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Limits of Temperature Tolerance of Leishmania Enriettii and Leishmania Hertigi in a Real-Time PCR Assay

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LIMITS OF TEMPERATURE TOLERANCE OF *Leishmania enriettii* and *Leishmania hertigi* IN A REAL-TIME PCR ASSAY

A Thesis

by

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SUMMARY

LIMITS OF TEMPERATURE TOLERANCE OF

Leishmania enriettii and *Leishmania hertigi*

IN A REAL-TIME PCR ASSAY

Christina Salinas, B.S., University of North Texas

Protozoans of the genus *Leishmania* are intracellular parasites found across five continents. They cycle between a promastigote form inside the digestive tracts of blood-feeding female sand flies and an amastigote form within mammalian host macrophages. Temperature is an important factor that must be defeated in order for the parasites' survival and continuance. This paper studies the limits of temperature tolerance by *Leishmania enriettii* and *Leishmania hertigi*. It was hypothesized that a correlation would exist between the limits of temperature tolerance to body temperature of their host. I explored growth characteristics at 21, 27, 29, 33, 35, 37, and 39° C. It was observed that both *Leishmania enriettii* and *Leishmania hertigi* amastigotes grow optimally at 33° C in this in vitro system. Also 37° C was the limit that the parasites could survive, and 39° C was the lethal temperature that could no longer support the growth of the parasites. Based on the results indicated in the paper, it can be concluded that limits of temperature tolerance can be correlated to body temperature of their hosts.

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INTRODUCTION

Infections caused by *Leishmania* spp. are considerable health problems in several countries across the continents of Africa, Asia, Europe, North America, and South America (World Health Organization, 1990). Every year nearly 350 million people are at risk, and there are about 12 million infections per year (Magill, 1995). Three human infection syndromes have been characterized depending on the type of infecting species and location of infection: 1) localized cutaneous lesions 2) mucocutaneous lesions of the nasopharyngeal region, and 3) severe visceral forms (Magill, 1995). Each year, 1.5 million people will develop one of the two forms of cutaneous infections, and another 500,000 people will acquire visceral leishmaniases (Ogg *et al.* 2003).

Parasites of the genus *Leishmania* possess a biphasic life cycle that consists of a promastigote form inside the digestive tracts of blood-feeding sand fly vectors and an amastigote form within mammalian host macrophages (Pral *et al.* 2003). Of the six genera of sand flies only *Lutzomyia* spp. (New World) and *Phlebotomus* spp. (Old World) support the parasite's life cycle (World Health Organization, 1990). Infections by parasites of *Leishmania* spp. are transmitted when infected female sand flies take a blood meal from a vertebrate host (Lewis, 1974; Souza *et al.* 2005).

Before 1940, *Leishmania* spp. were identified only in infections of humans, dogs, and rodents. However, it was not known that other species of *Leishmania* existed that could result in disease in other mammals (Lainson, 1997). At least six

strains have been identified from both the Old and New World that do not cause disease to humans, but in other mammals. *Leishmania enriettii* Muniz and Medina (Kinetoplastida: Trypanosomatidae) and *Leishmania hertigi* Herrer (Kinetoplastida: Trypanosomatidae) are two species found in neotropical regions that are non-infectious to humans (Lainson, 1997).

The purpose of this project was to study how *L. enriettii* and *L. hertigi* tolerate a range of elevated temperatures. I investigated how these parasites responded to a shift of cool to warm temperatures. The main aims of the project were to characterize optimal temperature for this in vitro system, by comparing parasite population densities and parasite divisions, and to determine the upper limits of temperature tolerance. I hypothesize that tolerance of high temperatures by *L. enriettii* and *L. hertigi* corresponds to body temperatures of their host.

LITERATURE REVIEW

Parasites

Leishmania enriettii was first isolated from domestic guinea pigs, *Cavia porcellus*, in 1946 by Medina in the State of Paraná, Brazil (Lainson, 1997). The parasite was named in 1948 by Muniz and Medina. The two observed unusually large amastigotes found in tumor-like skin lesions on the ears and testicles of laboratory guinea pigs. Infections in guinea pigs have displayed a range of symptoms, from self-curing lesions to chronic metastatic lesions (El-On, Witztum and Schnur, 1986; Lainson, 1997). Immediately after the discovery, rhesus monkeys, dogs, white mice, golden

hamsters, puppies, rats, hares, and human volunteers were inoculated by subcutaneous or intraperitoneal injections of promastigotes or amastigotes. Similar lesions did not develop in any of the cases (Belehu and Turk, 1976; Machado *et al.* 1994). Since its first appearance, there have been at least three publications of its recurrence in domestic guinea pigs, all occurring within the geographical region of Brazil (Adler and Halff, 1955; Machado *et al.* 1994; Thomaz-Soccol *et al.* 1996).

Leishmania enriettii has been a suitable model to study in the laboratory because the parasite grows easily and large identifiable amastigotes can be obtained in considerable numbers from infected sites. Also, the infection in guinea pigs closely resembles human cutaneous leishmaniases (El-On *et al.* 1986; Lainson, 1997) Despite its uses in the laboratory, much is unknown about its epidemiology. Currently no natural infections have been reported in humans or mammals other than the guinea pig. After the second reappearance of *L. enriettii* in guinea pigs, *Lutzomyia monticola* sand flies were captured near the area of its appearance. The sand flies were found resting on tree trunks and feeding on humans. A study was done in 1967 demonstrating the ability of *Lu. monticola* to support the growth of the parasite; however the sand fly was unable to transmit the parasite naive guinea pigs. Therefore the natural sand fly vector is still unknown (Machado *et al.* 1994; Thomaz-Soccol *et al.* 1996).

Leishmania hertigi was first reported in the Panamanian porcupine, *Coendou rothschildi* in 1971. It was described to cause infection of the skin and sometimes viscera of porcupines (Zeledon, Ponce and de Ponce, 1977). Herrer (1971) reported

the parasite was harmless to porcupines, and could be found in small numbers throughout the skin and viscera. In 1975, Herrer and Christensen proposed that the association of *L. hertigi* with porcupines was of an ancient origin because no host cell reaction has been observed, and they concluded by stating *L. hertigi* has developed a commensal relationship with porcupines rather than a parasitic one.

Between April 1965 and September 1974, a study was accomplished that demonstrated the infrequency of gross skin lesions among 13 Panamanian forest mammals with cutaneous leishmaniases. It was reported that 89% of the mammals infected with *Leishmania* parasites showed no skin lesions. *Leishmania hertigi* accounted for 50% of the cryptic infections observed. Results in the article suggested that cryptic leishmanial infections are a common phenomenon found among Panamanian forest mammals (Herrer and Christensen, 1975). The parasite has also been detected in porcupines in Brazil and Costa Rica (Zeledon *et al.* 1977). In 1978, publications proclaimed that the Brazilian strains were significantly different to the Panamanian strains based on morphological, biochemical, and serological characteristics (Croft, Schnur and Chance, 1978). Brazilian strains are now given the name *Leishmania deanei* (Lainson, 1997).

Taxonomy of Leishmania enriettii and Leishmania hertigi

Leishmania species originally were categorized based on symptoms, geographic occurrences, and location of promastigote development within the sand fly (Cupolillo, Momen and Grimaldi, 1998; Grimaldi and Schottelius, 2001). The genus *Leishmania* was subdivided into two subgenera, the *Leishmania* and the *Viannia*. Within the subgenera, complexes were grouped according to isoenzymatic profiling (World Health Organization, 1990). However taxonomy of *Leishmania* is much more complex and often disputed among scientists. Cupolillo *et al.* (1998) state that part of the difficulty is due to the enormous diversity within the genus. One difference is the selection of hosts. Some species are pathogenic to humans, while others are limited to lower orders of mammals.

Leishmania enriettii and *L. hertigi* are among the many species where classification is still under debate. WHO (1990) lists the two species under the subgenera *Leishmania*, but not under any of the five complexes. Cupolillo *et al.* (2000) have proposed a revised classification of *Leishmania* and *Endotrpanum* parasites based on a number of recent molecular studies. Based on these studies, it was suggested that various species of *Leishmania* are more closely related to the genus *Endotrypanum* than to other species of *Leishmania*. According to the results, two phylogenetic lineages were proposed separating the species that demonstrated more similarities with one another than to those that were most divergent. The two lineages are named "section" Euleishmania and "section" Paraleishmania. Euleishmania is comprised of both Old and New World species, which cluster

together based on biochemical and molecular analyses, including numerical zymotaxonomy, multilocus enzyme electrophoresis, measurement of the sialidase activity, PCR amplification and restriction enzyme digestion of rRNA, cloning and sequencing of the conserved region of minicircle kDNA (Cupolillo *et al.* 1998). *Leishmania enriettii* is among this lineage. Paraleishmania then was termed for species that clustered with *Endotrypanum*, including *Leishmania herreri*, *Leishmania deanei*, *Leishmania equatorensis*, *Leishmania colombiensis*, and *L. hertigi*, all of which are New World species. (Cupolillo *et al.* 2001; Grimaldi and Schottelius, 2001; Noyes *et al.* 2000).

Temperature and pH effects

All species of *Leishmania* undergo major stress upon transmission from a poikilothermic (non-temperature regulated) sand fly vector to the homeothermic (temperature regulated) mammalian host. The two main stresses are temperature and pH (Van der Ploeg, Giannini and Cantor, 1985; Zilberstein and Shapira, 1994). Within the sand flies, promastigotes can experience temperatures in the range of 22-28°C, while the temperature conditions of mammalian tissue can range from 31-39°C depending on species, but never exceeds 42°C (Clos and Krobitsch, 1999; Zilberstein and Shapira, 1994). Arends and McNab (2001) listed the energetics of eleven species of New World hystricognath ('caviomorph') rodents. Species of Caviidae, which include guinea pigs, had body temperatures in the range of 37.3-39.0° C, while

species of Erethizontidae, the New World porcupines, had a body temperature of 36.7° C.

In addition to temperature variability, parasites will experience drastic changes in pH. Although the pH value in the digestive tract of the sand fly is unknown, it has been estimated that the pH is similar to that of the mosquito (> 8.5) (Zilberstein and Shapira, 1994). In contrast, pH values inside phagolysosomes can range from 4.5-6.0 (Lukacs, Rotstein and Grinstein, 1991).

Questions have been raised as to what factors govern parasite tropism and clinical manifestations (Callahan *et al.* 1996; Clos and Krobitsch, 1999). Clos and Krobitsch (1999) proposed that temperature tolerance of *Leishmania* species offer clues to their distribution within their mammalian host. For example, *Leishmania mexicana*, *Leishmania major*, and *Leishmania braziliensis* are species commonly associated with human cutaneous lesions. These species have a temperature tolerance up to 35° C in vitro, which correlates with the maximum temperature of human skin. Viseralizing species such as *Leishmania donovani*, *Leishmania infantum*, *Leishmania chagasi*, and *Leishmania tropica* can withstand higher temperatures from 35-39° C, which compares to temperatures in the human abdominal cavity.

Previous studies have shown the effects of temperature on several *Leishmania* species (Berman and Neva, 1981; Greenblatt and Glaser, 1965; Krassner, 1965; Leon, Soares and Temporal, 1995). Callahan *et al.* (1996) studied promastigotes of visceral and cutaneous *Leishmania spp.* from both Old and New World. Eight different species were used at three elevated temperatures (30, 32, and 34° C), and compared to

growth at the control temperature (25°). Results indicated that visceral *L. donovani* amastigotes were able to grow in higher temperatures than the cutaneous species *L. major*, *L. tropica*, and *L. mexicana*. In addition, Old World cutaneous species had significantly better growth potential in higher temperatures than New World species.

Real-time quantitative PCR

Real-time PCR is an increasingly popular technique applied in Parasitology, and is currently being utilized for the detection and quantification of *Leishmania* spp. within sand fly vectors and vertebrate blood and tissue (Bell and Ranford-Cartwright, 2002; Bretagne *et al.* 2001; Gomez-Saladin, Doud and Maroli, 2005; Mary *et al.* 2004; Rolao *et al.* 2004; Schulz *et al.* 2003; Vitale *et al.* 2004). The real-time PCR system is designed using a 3′ quencher dye and a 5′ reporter dye. When the probe anneals to its target sequence, Taq DNA Polymerase will cleave the probe due to its 5′ nuclease activity. This results in separation of the reporter dye from the quencher dye, following an increase of fluorescence from the reporter dye. Primer extension will then continue down the template strand. Fluorescence intensity is directly proportional to the accumulation of PCR product produced (Wortmann *et al.* 2001).

Initial concentration of target DNA then can be determined by obtaining the number of cycles necessary to reach a threshold concentration. This cycle number is called the critical $_{\text{threshold}}$ (C_T) value. Quantification of parasites of unknown concentration can ultimately be determined by the generation of a standard curve, plotting the log concentrations of known standards against their C_T values. The initial

starting concentration of target DNA from samples can be determined with respect to their C_T values along the standard curve (Mehra and Hu, 2005).

Conventional PCR is limited in this aspect because only the end-stage product can be measured where primer template, enzyme, and dNTP components may be exhausted (Bell and Ranford-Cartwright, 2002). Quantification of parasites by microscopy is another method often used by researchers, but results often can be inconsistent and the method is time consuming (Rolao *et al.* 2004).

One of the first studies using real-time PCR for the diagnosis of leishmaniases was performed by Wortmann *et al.* (2001). The purpose of the study was to develop an assay that could detect all *Leishmania* species. The small subunit rRNA gene was the ideal choice for target primer and probe selection because it is conserved among all *Leishmania.* Alignment of 16S rRNA gene sequences from *Leishmania aethiopica, Leishmania guyanensis, L. chagasi, L. donovani, L. mexicana, L. major, L. tropica,* and *L. Viannia* complex were used to design primers and probe targets. Both cultured promastigotes and tissue biopsies were used to test the assay. Results showed that the assay could detect all strains tested and could distinguish *Leishmania* from the closely related Trypanosomes. With the use of this technology, health care providers are able to give rapid diagnostic results at the genus level.

Schulz *et al.* (2003) expanded on the idea of detection of *Leishmania* with real-time PCR by producing an assay that could differentiate between the three relevant *Leishmania* groups (the *L. donovani* complex, the *L. braziliensis* complex, and others). Leishmanial 18S rDNA sequences were targeted for the selection of

primers and probe binding sites. The sequence for 18S rDNA belongs to the multipcopy group, in that there is more than one copy per cell (Guilaume *et al.* 1992). The selection of rDNA for target primer and probe sites provide increased sensitivity and specificity. Discrimination was achieved by using different melting temperatures. Results indicated that the three clinically relevant groups could reliably be distinguished from one another. It is now possible to allow health care administrators to provide more effective courses of treatment directed towards distinct clinical manifestations. It is also possible to do direct examination on blood, bone marrow, and skin or liver biopsy specimens (Schulz *et al.* 2003).

MATERIALS AND METHODS

Parasites and culture conditions

Parasites of *L. enriettii* and *L. hertigi* were obtained from the American Type Culture Collection (ATCC number: 30035 for *L. enriettii* and 30286 for *L. hertigi*). Cultures were maintained at 22° C in 1X M199 media with Earle's salts, L-glutamine (Invitrogen). Sodium Bicarbonate (Fisher) was added and supplemented with 5% (v/v) heat-inactivated fetal bovine serum (GIBCO Life Technologies), 1M hepes buffer (pH 7.4) (Sigma Chemical Co., St. Louis, MO), 100X penicillin/streptomycin (GIBCO), 10 mM hypoxanthin (Sigma), 0.25% (v/v) bovine hemin (Sigma), and sterile filtered human urine. The pH was adjusted to 7.4 by 1M sodium hydroxide (Fisher), and then sterilized by passage through a 0.22 μm Corning 1L filter (Corning Inc.). Parasites were cultured in tissue culture flasks (25 cm^3) for a total of 5 mL and subpassaged every four to five days as necessary.

Parasite temperature sensitivity was assessed in vitro by comparing growth of both species at 21, 27, 29, 33, 35, 37, and 39°C.

Seeding Culture Flasks

Two culture flasks were initiated each with one species of *Leishmania*. Cultures were allowed to cultivate to a density of approximately 2.0 X 10^7 parasites/mL. Parasite quantification was determined using a Neubauer hemacytometer (Hausser Scientific). Parasites were first syringed 7 to 8 times using a 23 $gauge^{3/4}$ syringe needle to disrupt

any clumps of parasites. The hemacytometer slide was loaded with 10 µL of diluted sample (10-fold or 100-fold dilutions were used depending on parasite concentrations). Once concentrations were determined, a master flask was prepared. Media was added for a total volume of 40 mL. Next, 10 mL of culture was distributed to four flasks, each seeded with approximately 5 X 10^5 parasites/mL.

Sample Collection

After the initial seeding of each flask, 100 µL was immediately collected. Another sample was extracted from each flask approximately 5 hours after incubation in the given temperature. Each day thereafter, samples were extracted once each in the morning at the same time until the end of the experiment. Collected samples were kept at -80°C in polyprolene tubes until the final time point at which DNA could be extracted as described below.

DNA extraction

To extract the DNA from the samples, parasites were homogenized with 1.0 mL of DNA Stat-60 (Tel-Test Inc.) by passing several times through a sterile pipette tip. Next 200 µL of chloroform (Sigma) was added to each sample, and incubated at room temperature for 2-3 minutes. Samples were centrifuged at 14000 RPMs for 15 minutes at room temperature. After centrifugation, the aqueous layer containing the DNA was transferred to a fresh tube. Ten µL of 3M sodium acetate (Ambion), 1 µL of pellet paint (Novagen) was mixed with the DNA followed by the addition of 750

μL of isopropyl alcohol. Samples were stored at -20°C for at least 30 minutes, and then centrifuged at 10000 RPMs for 10 minutes. Pellet was resuspended in 1 mL of 75% nuclease free ethanol and centrifuged at 10000 RPMs for 5 minutes. Ethanol was decanted, followed by a brief spin, and residual ethanol was removed using a pipette. Finally the pellet was resuspended in 50 μL of nuclease free water (Ambion).

Selection of Primers and Probe

Gene sequences were searched by the NCBI database. Primers and probe binding sites were selected to target a region that is conserved for both strands of *L. enriettii* and *L. hertigi*. Fig. 1 shows the alignment of *Leishmanial* DNA polymerase from both species generated by the BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST) (Altschul *et al.*, 1990). Nucleotides 535 to 839 from the *L. enriettii* DNA polymerase sequence and nucleotides 543 to 847 from the *L. hertigi* DNA polymerase sequence contains the region that housed the primers and probe for detection of *Leishmania*. Total amplicon length is 304 bp. Both forward and reverse primers have a melting temperature of 60° C, while the probe has a melting temperature of 70° C. The following primers and probe were used: forward primer sequence: 5′-GGA TGG CAA GCG GAA GGC-3′; reverse primer sequence: 5′-TCA GCC GCT TCA CCT CGC-3′; and DNA Pol probe sequence: 5′CCT CTA CCC GTC GCT GAT TCA G-3′. The probe used in this procedure uses the reporter dye FAM

(6-carboxyfluorescein) at the 5′ end and the quencher dye TAMRA (6-carbody-N,N,N,N tetramethylrhodamine) at the 3′ end. The primers were tested using a standard PCR containing both *L. enriettii* and *L. hertigi* DNA.

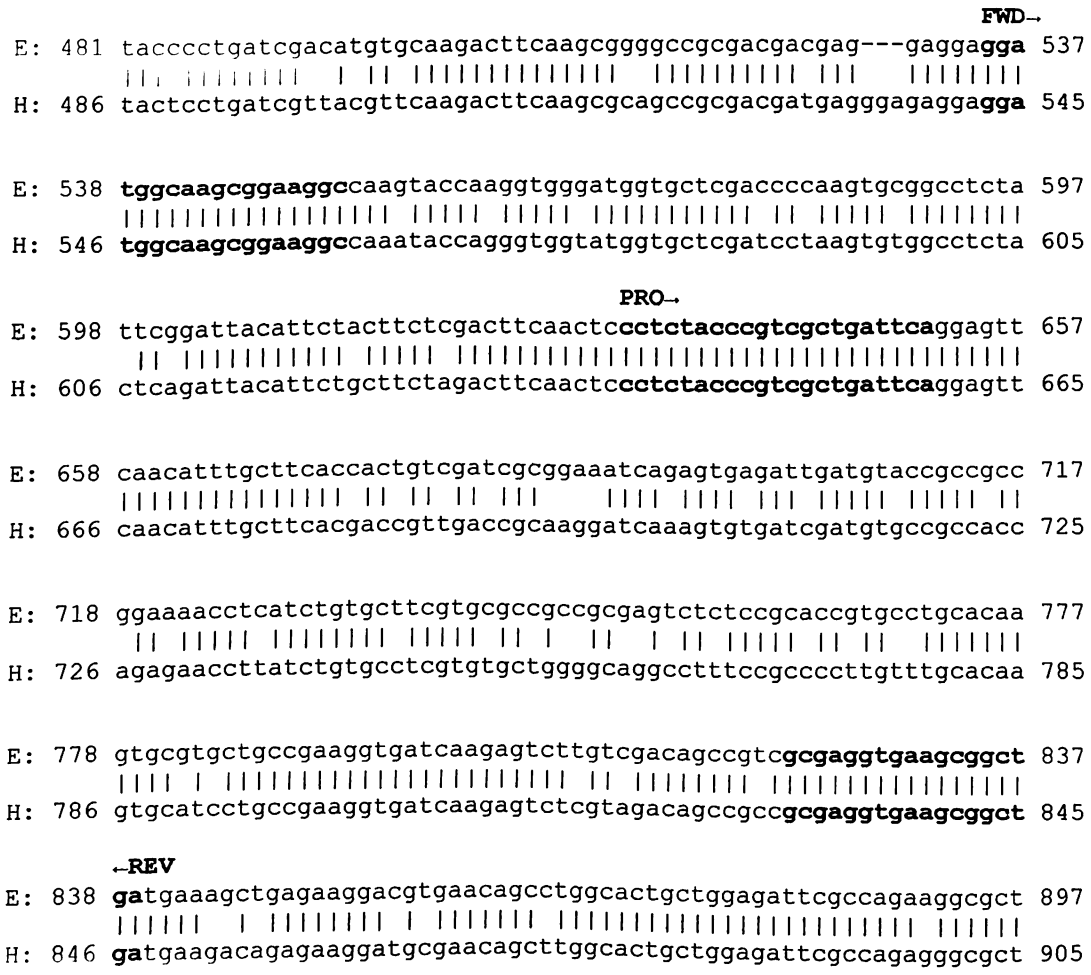


Fig. 1. DNA polymerase from *Leishmania enriettii* and *Leishmania hertigi* were aligned by the NCBI database using the BLAST algorithm (Altschul *et al.*, 1990). Numbers on the left and right of the sequence indicate the nucleotide number for that sequence. The vertical lines indicate homology between the two sequences. The sequence from *Leishmania enriettii* correspond to the top strand and the sequence from *Leishmania hertigi* is the bottom strand, labeled on the left with the letter E and H respectively. Primer and probe oligonucleotide descriptions as well as their designations are in bold and given above the alignment.

Taq-Man Assay

The ABI Prism ® 7700 Sequence Detection System was used for amplification and quantification of the *Leishmanial* DNA. Appendix B illustrates the SDS system. Optimal primer probe concentrations were determined using the checker board pattern of dilutions to determine the master mix final concentrations, 2X Taqman® Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 10 μM of forward primer (GGA TGG CAA GCG GAA GGC), 10 μM of reverse primer (TCA GCC GCT TCA CCT CGC), and 2.5 μM of TaqMan probe (CCT CTA CCC GTC GCT GAT TCA). Assays were performed in 25μL final reaction volumes with 2 μL of DNA sample. Nuclease free water (Ambion) was used as the no template control to ensure that there was no contamination in the master mix. All samples excluding those samples taken from the first day of the experiment were diluted 10 or 100 times with nuclease free water (Ambion) to ensure that each sample would fall within the standard curve. Each sample was amplified in duplicates, in a Micro-Amp® optical 96-well reaction plate (Applied Biosystems). The following settings were used during the reactions: 40 cycles of 50°C for 2 min, 95°C for 10 min, 95°C for 30 s, and 60°C for 1 min.

Standard Curve

Mass cultures of L. *hertigi* were used to establish standard curves. Parasite quantification was determined using a Neubauer hemacytometer. Extractions were

initiated from 2.0 X 10^6 parasites/mL. The culture was then diluted in 10-fold dilutions, ranging from 2.0 X 10^6 to 2000 parasites.

RESULTS

TaqMan Assay

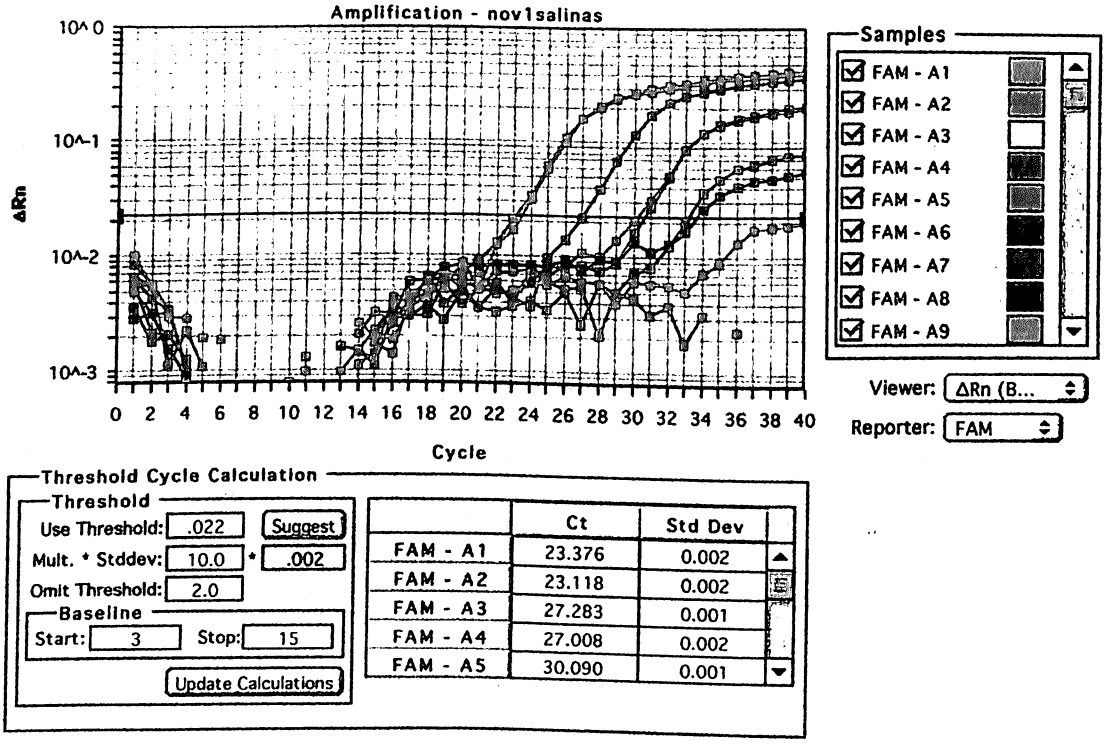
DNA polymerase amplification was achieved by using real-time PCR. Figures for each amplification are listed in Appendix A. Slopes for all amplifications ranged from -3.55 to -4.00, with efficiencies averaging 0.99. Any runs with slopes that were not within this range were repeated. Fig. 2A demonstrates an amplification plot of the serial dilutions, and Fig. 2B is an example of the standard curve for one of the amplification plots.

Parasite Growth for Leishmania enriettii

Table 1 shows the initial and maximal concentration of parasites/mL for *L. enriettii* at each of the studied temperatures. The numbers listed are the means of eight samples along with their standard error. Growth curves of *L. enriettii* at different temperatures are demonstrated in Figs. 3A-G. Each point is presented as a multiple of the original inoculum. All inocula were initially between 1.8 and 6.3 X 10^5 parasites/mL. (Refer to Table 1 for the initial inocula concentrations at different temperatures studied.) Cultures were maintained for 45 to 237 hours depending on temperature being studied and parasite viability. Growth of promastigotes was compared at 21, 27, and 29° C, while growth of amastigotes was compared at 33, 35, 37, and 39° C. Only viability of promastigotes was possible by microscopy because amastigotes are non-motile organisms. Transformation to amastigotes also was determined by observation using microscopy. Promastigotes had elongated slender bodies with long flagella.

Amastigotes were able to undergo complete transformation, their bodies were small and rounded, and no flagellum was present.

A



Figs. 2A-B. Example of an amplification plot and a standard curve obtained with the TaqMan assay. (A) Amplification plot of five 10-fold serially diluted DNA extracted from *Leishmania hertigi*. Range of $2.0 * 10^6$ to 200 parasites/mL. (B) Standard curve obtained by plotting the C_T against the input target quantity, plotted on a log scale. C_T represents the number of cycles needed to reach a threshold concentration.

B

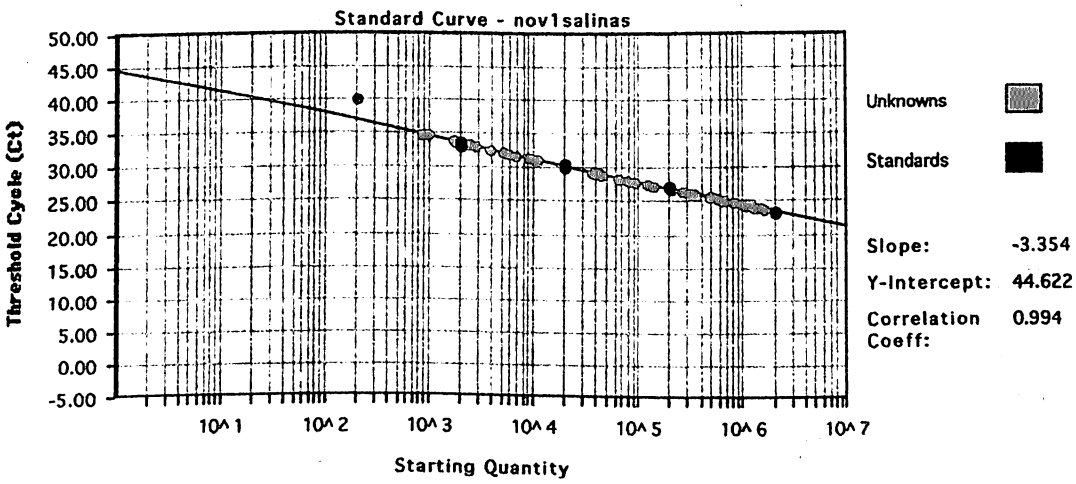


Fig. 2B. Standard Curve

Table 1. Initial and maximal concentrations of *Leishmania enriettii* at the different temperatures studied. Concentrations listed are the means from 8 DNA amplifications, along with their standard error.

Temperature (°C)	Initial Concentration (p/mL)	SEM	Maximum Concentration (p/mL)	SEM
21	293750	33270	1.59E+07	2709971
27	630000	111323	1.75E+07	3776877
29	189000	30381	1.88E+07	1508902
33	256250	31900	6862500	568500
35	270250	44176	1133750	85125
37	176250	13750	286250	39818
39	472500	37973	N/A	N/A

Cultures of *L. enriettii* at 21° C were maintained for 237 hours (Fig. 3A). Maximum concentration was 1.59 X 10^7 parasites/mL. Population increased about 60 times the original inoculum at around 237 hours. No initial lag phase was evident at this temperature, but growth was continuous throughout the experiment. Cultures were not maintained long enough to see parasites stabilize in stationary phase.

Promastigote growth at 27° C was maintained for 141 hours (Fig. 3B). Parasites immediately went into log phase until growth was slowed after 45 hours. Population density reached maximum growth of 1.75 X 10^7 parasites/mL at 117 hours. Parasites multiplied 33 times from the original inoculum. Parasites remained as promastigotes at this temperature.

Growth at 29° C (Fig. 3C) was maintained for 141 hours. Parasites went immediately into log phase until 117 hours, were they reached maximum growth of about 1.88 X 10^7 parasites/mL. Population density increased 88 times the original inoculum. While there was some transformation to amastigote-like organisms, most of the parasites remained in promastigote form.

Parasites of *L. enriettii* were maintained for 141 hours at 33° C (Fig. 3D). Again no initial lag phase was evident. Parasites went into immediate log phase until about 45 hours and reached maximum growth of about 6.8 X 10^6 parasites/mL. Population density increased 32 times the original inoculum size. After about 45 hours, promastigotes were completely transformed into amastigote-like organisms.

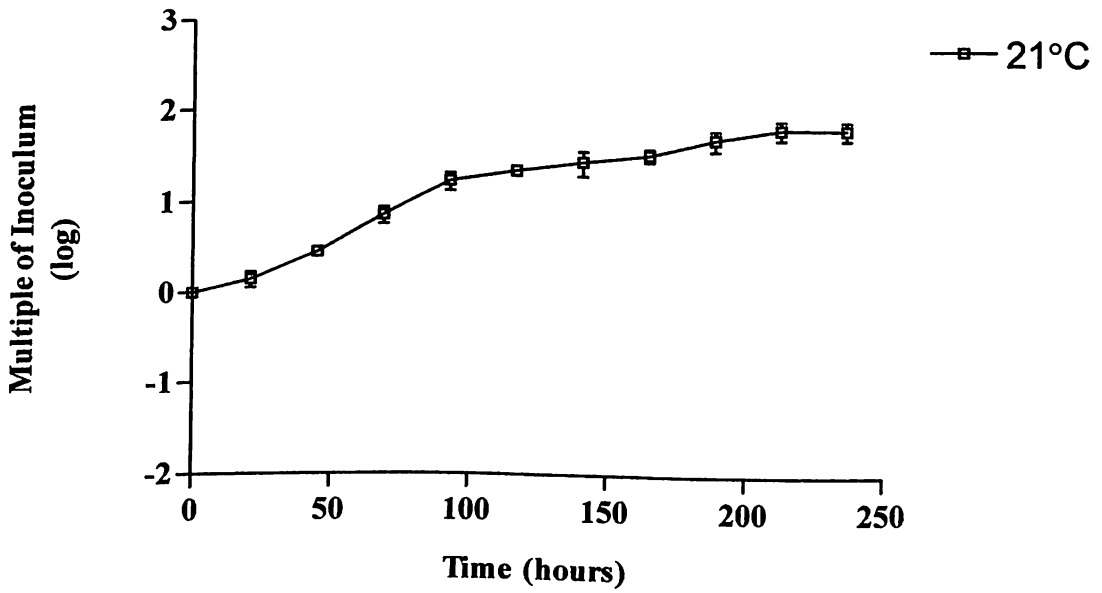
promastigotes began to completely transform into amastigote-like organisms. After 45 hours, complete transformation took place. Additional division occurred for about 48 hours, where amastigotes reach a maximum population of about 1.1 X 10^6 parasites/mL within 58 hours. Population size increased 5 times the original inoculum.

For growth at 37° C, cultures were maintained for 93 hours (Fig. 3F). Parasites never entered log phase but remained in a lag phase until 46 hours. Parasite population almost doubled the original inoculum. Maximum population reached about 2.9 X 10^5 parasites/mL at 46 hours. Parasites did transform completely into amastigote-like organisms within the first 22 hours.

At 39° C, *L. enriettii* were allowed to cultivate for 45 hours (Fig. 3G). Parasite death was immediate within the first hour and lysis of parasites occurred rapidly after two hours.

Maximum growth of *L. enriettii* (parasites/mL) at all temperatures studied is illustrated in Fig. 4. Open vertical columns signify promastigote growth, while closed bars represent amastigote growth. Also, maximum number of parasite divisions is shown in Fig. 5. Parasite division occurred at all temperatures except at 39° C.

A



Figs. 3A-G. Growth curves of *Leishmania enriettii* at different temperatures studied. Each point is presented as a multiple of the original inoculum. All inocula were initially between 1.8 and 6.3 X 10^5 parasites/mL. Cultures were maintained for 45 to 237 hours depending on temperature studied and parasite viability. The vertical bars indicate the standard error.

B

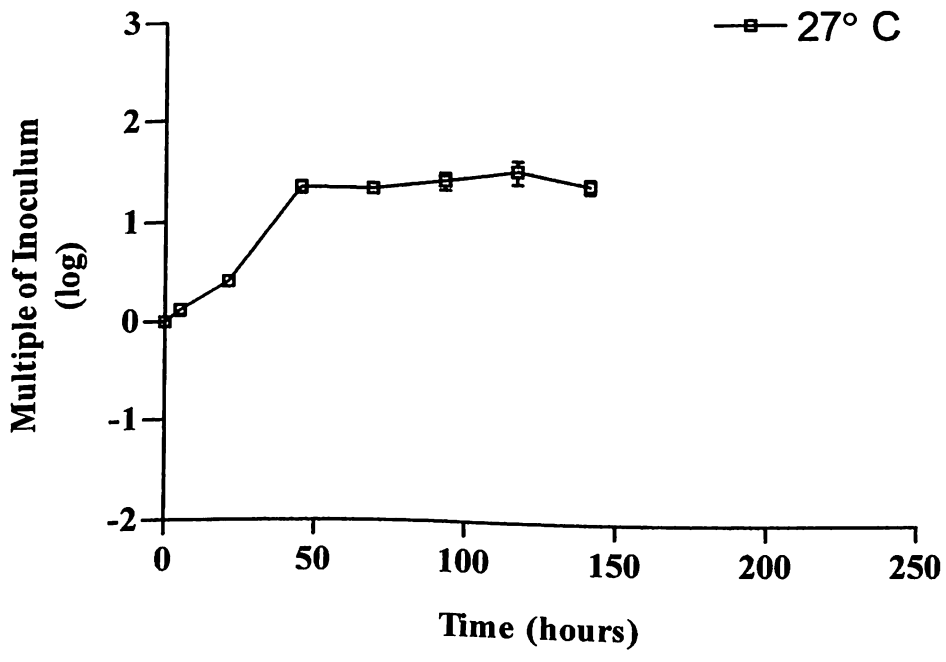


Fig. 3B. Growth of *Leishmania enriettii* at 27° C.

C

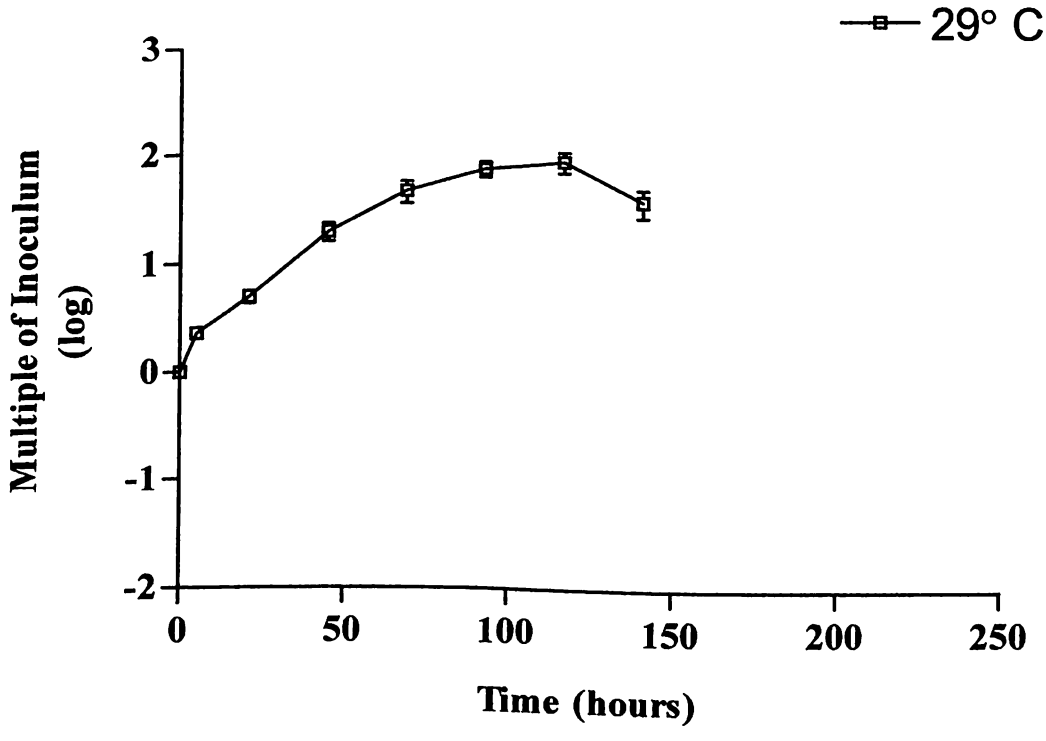


Fig. 3C. Growth of *Leishmania enriettii* at 29° C.

D

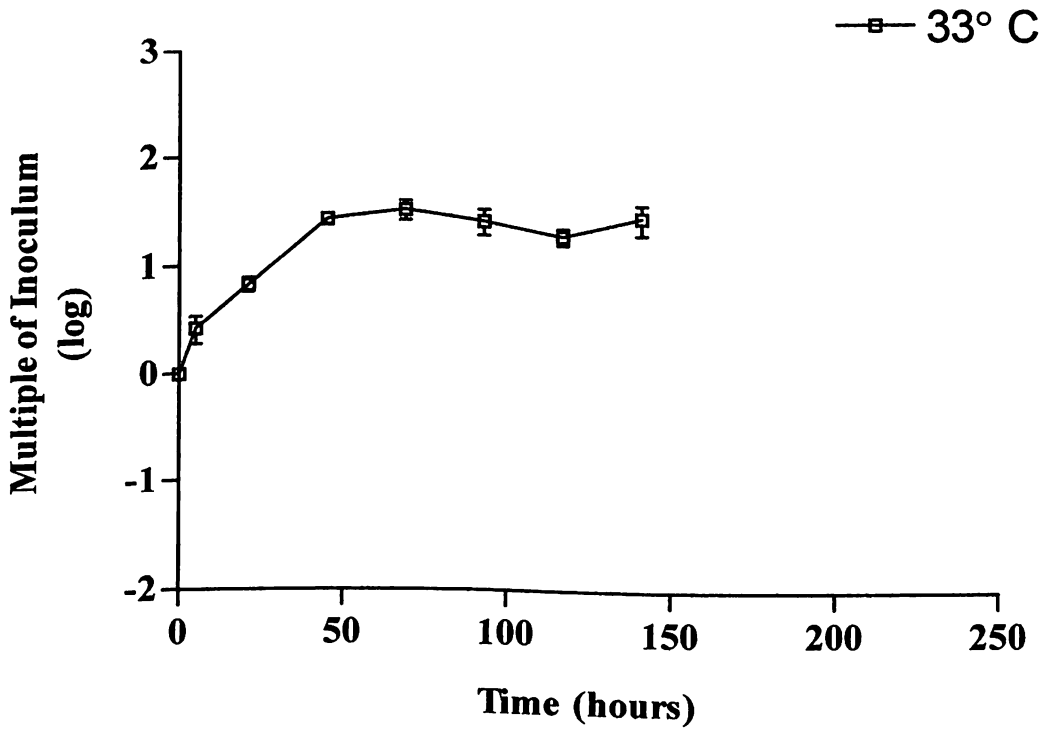


Fig. 3D. Growth of *Leishmania enriettii* at 33° C.

E

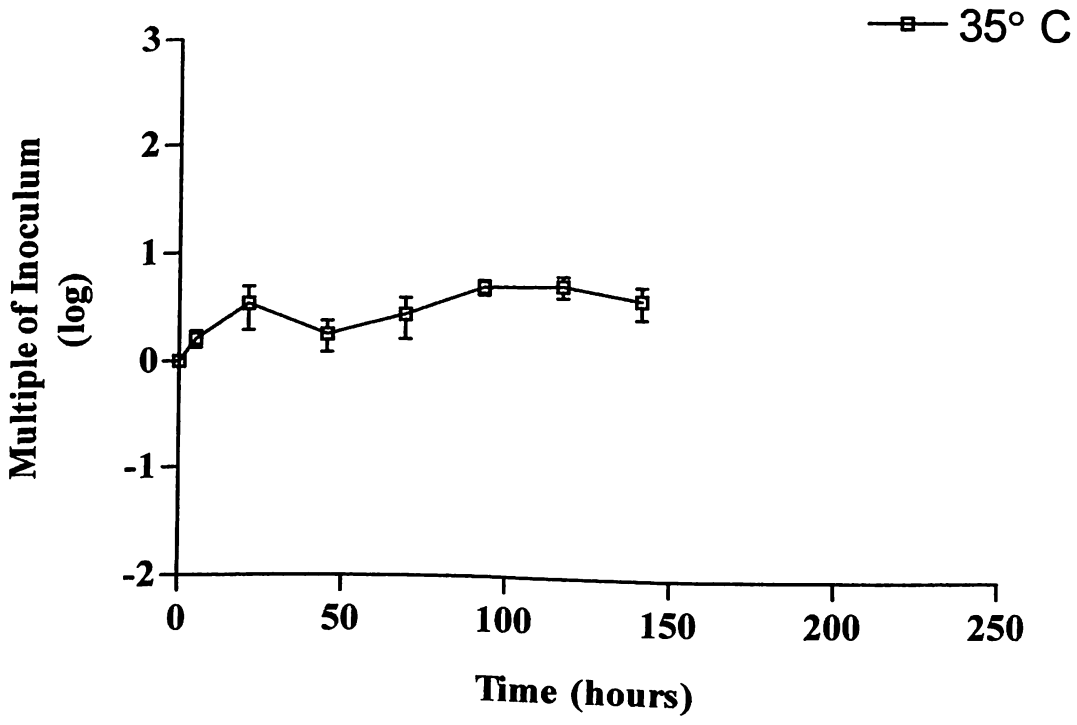


Fig. 3E. Growth of *Leishmania enriettii* at 35° C.

F

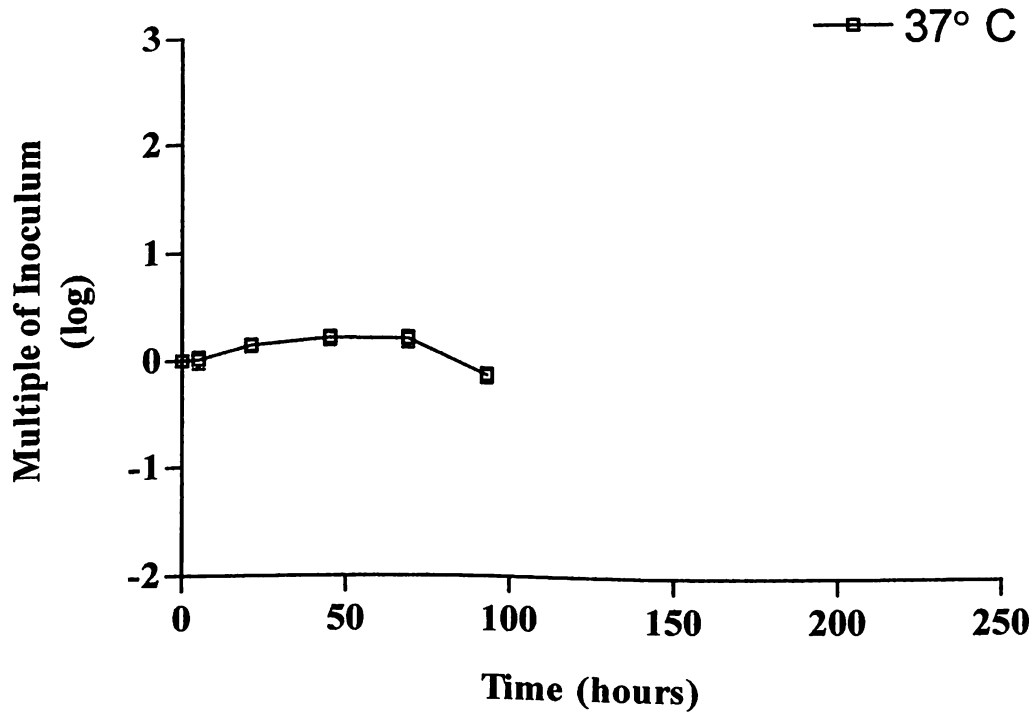


Fig. 3F. Growth of *Leishmania enriettii* at 37° C.

G

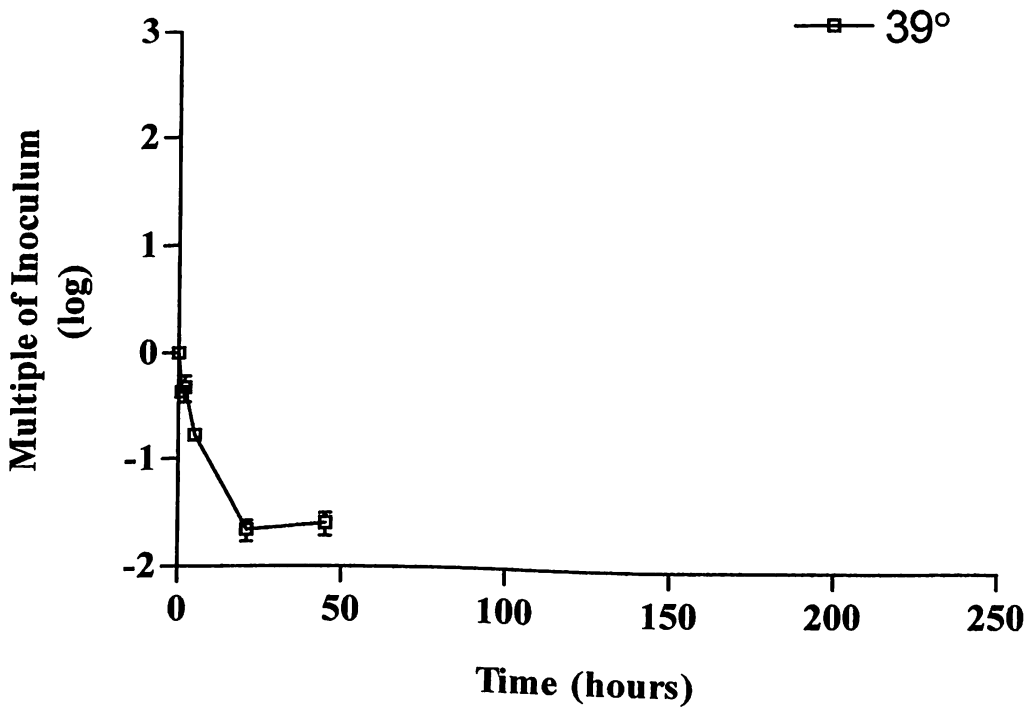


Fig. 3G. Growth of *Leishmania enriettii* at 39° C.

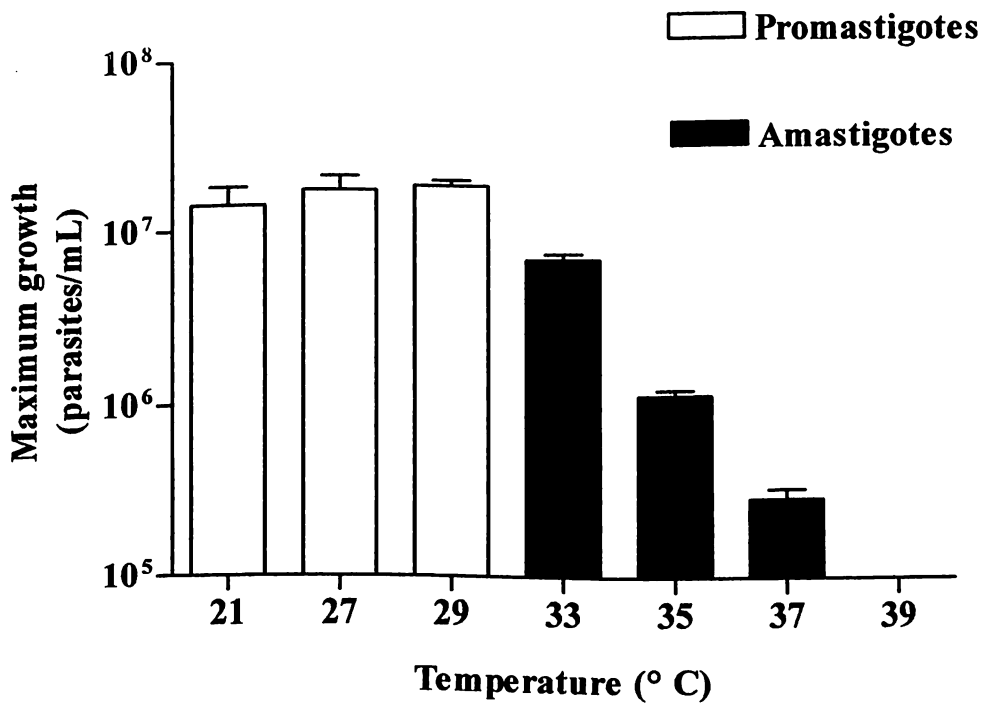


Fig. 4. Maximum growth (parasites/mL) of *Leishmania enriettii* at different temperatures studied. Open columns represent promastigote growth and closed columns represent amastigote growth. The vertical bars indicate the standard error.

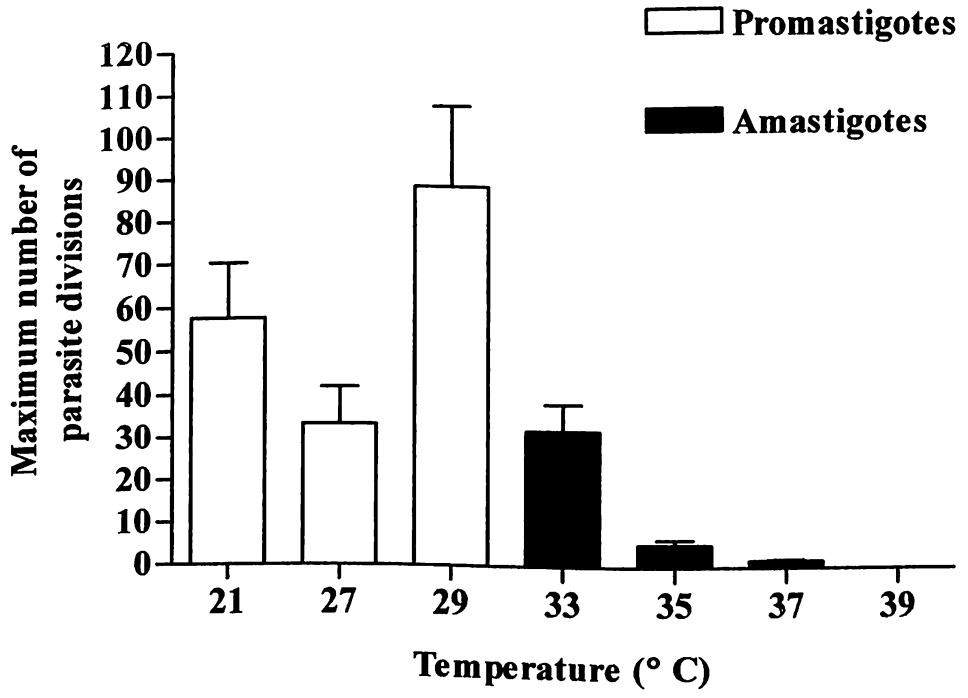


Fig. 5. Maximum number of parasite divisions for *Leishmania enriettii* at the different temperatures studied. Open columns represent promastigote growth and closed columns represent amastigote growth. The vertical lines indicate the standard error.

Parasite Growth for Leishmania hertigi

Initial and maximal parasite concentrations are given on Table 2 along with their standard error. Growth curves for *L. hertigi* at different temperatures are demonstrated in Figs. 6A-G. Each point is presented as a multiple of the original inoculum. All inocula were initially between 2.6 to 9.0 X 10^5 parasites/mL. (Refer to Table 2 for the initial inoculum concentrations at different temperatures studied.) Cultures were maintained for 45 to 189 hours depending on temperature studied and parasite viability. Again only promastigote viability was possible to estimate. Growth of promastigotes was compared at 21, 27, and 29° C, and growth of amastigotes was compared at 33, 35, 37, and 39° C. Unlike *L. enriettii*, *L. hertigi* promastigotes had different morphological shapes. Further morphological studies need to be evaluated. Amastigote transformation was also difficult to determine due to the variety of shapes observed. Complete transformation could not be evaluated. However estimations were given as to when the majority of amastigotes in culture were in a rounded form.

Leishmania hertigi cultures were maintained for 189 hours at 21° C (Fig. 6A). Parasites immediately entered log phase for the first 21 hours, then slowed to a gradual increase for another 72 hours. At 93 hours, maximum growth of 5.31 X 10^7 parasites/mL was achieved. On average, there was an increase of 85 times the original inoculum.

At 27° C, cultures were maintained for 141 hours (Fig. 6B). Parasites entered log phase at about 5 hours into incubation, and lasted another 64 hours. Maximum

growth of 4.21 X 10^7 parasites/mL was achieved near 69 hours. On average, this was 21 times the original inoculum size.

Leishmania hertigi cultures at 29°C were maintained for 141 hours (Fig. 6C). Parasites entered log phase after 5 hours and lasted for 16 hours. Maximum growth of 2.42 X 10^7 parasites/mL was achieved at 21 hours. On average, parasites multiplied 36 times their original inoculum size.

The growth curve for *L. enriettii* at 33° C is demonstrated in Fig. 6D. Cultures were maintained for 141 hours. Parasites entered log phase after 5 hours and continued another 40 hours. Maximum growth of 1.17 X 10^7 parasites/mL was achieved after 45 hours. On average, parasites multiplied 13 times their original inoculum size. It is estimated that transformation occurred after 45 hours.

Cultures in 35° C were cultivated for 141 hours (Fig. 6E). Parasites did not enter a log phase, but did achieve maximum growth of about 1.4 X 10^6 parasites/mL at 21 hours. On average, parasites multiplied 3 times the original inoculum size. It is estimated that transformation took place after 21 hours.

At 37° C parasites were incubated for 93 hours (Fig. 6F). Parasites did not enter log phase, but after 5 hours reached its maximum concentration of about 8.3 X 10^5 parasites/mL. On average, parasites multiplied 1.3 times the original inoculum size. It is estimated that transformation took place after 21 hours.

Fig. 6G shows the growth curve of *L. hertigi* at 39° C for 45 hours. Parasites did not enter log phase, but after 5 hours reached its maximum concentration of about

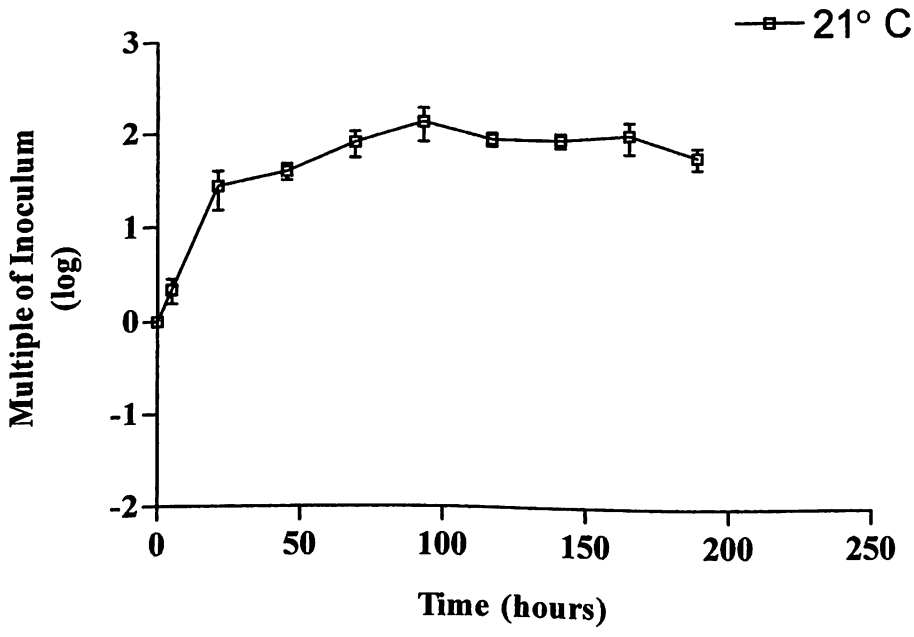
2.2×10^5 parasites/mL. On average, parasites multiplied almost twice the original inoculum size.

Maximum growth of *L. hertigi* (parasites/mL) at all temperatures studied is illustrated in Fig. 7. Open vertical columns signify promastigote growth, while closed bars represent amastigote growth. Also, maximum number of parasite divisions is shown in Fig. 8. Parasite division occurred at all temperatures studied.

Table 2. Initial and maximal concentrations of *Leishmania hertigi* at the different temperatures studied. Concentrations listed are the means of 8 DNA amplifications, along with their standard error.

Temperature (°C)	Initial Concentration (p/mL)	SEM	Maximum Concentration (p/mL)	SEM
21	521250	111507	5.31E+07	1.70E+07
27	262857	41157	4.21E+06	980876
29	653750	45667	2.42E+07	5631622
33	908750	49657	1.17E+07	2274156
35	465000	45943	1383750	111707
37	657500	66595	831429	178358
39	531429	81194	737500	215181

A



Figs. 6A.-G. Growth curves of *Leishmania hertigi at* different temperatures studied. Each point is presented as a multiple of the original inoculum. All inocula were initially between 2.6 and 9.0 X 10^5 parasites/mL. Cultures were maintained for 45 to 189 hours depending on temperature studied and parasite viability. The vertical bars indicate the standard error.

B

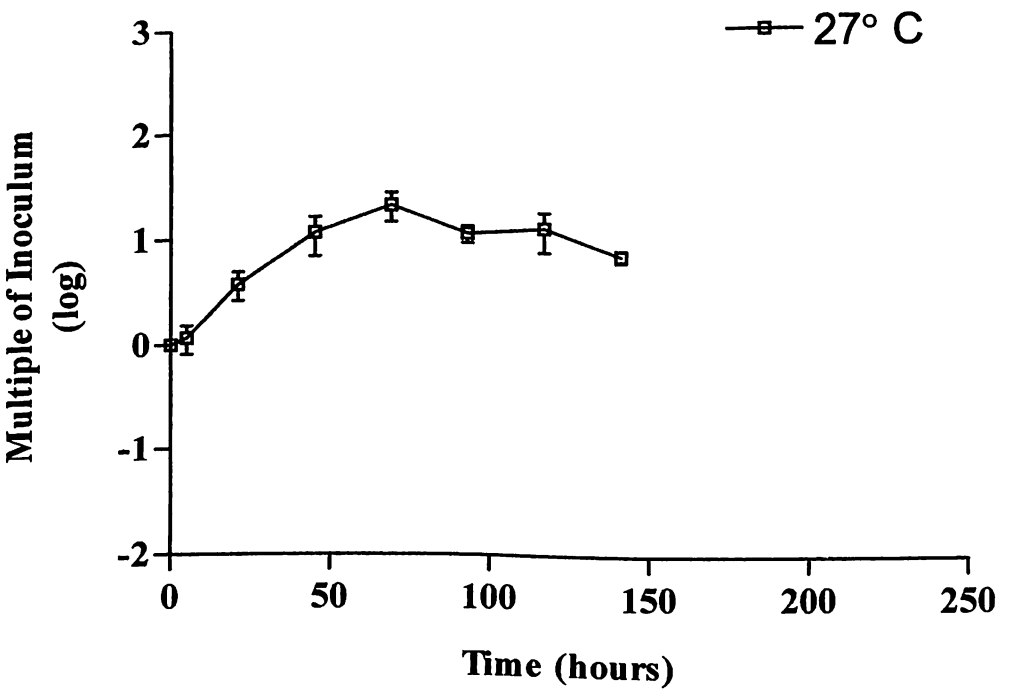


Fig. 6B. Growth of *Leishmania hertigi* at 27° C.

C

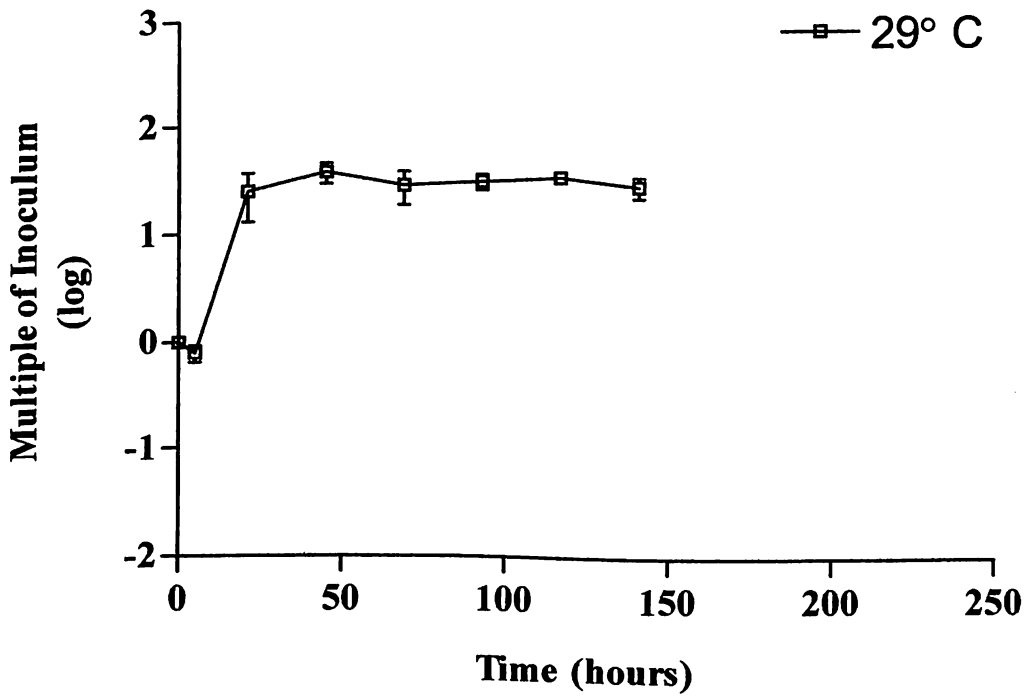


Fig. 6C. Growth of *Leishmania hertigi* at 29° C.

D

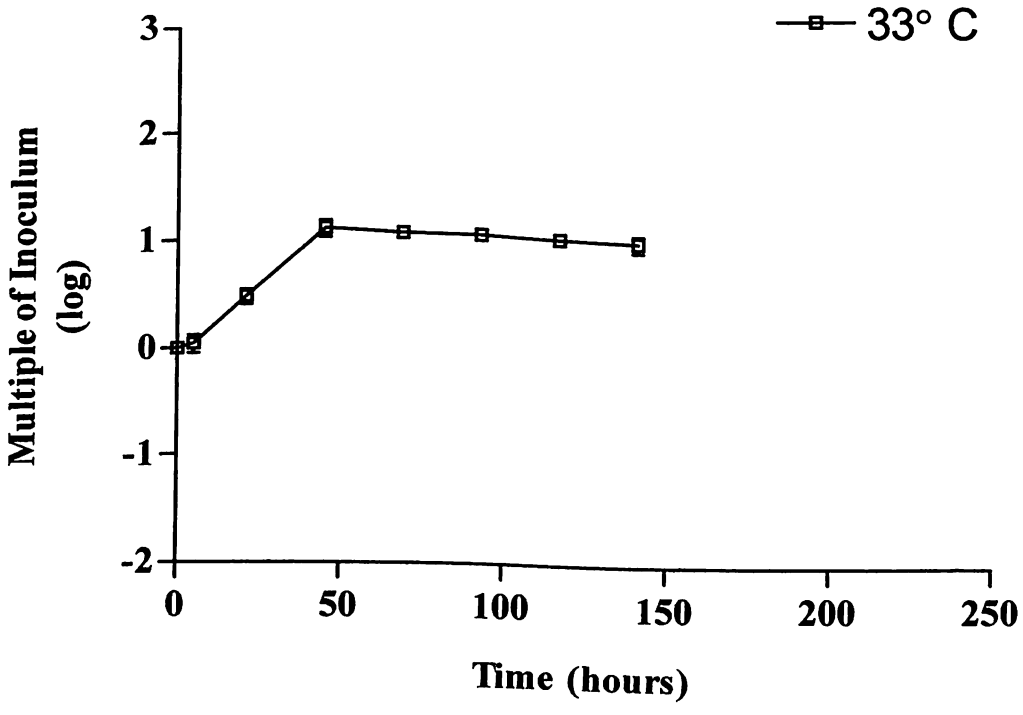


Fig. 6D. Growth of *Leishmania hertigi* at 33° C.

E

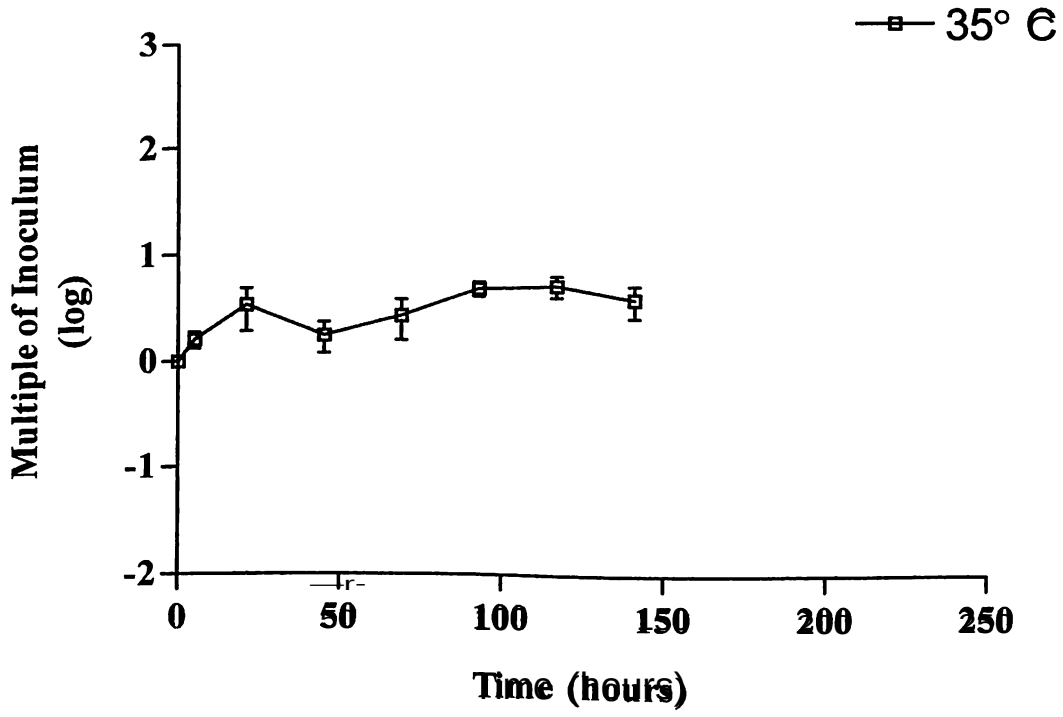


Fig. 6E. Growth of *Leishmania hertigi* at 35° C.

F

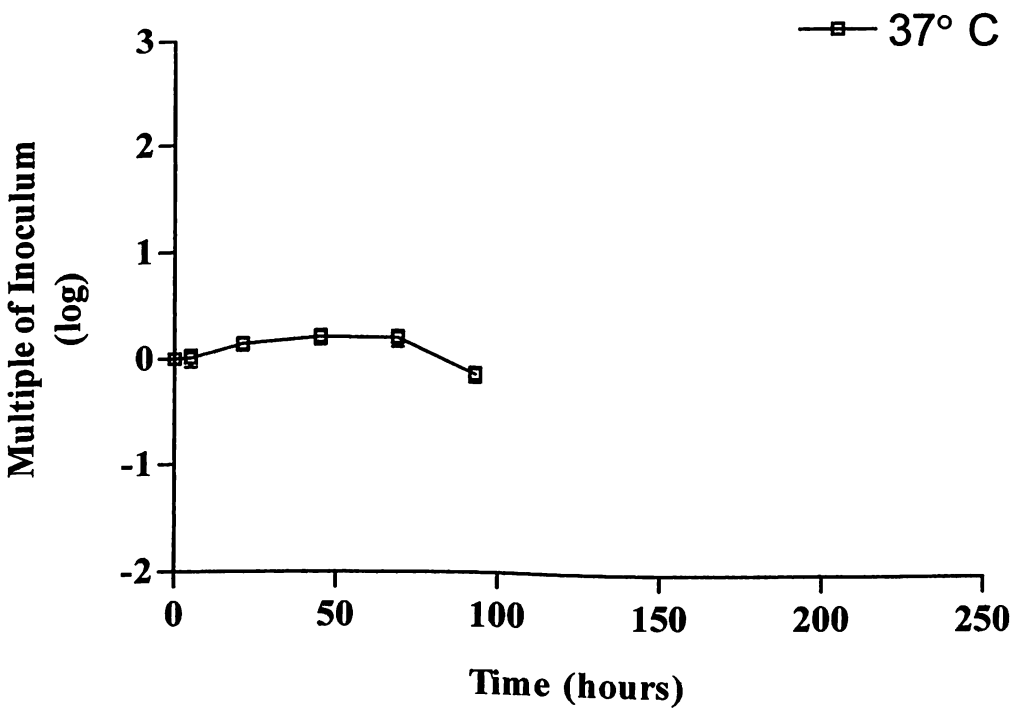


Fig. 6F. Growth of *Leishmania hertigi* at 37° C.

G

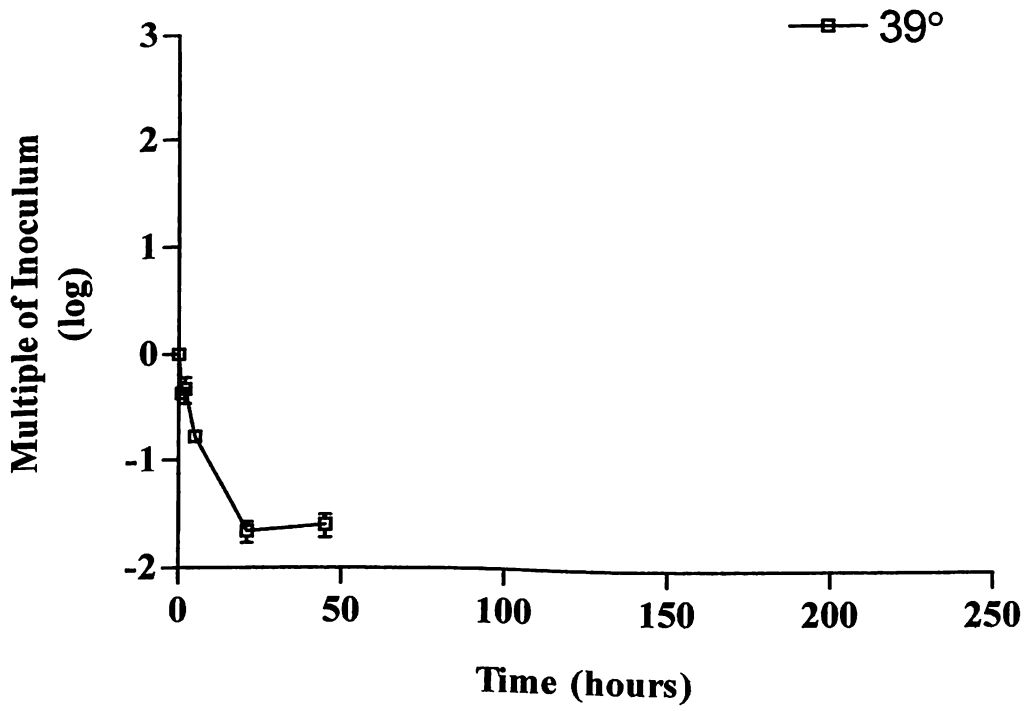


Fig. 6G. Growth of *Leishmania hertigi* at 39° C.

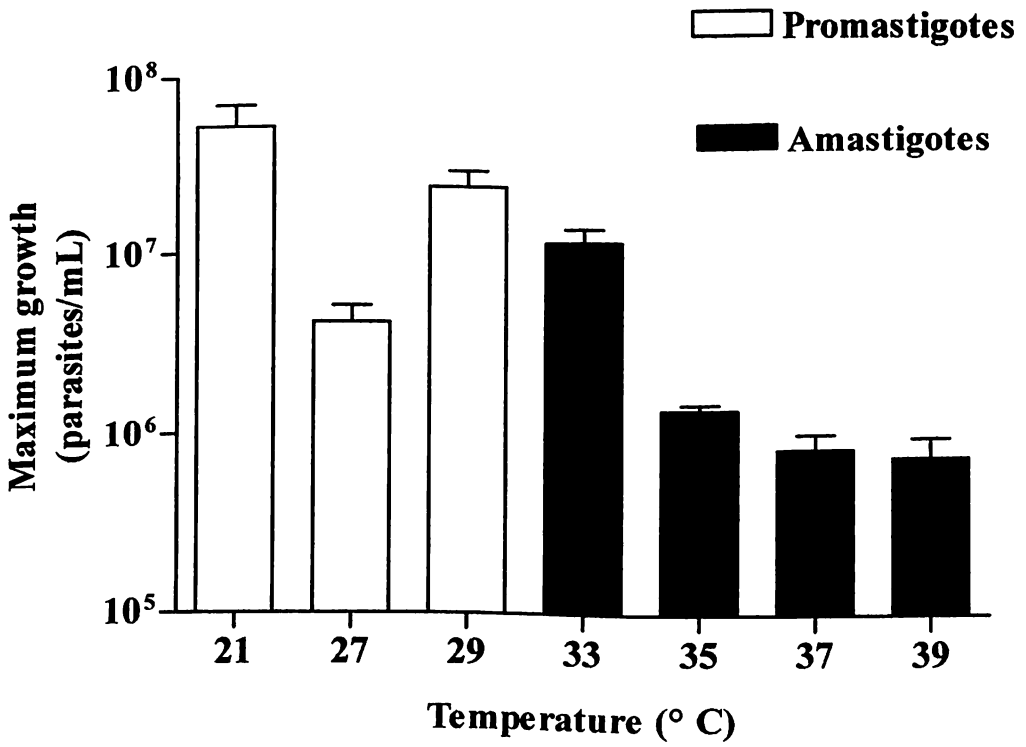


Fig. 7. Maximum growth (parasites/mL) of *Leishmania hertigi* at different temperatures studied. Open columns represent promastigote growth and closed columns represent amastigote growth. The vertical bars indicate the standard error.

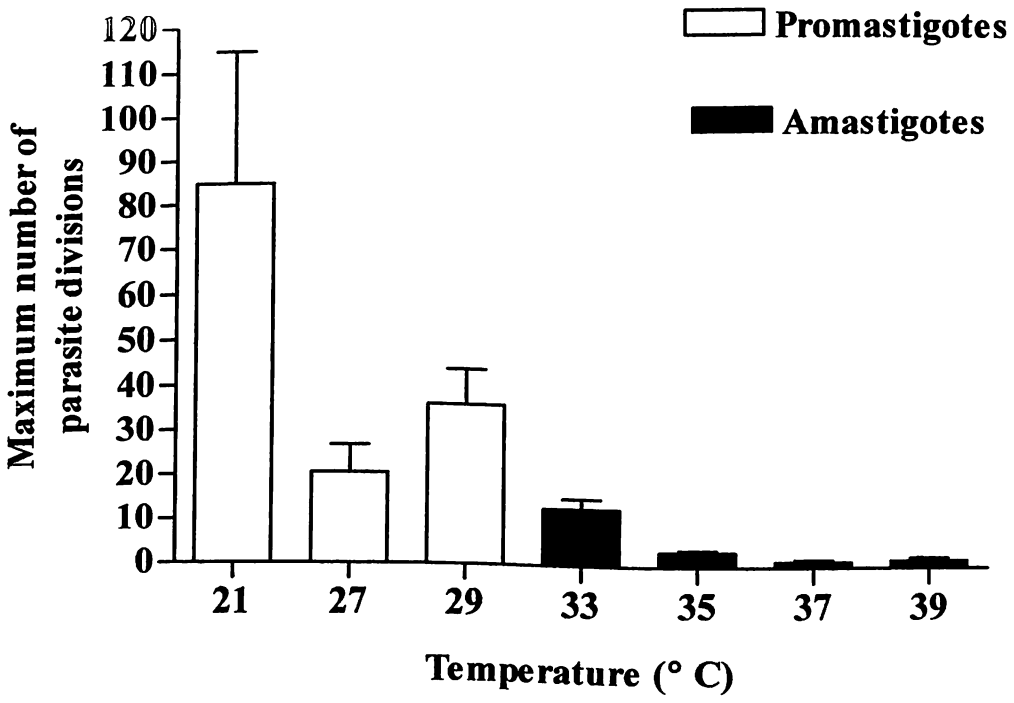


Fig. 8. Maximum number of parasite divisions for *Leishmania hertigi* at the different temperatures studied. Open columns represent promastigote growth and closed columns represent amastigote growth. The vertical bars indicate the standard error.

Discussion

Leishmania parasites face hostile environmental conditions such as dramatic temperature fluctuation, drastic pH changes, nutritional depletion in the sand fly, and host lytic and oxidative product formation (Sacks and Kamhawi, 2001; Zilberstein and Shapira, 1994). I have attempted to study one of the stresses faced by the parasites, which is increase of temperature from the sand fly host to the mammalian host. Temperature has been shown to induce the morphological transformation from the promastigote to amastigote form in axenic conditions (Ismaeel *et al.* 1998; Krassner, 1965; Leon *et al.* 1995). It also has been suggested that a correlation exists between skin/body temperature and the extent of the lesions caused by these parasites (Callahan *et al.* 1996). The purpose of this thesis project was to determine the upper limits of temperature tolerance by *L. enriettii* and *L. hertigi*, and to test whether or not body temperature of their mammalian host plays a role in their tolerance to high temperatures. With the use of a range of incubation temperatures and the incorporation of real-time PCR, growth curves were constructed to characterize temperature effects.

Based on the results, it was observed that parasites of *L. enriettii* were able to completely transform into amastigote-like organisms with temperature change alone. By phase contrast microcopy, inconclusive results were yielded if parasites of *L. hertigi* were able to undergo complete transformation with temperature change alone. All other variables in this in vitro system remained unchanged (pH, control temperature, and media). While the warmer temperatures were of more interest in

this study, growth studies also were done on a few cooler temperatures to demonstrate any differences between promastigote and amastigote growth. The American Type Culture Collection recommended that 25° C was the optimal temperature for growth of *L. enriettii* and *L. hertigi* promastigotes. Since 25° C was omitted from the study, the optimal temperature for promastigote growth can not be concluded for either *L. enriettii* or *L. hertigi*. It can be said that from Figs. 3A-C, 21° C had continuous but slow growth for a longer period of time, while at 29° C, growth was more rapid and reached the highest maximum growth. Also at 29° C, some transformation to amastigote-like organisms occurred. One reason this could have occurred is because of pH fluctuations in the media with increase of temperature. The pH of the media was never determined during the experiment.

Amastigote growth for *L. enriettii* was compared at 33, 35, 37, and 39° C (Figs. 3C-G). Complete transformation to amastigotes was evident after 45 hours for both 33 and 35° C. At 37° C, transformation was much more rapid and took place after 22 hours. Figures 4 and 5 show that of the elevated temperatures, 33° C had the most growth and number of divisions. However, only at 35° C, once promastigotes transformed to amastigotes, did additional division take place (Fig. 3E). At 33 and 37° C, amastigotes remained in a lag phase (Figs. 3D and 3F). *Leishmania enriettii* was able to withstand 37° C, but only underwent one division (Fig. 3G). One reason amastigotes were not able to enter exponential growth in the other temperatures besides 35° C could be that 35° C was their optimal temperature. Also it could be that other variables need to be changed in order to mimic in vivo conditions (Pan *et al.*

1993). Nutrients may have also been exhausted. Trager (1953) suggested that amastigotes may be more exacting in their nutritional requirements than promastigotes. Zilberstein and Shapira (1994) suggest that once promastigotes transform to amastigote-like organisms in elevated temperatures, their metabolism is reduced. This reduction, they suggest is a reason for parasite division to come to a halt. *Leishmania enriettii* was not able to tolerate 39° C, and rapid parasite death is apparent (Fig. 3G). While body temperatures of guinea pigs can range from 37.3-39.0° C, *L. enriettii* isolated from skin lesions of infected guinea pigs are limited to skin temperatures below 36° C (Berman and Neva, 1981) . The findings in this study agree with what is known about *L. enriettii* and skin temperature of guinea pigs. Thus in this in vitro system, *L. enriettii* demonstrated a temperature optimum of 33°C. The findings also suggest that *L. enriettii* could have adapted to high temperatures because the body temperatures of their host is so high. *Leishmania enriettii* has demonstrated that rapid death does not occur until 39° C, but survival in a range from 33-37° C is possible.

In this study, initial inoculum size remained constant for every temperature studied. Greenblatt and Glaser (1965) did extensive studies on morphology, activity, growth, respiration, phosphate metabolism, and retention of metabolites in *L. enriettii*. One of the findings indicated that division of parasite was not dependent on inoculum size. With the use of three inoculum sizes, Greenblatt and Glaser were able to obtain the same increase in the number of organisms each time.

Optimal temperature for promastigote growth cannot be inferred for *L. hertigi* because 25° C was not studied. However growth at 21° C produces maximum growth and number of divisions (Fig. 7 and Fig. 8).

Amastigote growth also was compared at 33, 35, 37, and 39° C. *Leishmania hertigi* growth curves (Figs. 6C-G) demonstrate that once transformation took place, amastigotes remained in a lag phase. Parasites might have been viable, but not capable of further divisions. The main difference between 33, 35, and 37° C was the time required for promastigotes to transform to amastigotes. At 35 and 37° C, promastigotes transformed more rapidly. Growth at 37° C (Fig. 6F), shows that after about 24 hours, there was slight growth, but amastigotes remained in a lag phase thereafter. At 39° C (Fig. 6G) promastigotes multiplied almost twice the original size after 5 hours, but quickly lysed due to the high temperature. This multiplication may not indicate new growth but rather completion of divisions initiated at time zero. Fig. 7 illustrates that maximum parasites/mL were about the same for 33, 35, and 37° C, but Fig. 8 demonstrates there was more growth at 33° C. Thus in this in vitro system, *L. hertigi* had a temperature optimum of 33° C. These results are consistent to what is known about New World species of *Leishmania.* In general, amastigotes of New World species have an optimal temperature range of 28 to 33° C, while Old World species prefer elevated temperatures of 37 to 39° C (Pan *et al.* 1993)

The findings also support the hypothesis in that the body temperature of the host corresponds to the limits of temperature tolerance by *L. hertigi.* Body temperature of porcupines was reported at 36.7° C in Arends and McNab (2001)

study. *Leishmania hertigi* amastigotes in this in vitro system were able to tolerate a range of 33-37° C. While death was evident at 39° C, it was not as rapid as the rate observed with *L. enriettii*. Parasites were able to complete one division. These findings are not surprising because *L. hertigi* has sometimes been found in the viscera of porcupines (Zeledon *et al.* 1997). Although the temperature of the viscera is unknown for porcupines, it appears *L. hertigi* has developed a thermotolerance to extreme temperatures.

There is no question that parasites of *Leishmania* species have developed a tolerance to extreme temperature change. Its survival and continuance is dependent on making the necessary adaptations to extreme environmental stresses. Each species could have developed their own limits of temperature tolerance depending on the body temperatures of their mammalian hosts. By this host characteristic, parasite coevolution with their host will persist. Additional studies using the in vitro system described in this project need to be demonstrated with other species of *Leishmania* to further develop this hypothesis.

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Appendix A. Real-time PCR results for *L. enriettii* and *L. hertigi* at 21, 27, 29, 33, 35, 37, and 39° C. Numbers reflect parasites/mL. Any gaps in data reflect that no signal was detected in that well. Samples were run in duplicates. E1-E4 and H1-H4 represent each trial for that experiment.

L. enriettii at 21° C.

Time (hours)	E1	E1	E2	E2	E3	E3	E4	E4
0	2.70E+05	2.80E+05	4.00E+05	4.40E+05	1.70E+05	1.80E+05	3.00E+05	3.10E+05
21	2.70E+05	4.40E+05	6.60E+05	4.30E+05	4.90E+05	3.70E+05	1.40E+05	2.30E+05
45	8.70E+05	8.40E+05	8.10E+05	7.80E+05	6.70E+05	5.90E+05	8.10E+05	7.50E+05
69	1.60E+06	2.00E+06	1.90E+06	1.40E+06	2.10E+06	2.60E+06	1.20E+06	1.50E+06
93	4.40E+06	2.40E+06	4.20E+06	4.30E+06	6.80E+06	4.10E+06	3.90E+06	4.30E+06
117	3.40E+06	5.00E+06	7.40E+06	6.60E+06	4.60E+06	4.40E+06	9.20E+06	9.20E+06
141	3.70E+06	3.60E+06	1.40E+07	1.40E+07	7.70E+06	1.30E+07	1.90E+06	1.30E+06
165	6.10E+06	3.60E+06	8.50E+06	1.30E+07	9.30E+06	9.20E+06	1.10E+07	1.10E+07
189	1.10E+07	1.10E+07	1.60E+07	2.20E+07	7.60E+06	5.50E+06	3.10E+06	3.80E+07
213	9.20E+05	1.10E+07	1.90E+07	1.90E+07	2.20E+07	1.50E+07	1.40E+07	2.60E+07

L. enriettii at 27° C.

Time (hours)	E1	E1	E2	E2	E3	E3	E4	E4
0	5.30E+05	6.20E+05	5.50E+05	1.40E+06	4.80E+05	4.70E+05	4.90E+05	5.00E+05
5	4.20E+05	7.30E+05	8.60E+05	9.10E+05	6.30E+05	9.90E+05	6.50E+05	8.40E+05
21	1.30E+06	2.10E+06	1.60E+06	2.30E+06	1.30E+06	1.20E+06	1.00E+06	1.40E+06
45	1.40E+07	9.20E+06	2.20E+07	2.40E+07	9.90E+06	1.00E+07	7.00E+06	1.10E+07
69	1.10E+07	1.50E+07	1.40E+07	1.10E+07	9.30E+06	1.20E+07	1.30E+07	1.20E+07
93	2.90E+07	1.80E+07	1.80E+07	1.30E+07	6.60E+06	1.40E+07	9.70E+06	1.10E+07
117	8.90E+06	1.50E+07	8.60E+06	9.00E+06	9.60E+06	2.70E+07	3.60E+07	2.60E+07
141	1.30E+07	1.20E+07	1.70E+07	1.50E+07	5.50E+06	1.20E+07	2.10E+07	1.40E+07

L. enriettii at 29° C.

Time (hours)	E1	E1	E2	E2	E3	E3	E4	E4
0	1.70E+05	1.70E+05	2.80E+05	3.60E+05	1.20E+05	1.20E+05	1.50E+05	1.40E+05
5	3.70E+05	3.50E+05	1.40E+05	1.40E+05	1.30E+05	1.30E+05	1.20E+05	1.40E+05
21	1.10E+06	1.50E+06	9.40E+05	1.10E+06	1.80E+06	1.60E+06	7.80E+05	8.20E+05
45	5.50E+06	4.30E+06	4.30E+06	4.20E+06	4.20E+06	3.00E+06	5.10E+06	3.60E+06
69	6.60E+06	9.20E+06	1.20E+07	9.30E+06	1.00E+07	1.20E+07	7.80E+06	9.80E+06
93	2.00E+07	2.00E+07	1.50E+07	1.80E+07	1.40E+07	1.10E+07	1.70E+07	1.80E+07
117	1.10E+07	1.90E+07	2.10E+07	2.30E+07	1.60E+07	1.60E+07	2.40E+07	2.00E+07
141	1.10E+07	7.40E+06	1.20E+07	1.10E+07	9.70E+06	7.90E+06	5.50E+06	2.00E+06

L. enriettii at 33° C.

Time (hours)	E1	E1	E2	E2	E3	E3	E4	E4
0	3.20E+05	3.50E+05	2.70E+05	3.30E+05	2.60E+05	2.80E+05	1.00E+05	1.40E+05
5	3.20E+05	3.50E+05	6.20E+05	6.10E+05	4.50E+05	4.40E+05	7.00E+05	7.10E+05
21	3.10E+06	2.80E+06	1.30E+06	1.20E+06	1.30E+06	1.10E+06	1.10E+06	1.10E+06
45	6.90E+06	7.40E+06	6.60E+06	5.30E+06	7.90E+06	6.80E+06	4.40E+06	4.00E+06
69	1.00E+07	6.50E+06	6.30E+06	6.30E+06	6.90E+06	4.60E+06	6.10E+06	8.20E+06
93	2.50E+06	4.50E+06	6.10E+06	4.20E+06	9.30E+06	4.30E+06	6.60E+06	4.40E+06
117	4.20E+06	3.90E+06	3.40E+06	3.20E+06	7.10E+06	5.90E+06	3.50E+06	2.80E+06
141	7.00E+06	4.50E+06	3.60E+06	4.40E+06	4.40E+06	3.80E+06	7.60E+06	6.90E+06

L. enriettii at 35° C.

Time (hours)	E1	E1	E2	E2	E3	E3	E4	E4
0	3.30E+05	4.90E+05	2.10E+05	2.60E+05	9.20E+04	1.40E+05	3.20E+05	3.20E+05
5	5.70E+05	4.70E+05	3.30E+05	3.40E+05	3.00E+05	3.10E+05	3.30E+05	3.00E+05
21	4.30E+05	3.60E+05	3.90E+05	4.20E+05	1.20E+06	7.70E+05	6.20E+05	4.40E+05
45	1.90E+05	2.70E+05	2.80E+05	2.40E+05	4.90E+05	3.80E+05	6.60E+05	2.30E+05
69	1.70E+05	1.80E+05	8.60E+04	9.60E+04	8.80E+05	5.70E+05	1.10E+06	1.00E+06
93	1.00E+06	1.20E+06	1.50E+06	1.20E+06	6.70E+05	1.10E+06	1.30E+06	1.10E+06
117	1.20E+06	1.20E+06	1.40E+06	1.60E+06	1.10E+06	9.80E+05	8.00E+05	6.40E+05
141			9.50E+05	9.50E+05	9.10E+05	1.20E+06	8.20E+05	6.90E+05

L. enriettii at 37° C.

Time (hours)	E1	E1	E2	E2	E3	E3	E4	E4
0	1.90E+05	1.80E+05	2.00E+05	2.10E+05	1.10E+05	1.20E+05	2.00E+05	2.00E+05
5	1.30E+05	1.30E+05	1.00E+05	1.20E+05	8.40E+04	2.20E+05	3.60E+05	2.70E+05
21	2.90E+05	4.00E+05	1.90E+05	1.50E+05	2.00E+05	2.20E+05	2.10E+05	2.10E+05
45	2.70E+05	3.80E+05	4.00E+05	3.00E+05	4.00E+04	3.30E+05	3.30E+05	2.40E+05
69	2.10E+05	2.00E+05	6.10E+05	3.80E+05	1.30E+05	1.90E+05	1.30E+05	2.30E+05
93	1.10E+05	3.20E+04	2.60E+05	1.30E+05	9.10E+04	9.30E+04	2.00E+05	1.20E+05

L. enriettii at 39° C.

Time (hours)	E1	E1	E2	E2	E3	E3	E4	E4
0	5.70E+05	5.30E+05	3.40E+05	3.70E+05	6.00E+05	5.80E+05	4.00E+05	3.90E+05
1	2.30E+05	1.70E+05	1.90E+05	1.60E+05	1.60E+05	1.60E+05	2.20E+05	2.40E+05
2	3.40E+05	3.50E+05	3.80E+05	2.70E+05	1.30E+05	1.60E+05	4.70E+04	2.70E+04
21	8.50E+04	7.70E+04	7.60E+04	6.40E+04	7.60E+04	5.90E+04	8.60E+04	8.00E+04
45	1.20E+04	9.60E+03	5.20E+03	2.00E+04	8.60E+03	7.60E+03	8.40E+03	6.70E+03

L. hertigi at 21° C.

Time (hours)	H1	H1	H2	H2	H3	H3	H4	H4
0	8.50E+05	1.10E+06	1.30E+05	3.10E+05	5.70E+05	4.90E+05	3.50E+05	3.70E+05
21	4.60E+05	1.00E+06	6.90E+04	1.70E+05	2.90E+06	2.00E+06	9.80E+05	1.10E+06
45	9.90E+06	1.10E+07	1.40E+07	8.10E+06	1.10E+07	4.20E+06	6.80E+06	3.30E+06
69	2.50E+07	3.00E+07	6.70E+06	2.20E+06	2.30E+07	8.20E+06	2.00E+07	2.60E+07
93	4.40E+07	4.70E+07	5.40E+05	8.80E+05	4.70E+07	3.50E+07	6.00E+07	6.70E+07
117	2.50E+07	3.80E+07	6.80E+06	9.20E+06	4.50E+07	5.90E+07	1.50E+08	9.20E+07
141	6.00E+07	4.60E+07	1.90E+07	1.60E+07	4.30E+07	4.10E+07	5.00E+07	2.10E+07
165	2.70E+07	1.60E+07	1.40E+07	2.10E+07	4.80E+07	5.40E+07	4.50E+07	4.20E+07
189	3.90E+07	7.20E+07	1.70E+06	1.30E+06	3.90E+07	2.70E+07	7.70E+07	9.90E+07
213	2.50E+07	2.40E+07	5.90E+06	5.00E+06	2.30E+07	2.20E+07	3.30E+07	4.70E+07

L. hertigi at 27° C.

Time (hours)	H1	H1	H2	H2	H3	H3	H4	H4
0	2.40E+05	2.70E+05	1.60E+05	1.20E+05		2.50E+05	3.70E+05	4.30E+05
5	1.10E+05	8.40E+05	1.30E+05	1.90E+05	1.50E+05	5.30E+05	2.00E+05	7.50E+04
21	1.50E+06	1.30E+06	1.10E+06	1.00E+06	4.50E+05	4.90E+05	7.40E+02	1.70E+03
45	5.30E+05	7.70E+05	4.70E+06	4.00E+06	1.80E+06	2.80E+06	9.60E+05	9.70E+05
69	7.00E+06	7.80E+06	6.00E+06	5.80E+06	9.00E+05	7.10E+05	2.70E+06	2.80E+06
93	3.40E+06	3.70E+06	4.70E+05	2.00E+05	3.30E+06	3.40E+06	6.10E+06	6.40E+06
117	4.30E+06	3.00E+06	1.50E+06	5.50E+06	3.60E+05	1.30E+05	3.10E+06	3.10E+06
141	1.10E+06	1.10E+06	5.70E+05	9.90E+05	2.20E+06	2.10E+06	3.80E+06	3.40E+06

L. hertigi at 29° C.

Time (hours)	H1	H1	H2	H2	H3	H3	H4	H4
0	6.10E+05	4.20E+05	7.30E+05	8.40E+05	7.20E+05	7.00E+05	6.70E+05	5.40E+05
5	4.80E+05	6.40E+05	8.60E+05	6.60E+05	2.20E+05	2.60E+05	4.70E+05	3.60E+05
21	3.20E+06	5.10E+06	4.50E+06	1.00E+06	7.40E+06	5.40E+06	4.30E+07	4.70E+07
45	4.30E+06	7.20E+06	2.00E+07	1.60E+07	5.20E+07	3.50E+07	3.50E+07	2.40E+07
69	3.50E+07	3.10E+07	7.00E+06	4.50E+06	2.20E+06	7.10E+06	1.50E+07	2.10E+07
93	2.00E+07	1.60E+07	1.20E+07	1.20E+07	1.50E+07	1.60E+07	3.10E+07	2.90E+07
117	1.50E+07	2.30E+07	2.30E+07	2.30E+07	2.60E+07	3.00E+07	1.70E+07	1.50E+07
141	9.30E+06	1.10E+07	7.60E+06	8.90E+06	3.80E+07	3.60E+07	1.70E+07	1.40E+07

L. hertigi at 33° C.

Time (hours)	H1	H1	H2	H2	H3	H3	H4	H4
0	1.00E+06	1.10E+06	1.00E+06	1.00E+06	8.90E+05	7.30E+05	7.10E+05	8.40E+05
5	6.80E+05	8.70E+05	9.60E+05	1.00E+06	6.70E+05	4.70E+05	1.30E+06	1.90E+06
21	2.30E+06	1.50E+06	1.90E+06	2.50E+06	5.00E+06	2.90E+06	2.70E+06	2.50E+06
45	6.50E+06	9.80E+06	1.30E+07	2.60E+07	1.20E+07	1.30E+07	6.60E+06	6.70E+06
69	1.70E+07	1.40E+07	7.80E+06	9.00E+06	1.30E+07	9.60E+06	9.20E+06	6.60E+06
93	1.00E+07	1.40E+07	6.80E+06	7.70E+06	1.10E+07	1.20E+07	1.10E+07	1.00E+07
117	1.00E+07	6.40E+06	1.00E+07	8.60E+06	1.10E+07	9.80E+06	1.00E+07	9.60E+06
141	1.00E+07	7.70E+06	5.80E+06	8.00E+06	1.50E+07	1.30E+07	5.20E+06	5.40E+06

L. hertigi at 35° C.

Time (hours)	H1	H1	H2	H2	H3	H3	H4	H4
0	3.30E+05	3.10E+05	4.40E+05	5.20E+05	6.40E+05	6.50E+05	4.50E+05	3.80E+05
5	2.90E+05	3.00E+05	8.50E+05	8.70E+05	3.50E+05	3.50E+05	5.80E+05	4.30E+05
21	1.40E+06	1.50E+06	1.90E+06	1.70E+06	8.90E+05	8.00E+05	1.20E+06	1.10E+06
45	1.60E+06	1.50E+06	1.30E+06	1.30E+06	1.10E+06	8.70E+05	1.50E+06	1.90E+06
69	1.10E+06	1.10E+06	1.90E+06	1.80E+06	1.30E+06	1.20E+06	1.30E+06	1.00E+06
93	1.50E+06	1.40E+06	1.30E+06	1.00E+06	9.70E+05	9.30E+05	1.60E+06	1.40E+06
117	7.40E+05	9.30E+05	7.80E+05	7.10E+05	1.10E+06	1.10E+06	1.50E+06	1.20E+06
141	6.60E+05	7.90E+05	6.00E+05	1.10E+06	1.20E+06	1.20E+06	2.50E+06	

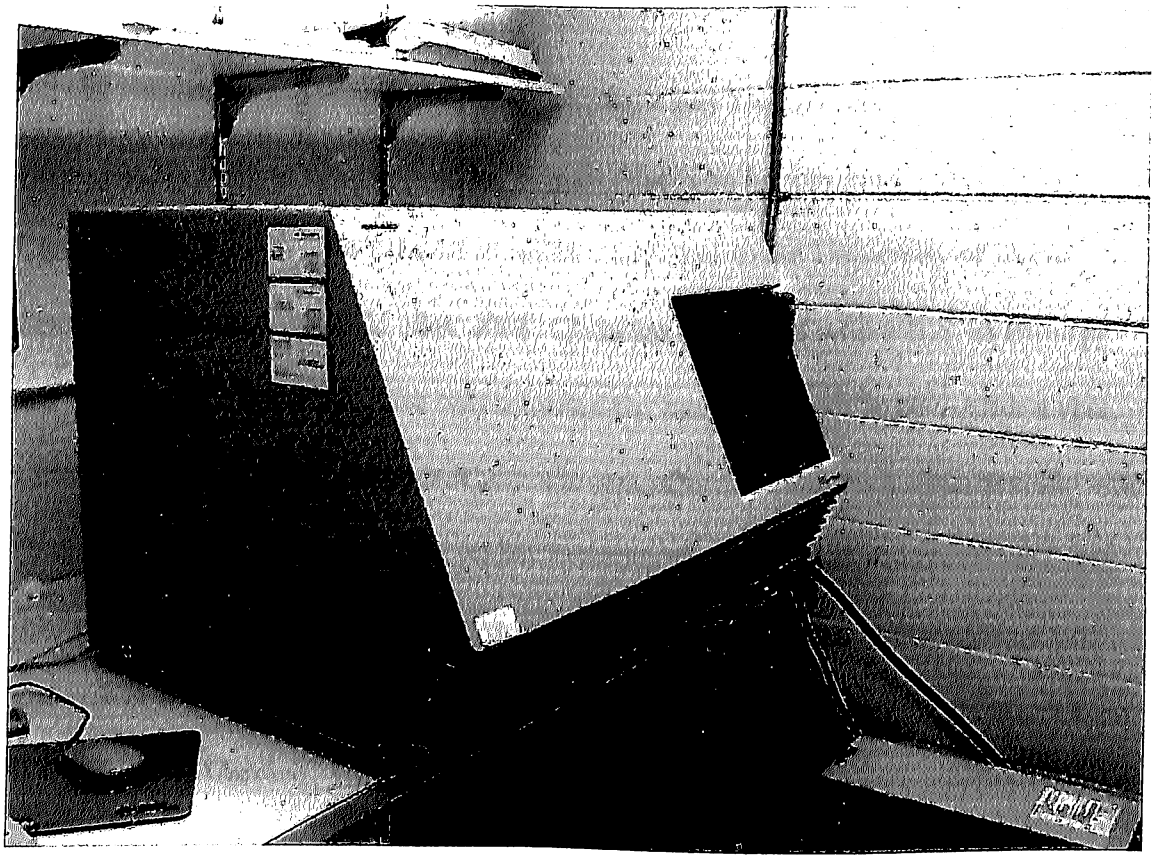
L. hertigi at 37° C.

Time (hours)	H1	H1	H2	H2	H3	H3	H4	H4
0	5.20E+05	5.50E+05	6.40E+05	6.90E+05	8.70E+05	1.00E+06	5.20E+05	4.70E+05
5	1.10E+06	1.80E+06	5.80E+05	6.60E+05	5.10E+05	6.50E+05	5.20E+05	
21	3.00E+05	2.40E+05	2.70E+05	3.50E+05	1.80E+05	1.20E+05	1.10E+05	8.60E+04
45	5.30E+05	6.30E+05	7.00E+05	5.70E+05	2.70E+05	2.70E+05	1.90E+05	1.90E+05
69	4.30E+05	3.70E+05	2.40E+05	2.10E+05	2.20E+05	1.50E+05	2.90E+05	3.30E+05
93	2.60E+05	3.00E+05	5.00E+05	2.20E+05	3.60E+05	3.70E+05	1.30E+05	1.00E+05

L. hertgi at 39° C.

Time (hours)	H1	H1	H2	H2	H3	H3	H4	H4
0	5.10E+05		3.70E+05	3.90E+05	7.80E+05	8.90E+05	3.90E+05	3.90E+05
1	5.30E+05	5.20E+05	2.70E+05	2.50E+05	1.10E+06	9.30E+05	7.20E+05	6.90E+05
2	2.30E+05	2.50E+05	5.90E+05	4.90E+05	5.90E+05	4.50E+05	1.30E+06	2.00E+06
21	1.40E+05	1.30E+05	8.60E+04	8.00E+04	9.00E+04	9.90E+04	8.60E+04	8.50E+04
45	2.70E+04	2.70E+04	7.70E+03	7.10E+03	1.70E+04	2.00E+04	9.40E+03	1.60E+04

Appendix B. ABI Prism ® 7700 Sequence Detection System



VITA

Christina Salinas, daughter of Jose Oscar and Thelma Salinas, was born on February 21, 1980 in Corpus Christi, Texas. She is an honors graduate from Mary Carroll High School. Her interest in science took her to the University of North Texas, in Denton, Texas. In May 2003, Christina graduated with a B.S. in Biology and a minor in Chemistry. She was awarded the honors of cum laude. After a year, Christina moved to San Antonio where she was offered a Research Assistantship with Dr. Sara F. Kerr. While working on her masters, she partook in extensive fieldwork studies on the ecology of *Leishmania mexicana*. For her thesis, she conducted her research at the Southwest Foundation for Biomedical Research under the supervision of Dr. Ricardo Carrion. Her thesis involved the study of temperature tolerance by *Leishmania enriettii* and *Leishmania hertigi* using a real-time PCR assay.