The Effect of Temperature on *Leishmania* (Kinetoplastida: Trypanosomatidae) Development in Sand Flies

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ABSTRACT The spread of leishmaniasis to areas where it was previously considered nonendemic has been recently found in the New and Old Worlds, and climate changes are suspected as a crucial factor responsible for this spread. Ambient temperature is known to significantly affect the metabolism of sand flies and their developmental times, but little is known about the effect of temperature on the *Leishmania* life cycle in vectors. This study assesses the effect of temperature on the development of two closely related New World *Viannia* species, *Leishmania braziliensis* and *Leishmania peruviana*, in the permissive vector *Lutzomyia longipalpis*, and on the development of New and Old World *Leishmania infantum* in its natural vectors *Lu. longipalpis* and *Phlebotomus perniciosus*, respectively. The mountain species *L. peruviana* developed well in sand fly females kept at 20°C, whereas at 26°C, most infections were lost during the defecation of bloodmeal remains; this suggests an adaptation to the slower metabolism of sand flies living at lower ambient temperature. On the contrary, *L. infantum* and *L. braziliensis* developed well at both temperatures tested; heavy late-stage infections were observed in a majority of sand fly females maintained at 20°C as well 26°C. Frequent fully developed infections of *L. infantum* and *L. braziliensis* at 20°C suggest a certain risk of the spread of these two *Leishmania* species to higher latitudes and altitudes.

KEY WORDS sand fly, leishmaniasis, *Viannia*, climate change

Leishmania (Kinetoplastida: Trypanosomatidae) are digenetic parasites causing leishmaniasis; their only proven vectors are phlebotomine sand flies (Diptera: Phlebotominae). *Leishmania* infecting mammals belong to two subgenera, *Leishmania* and *Viannia*, which differ not only in their distribution but also in their development in the sand fly vector (reviewed by Lainson 2010).

The two *Viannia* species chosen for this study, *Leishmania braziliensis* and *Leishmania peruviana*, are phylogenetically closely related but differ in the clinical outcome of the disease and occur in very distinct biotopes. Whereas *L. peruviana* causes only cutaneous leishmaniasis and its distribution is restricted to the Andean mountains, *L. braziliensis* can progress to mucocutaneous lesions and is widely distributed in the lowlands of Latin America (Lainson 2010). The third parasite species tested, *Leishmania infantum*, is the causative agent of visceral and cutaneous leishmaniasis in many countries of the Old World, as well as the New World, where it is known as *Leishmania chagasi*. In the Old World, *L. infantum* is transmitted by *Phlebotomus* sand flies, including *Phlebotomus perniciosus* (reviewed by Killick–Kendrick 1999). Because of European colonists and their dogs, it was introduced to Latin America (Mauricio et al. 2000), where it adapted to the local permissive sand fly *Lutzomyia longipalpis* (Volf and Myskova 2007).

Recently, *L. infantum* has spread to new areas and higher latitudes, where it was previously considered nonendemic. For example, a new focus of human visceral leishmaniasis was detected in the city of Posadas (northeast Argentina) (Salomon et al. 2008). In Europe, the northward spread of *L. infantum* has been recorded in northern Italy (Maroli et al. 2008) and Catalonia, Spain (Ballart et al. 2012). A progressive increase in *L. infantum* seroprevalence in dogs has been observed in the foothills of the French Pyrenees (Dereure et al. 2009) and in the Alpujarras region in Spain (Martín–Sánchez et al. 2009).

The spread of leishmaniasis may be enhanced by several factors, including human-made and environmental changes, immune status, and drug resistance (reviewed by Dujardin 2006). In Europe, climate changes are considered a crucial factor responsible for the spread of the disease. Ready (2008) stressed that changes of climate and ambient temperature can affect the distribution of leishmaniases via sand fly abundance or via the effect of temperature on parasite development in the vector. Ambient temperature significantly affects the digestion, metabolic processes, and developmental times of sand flies (Benkova and Volf 2007), but there is only one publication about the effect of temperature on the *Leishmania* life cycle in vectors: Rioux et al. (1985) demonstrated that *L. infantum* develops in the digestive tract of *Phlebotomus*

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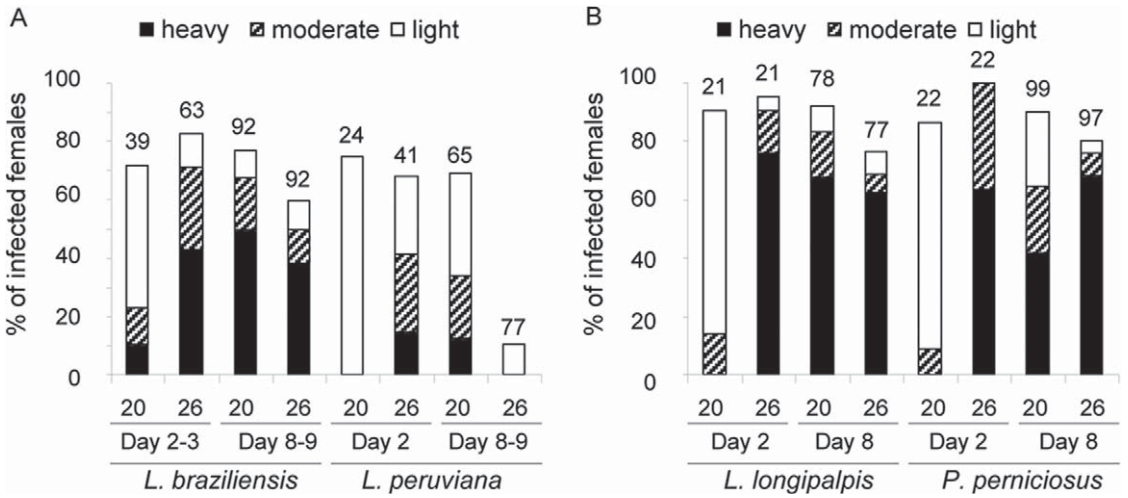

Fig. 1. Development of *Leishmania* strains in sand flies maintained at 20°C and 26°C. (A) *Leishmania braziliensis* and *Leishmania peruviana* in *Lutzomyia longipalpis*. (B) *Leishmania infantum* in *Lu. longipalpis* and *Phlebotomus perniciosus*. Intensities of infection were estimated as: light (<100 promastigotes/gut), white bar; moderate (100–1,000 promastigotes/gut), striped bar; and heavy (>1,000 promastigotes/gut), black bar. Numbers above each bar indicate the number of dissected females. Numbers on the "x" axis: 20 = 20°C; 26 = 26°C.

ariasi better at higher temperatures compared with the lower temperatures tested.

Therefore, the current study is focused on the effect of temperature on the development of three different *Leishmania* species; *L. infantum* was tested in its two natural vectors, *P. perniciosus* and *Lu. longipalpis*, whereas the development of two *Viannia* species, *L. braziliensis* and *L. peruviana*, was compared in their unnatural vector *Lu. longipalpis*, a permissive sand fly species frequently used as a laboratory model.

Materials and Methods

Sand Flies and Parasites. Laboratory colonies of *P. perniciosus* (Murcia, Spain) and *Lu. longipalpis* (Jacobina, Brazil) were reared at 25–26°C under standard conditions. Three *Leishmania* species were used: *L. infantum* (MCAN/PT/2005/IMT373), *L. braziliensis* (MHOM/PE/1993/LC2177), and *L. peruviana* (MHOM/PE/1990/HB86).

Experimental Infections. Sand fly females were fed through a chick-skin membrane on heat-inactivated rabbit blood containing 1×10^6 (for *L. infantum*) or 5×10^6 (for *L. braziliensis* or *L. peruviana*) promastigotes per milliliter of blood. Blood-fed females were maintained at 20 or 26°C, and on days 2–3 and 8–9 post-bloodmeal (PBM), either examined under a light microscope or used for DNA isolation. Microscopically, parasite loads were graded into three categories as described by Myskova et al. (2008). Infection rates (percentage of infected females) and intensities of infection were compared by the χ^2 test (S-PLUS 2000).

Real-Time Polymerase Chain Reaction. Extraction of total DNA from infected females was performed using a High Pure PCR Template Preparation Kit (Roche, Czech Republic) according to the manufacturer's instructions. DNA was eluted in 100 μl elution buffer and stored at −20°C. Quantitative polymerase chain reaction was performed by the SYBER Green detection method (iQSYBER Green Supermix, Bio-Rad, Hercules, CA). DNA of *L. braziliensis* and *L. peruviana* was amplified according to method by Castilho et al. (2008) and *L. infantum* according to the method by Mary et al. (2004). Statistical evaluation was performed by the Kruskal–Wallis test and Mann–Whitney *U* test (STATISTICA 6.1, StatSoft).

Sand Fly Defecation. *P. perniciosus* and *Lu. longipalpis* females were fed through a chick-skin membrane on heat-inactivated rabbit blood, and the method described by Benkova and Volf (2007) was used to compare their defecation times at different temperatures. Briefly, fully blood-fed females were individually placed in small glass vials, maintained at 20 or 26°C and checked twice a day under a binocular microscope for defecation. Data were evaluated by the Mann–Whitney *U* test (STATISTICA 6.1).

Results and Discussion

Experimental infections of *L. braziliensis* and *L. peruviana* were studied in *Lu. longipalpis* to compare the development of these two *Viannia* species at different ambient temperatures (20 vs. 26°C). On days 2 and 3 PBM, the infection rate was comparable for all four parasite–temperature combinations studied. However, at 26°C, both *Viannia* species multiplied faster, resulting in more numerous heavy infections than at 20°C (*L. braziliensis:* χ^2 test $P < 0.00002$; *L. peruviana:* χ^2 test $P < 0.0005$) (Fig. 1A).

On days 8 and 9 PBM, *L. braziliensis* developed well in both temperatures tested. Despite the fact that the infection rate was significantly higher at 20°C (χ^2 test $P < 0.02$), the intensity of *L. braziliensis* infections

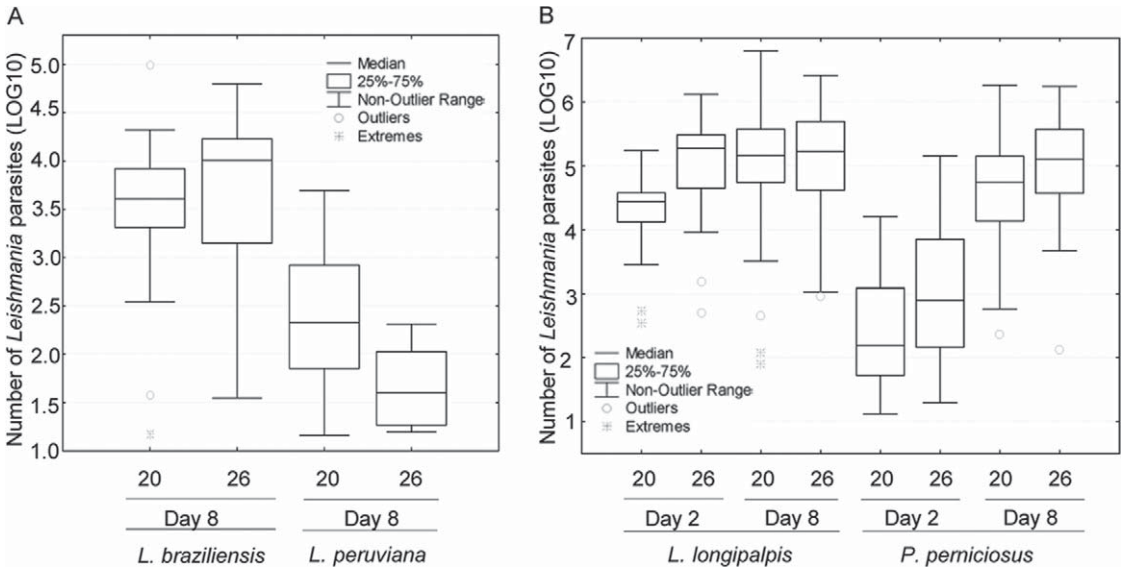

Fig. 2. Analysis of *Leishmania* parasites loads in infected sand flies maintained at 20 and 26°C by quantitative polymerase chain reaction. (A) *L. braziliensis* and *L. peruviana* in *Lu. longipalpis.* (B) *L. infantum* in *Lu. longipalpis* and *P. perniciosus.* Numbers on the "x" axis: 20 = 20°C; 26 = 26°C.

measured by quantitative polymerase chain reaction (parasite loads) did not significantly differ between the two temperatures tested (Figs. 1A and 2A). In contrast, *L. peruviana* developed well only in *Lu. longipalpis* females maintained at 20°C, whereas at 26°C, its infection rates and parasite loads were extremely low on days 8–9 PBM (Figs. 1A and 2A).

These experiments revealed that early stage infections of *L. peruviana* thrive at 26°C, but that almost 100% of infections are then lost between days 2 and 8. This is the time when sand fly females defecate and parasites might be expelled with bloodmeal remnants. We studied the timing of *Lu. longipalpis* defecation and found that it is affected by the ambient temperature. The lower temperature tested delayed defecation for ≈3 d. Whereas at 26°C, >80% females defecated by day 3 PBM (72 h), at 20°C, it took ≈6–7 d PBM (160–168 h) for a similar percentage of females to defecate (Fig. 3). Delayed defecation at 20°C provided more time to parasites to become established in the sand fly gut. Because of the fact that *L. peruviana* is a geographically restricted mountain species, we suppose that it is adapted to the slower metabolism of sand flies living in lower ambient temperatures.

The development of *L. infantum* was compared in its two natural vectors, *Lu. longipalpis* and *P. perniciosus,* and at two ambient temperatures, 20 and 26°C. In all vector–temperature combinations tested, *L. infantum* developed well, producing high infection rates and heavy late-stage infections on day 8 PBM (Figs. 1B and 2B). In early stage infections (day 2 PBM), however, the temperature affected the intensity of infections, with lower parasite loads observed at 20°C in both vectors (Figs. 1B and 2B). This suggests that the lower ambient temperature resulted in slower parasite growth within the bloodmeal, but did not have any negative effect on the establishment of *L. infantum* within the midgut. Fully developed heavy late-stage infections were frequently observed at both temper-

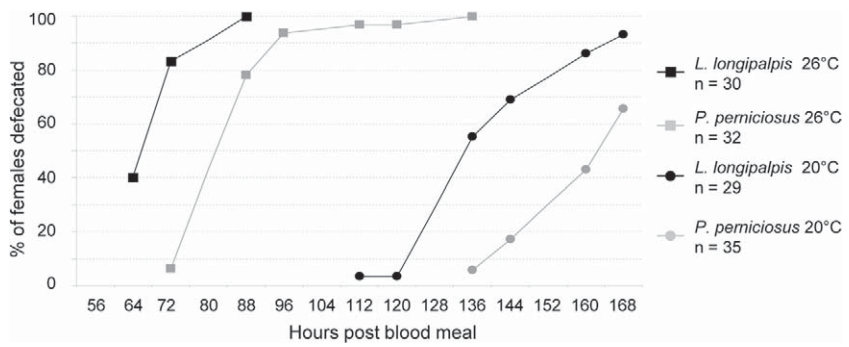

Fig. 3. Defecation of blood-fed *Lu. longipalpis* and *P. perniciosus* females maintained at 20 and 26°C.

atures tested and both vectors, *Lu. longipalpis* and *P. perniciosus* (Figs. 1B and 2B).

Similarly to *Lu. longipalpis*, lower ambient temperature also delayed defecation in *P. perniciosus* (Fig. 3). Whereas at 26°C, almost 90% of females defecated by day 4 PBM (96 h), at 20°C, the defecation was delayed for >3 d and only two-thirds of *P. perniciosus* females were defecated by day 7 PBM (168 h). Comparison of two vectors revealed that at both temperatures tested, *Lu. longipalpis* digest the bloodmeal faster and defecate significantly earlier than *P. perniciosus* (T = 20°C: Mann–Whitney *U* test $P < 0.0001$; T = 26°C: Mann–Whitney *U* test $P < 0.0001$) (Fig. 3). This finding corresponds with the previous observations of Volf and Killick–Kendrick (1996), who showed that at 25°C, *Lu. longipalpis* digest the bloodmeal more quickly than *P. perniciosus* and other four sand fly species tested.

Recent findings of new leishmaniases foci in Latin America and Europe urge for more data on the development of *Leishmania* in their vectors. Here we demonstrate the ability of *L. infantum* and *L. braziliensis* to develop heavy late-stage infections in sand flies even at 20°C. This could be an important factor enabling the spread of leishmaniases into new areas. Therefore, the risk of spread of these two *Leishmania* species to higher latitudes and altitudes should not be neglected.

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