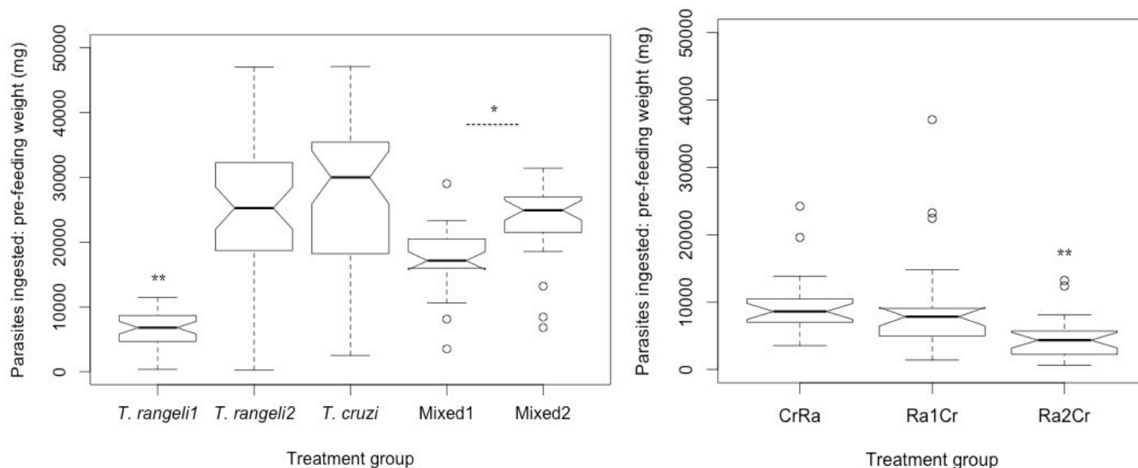
*Blood ingested and blood:weight ratio*

The volume of blood in the infective meals was significantly different between the first and second feedings, as were the blood:weight ratios (Kruskal Wallace, blood, $p = 3.909e-14$; blood:weight: $p < 2.2e-16$, Figures 4.1a and 4.1b). The pre-feeding weights were significantly higher in the groups that took a

second infective meal (Ra1Cr, Ra2Cr and CrRa). It should be mentioned, that most of those insects had completed their imaginal molt into the adult stage by the second meal, and adults are larger than nymphs, but tend to eat less [5,6].

*Parasites ingested and parasite:weight ratio*

Both the estimated number of parasites ingested and the number of parasites ingested per mg of insect biomass were different between treatments (Kruskal Wallis, p < 2.2e-16 for both,). In the first infective meal, the *T. rangeli*1 treatment, which was fed with a lower dose of *T. rangeli,* consumed significantly fewer parasites in total and per mg of biomass than all other groups (KruskalMC, p < 0.01, Figure 4.2a). The Mixed 1 group, which was fed with a




**Figures 4.2a and b. Distribution of the ratios of the estimated number of parasites ingested to the pre-feeding weight in each treatment group. 4.2a (left) is the distribution of ratios in the first infective feeding. 4.2b (right) are the ratios for the second infective feeding. A point in the Mixed2 group of 90,482 parasites is not shown. *p < 0.05; **p < 0.01.**

lower concentration of *T. rangeli* and a normal concentration of *T. cruzi* consumed significantly fewer parasites in total and per mg of biomass than the Mixed 2 group (KruskalMC, p < 0.05), which was fed blood with a normal concentration of both parasites.

In the second infective meal, there were no differences between groups in the estimated number of parasites ingested, but there was a difference in the number of parasites ingested per mg of insect biomass, (Kruskal Wallis, p = 2.48e-04, Figure 4.2b), with the Ra2Cr group ingesting significantly fewer parasites (*T. cruzi* only) per mg of biomass than the other groups (KruskalMC, p < 0.01).

*Infection confirmation*

| Treatment | DMO | PCR | Both | Total |
|---|---|---|---|---|
| *T. cruzi* | 4 | 8 | 2 | 10 |
| *T. rangeli1* | 2 | NA | NA | 2 |
| *T. rangeli2* | 1 | NA | NA | 1 |
| Mixed1 | 4 | 7 | 1 | 10 |
| Mixed2 | 13 | 7 | 3 | 17 |
| CrRa | 5 | 6 | 1 | 10 |
| Ra1Cr | 3 | 5 | 2 | 6 |
| Ra2Cr | 3 | 10 | 0 | 13 |

**Table 4.2. Infection confirmations.**

Infections were confirmed in just a small subset of the insects through direct microscopic observation (DMO) and/or PCR (Table 4.2). All *T. rangeli* infections were confirmed only by DMO. 100% of samples run in PCRs were positive.

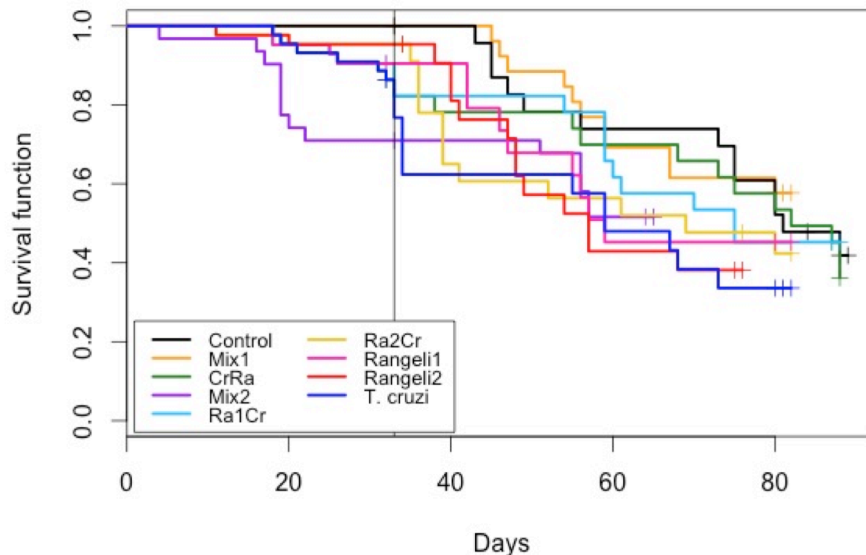



**Figure 4.3. K-M survival curves. Y axis displays cumulative survival and x axis displays days post-infection. The vertical line indicates 33 days post-infection.**

*Survival*

There was no difference between treatment groups in survival function (Figure 4.3).

The final Cox model included the main effects of the

following treatments, with the earlier time period (before 32-34 days) denoted with an "E" at the end of the treatment name, and the later time period (after 32-34 days) denoted with an "L." This model was the strongest model that fit the PH assumption. Complete model outputs are in Appendix 4.

- *T. cruzi* L
- *T. rangeli1* L
- *T. rangeli2* E
- *T. rangeli*2 L
- Mixed2 E
- Ra1Cr L
- Ra2Cr L
- CrRa L

I also included main effects of parasites ingested and interaction effects of parasites ingested with the *T. rangeli2* (E and L), Mixed2E, Ra1CrL, and Ra2CrL. Results are presented below, separated by research question.

*Treatment main effects:* T. rangeli *dose in single and mixed infections*

As a reminder, the "*T. rangeli1"* and "Mixed1" treatments were infected with the low dose of *T. rangeli*, while the "*T. rangeli2"* and "Mixed2" treatments were infected with the "normal" dose, which is comparable to that used in my other experiments.

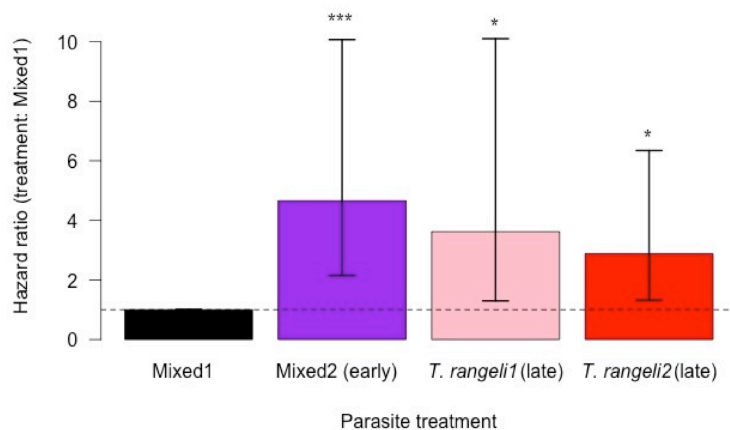



**Figure 4.4. Hazard ratios for *T. rangeli* dose treatments. Ratios are the hazard of a given treatment to the Mixed1 group hazard, shown in black. The dotted horizontal line is to indicate where a hazard ratio of 1 is. *p < 0.05; ***p < 0.001**

Treatment groups infected with just *T. rangeli* had a higher hazard than the Mixed1 group after 33 days, at both the low and normal doses (*T.* rangeli1: $e^{\beta} = 3.58$, p = 0.01; *T. rangeli*2: $e^{\beta} = 2.80$ p = 0.01, Figure 4.4). The Mixed2 group had a significantly higher hazard ratio before 33 days (Mixed2 (early): $e^{\beta} = 4.64$, p = 9.38e-05, Figure 4.4).

*Main effects of treatment: co- infection timing and order at low and normal* T. rangeli *doses*

Insects infected with the low dose of *T. rangeli* in simultaneous and delayed co-infections did not have an increased hazard ratio (Figure 4.5). *R. prolixus* infected simultaneously with *T. cruzi* and *T. rangeli* at the normal dose had an increased hazard ratio only in the early period (Figure 4.4). Insects infected first with the normal dose of *T. rangeli* and later infected with *T. cruzi* (Ra2Cr) had a significantly higher hazard ratio after the *T. cruzi* infection ($e^{\beta} = 5.45$, p = 3.05e-4, Figure 4.5), but insects infected in the opposite order (CrRa) did not have a higher hazard ratio after the second infection.

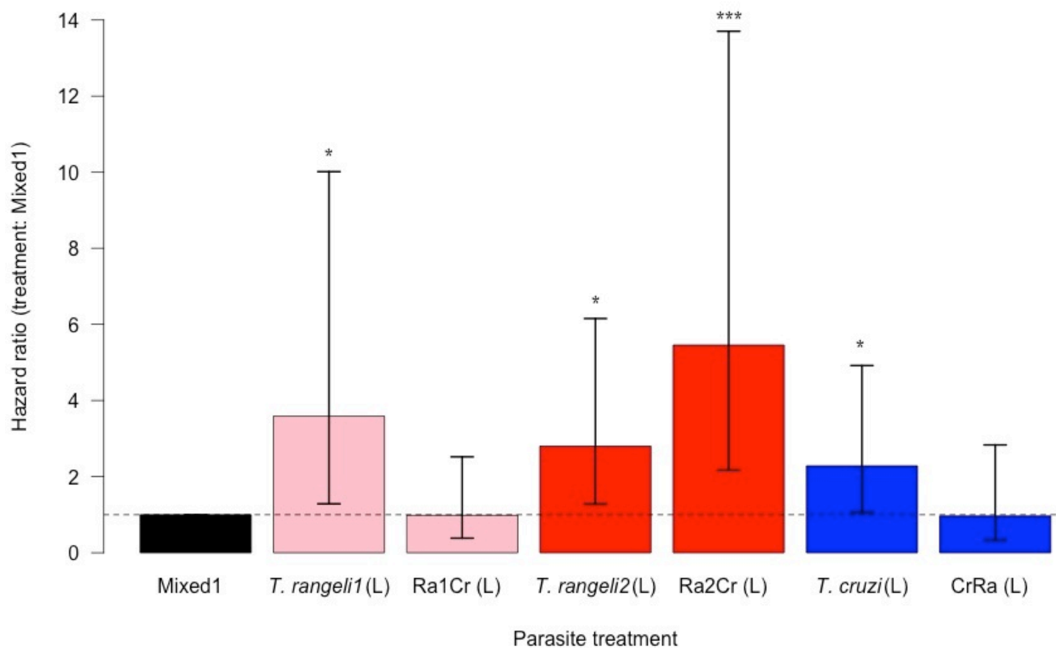



**Figure 4.5. Hazard ratios of single infections and related delayed co-infections after the 2nd infection (L = Late). *p <0.05; ***p < 0.001.**

*Single vs. mixed infection:* T. cruzi *variation*

*R.* prolixus infected with T*. cruzi* alone had an increased hazard ratio after 32 days ($e^{\beta}$ = 2.28, p = 0.03, Figure 4.5), while simultaneous co-infection with a low dose of *T. rangeli* or a delayed infection with a higher dose of *T. rangeli* following *T. cruzi* infection did not significantly change the hazard ratio.

*Interaction of treatment with parasite dose*

Three mixed infection treatment groups significantly interacted with parasite dose: Mixed1, Mixed2 and Rangeli1Cruzi. Hazard increased with dose for the group Mixed1 group ($e^{\beta}$ = 1.42, p = 0.01), while it decreased with dose for the other groups (Mixed2: $e^{\beta}$ = -1.79, p = 1.70e-04; Ra1Cr: $e^{\beta}$ = -0.83, p = 0.01). For Ra1Cr, this represents the dose of *T. cruzi* only.

### 4.4 Discussion

Some clear patterns emerged in this experiment in relation to the effects of single vs. mixed infections, infection timing, order and *T. rangeli* dose (Table 4.3).

| | | Single Infection | *Mixed infections* Simultaneous | *T. cruzi* 1st | *T. rangeli* 1st |
|---|---|---|---|---|---|
| *T. rangeli dose* | **low** | + | = | na | = |
| | **high** | + | + | = | + |

**Table 4.3. Patterns revealed in hazards analysis relative to the Mixed1 group, which had similar survival to the control group. A plus sign indicates a significantly higher hazard than the Mixed1 group and an equal sign signifies that there was no significant difference.**

T. cruzi *and* T. rangeli *(low dose) mono-infections: better to have a co-infection*

*T. rangeli* mono-infection increased hazard over time regardless of dose, suggesting that *T. rangeli* dose does not influence survival when infecting alone (alone, meaning without *T. cruzi*). This result was unexpected, as I had predicted that dose would have an effect on insect survival. However, *T. rangeli* infections at a low dose did not have increased hazard in co-infections with *T. cruzi*, in both the simultaneous and the delayed low dose co-infections. This pattern also occurred with the *T. cruzi* mono-infection; the hazard increased after 32 days for the mono-infection, but not for the delayed co-infection. This suggests that an advantage could be conferred by *T. cruzi*-*T. rangeli* co-infection, as I observed in Chapter 3, but here I also observed that it was independent of infection timing and order when the *T. rangeli* dose was low. It is thought that *T. rangeli* circulates in low parasitemias in its mammal hosts [7–9], and thus the low dose in this experiment is possibly more representative of the doses received by the insects in the wild. In fact, for many years after its discovery, researchers struggled to generate a high parasitemia in

experimentally infected laboratory animals [8]. Thus, co-infection with *T. cruzi* could be one way that virulent (for the insect) strains of *T. rangeli* and also *T. cruzi* are maintained in natural populations. Alizon [10] predicted that when the overall virulence of a co-infection is lower than the virulence of the individual parasites, it could lead to persistence of virulence on evolutionary timescales. This has been observed in plant populations [11], helminth-microparasite co-infections [12,13], and between different bacteria species [14].

T. rangeli *at high doses: better to have a* T. cruzi *background infection*

My results suggest that at higher *T. rangeli* doses, infection order and timing are important. As mentioned above, insects with an established *T. cruzi* infection did not experience an increased hazard after the *T. rangeli* infection, which was at the normal *T. rangeli* dose. This group was the only treatment group with a high *T. rangeli* infection dose that did not have an increased hazard ratio at some point in the experiment, further supporting the idea that *T. cruzi* co-infection may protect the insect from harmful effects of *T. rangeli.*

*Hazard timing and* T. rangeli

In the simultaneous co-infection, hazard increased right after infection and then stabilized after the next blood meal. In single infections and also in delayed co-infections, hazard did not increase until after the next blood meal. This suggests that the hazard is more intense in a simultaneous co-infection, but also possibly just temporary until an equilibrium is reached between the two infections.

*Comparing across experiments*

The results from this experiment support the idea that a *T. cruzi*-*T. rangeli* co-infection confers an advantage to the insect over a mono-infection, as observed in Chapter 3. In Chapter 3, I did not observe the decrease in survival early in the experiment, which could be attributed to a *T. rangeli* strain difference, as I did use a different strain of *T. rangeli* in that experiment. However, in spite of the decrease in survival early on in the mixed group in this experiment, its survival function is not significantly different than the control group, as in Chapter 3. Additionally, there were other differences between the two experiments that could partially explain this difference. Most importantly, all the insects in the experiment presented in Chapter 3 were mated females, while insects in this experiment were virgins of both sexes. These differences would not affect anything in the early part of the experiment, up to the first blood meal post-infection, however, and further experiments should be carried out using mated insects.

As discussed in Chapter 3, the effect of insect stage at infection could play a role in the survival outcomes I observed, especially with the *T. cruzi* infections. An obvious missing element in this experiment was a treatment investigating *T. cruzi* dose. Unfortunately, due to limitations in the number of insects available, I could not include any more treatment groups in the experiment.

Finally, a limitation of this experiment is that few insects were checked for infection. However, as insects were checked randomly for infection, and all samples run in the PCR

did come out positive, I do not think the presence of uninfected insects influenced the results.

### 4.5 Summary

As with all biological interactions, *T. cruzi*-host infections begin under an array of possible conditions. In this experiment, I observed that some of these conditions do influence the survival outcome for the invertebrate host. Interestingly, these external conditions seemed to be entangled and dependent on one another; it was almost always better to have a co-infection at lower *T. rangeli* doses. However, at high *T. rangeli* doses, it was better to be infected with *T. cruzi* first, suggesting an interaction between dose, order and timing. Thus, although in this chapter, I sought to understand the influence of infective dose, co-infection order, and co-infection timing on the invertebrate host, an unexpected and yet remarkable result was the revelation of a connection between tangible factors (dose) and intangible factors (time and order) to an outcome on another level, host fitness.

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# Chapter 5

## What are the "true" effects of *T. cruzi* and *T. rangeli*?

### 5.1 Introduction

As I have alluded to in the preceding chapters, the orthodox view in the study of triatomine-trypanosome interactions is that *T. rangeli* is pathogenic to its vector and *T. cruzi* is not. For *T. rangeli,* this idea is supported by an overwhelming number of studies on the triatomine species *Rhodnius prolixus*, which is the laboratory model for this system [1]. For *T. cruzi,* the majority of studies were carried out with the triatomine species *Triatoma infestans* [2–9].

My results presented in chapters 1-4 do not support this view of triatomine-trypanosome interactions, and suggest that the outcome of trypanosome infection in triatomines lies on a spectrum ranging from no effect to highly negative, depending on properties unique to each insect-parasite pair.

Here, I review the published studies of these vector-parasite relationships that do and do not support the orthodoxy. The effect of *T. cruzi* on triatomines was reviewed by Schaub [10], and so the majority of my review is dedicated to *T. rangeli.*

### 5.2 Background

*T. rangeli* was discovered in 1920 by Tejera [11], who found it infecting a triatomine bug alongside *T. cruzi*. After determining that *T. rangeli* was not a human disease agent [12–14],

scientific attention turned toward the negative effects of *T. rangeli* on its invertebrate host, and a large volume of studies subsequently accumulated on the subject.

Identifying vector control strategies is a critical challenge in Chagas disease prevention efforts [15–17], and the idea that *T. rangeli* is pathogenic to triatomine bugs rapidly gained traction due to the possibility that it could be used as a potential biological control agent to interupt vector-borne *T. cruzi* transmission. However, although some researchers proposed that *T. rangeli* could be a limiting factor for triatomine populations [18,19], there are conflicting points of view. Professor Gunter Schaub, arguably one of the leading experts in triatomine-trypanosome interactions, writes in his review of trypanosome-triatomine interactions that even if *T. rangeli* slightly regulates triatomine populations, it is not pathogenic enough to be used as a biological control agent [20].

There is no empirical evidence that *T. rangeli* negatively affects bugs outside of the laboratory setting, and *T. rangeli* has been found infecting many different species of field-caught triatomines of all developmental stages, with no mention of apparent sickness or augmented death [21–34]. Marinkelle [9] reported an observed increase in mortality in field-caught *R. prolixus* infected with *T. rangeli,* but after careful review of the results presented in his paper, this conclusion is not supported by the data presented in the paper (see section 5.3 for a detailed analysis). Furthermore, although initial studies reported strong negative effects of *T. rangeli* on triatomine survival, experimental concerns (ranging from highly inbred bugs to environmental controls) call the validity and generality of these effects into question.

Here, I review the published studies examining the effect of *T. rangeli* on triatomine bugs. I identify potentially confounding factors, which I address under the heading of four general problems I identified (1) inconsistencies in experimental design and presentation of results; (2) limited triatomine species diversity and/or *T. rangeli* lineages; (3) premature declarations of pathogenicity and (4) sparse and conflicting studies. As in Chapter 3, I refer to pathogenicity as defined Shapiro[35] for invertebrate pathology, i.e. the all or nothing ability to produce disease. I use the Read [36] definition of virulence, the quantifiable decrease in host fitness after infection.

### 5.3 Inconsistencies in experimental design and presentation of results

*Laboratory experiments*

The first published reports of *T. rangeli* pathogenicity in triatomines were from three laboratory experiments [37–40] carried out between 1956- 1965, and one study of field-caught bugs in 1968 [18]. These were important and pioneering works on the effect of *T. rangeli* on triatomines, but unfortunately they also have several fundamental problems. The laboratory studies observed increased mortality in *R. prolixus* that were fed a *T. rangeli*-infected blood meal shortly after emerging as first instars. All studies used the "El Tocuyo" parasite strain and insects from highly inbred laboratory colonies [37,41,42]. The insects used by Grewal in 1967 [38,39] and Tobie in 1965 [40] were strains from colonies founded 43 and 53 years prior to their experiments, respectively [1,43], and were thus highly inbred. Grewal [39] did not include any control data in the presentation of his results, rendering the interval validity of the experimental results questionable. Each study

used a different infective dose of *T. rangeli*, with the study using the highest dose reporting the highest mortality [37]. The external conditions under which Gómez [37] reared his insects colonies and carried out his experiments were potentially stressful for the insects: colonies were kept at 21° +/- 4°C and insects used in the experiment were transferred into jars kept in a 30° +/- 0.5° water bath. This move was accompanied by a 40% jump in humidity. The recommended temperature range for rearing triatomines is 27-30°C [44], and temperatures below 22°C can lead to adverse effects similar to those observed in insects infected with *T. rangeli* (deformities, molt problems and increased mortality; 29). The cool temperatures combined with the sudden change in temperature and humidity could have played a role in the death rates observed by Gómez, who also observed higher mortality in his control groups than Tobie (36% mortality vs. 18% mortality; [40]).

Other confounding factors in experimental design in later studies include insects that were periodically anesthetized for colony maintenance [45] and problems related to infection route. Two studies reported augmented negative effects in hemocoelically inoculated insects [45,46], that were not observed in orally infected insects in the same experiment, suggesting problems stemming from the parasite injection procedure. Additionally, many studies that employed the oral infection technique did not take into account the quantity of blood consumed by each insect in the infective meal [19,37,39,40,47]. Complete engorgement is required to initiate and execute molting in triatomines [48], and thus it is possible that molting problems could have been associated with small blood meals instead of or in addition to *T. rangeli* infection. Tobie [49] observed mortality in several uninfected *R. prolixus* due to difficulties in feeding and molting, and she also suggests that these

symptoms are not always due to *T. rangeli* pathogenicity. If this were the case, and *T. rangeli* caused pathogenicity only when the insect did not take a complete blood meal, it should be considered as sub-pathogenic, like *T. cruzi* [10], meaning it is pathogenic only under suboptimal conditions.

*Field experiments*

The study of field caught bugs presents conclusions that are not consistent with the results presented in the paper. In fact, these conclusions are so strange that I would not include this study in the review but for the fact that it is still very often cited and also the only published study to date looking at mortality in *T. rangeli*-infected, field-caught bugs [18]. In this paper, published in 1968, the author reports 87% insect mortality in a group of field-caught *R. prolixus* over the course of 6 weeks, and implies that the mortality rate was due to *T. rangeli* infection. However, of the insects included in this 87%, flagellates were found in 8.7% of them. The other 79.3% were assumed by the author to be infected with *T. rangeli* only because they had died. Moreover, the majority of parasites were found in insect feces, where *T. rangeli* is often confused with *T. cruzi,* as it is *T. cruzi* that is transmitted with triatomine feces; *T. rangeli* is transmitted in triatomine saliva, and thus its presence in triatomine feces is incidental. Out of the remaining 13% of insects (those that survived and thus not included in the 87% mortality), 10.1% of them were also found to be infected, meaning that a higher percentage of surviving insects were infected than those that died, strongly suggesting that infection rate was not associated with mortality.

*Summary*

The early studies of *T. rangeli* and triatomines suggested that *T. rangeli* could be pathogenic to the triatomine species *R. prolixus.* However, more studies with different species of triatomines and improved experimental design were needed to confirm this result and test if it is applicable to other triatomine species. Fortunately, a series of experiments were undertaken several years after those first findings, all of which used controls, paid attention to environmental conditions, and reported the data and results coherently. These studies observed decreased survival in *R. prolixus*, *R. robustus* and *R. neglectus* when experimentally infected with *T. rangeli* [47,50], but not in *R. pictipes* and *R. nasustus* infected with *T. rangeli* [50,51]. These studies lend support to the idea that *T. rangeli* is pathogenic to laboratory *R. prolixus* populations, and provided evidence that it may also negatively affect *R. robustus* and *R. neglectus*. Additionally, these studies importantly suggested that *T. rangeli* may not be pathogenic to every one of its vector species. However, more studies like these are needed with other *Rhodnius* species, as detailed below, and additionally to test the influence of variables such as insect stage and parasite strain on the outcome of *T. rangeli* infection.

### 5.4 Few investigations of different triatomine species and *T. rangeli* lineages

14 out of the 18 (78%) publications I reviewed on *T. rangeli*- triatomine interactions were carried out with the same triatomine species, *R. prolixus* (Table 5.1). Two studies have been carried out on *R. robustus*, and one study has been carried out on *R. nasustus, R. neglectus,* and *R. pictipes.* 15 of the 17 studies on *R. prolixus* were carried out with insects from laboratory colonies, which are commonly inbred.

| Triatomine species | Aspect studied | Stage infected | Infection route | Pathogenic? | Citation | Notes |
|---|---|---|---|---|---|---|
| *R. prolixus* | survival | 1st instar | Oral | Yes | [37,38] | No control data reported |
| *R. prolixus* | survival | 1st instar | Oral | Yes | [39] | |
| *R. prolixus* | survival | Adults | Oral | No | [39] | |
| *R. prolixus* | survival | 1st instar | Oral | Yes | [36] | Climate problems |
| *R. prolixus* | survival | 3rd instar | Oral | Yes | [36] | Dose-dependent |
| *R. prolixus* | survival | Field-caught | N/A | No* | [18] | Results do not agree with conclusions |
| *R. prolixus* | survival | 4th, 5th, adult | Both | Yes | [44] | |
| *R. prolixus* | survival | 1st instar | Oral | Yes | [46] | |
| *R. prolixus* | survival | All stages | Oral | Yes | [19] | |
| *R. prolixus* | survival | 5th instar | Both | Yes | [57] | Only in intracoelomatic infections |
| *R. prolixus* | survival | 5th instar | Oral | Yes | Ch3,4 | This thesis |
| *R. robustus* | survival | 1st instar | Oral | Yes | [46] | |
| *R. nasustus* | survival | 1st instar | Oral | No | [49] | |
| *R. neglectus* | survival | 1st instar | Oral | Yes | [49] | |
| *R. pictipes* | survival | 1st instar | Oral | No | [49] | |
| *R. prolixus* | reproduction | 5th, adult | Both | Yes | [44] | |
| *R. prolixus* | reproduction | 3rd instar | Both | Yes | [59] | |
| *R. prolixus* | feeding behavior | Field-caught | N/A | Yes | [55] | |
| *R. prolixus* | feeding behavior | 4th, 5th ,adult | NS | No* | [56] | Sample size of 7 insects |
| *R. robustus* | feeding behavior | 4th, 5th ,adult | NS | No* | [56] | Sample size of 4 insects |
| *R. prolixus* | excretion | 5th and adult | Both | Yes | [44] | More severe in females & intracoelomatic infections |
| *R. prolixus* | excretion | 4th instar | intra-coelomatic | No | [52] | |
| *R. neglectus* | survival | 4th instar | Oral | ** | [53] | |
| *Panstrongylus megistus* | survival | 4th instar | Oral | ** | [63] | |

| | | | | | |
|---|---|---|---|---|---|
| *Triatoma braziliensis* | survival | $4^{th}$ instar | Oral | ** | [53] |
| *T. infestans* | survival | $4^{th}$ instar | Oral | ** | [53] |

**Table 5.1 Summary of the studies reviewed. *Reported as pathogenic in original paper, but does not fit with the definition of pathogenicity used here. ** Due to the absence of sample size and overall mortality data in the publication it was not possible to determine if there was a pathogenic effect. For this reason, the study was excluded from my review.**

Investigating just *T. rangeli* in *R.* prolixus is not only limiting in the triatomine species, but also in the *T. rangeli* lineage. *T. rangeli* is classified into two lineages, KP1(+) and KP1(-)[54,55]. These lineages are co-adapted with species of the triatomine genus *Rhodnius* [54], and can invade the salivary glands of only those species to which they are adapted. This means that most studies of *T. rangeli*-triatomine interactions have been on just one *T. rangeli* lineage. Moreover, studies carried before these lineages were published (around the year 2002), could have used mismatched triatomine species and *T. rangeli* lineages. Indeed, several older studies report that their laboratory *T. rangeli* strain rarely invaded insect hemolymph [37,45,49], and Tobie [49] noticed that laboratory strains from Panamá, where the main vector is *R. pallescens*, which is in the KP1(-) group, were not able to invade the salivary glands of *R. prolixus*, which belongs to the KP1(+) group.

There is currently just one published study (and corresponding thesis) investigating *Rhodnius*-*T. rangeli* interactions using the KP1(-) lineage (Table 5.1). The authors found no significant difference in survival between *T. rangeli*-infected insects and control insects in two out of three triatomine species investigated [50,51], leading me to ask if there is a difference in the effect of *T. rangeli* KP1(-) on its triatomine hosts. If KP1(-) is less virulent and/or pathogenic, it could be another reason (in addition to co-infection with *T.* cruzi) why high prevalences of *T. rangeli* infection and *T. cruzi*-*T. rangeli* co-infections are found

in *R. pallescens* in parts of the Panamá around the Panamá Canal [23,56,57]. Thus, further studies of *T. rangeli* KP1(-) infection in different species of *Rhodnius* could be beneficial to the study of Chagas disease transmission dynamics in regions such as the Panamá Canal area, where the main Chagas disease vector is a *Rhodnius* species of the KP1(-) group.

### 5.5 Premature declarations of pathogenicity

*Feeding behavior*

Alterations in feeding behavior have been observed in several studies of triatomines infected with *T. rangeli* [50,51,58,59]. However, this does not necessarily produce disease and thus by definition, is not pathogenicity. *T. rangeli*-infected insects would need to experience effects such as starvation or the inability to consume enough blood to produce fertile eggs. Slight feeding behavior changes such as increased probing and feeding time increases the chance of producing host irritation and getting swatted off or predated on, and smaller blood meals may delay molting, and in turn, delay time until the insect is reproductively mature. However, this is not necessarily disease, nor definitive evidence of *T.* rangeli pathogenicity. Definitive evidence would be proof of a connection between alterations in feeding behavior and decreased fitness.

### 5.6. Sparse and conflicting studies

*Insect stage at infection*

Triatomines face unique challenges throughout their nymphal stages, and *T. rangeli* infection may affect them differently at different stages, as discussed in Chapter 3 in relation to *T. cruzi* infection. Based on the physiological demands faced at each stage, I

would predict that mortality would be most severe in insects infected in in the first stage, where the infected blood meal is the first blood meal of their lives, or in the 5th stage, where insects must molt into adults with wings and reproductive organs. However, results reported in the literature regarding insect stage are conflicting. There is just one published study [19] comparing the mortality of insects infected with *T. rangeli* at each stage, and the author did not find any significant difference between stages. On the other hand, Gomez [37] observed increased mortality in insects infected in the first stage, and others have observed less severe outcomes in insects infected as 5th instars and adults [40,60]. Therefore, the importance of insect stage at infection is currently unclear, and is likely variable, depending on an individual insect's background health, and the *T. rangeli* dose ingested.

*Reproduction and sex bias*

Despite the direct influence of reproduction on triatomine population dynamics, and potentially, Chagas disease transmission, I found just two published studies on the effect of *T. rangeli* on triatomine reproduction, both in *R. prolixus*. Watkins [45] found that *T. rangeli*-infected *R. prolixus* produced fewer viable eggs than uninfected insects, but she did not take into account the influence of blood meal size on egg production, which are directly linked [61], meaning the difference in viable egg production could have been due to small blood meals. Fellet *et al.* [62] repeated the experiment using the E-value [61], and she also observed decreased reproduction in *T.* rangeli-infected insects, as I observed in the experiment described in Chapters 3. Therefore, there is evidence that *T. rangeli* decreases

reproduction in laboratory-infected *R. prolixus*, but there is no published study on other species or free-living insects.

Finally, I did not find any studies designed to specifically investigate the effect of *T. rangeli* on males and females, although there are brief mentions of increased pathogenicity in females in studies designed around other questions [40,45]. Thus, a reduction in fecundity in *R. prolixus* infected with *T. rangeli* in the laboratory is the only effect unique to females that can be cautiously attributed to *T. rangeli* infection.

**5.7 Final thoughts**

T. rangeli *pathogenicity: what we actually know*

Schaub [63] proposed that the pathogenicity observed in *T. rangeli*–infected triatomines could be due to artificial combinations of parasites and insects in laboratory experiments. However, considering the volume of studies in which some kind of pathogenic effect was observed, it seems unlikely that all results are solely products of mismatched hosts and parasites. Based on the review of the literature presented here, it seems that there is sufficient data to support the idea that *T. rangeli* may raise death rates in *R. prolixus,* at least in a laboratory setting. There are less data on reproduction, but existing data support the idea that *T. rangeli* could also lower birth rates.

On the other hand, I do not think there is sufficient evidence to support the assumption that *T. rangeli* is pathogenic to all of its vector species. "*T. rangeli* is pathogenic to its vector" implies pathogenicity to more than one of its vector species, an idea that requires more

standardized studies on survival and reproduction in different triatomine species. *T. rangeli* pathogenicity must also be tested in a representative number of field-caught triatomines to understand its true effects. Finally, the use of mathematical models to make predictions about the influence of birth and death rates of *T. rangeli*-infected insects on long term *R. prolixus* population dynamics and *T. cruzi* transmission will also provide more insight into the broader effect of *T. rangeli* infection in triatomines.

T. cruzi *vs.* T. rangeli: *refining the orthodoxy*

As I discussed in Chapter 3, there are relatively few studies of the effect of *T cruzi* on *Rhodnius*, and most of them found that *T. cruzi* has a pathogenic effect independent of external stressors [58,62,64]. The orthodox view of the relative virulence of *T. cruzi* and *T. rangeli* rests on these studies in addition to the studies reviewed in this chapter, all of which were carried out with just one of the parasites in each study. I found just two published studies comparing the effect *T. cruzi* and *T. rangeli* infection on triatomines in the same experiment (single infections, not co-infections). As in my experiments, these studies (also carried out with *R. prolixus*), found negative effects in insects infected with either parasite [58,62]. Therefore, in terms of the pathogenicity *T. cruzi* and *T. rangeli* for triatomines, I propose that the accepted orthodox be transformed into a more nuanced, evidence-based heterodox that takes into account the diverse set of variables and contexts influencing the outcome of each infection. First, I propose that there is sufficient evidence to make this comparison just for *R. prolixus* infected in the laboratory, as the effect of either parasite on other species of *Rhodnius* or on wild-caught triatomines is virtually unknown. Second, I predict that the virulences of *T. cruzi* and *T. rangeli* mono-infections in

experimentally infected *R. prolixus* lie on an overlapping spectrum, with *T. cruzi* extending further into the lower virulence ranges and *T. rangeli* extending further into the higher ranges.

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## Conclusion

In the collection of work presented here, I took *Rhodnius prolixus,* the insect vector of *Trypanosoma cruzi,* a human pathogen, and I framed it as a parasitized host with the potential to experiences fitness consequences of infection. I asked questions rooted in principles of ecology and evolutionary biology; namely*, can* T. cruzi *regulate* R. prolixus *populations analogously to top down predators?* And, *does competition for resources between* T. cruzi *and* T. rangeli *in a co-infection change the effect of* T. cruzi *on the* R. prolixus*?*

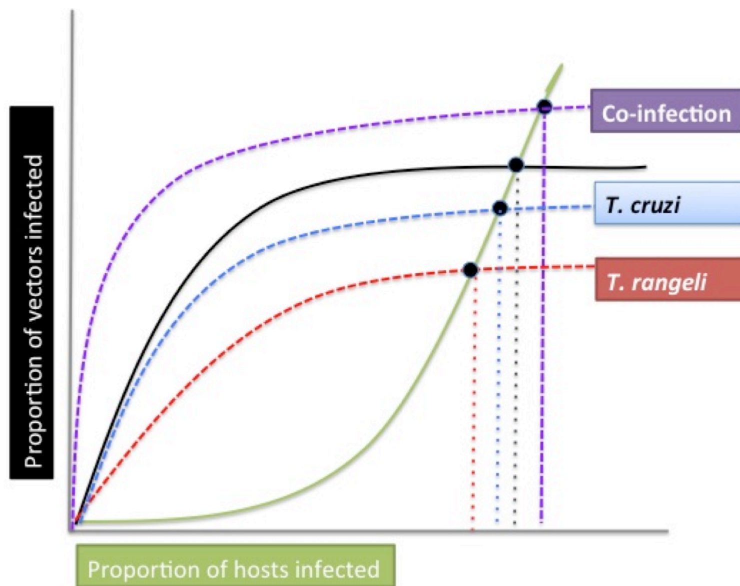


**Figure B. Graphical representation of the potential for *T. cruzi*, *T. rangeli*, and *T cruzi-T. rangeli* co-infection to affect the proportion of mammal hosts infected with *T. cruzi*, based on results presented in this thesis. Lines represent zero growth isoclines. Each intersection represents stable *T.* cruzi transmission. The solid lines represent infected vectors that do not experience consequences of *T. cruzi* infection (black) and mammal hosts infected with *T. cruzi* (green). The dotted lines represent insects infected with *T. rangeli* (red), *T. cruzi* (blue), and *T. cruzi-T. rangeli* co-infection (purple), according to the findings presented in Chapters 1-4.**

I predicted that *R. prolixus* individuals would experience negative consequences from *T. cruzi* infection, reflected in their survival, reproduction and overall fitness. I hypothesized that these consequences would be variable, as they are in vertebrate hosts infected with *T. cruzi*. This is indeed what I found, with *T. cruzi*-infected *R. prolixus* having different survival, reproduction, and fitness, depending on *T.*

*cruzi* strain; some strains were quite virulent while others had no apparent effect. On a population level, this could lead to unstable transmission of Chagas disease, and potentially a decreased proportion of mammal hosts infected as shown in Figure B. This outcome was as I expected, as it reflects the variability observed in vertebrate hosts infected with *T. cruzi*.

I also predicted that insects co-infected with *T.* cruzi and T*. rangeli* would have lower fitness than those infected with one parasite. Unexpectedly, I observed that *T. cruzi*-*T. rangeli* co-infection can confer a fitness advantage to *R. prolixus*, depending on a combination of infection order, timing and *T. rangeli* dose. I had expected a co-infection to lower vector fitness, and I predicted that it could potentially lower the proportion of mammal hosts infected. However, co-infection seemed to ameliorate the negative effects of a single infection, which could, on a population level, lead to an increased proportion of infected mammal hosts, as shown by the purple line in Figure B.

Overall, my results suggest that *T. cruzi* infection could regulate vector populations, but also that co-infection with *T.* rangeli could cancel out a negative effect of *T. cruzi* or *T. rangeli* single infections. Figure B illustrates a simplified visual representation of one way that these scenarios could be change the proportion of vectors in a population transmitting *T. cruzi* and *T. rangeli,* and how this could be reflected in the proportion of infected mammal hosts.

Clearly, more studies are needed to replicate these patterns. However, even if they were replicated and confirmed, the potential for *T. cruzi* to regulate triatomine populations would still depend on the composition of parasite species and virulence in the infected vector population. If enough insects were infected with virulent strains, *T. cruzi* and *T. rangeli* could regulate triatomine populations and lead to a lower proportion of *T. cruzi*-infected mammal hosts, as represented in Figure B. Conversely, abundant co-infections could result in a higher proportion of hosts infected.

Therefore, more data is needed to understand the virulence of these parasites in field populations. My results suggest that parasite strain, dose, infection timing, sequence and co-infection with *T. rangeli* can influence the outcome of *T. cruzi*-*R. prolixus* interactions, but this does not tell us how virulent strains come to be in the first place. Is it predictable or random? Are there vertebrate host characteristics that increase the likelihood of a *T. cruzi* strains being virulent to a triatomine? The answer to these questions could lead to a more real-world application of the knowledge of trypanosome-triatomine interactions.

To conclude, I set out to refine the orthodoxy of triatomine-trypanosome interactions and add more specificity to the body of knowledge that is drawn upon for the control of Chagas disease and other vector-borne diseases. By applying tenets of ecology and evolutionary biology, it was revealed that *R. prolixus*-*T. cruzi* interactions lie on a virulence spectrum ranging from no virulence to high virulence, and dependent on several contexts. As I emphasized in Chapter 5, caution must be taken when translating observations in laboratory experiments with limited species diversity and artificial conditions to their

meaning in the real system, and thus, the central idea that can confidently be taken from this work is that *T. cruzi* does not follow a different set of rules when infecting its invertebrate hosts; each outcome is inherently intricate and nuanced. These results illustrate the complexity of species interactions, and the astonishing power of experimental biology to reveal interactions across both biological and spatio-temporal scales.

# Appendix 1

## A1.1 Photos of insects that experienced molting problems

**Image A1c and A1d. *R. prolixus* individual with incomplete wing expansion after molting to the adult stage from the 5th instar. Top view (left) and side view (right). Image credit: JK Peterson**

**Image A1a and A1b. *R. prolixus* individual with head and thorax doubled over from an unsuccessful attempt to molt from the 5th instar to the adult stage, resulting in death. Top view (left) and side view (right). Image credit: JK Peterson.**

## A1.2 Cox model output and interpretation aid

| Variable | | *Regression coefficient (β)* | *Hazard ratio $e^{\beta}$* | *Standard Error of β* | *p-value* | *95% CI for hazard ratio* | |
|---|---|---|---|---|---|---|---|
| | | | | | | **lower** | **upper** |
| Cas15 | early | 1.825 | 6.206 | 0.894 | 0.041* | 1.075 | 35.82 |
| | late | 1.729 | 5.637 | 0.489 | 4.0e-04*** | 2.162 | 14.69 |
| Cas20 | early | 1.919 | 6.814 | 0.910 | 0.035* | 1.144 | 40.57 |
| | late | 0.754 | 2.125 | 0.648 | 0.244 | 0.596 | 7.576 |
| Gal61 | early | 1.421 | 4.141 | 0.921 | 0.122 | 0.681 | 25.18 |
| | late | 0.682 | 1.979 | 0.486 | 0.160 | 0.763 | 5.134 |
| SO-8 | early | 1.699 | 5.472 | 0.918 | 0.064 | 0.905 | 33.08 |
| | late | 0.769 | 2.159 | 0.604 | 0.202 | 0.660 | 7.055 |
| Sebas1 | early | -0.359 | 0.697 | 1.024 | 0.725 | 0.093 | 5.192 |
| | late | -1.686 | 0.185 | 1.073 | 0.116 | 0.022 | 1.517 |
| Blood-weight | | -2.004 | 0.134 | 0.639 | 0.001** | 0.038 | 0.471 |
| Gal61E : Blood-weight | | 1.547 | 4.701 | 0.807 | 0.055 | 0.966 | 22.87 |
| Cas20E: Blood-weight | | 2.036 | 7.664 | 0.756 | 0.007** | 1.741 | 33.73 |
| SO-8E : Blood-weight | | 0.756 | 2.129 | 0.769 | 0.325 | 0.471 | 9.618 |
| Cas15E: Blood-weight | | 1.391 | 4.020 | 0.786 | 0.076 | 0.861 | 18.76 |
| Episode:Blood-weight | | 2.959 | 19.28 | 0.708 | 2.9e-05*** | 4.806 | 77.38 |

**Table A1.1. Cox model output. "Early" and "late" represent before and after 28 days post-infection. Episode represents the two time periods examined, described above. Episode:blood-weight hazard is significantly high due to their being no deaths in the control group in episode 0. P-value notation: °below 0.1; *below 0.05; ** below 0.01; *** below 0.001**

*Cox PH model interpretation*

The Cox PH model yields a regression coefficient $\beta$, for which the exponential ($e^{\beta}$) expresses a hazard ratio of a given covariate to the hazard of a pre-determined comparative group. For main effects of categorical covariates, $e^{\beta}$ represents the hazard of the given categorical covariate to the hazard of the group designated as the comparative group, in this case, the control treatment group, at fixed values of all other covariates. When $e^{\beta}$ is larger than one, it means that the given variable has a hazard $e^{\beta}$ times higher than the comparative group hazard. When the hazard ratio is between 0-1, it means that its hazard is smaller than the comparative group, reduced by 1-$e^{\beta}$.

For main effects of discrete variables, $e^{\beta}$ represents the hazard at one unit of a given variable to the hazard at 0 units, with all other covariates fixed. For interaction effects, the $\beta$ value given for the interaction must first be subtracted from or added to the main effects $\beta$ before taking the exponent, which represents the hazard at one unit below or above the mean for the categorical covariate in question.

## Appendix 2

### A2.1 Detailed description of LSSP-PCR methods

The LSSP-PCR was carried out as described in [49,50], with an initial PCR to amplify a 330 bp *T. cruzi* kDNA fragment that was used as the template for the subsequent LSSP-PCR. For the first PCR, we amplified *T. cruzi* kDNA with the primers 121(5' - AAATAATGTACGGG(T/G)GAGATGCATGA-3') and 122 (5'- GTTCGATTGGGGTTGGTGTAATATA-3´) [51]. The final reaction volume of the PCR was 50 μL, consisting of 50 mM KCl, 10 mM Tris–HCl, 0.1% Triton X-100, 1 μL of DNA template (10 ng/μL), 37 pmol of each primer, 200 μM of deoxyribonucleotide triphosphates (dNTPs), 1.5 mM of $MgCl_2$ and 2.5 U of Taq DNA polymerase. The PCR was run at an initial temperature of 94°C for 3 min, followed by 35 cycles at 94°C for 45 s, 63°C for 45 s, 72°C for 45 s and a final cycle at 72°C for 10 min.

For the LSSP-PCR, 20 μL of the PCR product was run on a 1.5% low melting point agarose gel stained with ethidium bromide. Bands from the gel corresponding to 330 bp were cut out, and diluted to 1:10 in double-distilled water. One microliter of this dilution was used as a template for the LSSP-PCR reaction. The LSSP- PCR was run in a final reaction volume of 25 μL using 120 pmol of the 121 primer, 4 U of Taq polymerase, 200 μM of each dNTP, 1.5 mM of $MgCl_2$, 50 mM of KCl, 10 mM Tris–HCl and 0.1% Triton X-100. The amplification cycle consisted of 3 min of initial denaturing at 94°C, followed by 35 cycles of 94°C for 45 s, 30°C for 45s, 72°C for 45s and a final cycle at 72°C for 10min, as in [48]. 20 μL of the amplification products from each of the stocks were analyzed in polyacrylamide gels

visualized with silver staining. LSSP-PCR for each of the strains was performed in duplicate for the 2013 samples.

## 2.2 Full Cox PH model output

| Variable | *Regression coefficient (β)* | *Hazard ratio* $e^{\beta}$ | *Standard Error of β* | *p-value* | *95% CI for hazard ratio* | |
|---|---|---|---|---|---|---|
| | | | | | **lower** | **upper** |
| Cas15 | -0.509 | 0.600 | 0.254 | 0.045* | 0.364 | 0.989 |
| Gal61 | 0.610 | 1.841 | 0.305 | 0.045 | 1.012 | 3.348 |
| Sebas1 | -0.310 | 0.732 | 0.274 | 0.257 | 0.427 | 1.255 |

**Table A2.1. Cox PH model output. Hazard ratios represent the hazard of each treatment to the control group hazard. *p < 0.05**

# Appendix 3

## A3.1 Cox model outputs

| Covariate | Regression coefficient (β) | Hazard ratio $e^{\beta}$ | Standard Error of β | p-value | 95% CI for hazard ratio | |
|---|---|---|---|---|---|---|
| | | | | | lower | upper |
| *T. cruzi* | 0.778 | 2.178 | 0.329 | 0.018* | 1.142 | 4.155 |
| *T. rangeli* | -0.135 | 0.873 | 0.290 | 0.500 | 0.494 | 1.543 |
| Mixed | 0.227 | 1.255 | 0.338 | 0.640 | 0.647 | 2.435 |
| Blood:weight ratio | 0.231 | 1.260 | 0.073 | 0.001** | 1.092 | 1.454 |
| *T. cruzi*-blood:weight | -0.164 | 0.847 | 0.104 | 0.114 | 0.690 | 1.040 |
| *T. rangeli*-blood:weight | -0.050 | 0.951 | 0.092 | 0.588 | 0.793 | 1.140 |
| Mixed-blood:weight | -0.292 | 0.105 | 0.105 | 0.005** | 0.606 | 0.918 |

**Table A3.1 Cox PH model output for model run with treatment-blood:weight interactions and the control group as the comparative group. *p < 0.05; **p < 0.01.**

| Covariate | Regression coefficient (β) | Hazard ratio $e^{\beta}$ | Standard Error of β | p-value | 95% CI for hazard ratio | |
|---|---|---|---|---|---|---|
| | | | | | lower | upper |
| *T. cruzi* | 0.125 | 1.134 | 0.493 | 0.798 | 0.431 | 2.980 |
| *T. rangeli* | -0.491 | 0.611 | 0.315 | 0.119 | 0.329 | 1.135 |
| parasites ingested (absolute number) | -0.405 | 0.666 | 0.310 | 0.190 | 0.363 | 1.223 |
| *T. cruzi*-parasites | 1.529 | 4.615 | 0.683 | 0.025* | 1.209 | 17.61 |
| *T. rangeli*-parasites | 1.592 | 4.913 | 0.463 | 0.0005*** | 1.979 | 12.19 |

**Table A3.2 Cox PH model output for model run with treatment-absolute parasite dose interactions and the mixed group as the comparative group. *p < 0.05; ***p < 0.001.**

| Covariate | Regression coefficient (β) | Hazard ratio $e^{\beta}$ | Standard Error of β | p-value | 95% CI for hazard ratio | |
|---|---|---|---|---|---|---|
| | | | | | lower | upper |
| *T. cruzi* | 0.531 | 1.701 | 0.505 | 0.293 | 0.631 | 4.587 |
| *T. rangeli* | -0.085 | 0.918 | 0.304 | 0.779 | 0.505 | 1.668 |
| parasites ingested ( parasite species dose) | -0.405 | 0.666 | 0.309 | 0.190 | 0.363 | 1.223 |
| *T. cruzi*-parasites | 1.529 | 4.615 | 0.683 | 0.025* | 1.209 | 17.61 |
| *T. rangeli*-parasites | 1.592 | 4.913 | 0.463 | 0.0005*** | 1.979 | 12.19 |

**Table A3.3 Cox PH model output for the model run with treatment- parasite species dose interactions and the mixed group as the comparative group. *p < 0.05; ***p < 0.001.**

# Appendix 4

## A4.1 Kruskal Wallis comparisons between treatment groups for components of the infective blood meal

*Parasite:weight ratios*

| | *T. cruzi* | *T. rangeli1* | *T. rangeli2* | Mixed1 | Mixed2 | CrRa | Ra1Cr | Ra2Cr |
|---|---|---|---|---|---|---|---|---|
| *T. cruzi* | | | | | | | | |
| *T. rangeli1* | T | | | | | | | |
| *T. rangeli2* | T | T | | | | | | |
| Mixed1 | F | T | F | | | | | |
| Mixed2 | F | T | F | F | | | | |
| CrRa | T | F | T | F | T | | | |
| Ra1Cr | T | F | T | F | T | F | | |
| Ra2Cr | T | F | T | T | T | F | F | |

**Table A4.1. Results from KruskalMC comparisons between treatments of the number of parasites ingested per mg of biomass. T = TRUE, $p < 0.05$. F = FALSE, $p > 0.05$.**

*Blood:weight ratios*

| | *T. cruzi* | *T. rangeli 1* | *T. rangeli2* | Mixed1 | Mixed2 | CrRa | Ra1Cr | Ra2Cr | Control |
|---|---|---|---|---|---|---|---|---|---|
| *T. cruzi* | | | | | | | | | |
| *T. rangeli1* | F | | | | | | | | |
| *T. rangeli2* | F | F | | | | | | | |
| Mixed1 | F | F | F | | | | | | |
| Mixed2 | F | F | F | F | | | | | |
| CrRa | T | T | T | T | T | | | | |
| Ra1Cr | T | T | T | T | T | F | | | |
| Ra2Cr | T | T | T | T | T | F | F | | |
| Control | F | F | F | F | F | T | T | T | |

**Table A4.2. Results from KruskalMC comparisons between treatments of the volume of blood ingested per mg of biomass. T = TRUE, $p < 0.05$. F = FALSE, $p > 0.05$.**

*Total volume of blood ingested in infective meal*

| | *T. cruzi* | *T. rangeli 1* | *T. rangeli 2* | Mixed1 | Mixed2 | CrRa | Ra1Cr | Ra2Cr | Control |
|---|---|---|---|---|---|---|---|---|---|
| *T. cruzi* | | | | | | | | | |
| *T. rangeli1* | F | | | | | | | | |
| *T. rangeli2* | F | F | | | | | | | |
| Mixed1 | F | F | F | | | | | | |
| Mixed2 | T | T | F | F | | | | | |
| CrRa | F | F | T | T | T | | | | |
| Ra1Cr | F | F | T | T | T | F | | | |
| Ra2Cr | T | T | T | T | T | F | F | | |
| Control | F | F | F | F | F | F | F | T | |

**Table A4.3. Results from KruskalMC comparisons between treatments of the volume of blood ingested. For the delayed treatments, their second infective meal is the one in the analysis for the treatment group. T = TRUE, $p < 0.05$. F = FALSE, $p > 0.05$.**

*Estimated number of parasites ingested in infective meal*

| | *T. cruzi* | *T. rangeli1* | *T. rangeli2* | Mixed1 | Mixed2 | CrRa | Ra1Cr | Ra2Cr |
|---|---|---|---|---|---|---|---|---|
| *T. cruzi* | | | | | | | | |
| *T. rangeli1* | T | | | | | | | |
| *T. rangeli2* | F | T | | | | | | |
| Mixed1 | F | T | T | | | | | |
| Mixed2 | F | T | F | T | | | | |
| CrRa | F | T | F | F | T | | | |
| Ra1Cr | F | T | F | F | T | F | | |
| Ra2Cr | T | F | T | F | T | F | F | |

**Table A4.4. Results from KruskalMC comparisons between treatments of the estimated number of parasites ingested. T = TRUE, $p < 0.05$. F = FALSE, $p > 0.05$.**

## A4.2 Cox model output

| *Covariate* | *Regression coefficient (β)* | *Hazard ratio* $e^{\beta}$ | *Standard Error of β* | *p-value* | *95% CI for hazard ratio* | |
|---|---|---|---|---|---|---|
| | | | | | **lower** | **upper** |
| *T. cruzi* Late | 0.825 | 2.284 | 0.391 | 0.034* | 1.060 | 4.919 |
| *T. rangeli*1 Late | 1.278 | 3.589 | 0.523 | 0.014* | 1.286 | 10.01 |
| *T. rangeli*2 Early | -1.169 | 0.310 | 0.745 | 0.116 | 0.071 | 1.338 |
| *T. rangeli*2 Late | 1.031 | 2.804 | 0.401 | 0.010* | 1.277 | 6.155 |
| Mixed2 Early | 1.536 | 4.647 | 0.393 | 9e-05*** | 2.149 | 10.04 |
| Ra1Cr Late | -0.016 | 0.983 | 0.480 | 0.972 | 0.383 | 2.522 |
| Ra2Cr Late | 1.696 | 5.457 | 0.469 | 3e-04*** | 2.173 | 13.70 |
| CrRa Late | -0.030 | 0.969 | 0.546 | 0.954 | 0.331 | 2.831 |
| parasites ingested | 0.354 | 1.425 | 0.142 | 0.012* | 1.078 | 1.884 |
| *T. rangeli*2 E : parasites | -0.285 | 0.751 | 0.860 | 0.740 | 0.139 | 4.058 |
| *T. rangeli*2 L: parasites | -0.168 | 0.845 | 0.488 | 0.730 | 0.324 | 2.203 |
| Mixed2 E : parasites | -1.796 | 0.165 | 0.477 | 2e-04*** | 0.064 | 0.423 |
| Ra1Cr L: parasites | -0.838 | 0.432 | 0.352 | 0.017* | 0.216 | 0.861 |
| Ra2Cr L: parasites | 0.366 | 1.443 | 0.340 | 0.281 | 0.105 | 1.493 |

**Table A4.5 Cox PH model output for model run with Mixed1 group as the comparative group. E/Early and L/Late represent before and after 33 days, for the Mixed2; 32 days for *T. cruzi* and *T. rangeli1*; 34 days for *T. rangeli2*; and before and after the second infection for Ra1Cr,Ra2Cr and CrRa. *p < 0.05; ***p < 0.001.**