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Phlebovirus, Leishmania et phlébotomes : interactions et impacts sur la biologie et la transmission de ces agents pathogènes

Présentée par Lison LAROCHE
Le 15 décembre 2022

Sous la direction de Anne-Laure BAÑULS
& Rémi CHARREL
Et le co-encadrement de Jorian PRUDHOMME
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Presented by Lison LAROCHE
December 15, 2022

Directed by Anne-Laure BAÑULS
& Rémi CHARREL
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« Quand y'a larve, y'a larve. Quand y'a pas larve, y'a pas larve… »

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Auteur(e) anonyme



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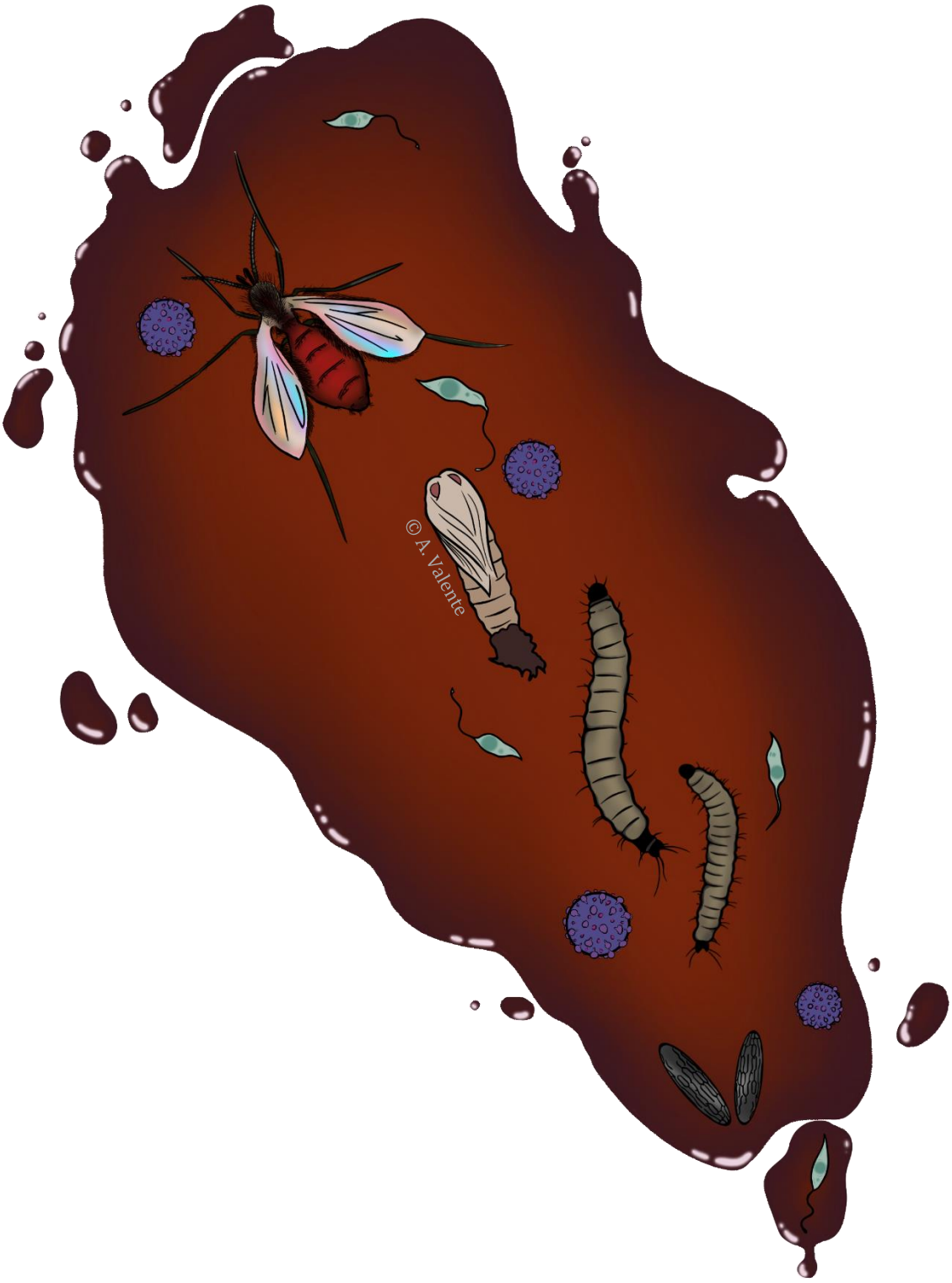
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PREAMBLE

Vector-borne diseases are caused by pathogens (*i.e.* parasites, viruses, bacteria) which are inoculated and spread by vectors. They are responsible for more than 17% of infectious diseases and cause more than 700 000 deaths each year worldwide. Vectors are hematophagous arthropods that actively transmit (mechanically or biologically) the pathogen from one vertebrate to another. They are mainly blood-sucking insects and mites, such as sand flies, mosquitoes or ticks. The epidemiology of vector-borne diseases depends on several factors: vectorial capacity, vectorial competence and vector distribution; pathogen infectivity, its host specificity and resistance to drugs; human activities and environmental conditions (*e.g.* climate). Vector-host-pathogen interactions can be modulated by these factors, therefore it is necessary to understand them to prevent the spread of vector-borne diseases. This thesis focuses on vector-pathogen interactions between sand fly, *Leishmania* and *Phlebovirus*. Sand flies are distributed over most intertropical regions of the world and responsible for pathogen transmission. They transmit parasites of the genus *Leishmania*, responsible for leishmaniasis, a disease which can be lethal if left untreated. Sand flies also transmit viruses of the genus *Phlebovirus*, which are responsible for infectious diseases expanding over the Mediterranean basin. Thus, the objective of this work was to investigate the interactions and impact of the parasite *Leishmania infantum* and the *Phlebovirus* Toscana virus, on the transmission dynamics and biology of their common vector, *Phlebotomus perniciosus*. This thesis is divided into five parts: an introduction contextualizing the study and presenting the general and specific objectives, a first chapter describing the setting up of sand fly colony and experimental infections in the laboratory, a second chapter on the study of vector-parasite interactions and a third chapter on vector-virus interactions describing, for the first time, the cycle of Toscana virus within the vector. The thesis concludes with a general discussion confirming the necessity to consider the vector, pathogen, host and environment through their interactions to prevent emergence of vector-borne diseases in a sustainable manner.


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INTRODUCTION

1. Phlebotomine sand flies

a. Taxonomy

Sand flies are insects belonging to the order Diptera, suborder Nematocera, family Psychodidae and subfamily Phlebotominae (1). Out of the approximately 1000 species identified and described worldwide, at least 98 have been defined as potential and proven vectors of pathogens (2,3). The vectors are mainly of *Phlebotomus* (Old World) and *Lutzomyia* (New World) genera, but recent studies have shown that the genus *Sergentomyia* can also be a potential vector (4). Generally, the identification of sand flies is based on morphological analyses by observation of: external characteristics (male genitalia, antenna, etc.) or internal structures (female spermathecae, cibarium, and pharynx) (5,6). There are, more recent methods to identify and classify sand fly species, although they are still expensive and cannot be applied globally. These modern methods include chromosome analysis, molecular and phylogenetic analysis (DNA barcoding and Next-Generation Sequencing), and mass spectrometry with MALDI-TOF (7,8).

b. Geographical distribution

Sand flies are widespread in warm regions such as the Mediterranean basin, South America, Asia and Africa (**Figure 1**). They have a global distribution between latitudes 50°N and 40°S, excluding New Zealand and the Pacific Islands (9). Two important factors in the sand fly development are humidity and temperature. This explains why they can only be found in areas with temperatures above 15.6°C for at least several months a year (10). Nevertheless, modeling studies suggest that climate change may lead to expand their distribution, which has already been described for other arthropod vectors (11–13). Evidence of increasing risk of sand fly species establishment in Europe, particularly the Atlantic coast and inland regions of Germany, Switzerland and Austria have been shown (14).

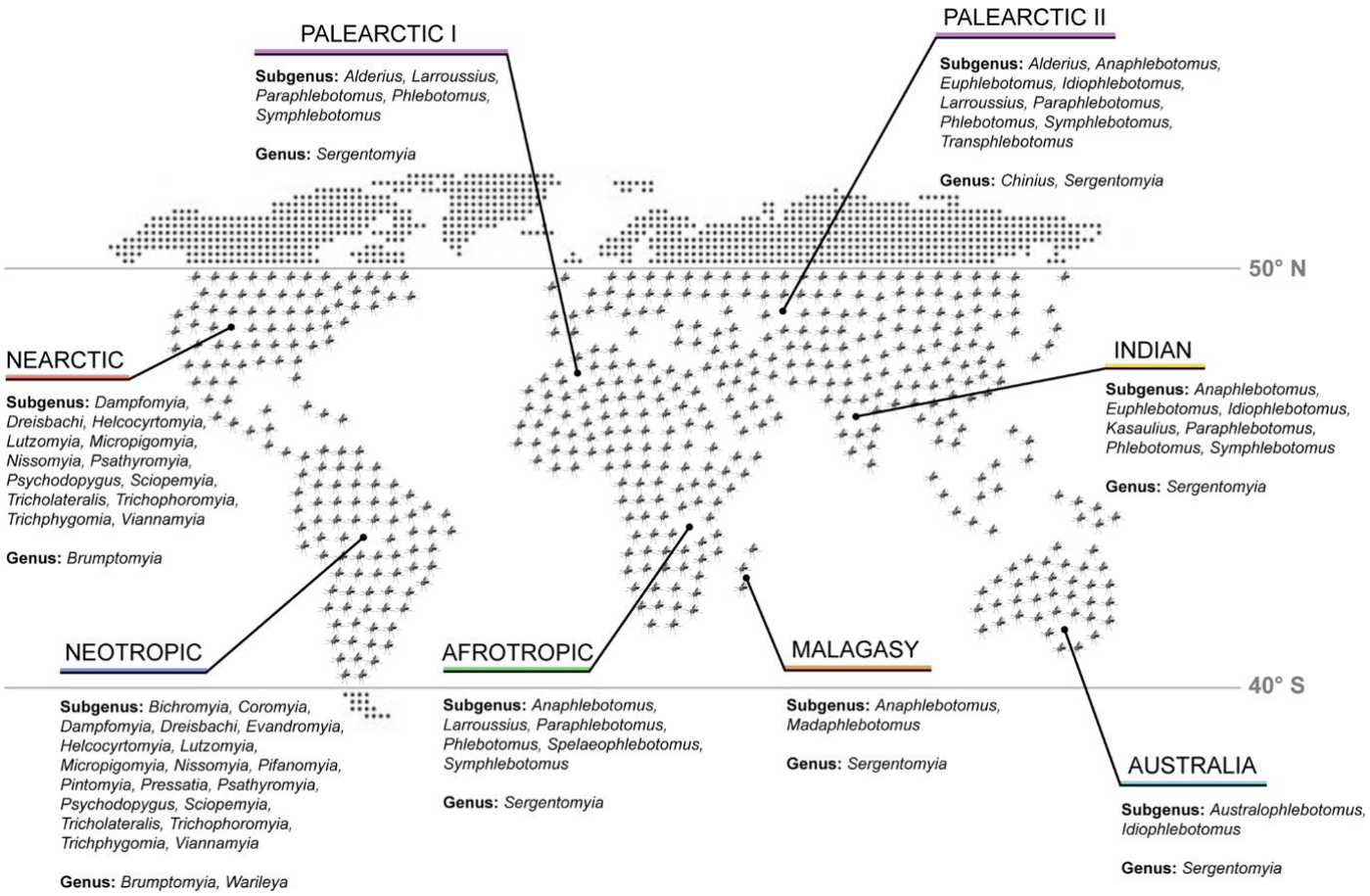


Figure 1. Sand fly distribution map by genus and subgenus (from Cecílio P *et al.* 2022, (9))

c. Biology and ecology

i. Adult morphology

Sand flies are tiny (2 to 3 mm) and humpbacked insects, with a hairy appearance due to a silk abundance on the head and thorax. The adult color varies from light beige to black. They have long slender legs and lanceolate wings with characteristic venation, held upright in a V-shape above them at rest (15) (**Figure 2**). They have a bumpy flight, with successive jumps interrupted by brief rest periods, preceded by frequent changes of direction. Due to this behavior, they are called "*sknipes*" in Greek, which means tipsy (16).



Figure 2. Blood fed female sand fly (Photo © IRD – N. Rahola, on the skin of L. Laroche)

ii. Sand fly life cycle

Eggs are 300-400µm long, elongate, dark brown and shiny, with fine surface markings (useful in classification and identification of species (17)). The breeding sites are not currently identified, but the larvae development requires environments rich in organic matter, such as animal burrows or rock crevices (18). Eggs take an average of eight days to hatch, followed by four larval stages and after 20 to 30 days, the pupae (**Figure 3**). Depending on the environmental conditions (*e.g.* temperature, photoperiod), a diapause can be observed for the eggs or the last (fourth) instar larva (19). Approximately 10 days are needed from the pupae to the adult emergence. Males emerge before females (20). During the first 24 hours of life, to become sexually mature, the male's external genitalia rotate 180° (21). For mating, the male performs a courtship ritual, which consists of folding the abdomen and flapping the wings in jerky movements near the female (22). The mating duration can vary from two to 25 minutes and occur before or after the blood meal depending on the species (23). Both females and males feed on sweet plant meals for energy, such as flower nectar or aphid honeydew (24). Only females take blood meals necessary for eggs maturation which they will lay about seven days later (20-50 eggs per female per gonotrophic cycle). Most of the sand fly species are active from dusk to dawn, during the day they usually rest in cool, moist environments (25). The flight of sand flies is silent and generally short-range, it can vary according to the species, however, the maximum dispersion rarely exceeds one kilometer (26). The adult lifespan depends on environmental

conditions and varies between two weeks and two months for females but is shorter for males (19).

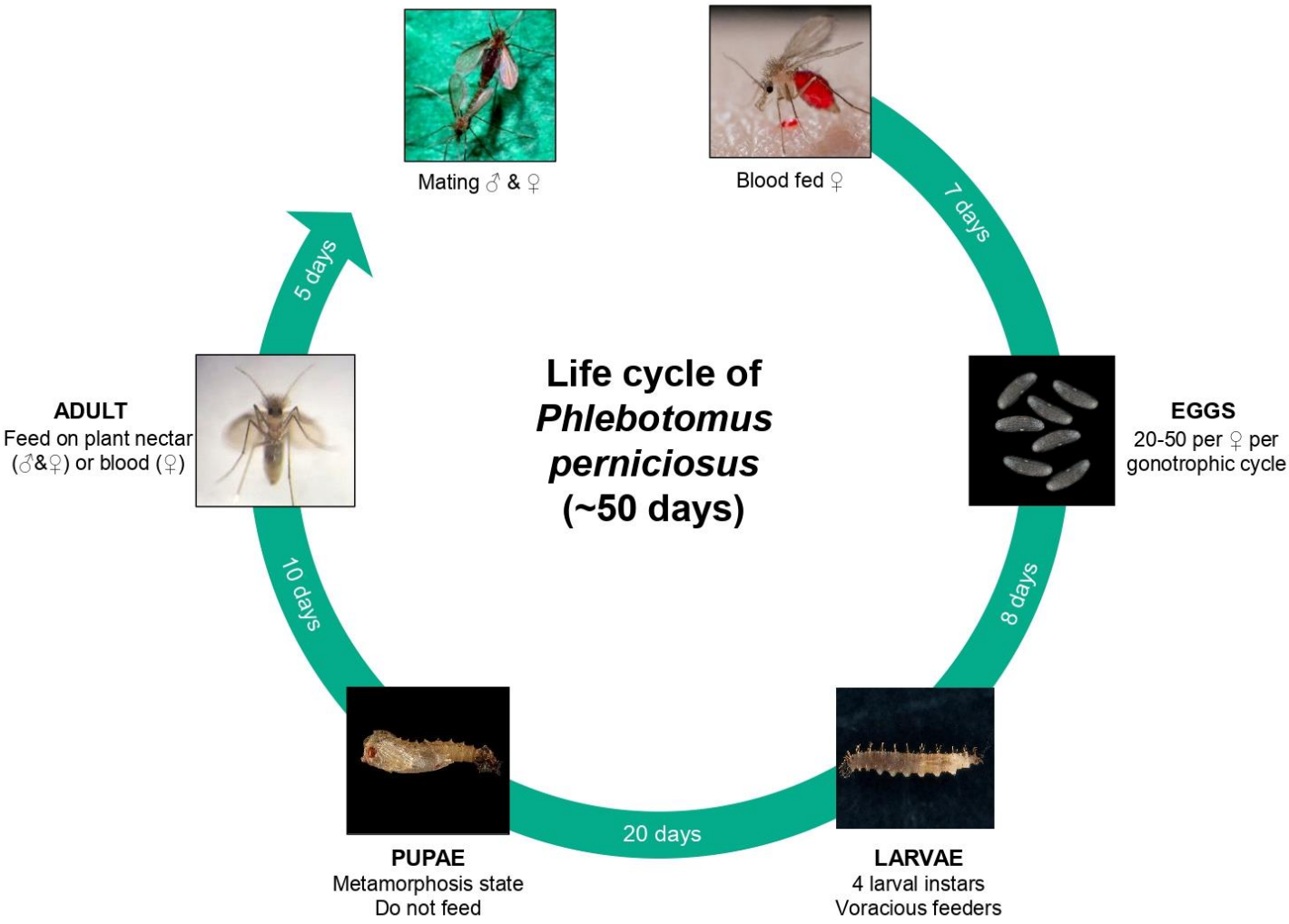


Figure 3. Sand fly *Phlebotomus perniciosus* life cycle (Photo © IRD – N. Rahola, P. Landmann & L. Laroche)

d. Public health importance

Female sand flies are telmophages, they feed on pools of blood formed by lacerating a vertebrate skin with a serrated proboscis (27). They are important vectors of various animal and human pathogens such as the bacteria *Bartonella bacilliformis*, some Phleboviruses and parasites of the genus *Leishmania*. Thus, sand flies can affect human health and contaminate domestic and wild animals by transmitting pathogens (28). The sand fly vector presence is considered to be a risk factor for the emergence of sandfly-borne diseases. Therefore, their occurrence and any changes in their distribution are important to determine the potential

change in these diseases' distribution or expansion (14). Moreover, they are a nuisance due to their painful bites which can lead to allergic reactions. Several methods are available to control sand flies, particularly the use of insecticides. These methods focus on adults, as their larval biotopes remain relatively unknown. One of the approaches is the use of chemical methods, such as indoor residual spraying and insecticide-impregnated nets (29). However, insecticides resistance or tolerance has already been reported (30). It is therefore necessary to find control alternatives, such as the use of entomopathogenic fungi, attractive toxic sugar-baits or integrated pest management, combining different methods (31,32).

e. Sand fly rearing: current situation

Laboratory colonies are necessary for experimental study of vector competence and biology, but also to test new control methods (33). They are necessary in genetic studies and controlled observations on behavior. However, setting up and maintaining a colony are subject to many constraints, they are time consuming and labor intensive with specific laboratory skills. In the last few years, rearing techniques have been developed and improved, and sand fly colonies have become more common, allowing many significant advances (34). Subsequently, this has contributed significantly to the study of sandfly-borne diseases. According to the Global Sandfly Colony Database, 90 colonies of 21 different species in 35 laboratories from 18 countries were identified around the world in 2017 (34). Currently, there are only two colonies in France: Toulouse (National veterinary school) and Montpellier (French National Research Institute for Sustainable Development).

2. *Leishmania* parasites

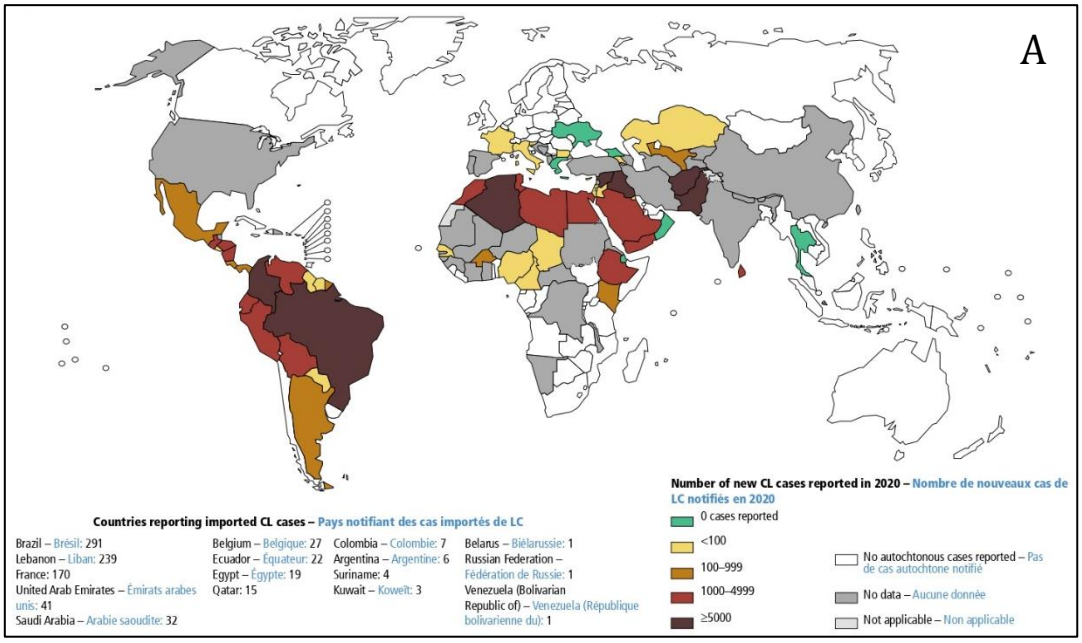
a. Taxonomy

Leishmania are protozoa belonging to the order Kinetoplastidae and the family Trypanosomatidae. Like all Kinetoplastidae, *Leishmania* have a nuclear genome characterized by a single mitochondrion (the kinetoplast) containing thousands of circular minicircle DNAs and a smaller number of maxicircle DNAs (35). These flagellates are obligate intracellular parasites of their host macrophages (36). The genus *Leishmania* is divided into two subgenera based on their developmental pattern in the sand fly's gut: *Leishmania* (Suprapylorian) and *Viannia* (Peripylorian) (37). Suprapylorian parasites develop anterior to the pylorus in the midgut, and Peripylorian parasites in the hindgut and the pylorus (1). The subgenus *Leishmania* is present in the New and Old World, while the subgenus *Viannia* is restricted to the New World.

Worldwide, 31 *Leishmania* species are known to be mammalian parasites, of which more than 20 species are pathogenic to humans (38). Natural transmission is zoonotic or anthroponotic. There are three main disease clinical forms in humans: Visceral Leishmaniasis (VL), also known as kala-azar, the most severe form of the disease, lethal without treatment; Cutaneous Leishmaniasis (CL), the most common form; and Muco-Cutaneous Leishmaniasis (MCL).

b. Geographical distribution

Leishmaniasis is among the top ten neglected tropical diseases in the world. It is endemic in more than 90 countries and mainly in poor and developing countries. The disease impacts 12 million people and 350 million are considered at risk. The annual global incidence is estimated at 0.2-0.4 million cases of VL and 0.7-1.2 million cases of CL, and around 60,000 deaths each year are due to both CL and VL (39). New VL cases occur mainly in Brazil, East Africa and Southeast Asia (40). Unlike VL, CL is more widespread and occurs in Central and South America, the Mediterranean region and Western Asia (**Figure 4**).



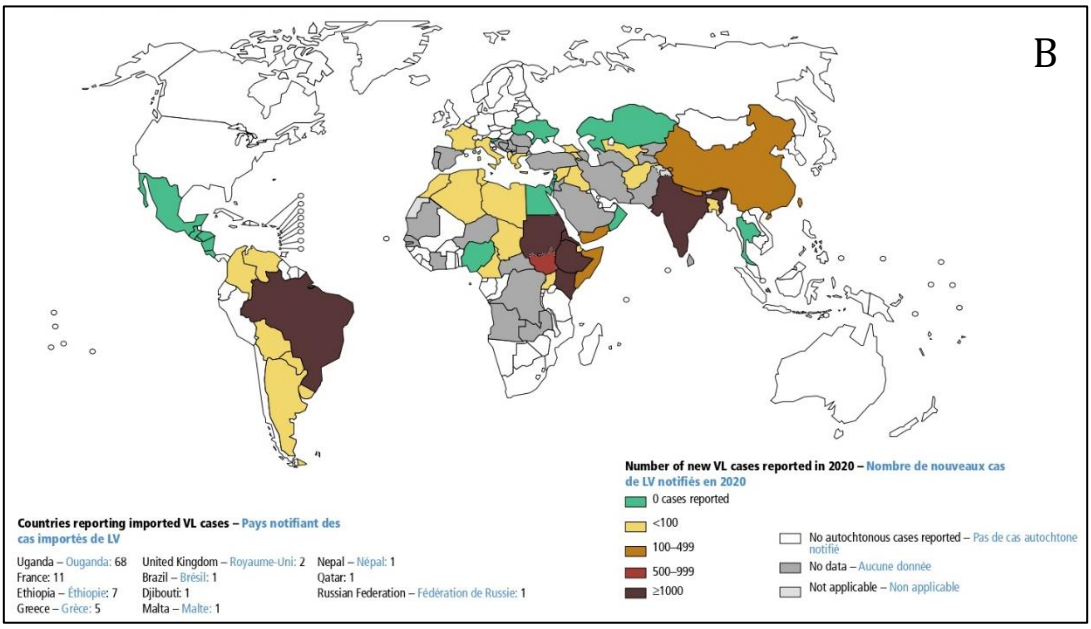


Figure 4. Status of endemicity of Cutaneous Leishmaniasis (A) and Visceral Leishmaniasis (B) worldwide in 2020 (from Ruiz-Postigo JA *et al.* 2021, (41))

Leishmaniasis occurs mainly in remote rural villages, with poor housing and little or no access to modern health facilities. In endemic areas, the diagnosis of leishmaniasis places a heavy financial burden, both at individual and community level (42). There is a risk of outbreaks in endemic areas and of spread to previously free areas due to human activities, such as deforestation, agricultural development, mobility from rural to urban areas, cross-border movements, and urbanization (43). Furthermore, in the context of current global changes, the risk of emergence and re-emergence of leishmaniasis also increases, as it is correlated to the expansion of sand fly distribution (44).

In the Mediterranean basin, the main *Leishmania* species responsible of VL are *Le. donovani* and *Le. infantum*; the CL is mainly due to *Le. tropica* and *Le. major*, associated with dermotropic *Le. infantum* strains and the *Le. infantum*/*Le. donovani* natural hybrids (45,46) (**Figure 5**). In this area, we observed a progressive augmentation in CL and VL incidence over the last two decades. Although the parasite endemicity is low in some Mediterranean countries, it could become a serious public health problem, requiring regular entomological surveillance in these countries but also in neighboring temperate countries (47–49).

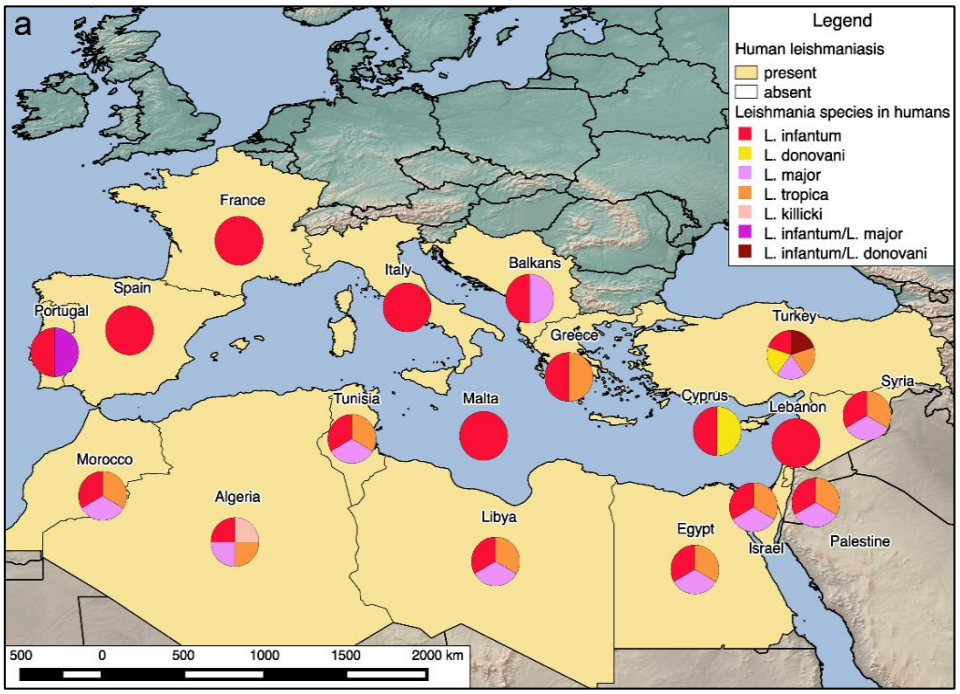


Figure 5. Distribution of *Leishmania* anthroponotic species in the Mediterranean basin (from Moriconi M *et al*. 2017 (45))

c. Biology and ecology

i. Leishmania *life cycle*

Leishmania have a heteroxenous life cycle involving two actors: the sand fly vector and the vertebrate host. First, a female sand fly ingests the parasite immobile form (amastigote) during a blood meal on an infected host. Amastigotes are mainly found in the circulating macrophages of the host's immune system. Inside the vector they will transform into procyclic promastigotes, which start the first multiplication cycle (**Figure 6, 1**). Subsequently, the procyclics turn into motile nectomonads that escape the peritrophic matrix and begin to attach to the sand fly midgut to avoid expulsion via defecation (three to four days after the blood meal). A part of the nectomonads differentiate into haptomonads and attach to the stomodeal valve, the other part transform into leptomonads, which divide efficiently and establish the infection of the midgut six to eight days after the infectious blood meal (**Figure 6, 2-3a-3b**). Leptomonads then differentiate into infectious non-dividing metacyclic promastigotes that accumulate at the stomodeal valve 10-12 days after blood meal, ensuring the availability of a large number of parasites for transmission to a new host **(Figure 6, 4-5**) (9,50). After the infectious sand fly bite, the metacyclics injected are phagocytosed by the host macrophages, in which they turn into amastigote form. Then, amastigotes multiply in the cells leading to their burst. The cycle continues with the colonization of new macrophages which lead to the infection progression in

tissues depending on the *Leishmania* species, and also on the physiological and immunological state of the host (51).

The sand fly vector competence is characterized by the ability of the parasites to resist the defenses present in the gut (50). Although several *Leishmania* species can infect a particular vector species, there are specificities between some sand fly/*Leishmania* pair (1). These specificities depend on the parasite ability to resist digestive enzymes in the gut, to escape the peritrophic matrix and the defecation of the sand fly (52). Depending on the species, the time required for parasites to complete their development cycle in the vector is approximately 6–9 days (50). Currently, 10 proven, or potential, vector species are indigenous in the Mediterranean basin: *Ph. perniciosus, Phlebotomus papatasi, Phlebotomus tobbi, Phlebotomus alexandri, Phlebotomus mascittii, Phlebotomus ariasi, Phlebotomus neglectus, Phlebotomus perfiliewi, Phlebotomus sergenti* and *Phlebotomus similis* (53).

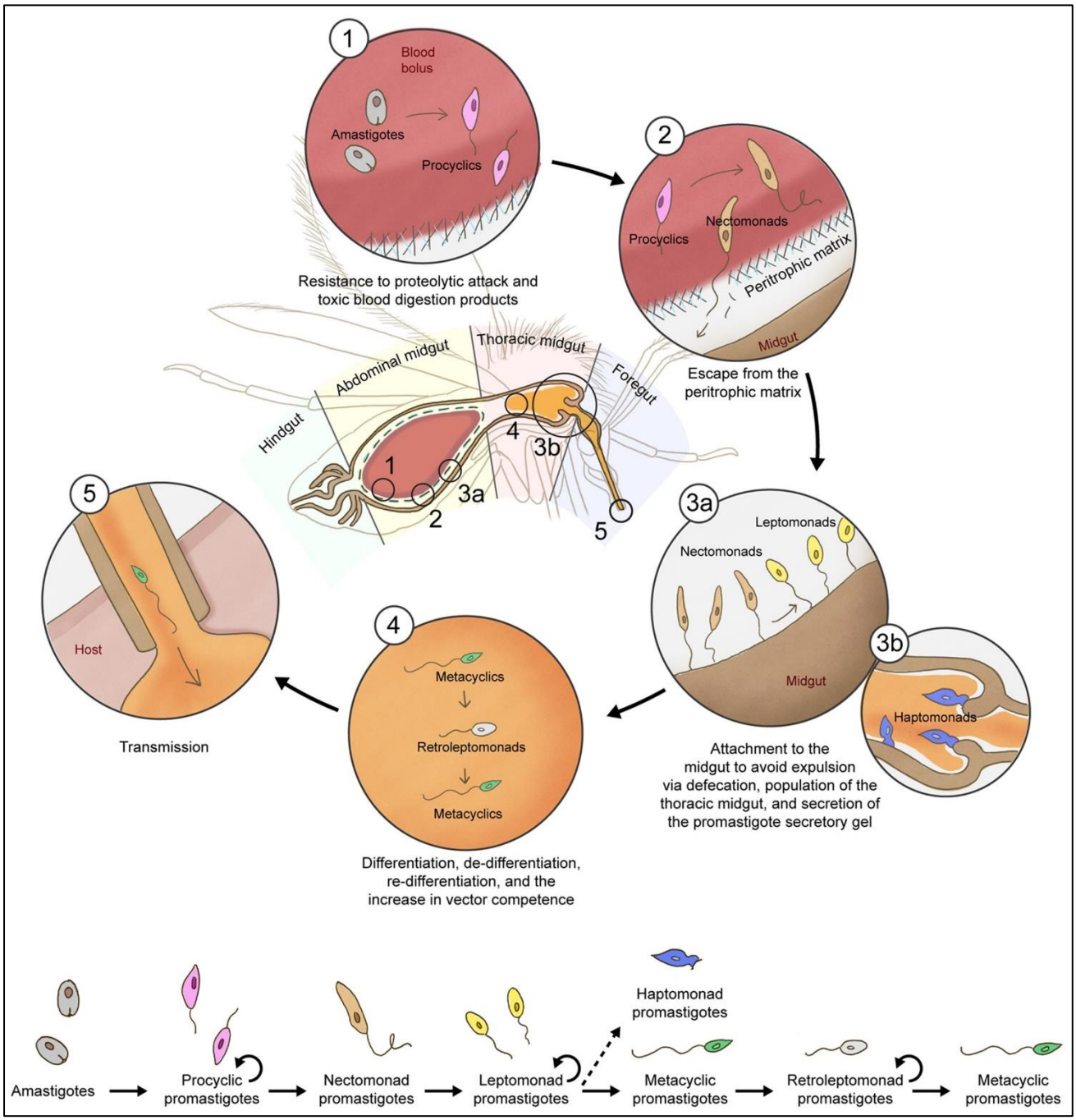


Figure 6. Schematic representation of the different forms of *Leishmania* parasites within the sand fly vector the main barriers they encounter during infection process; with a linear life cycle of the different parasite forms in the vector, the circular arrows represent the replicative parasite forms (from Cecílio P *et al.* 2022 (9))

ii. Reservoirs

Hosts allowing the parasite maintenance, amplification and dissemination are considered as reservoirs, as opposed to accidentally infected hosts that do not play a role in pathogen maintenance (54). For anthroponotic leishmaniasis, humans are the main reservoir, whereas for

zoonotic leishmaniasis, domestic dogs, small mammals, especially wild rodents and carnivores, remain the major reservoirs (55). Moreover, asymptomatic infected individuals could constitute potential reservoirs favoring the transmission cycle maintenance (56). In the Mediterranean basin, humans are the primary reservoir hosts in VL caused by *Le. donovani* and CL caused by *Le. tropica*; and wild or domestic animals are the main reservoirs of *Le. infantum* and *Le. major* (45).

iii. Transmission pathways

Leishmania are usually transmitted between hosts through the sand fly bite. However, in recent decades, evidence has accumulated that other transmission pathways exist such as direct contact with infected tissue (57). Since the development of organ transplantation and blood transfusion techniques, microorganisms such as *Leishmania* can be transmitted during these procedures. *Leishmania* infections have been observed in patients after kidney or heart transplants, but also after contamination by objects (syringes), among drug addicts or more rarely among clinical-medical personnel (58–62). In VL, vertical transmission from an infected mother to her newborn has also been described (63,64).

iv. Vector-parasite interactions

Under experimental conditions, it has been shown that *Leishmania* parasites can manipulate the sand fly feeding behavior to promote its own transmission (65). In addition, the development of *Le. infantum* and *Leishmania mexicana* in the gut of *Lutzomyia longipalpis* is likely to cause a fitness cost by reducing the sand fly longevity (66). Another experimental study showed that *Le. infantum* and *Le. major* infections significantly decrease longevity and fecundity (egg production reduction) of their natural vectors *Ph. papatasi* and *Phlebotomus langeroni* (67). Changes in these life history traits may influence the vectorial capacity and the transmission dynamics.

d. Public health importance

i. Clinical forms

The various clinical manifestations are associated with the host immune response, the *Leishmania* species and their cellular tropism. Usually, host immune defenses may be sufficient to limit the parasite development, resulting in asymptomatic infections (68). However, clinically symptoms occur when the host immune system cannot control parasite growth. Primary infection always involves parasite multiplication at the inoculation site with an incubation

period from one to four months, which make difficult to identify the geographic location of infection (69). In CL, skin lesions appear around the bite site, and in VL, spleen, liver and other lymphoid tissues are affected. Although less frequent, MCL can cause severe mucosal damage to the nasal, oral and pharyngeal cavities and surrounding tissues. Due to persisting scars, even after treatment, CL and MCL forms are responsible for significant psychosocial impacts and social stigmatization (70).

ii. Diagnostic, treatment and prevention

The diagnosis of leishmaniasis depends on the disease clinical forms and the species involved. In VL, diagnosis is made by combining clinical signs with parasitological or serological tests (such as rapid diagnostic tests). In CL and MCL serological tests have limited value, clinical manifestations with parasitological tests confirms the diagnosis (71). In humans, leishmaniasis is a curable disease, although there are risks of relapse in case of immunosuppression. The choice of treatment depends on the disease form, the parasite species and the geographical location. There are physical treatments by cryotherapy or surgery but chemical treatments like pentamidine are used as first line (72). In general, antileishmanial drugs, such as amphotericin B or antimonial, can be toxic and cause undesirable side effects that can lead to death (73). Moreover, *Leishmania* drug-resistance has been observed (74). Currently, no effective vaccine is available on the market for humans, while effective vaccines have already been developed for dogs (75,76). Vector control can limit disease transmission by reducing the sand fly number, but remains difficult due to the vector ecology and its particular habitat types (77).

e. Experimental infection studies

The first experimental infections for leishmaniasis study took place in the first half of the 20th century and, since the 2000s, the number of experimental infection studies has increased and allowed to identify new and potential sand fly vector species (78,79). Competence, vectorial capacity and the factors influencing them have been studied in detail through experimental infections and provide new parameters for modelling parasite transmission dynamics (80,81). It is also possible to co-infect with several *Leishmania* species, as well as between *Leishmania* and other pathogens (82–84). These studies highlight that pathogens transmitted by sand flies may be involved in modulating the host response and therefore the outcome of *Leishmania* infection.

3. Sandfly-borne phleboviruses

a. Taxonomy

The sandfly-borne viruses of the genus *Phlebovirus* belong to the family Phenuiviridae within the order Bunyavirales (85). According to the International Committee on Taxonomy of Viruses, 66 phleboviruses are recognized worldwide, of which more than 40, including 11 species associated with human disease, have been isolated or detected in sand flies (86,87). Due to the continuous discovery of phleboviruses, such as Ntepe and Drin viruses, the taxonomy is evolving rapidly (88–90).

Phleboviruses are enveloped viruses with a negative sense single stranded trisegmented ribonucleic acid (RNA). The viral particles have a diameter of 80 to 120 nm and are composed of helical nucleocapsids containing the tripartite genome: Large (L) segment (6.4 kb) that codes for the RNA-dependent RNA polymerase (RdRp), Medium (M) segment (3.2 kb) which encodes envelope glycoproteins (Gn and Gc) and nonstructural protein (NSm), and Small (S) segment (1.7 kb) that encodes nucleoprotein (N) and another nonstructural protein (NSs) with an ambisense strategy (91) (**Figures 7 & 8**). While structural proteins have been well characterized in all viral families, NS proteins have multiple functions (*e.g.* virulence and evasion of the host immune system) that may differ between viral species (92–94). The segmented nature of the *Phlebovirus* genome allows for recombination and reassortment events, which could generate new viruses and lead to new disease emergences (95). *Phlebovirus* are mainly transmitted by the bite of sand fly, mosquito, and tick species. In the Old World, sand flies are vectors of several phleboviruses including human pathogens, such as *Toscana phlebovirus* (TOSV), *Naples phlebovirus* (SFNV) and *Sicilian phlebovirus* (SFSV) (96).

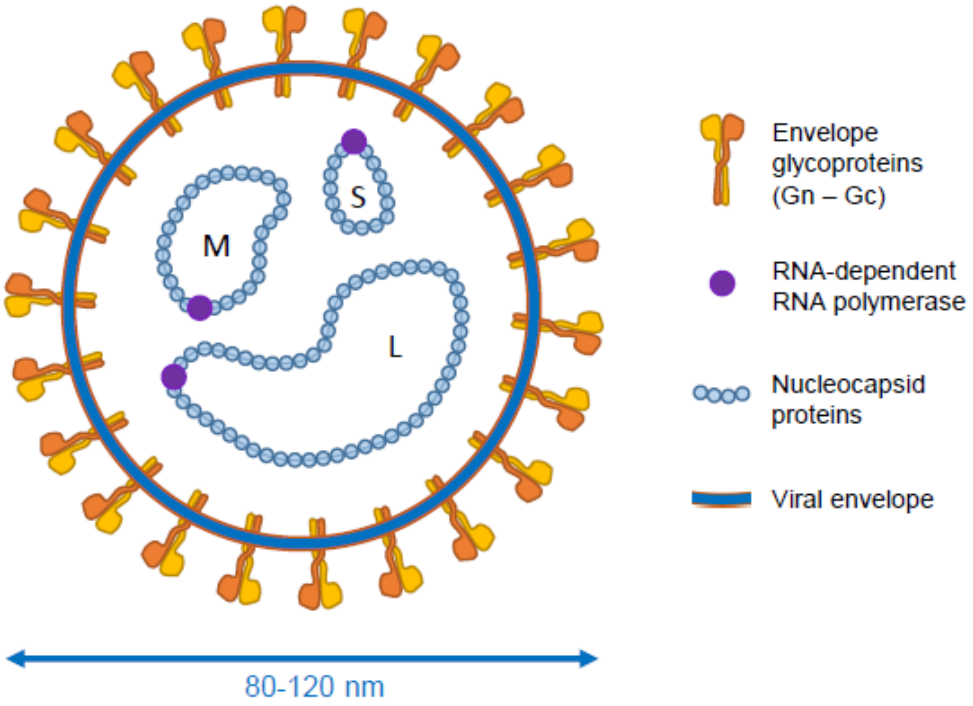


Figure 7. *Phlebovirus* schematic representation. Nucleocapsid proteins coat the genomes with single-stranded antisense RNA and, together with RNA-dependent RNA polymerase, form ribonucleoproteins on each segment. The genomic segments are packaged into the viral envelope studded with viral glycoproteins (Gn and Gc). Small, medium and large genomic segments are labeled S, M and L, respectively.

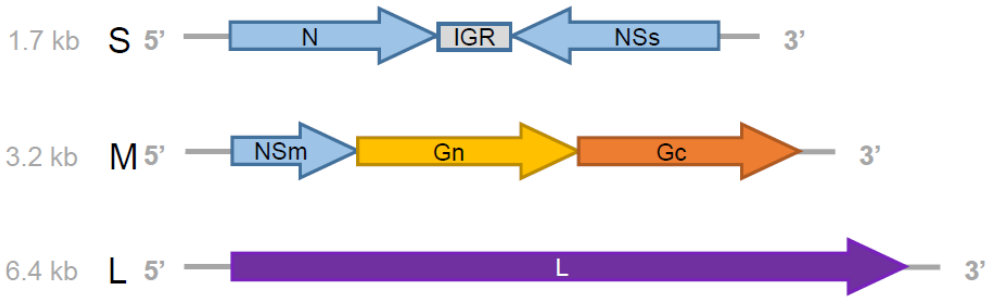


Figure 8. *Phlebovirus* genome organization. N: nucleoprotein, IGR: intergenic region, NSs: nonstructural S segment protein, NSm: nonstructural M segment protein, Gn and Gc: surface glycoproteins, L: RNA-dependent RNA polymerase encoding part (adapted from Leventhal, SS. *et al*. 2021 (97))

b. Geographical distribution

The geographical distribution of sandfly-borne phleboviruses is wide and common to the presence of vectors belonging to the genera *Lutzomyia* in Central America, *Phlebotomus* and

Sergentomyia in Africa, Europe and Central Asia (98,99). The vector-virus interactions constrain the geographic distribution and play a role in the inter-epidemic maintenance and the *Phlebovirus* evolution (100). The *Phlebovirus* species distribution in the Mediterranean area is shown in **Figure 9**; the two most represented species in this region are TOSV and SFSV.

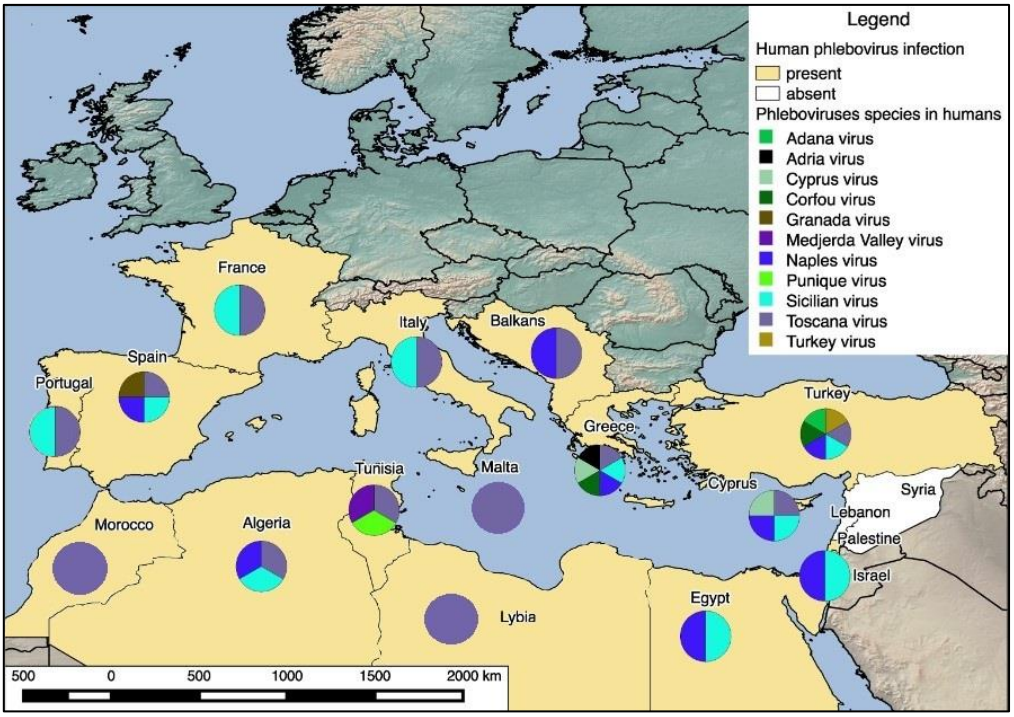


Figure 9. Distribution of anthroponotic *Phlebovirus* species in the Mediterranean area (adapted from Moriconi M *et al.* 2017 (45,101–103))

However, it is possible that a larger number of unidentified phleboviruses circulates in the Mediterranean region. Indeed, in the last decades, advances in molecular diagnosis allowed great progress in the identification and characterization of new species (90,104). Thus, phleboviruses may be responsible for significantly more human diseases than previously thought. For instance, it is possible to estimate the circulation area of a virus by investigating its potential new vectors, as we realized for the TOSV in the following review.

Article 1

Broader geographical distribution of Toscana virus in the Mediterranean region suggests the existence of larger varieties of sand fly vectors

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This review highlights the lack of information on the biology and epidemiology of Toscana virus (TOSV), a virus species within *Sandfly Fever Naples Virus*. Currently, there is little information on the natural cycle of TOSV, its reservoirs and vectors. It has been shown recently that TOSV circulates in a much wider area than suspected ten years ago. Indeed, the hypothesis that other sand fly species may be involved in the natural cycle of TOSV is investigated here. However, further studies are necessary to investigate whether the species suspected in this study or other species might be competent for TOSV transmission.

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Opinion

Broader Geographical Distribution of Toscana Virus in the Mediterranean Region Suggests the Existence of Larger Varieties of Sand Fly Vectors

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Abstract: Toscana virus (TOSV) is endemic in the Mediterranean basin, where it is transmitted by sand flies. TOSV can infect humans and cause febrile illness as well as neuroinvasive infections affecting the central and peripheral nervous systems. Although TOSV is a significant human pathogen, it remains neglected and there are consequently many gaps of knowledge. Recent seroepidemiology studies and case reports showed that TOSV's geographic distribution is much wider than was assumed a decade ago. The apparent extension of the TOSV circulation area raises the question of the sandfly species that are able to transmit the virus in natural conditions. *Phlebotomus (Ph.) perniciosus* and *Ph. perfiliewi* were historically identified as competent species. Recent results suggest that other species of sand flies could be competent for TOSV maintenance and transmission. Here we organize current knowledge in entomology, epidemiology, and virology supporting the possible existence of additional phlebotomine species such as *Ph. longicuspis, Ph. sergenti, Ph. tobbi, Ph. neglectus, and Sergentomyia minuta* in TOSV maintenance. We also highlight some of the knowledge gaps to be addressed in future studies.

Keywords: Toscana virus; sand fly; *Phlebotomus*; *Sergentomyia*; Mediterranean area; *Phenuiviridae*; Bunyavirales; *Sandfly fever Naples phlebovirus*; arbovirus; arthropod-borne; sandfly; phlebotomine

1. Introduction

The genus *Phlebovirus* includes 58 viruses classified into a ten-species complex: Bujaru, Candiru, Chilibre, Frijoles, Punta Toro, Rift Valley fever, Salehabad, Sandfly fever Naples, Severe fever with thrombocytopenia syndrome, and Uukuniemi phleboviruses. The Sandfly fever Naples phlebovirus species comprises thirteen viruses, including Toscana virus (TOSV) [1]. TOSV has a tropism for central and peripheral nervous systems and is responsible for meningitis and encephalitis in the Mediterranean region [2].

Phleboviruses are transmitted to humans by the bite of an infected female sand fly during the blood meal. There is still little information on the development cycle of phleboviruses in the vector [3].

During the last decade, the known geographical area where TOSV circulates has increased considerably. With recent entomological studies, seroepidemiological studies, and case reports, the

known distribution of TOSV is now extended in Central and Eastern Europe, North Africa, and Turkey.

We suggest that the existence of a larger diversity of sand fly vectors may explain the TOSV circulation in different geographical regions, which is supported by the recent identification of the virus in phlebotomine species that were not previously considered as TOSV vectors.

Here we present a comprehensive analysis of recent data suggesting that additional species of sand flies are involved in natural cycles to explain the recently revealed broader geographical distribution of TOSV in the Mediterranean region.

2. Toscana Virus

2.1. Overview on Toscana Virus

TOSV is an enveloped, tri-segmented RNA arbovirus which belongs to the genus *Phlebovirus*, the family *Phenuiviridae*, and the order Bunyavirales [4]. At the family level, genomes are comprised of three unique molecules of negative or ambisense single-stranded RNA, designated L (large), M (medium), and S (small) to a total of 11 to 19 kb. Within each genus, viruses share similar segment and structural protein sizes, as well as characteristic terminal sequences at the 3′ and 5′ ends of each of the three segments. TOSV distribution and abundance are highly dependent on its arthropod vector. Human cases are observed during the warm season, with a peak during the hottest months in relation with sand fly activity. This virus shows a peculiar neurotropism and causes central nervous system (CNS) diseases in infected individuals [2,5]. Like other arboviruses, most TOSV infections in humans are asymptomatic [6]. So far, 864 symptomatic human cases (834 autochthonous and 30 imported) have been reported in residents of different Mediterranean countries and in tourists and travelers returning from endemic regions [7–10].

Genetic studies have identified the existence of three lineages of TOSV (A, B, and C) [11] (Figure 1). To date, there is no difference in virulence or clinical manifestations depending on the genetic lineage. At least two lineages co-circulate in France [12], Turkey [13], and Croatia [14]. Further studies are required in order to have more information about the genotype distribution and their association with vector distribution. The known geographical distribution of TOSV in the Mediterranean basin raises the question of whether the demonstrated vectors, *Phlebotomus (Ph.) perniciosus* and *Ph. perfiliewi*, can explain this distribution or whether other species could be implicated.

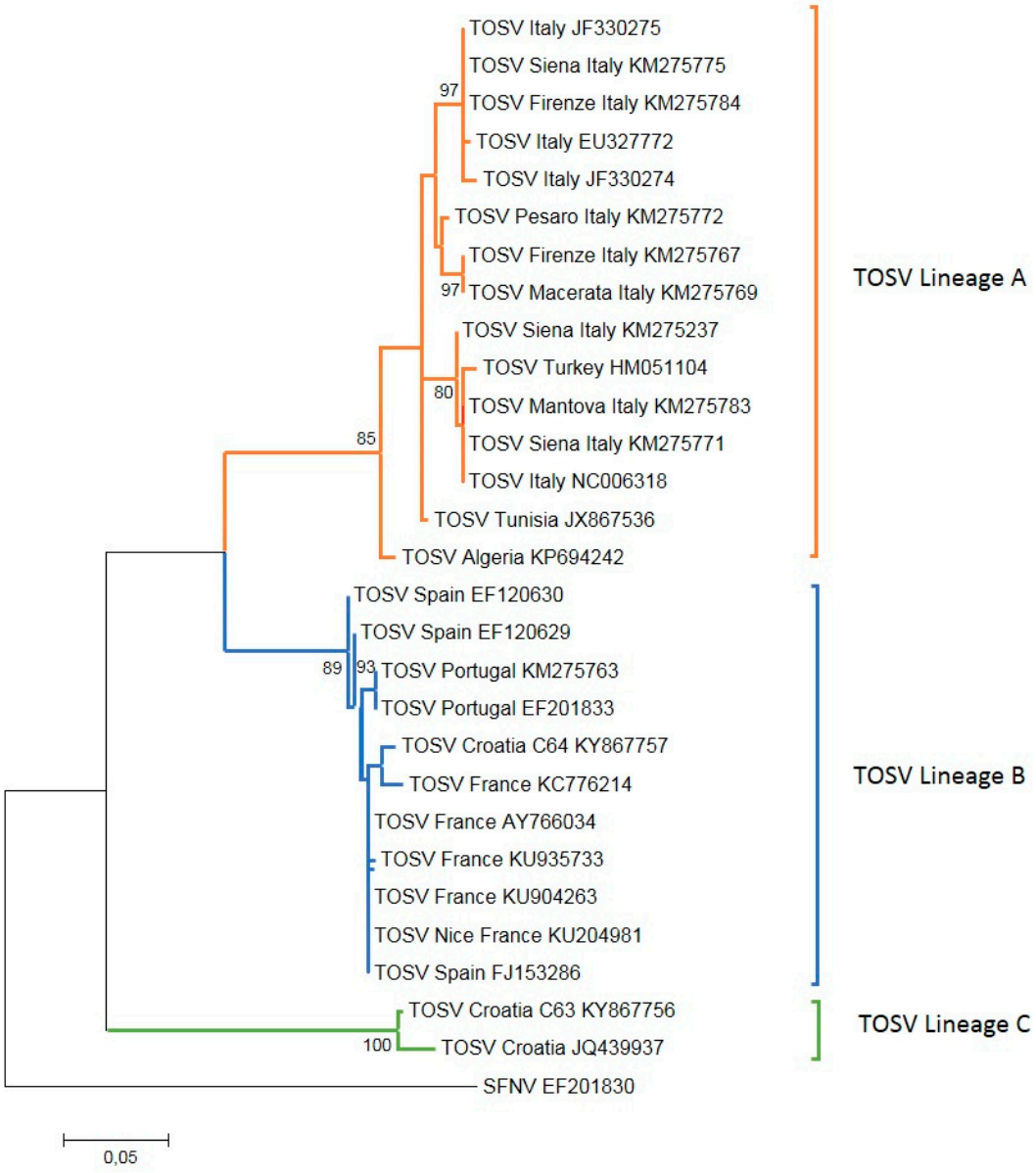


Figure 1. Phylogenetic analysis of Toscana virus (TOSV) based on the nucleocapsid protein gene. Maximum likelihood analysis at nucleotide level was performed using the MEGA 6.06 software program [15]. Bootstrap values are indicated and correspond to 1000 pseudoreplications.

The knowledge of the vectors and geographical distribution of TOSV is increasing in parallel with the number of studies. The current TOSV distribution includes South European countries (Italy, Spain, France, and Portugal) [16–19], East Mediterranean countries (Turkey and Cyprus) [13,20], Balkan countries (Greece, Croatia, Bosnia and Herzegovina, Kosovo, and Bulgaria) [21], and North African countries (Tunisia, Morocco, and Algeria) [22]. The geographical distribution of TOSV in the Mediterranean basin raises the following question: Can the known vectors, *Ph. perniciosus* and *Ph. perfiliewi*, explain this distribution, or are other species implicated?

2.2. Epidemiology of Toscana Virus

Seroprevalence studies demonstrate that human populations are exposed to TOSV in Italy, Spain, France, Portugal, Turkey, Malta, Cyprus, Greece, Croatia, Bosnia and Herzegovina, Kosovo, Bulgaria, Tunisia, and Algeria [7,22–36]. Together, the results of serological surveillance studies and case reports showed that the TOSV geographic distribution is much wider than believed a decade ago; in particular, the presence of TOSV has been recently documented in North Africa and the Balkan countries [31,32,34–36] (Figure 2).

High rates of TOSV-neutralizing antibodies (17.2–59.4%) have been identified in the healthy population of Tunisia, with geographical variation [35,36]. Similar results were obtained in Algeria with a prevalence up to 50% in Draa El Mizan [22]. Although the studies are geographically limited within Tunisia and Algeria, the high rates of neutralizing antibodies emphasize the massive circulation of TOSV in these countries (Figure 2).

In Kosovo, 5.5% of blood donors possess IgG against TOSV [32]. Higher rates were reported in healthy residents of Croatia, with the highest values in coastal areas and Croatian islands [31]. Another study identified the presence of TOSV antibodies in patients from Bosnia and Herzegovina [33]. Recently, anti-TOSV IgGs were found in 24.4% of healthy residents of Bulgaria [34] (Figure 2).

2.3. Pathways of TOSV Transmission and Maintenance

Transovarial and venereal pathways of TOSV transmission have been demonstrated experimentally with *Ph. perniciosus* colonies [37–39]. These experimental results were complemented by the observation in nature of equal rates of TOSV infection in male and female sand flies, suggesting the existence of transovarial (vertical) and/or venereal (horizontal) transmission during mating [40–43]. Additionally, the survival of TOSV in overwintering *Ph. perniciosus* larvae underlined the capability of TOSV for maintenance during diapause [37]. Co-feeding transmission of TOSV during sugar meal was also suggested experimentally in males and females since it was demonstrated in *Ph. perniciosus* with Massilia virus, a relative of TOSV also transmitted by *Ph. perniciosus* in nature [44]. Future studies need to be conducted with TOSV and other species belonging to the *Phlebotomus* genus in order to confirm that viral transmission is possible with other species.

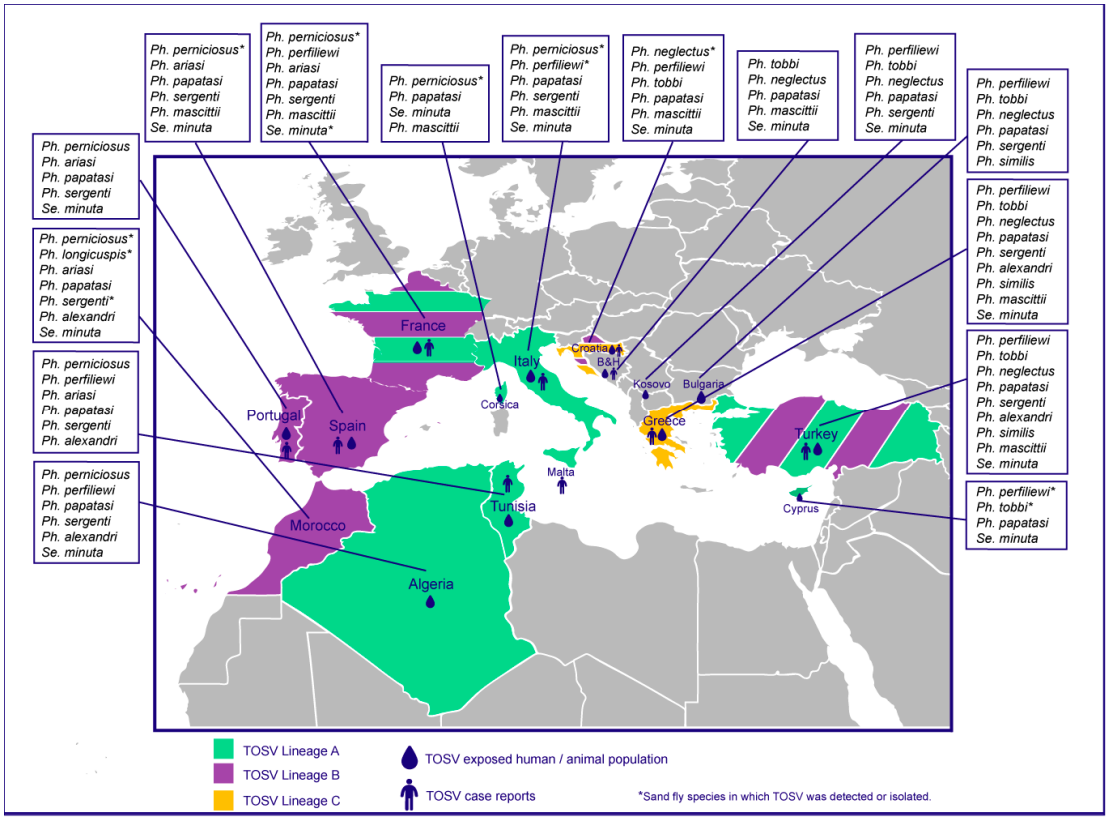


Figure 2. Geographical distribution of Toscana virus (TOSV) and reported distribution of predominant sand fly species in the countries where TOSV is present.

3. Sand Flies

3.1. Overview on Sand Flies

Sand flies are tiny blood-feeding (hematophagous) insects belonging to the order Diptera, family Psychodidae, and subfamily Phlebotominae. Approximately 900 sand fly species are described, of which 70 have been identified as potential vectors of *Leishmania* [45] and a few species were associated with *Phlebovirus* and other viruses [46]. Each species can be identified by the characteristic shape of their cibarium, pharynx, and reproductive organs [47].

Both genders feed on honeydew, plant sap, and aphid secretions [48], but only females take a blood meal, necessary for egg maturation. Sand flies present a crepuscular and nocturnal activity [49]. They are very abundant in warm regions (e.g., Mediterranean basin, Asia, Africa, South America), but their range is very wide (between 50° N and 40° S). They occur on all continents but have not been reported in New Zealand or the Pacific Islands. Their altitudinal distribution ranges from sea level to 3500 m above sea level (Afghanistan: *Ph. rupester*) [50–52]. Modeling studies suggest an expansion of the range of different *Phlebotomus* species in Europe due to the influence of environmental and climate change (*Ph. ariasi*, *Ph. mascittii*, *Ph. perniciosus*, *Ph. neglectus,* and *Ph. perfiliewi*) allowing the colonization of new habitats [53,54].

However, this insect remains relatively unknown from a biological and ecological point of view (unknown breeding sites, poorly known food preferences, etc.).

3.2. Sandflies as TOSV Vectors

Sandfly activity is seasonal in the Mediterranean countries. They are present from May to October due to low temperatures in winter. The average annual temperatures and the latitude influence the emergence periods and the seasonal dynamics of these insects (with a peak in July and August) [49]. Their activity and abundance are dependent on environmental and climatic conditions and may vary depending on the species and the location of capture.

The first isolation of TOSV was obtained from *Ph. perniciosus* in central Italy, in 1971 [55]. Subsequently, other strains were isolated from *Ph. perfiliewi* [56]. The isolation or detection of TOSV in sand flies started to be reported not only in Italy but also in other Mediterranean countries. *Ph. perniciosus* was identified as vector species of TOSV in Southern France [12], the South-West region of Madrid, Spain [57], and Morocco [58]. Additionally to *Ph. perniciosus, Ph. longicuspis* and *Ph. sergenti* are identified as potential vectors of TOSV in Morocco [59]. Another study reported TOSV presence in *Se. minuta* in France which feeds preferentially on cold-blooded vertebrates [60]. More recently, TOSV was detected from two pools of *Ph. neglectus* in Croatia [14]. In Cyprus, TOSV was detected in one pool of *Ph. perfiliewi* and in tow pools of *Ph. tobbi* [61].

In some other studies, the infected vectors were identified at the genus level only, thus preventing implication of a given species. This was the case in Spain (where almost 70% of captured insects were *Ph. perniciosus) [62]*, Corsica (where *Ph. perniciosus* and *Se. minuta* were largely predominant in equal proportions) [63], Algeria (where six species were morphologically identified: *Ph. perfiliewi* (51.4%), *Ph. perniciosus* (36.7%), *Ph. longicuspis* (2.6%), *Ph. papatasi* (6.5%), *Ph. sergenti* (0.5%), and *Se. minuta* (2.3%)) [22], and in Tunisia (where *Ph. perniciosus* was the most abundant species (71.74%)) [64] (Figure 2).

To date, only TOSV lineage A has been recorded in *Ph. perfiliewi* [56]. However, at this stage of knowledge, the correlation of TOSV genetic lineage and phlebotomine species is poorly supported (Figure 2). *Ph. perniciosus* can transmit TOSV strains belonging to lineage A and lineage B [12,55,57,58]. Lineage A was also detected once in *Se. minuta* [60]. In addition to *Ph. perniciosus, Ph. longicuspis, Ph. sergenti,* and *Ph. neglectus* are possible vectors of TOSV strains belonging to lineage B [11,59]. So far, TOSV strains belonging to lineage C have only been recorded in Croatia and Greece, where they are assumed to be transmitted by *Ph. neglectus* [14] (Figure 2).

In regions where known vector species (e.g., *Ph. perniciosus* and *Ph. perfiliewi*) are in the minority or even totally absent (e.g., Bosnia and Herzegovina), seroprevalence rates observed in humans and animals suggest that alternative species could be competent (e.g., *Ph. neglectus* in Bosnia and Herzegovina). It is therefore important to study the specificities for each country and species in order to better understand the current expansion of TOSV together with its demonstrated and putative vectors.

Climate change affects the geographical distribution of many sand fly species [65,66]. As a consequence, the geographical distribution of sand flies has expanded towards Northern Europe during the last decade. For instance, populations of *Ph. perniciosus* are now present in new regions in Germany [54,67]. Consequently, TOSV could also spread to these newly colonized regions where competent species are present [68].

Despite the lack of knowledge for many species of sand flies, those belonging to the genus *Phlebotomus* have benefited from recent efforts from the European Center for Disease Prevention and Control (ECDC) in terms of species inventory and mapping. However, these efforts should be pursued and completed by abundance studies [69]. Regarding *Sergentomyia* species, very little is known, nevertheless these species deserve more attention due to their possible association with TOSV transmission [60] and their putative role in *Leishmania* transmission [70–73].

4. Vertebrates as Possible Reservoir or Amplification Host

The conditions required for a given species to be an efficient reservoir or amplifying host are (i) to be able to generate high and/or sustained viremia for transmission to the competent sand fly species and (ii) to present a geographical distribution equal to, or larger than, that of the disease.

In humans the short duration of viremia together with the absence of persistent infection preclude any role in TOSV maintenance in nature. TOSV was isolated once from the brain of one wild caught bat (*Pipistrellus kuhlii*) in Italy where TOSV-infected *Ph. perniciosus* and *Ph. perfiliewi* were present [56]. This is the first and the only record of TOSV identification from bats.

Many seroprevalence studies testing vertebrate sera have described the presence of variable rates of antibodies directed against TOSV. These studies showed that TOSV circulates in the following countries: Greece [74], Spain [75], Portugal [76,77], France (Corsica island) [78], Tunisia [79], Algeria [80], and Kosovo [81], and can infect dogs, cats, cattle, and sheep. One study reported the presence of TOSV RNA detection in dogs in Portugal [76] and another study detected TOSV RNA in goat serum in southern Spain [75]. These results are very likely to be anecdotic. So far, there is no evidence for a vertebrate species acting as reservoir or amplifying host in the natural cycle of TOSV. Considering that dogs are a reservoir for sand-fly-borne *Leishmania infantum*, they are good candidates as natural reservoir hosts for TOSV. The fact that neutralizing antibodies against TOSV have been repeatedly described in dogs (4.3–8.4% in studies conducted in France, Portugal, Tunisia, Algeria, Greece, and Cyprus) is likely to reflect exposure rather than being indicative of a more peculiar role in the natural cycle [74,76–80]. However, two studies conducted in dogs from Mediterranean Anatolia (Turkey) reported the presence of TOSV RNA in the blood of approximately 3% and 10% of tested dogs, respectively, of which some were co-infected with *Leishmania* parasites [82,83]. Co-infection could contribute to this apparent active replication of TOSV. More recently, TOSV was detected in the brain and kidney from a greater flamingo (*Phoenicopterus roseus*), a great white pelican (*Pelecanus onocrotalus*), and a black stork (*Ciconia nigra*) in Turkey [84]. Nevertheless, the role of these animals in the life cycle of TOSV remains unknown. Currently, no data support the hypothesis that humans and/or any other vertebrates are the reservoirs of sand-fly-borne phleboviruses, due to the short duration of the viremia and the lack of persistent infection. The consideration of the vector as the reservoir of phleboviruses is currently under debate [60].

5. Conclusions

Here we highlight that there is very limited information on the biology and epidemiology of TOSV, its reservoirs, and its vectors. As there is neither a vaccine nor a specific treatment, the control of TOSV infections can only be achieved through sand fly control measures (indoor and outdoor

residual spraying, attractive toxic sugar baits, etc.) or personal protection against bites (skin repellents, impregnated bed nets, etc.). In order to be able to target regions where these measures could be promoted, it is necessary to know the geographical distribution of populations at risk through seroepidemiological studies and the surveillance of neuro-invasive TOSV infection cases. The recent demonstration that TOSV circulates in a much broader area than suspected a decade ago has raised questions about the possibility that additional phlebotomine species (at least *Ph. longicuspis, Ph. sergenti, Ph. tobbi, Ph. neglectus, and Sergentomyia minuta)* may be involved in the TOSV natural cycle. Whether these species or others could be competent for the transmission of TOSV merit further study. Accordingly, it is necessary to set up specific studies to address this question (i) in the natural environment and (ii) under experimental conditions using sand fly colonies in insectarium.

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c. Biology and ecology

i. Natural cycle

The majority of arthropod-borne viruses follow the same development processes in the vector (**Figure 10**). The virus is ingested during the blood meal on an infected host, it multiplies in the midgut and if a certain amount of virions manages to escape the vector defense barriers, there will be a progressive dissemination through the midgut to the haemocoel (105). Among the different insect defense barriers, RNA interference (RNAi) mechanism is the major innate immune pathway controlling pathogens infection and transmission (106–108). However, other barriers are involved, such as the peritrophic matrix, digestive enzymes and the vector's microbiota, which can establish a competitive interaction with pathogens (109). After escaping these defense barriers, virions infect other tissues such as the salivary glands, ovaries or neural system. Thus, the infected vector becomes competent to transmit the virus to naive hosts (110). In the Mediterranean area, most of the competent sand fly vectors of *Phlebovirus* are *Phlebotomus* species, such as: *Ph. perniciosus, Ph. perfiliewi, Ph. papatasi, Ph. sergenti, Ph. neglectus. Ph. tobbi* and *Ph. longicuspis* (98,111).

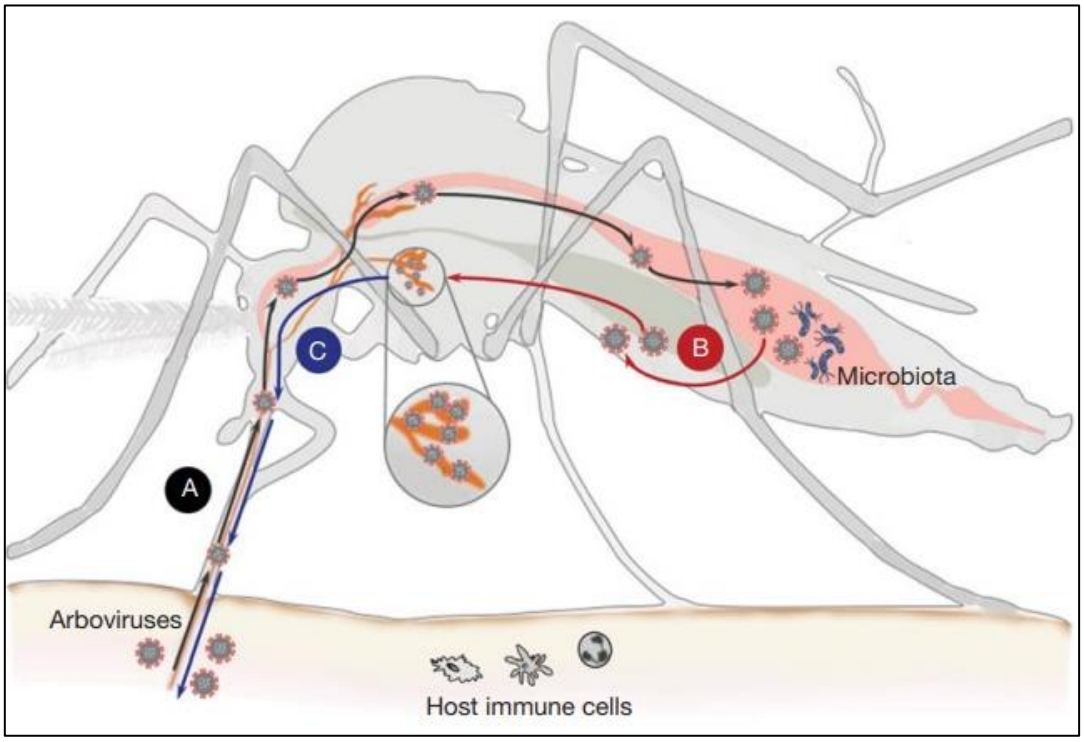


Figure 10. Schematic overview of arthropod-borne virus (arbovirus) natural cycle in a competent vector. (A) Absorption of virions during a blood meal on an infected host; (B) Virions spreading from the midgut epithelium into the haemolymph; (C) Virions in the salivary

glands of the vector are transmitted to a naive host during the blood meal (from Wu P *et al.* 2019 (110))

However, the natural cycle of *Phlebovirus* remains poorly known and is still very little studied. Some phleboviruses, such as TOSV, Arbia virus (ARBV) or Rift Valley Fever virus (RVFV), are maintained by transovarial transmission within the vector, which allows infectious virions transmission from the vector to its offspring (112–114). This mechanism has important ecological implications allowing viruses to persist during periods when adult vectors are absent (*e.g.* winter) or when susceptible vertebrate hosts are not available (98). Venereal transmission is also suggested for *Phlebovirus* and has been demonstrated from a transovarially TOSV-infected male to a virgin female of *Ph. perniciosus* (115,116). The mean *Phlebovirus* extrinsic incubation period (EIP), the time from virus ingestion until the sand fly is able to transmit, is at least a week (117). For example, the RVFV EIP is around 14 days. This period can be influenced by different parameters, such as the temperature, genetic factors and host barriers (118,119). Knowledge of EIP and vector lifespan is important for understanding the dynamics of sandfly-borne virus transmission.

ii. Reservoirs

To be an optimal arbovirus reservoir, the host must be susceptible to infection and develop extensive and sustained viremia. In addition, there must be frequent contact between vector and reservoir host (120). Because of the short viremia duration and the lack of persistent infection in humans, there is no evidence that humans and/or any other vertebrates are the reservoir of sandfly-borne phleboviruses (111). Nevertheless, a recent study revealed high TOSV seroprevalence rates in some birds, raising the question of a potential role of birds as amplifying hosts in TOSV natural cycle (121). However, the majority of *Phlebovirus* strains are transmitted and maintained by sand flies; their ecological distribution and evolutionary divergence seem to be highly dependent on their vectors (122). The vector could be considered as reservoir, but further studies are needed to confirm these assumptions (95).

d. Public Health importance

i. Clinical forms

In the Mediterranean basin, endemic phleboviruses, such as TOSV, SFNV, and SFSV, are responsible for mild infections but can cause complication and severe clinical manifestations

(123). Most of the time, symptoms are flu-like such as fever, headache, malaise, myalgia, retro-orbital pain, but also photophobia. Incubation periods vary between four and eight days, and the patient usually recovers within a week (124). Among the phleboviruses, TOSV can cause neuroinvasive infections affecting the central and peripheral nervous systems. A total of 6 mortal cases were reported due to TOSV infections (125,126). The incubation period for these neuroinvasive cases is longer (estimated at 12 days (127)) and, according to a modeling study, the longer the incubation period of *Phlebovirus*, the more the virus will spread among vector and host populations (128).

Article 2

Incubation Period for Neuroinvasive Toscana Virus Infections

Lison Laroche*, Frédéric Jourdain*, Nazli Ayhan, Anne-Laure Bañuls, Rémi N. Charrel, Jorian Prudhomme

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Toscana virus (TOSV) is an emerging pathogen in the Mediterranean area transmitted through the bites of female sand flies. TOSV can be neuroinvasive and is a major cause of meningitis and encephalitis in endemic areas during summer. Knowledge on the incubation period of this virus is needed to optimize surveillance and control of these diseases. However, information regarding the incubation period of TOSV is heterogeneous and rarely referenced. This paper is a systematic review on symptomatic travel-related TOSV infections conduced with the aim of estimating the incubation period with censored time-to-event models. A total of 24 imported cases of TOSV meningoencephalitis infections have been found in 22 case reports. The analyses showed a median incubation period of 12.1 days (95% CI: 10.2–14.2). This evidence-based estimate of the TOSV incubation period highlight the need to standardize the data collection for the diagnosis of infected patients.

Incubation Period for Neuroinvasive Toscana Virus Infections

Lison Laroche,[1] Frédéric Jourdain,[1] Nazli Ayhan, Anne-Laure Bañuls, Rémi Charrel, Jorian Prudhomme

Toscana virus (TOSV) is an emerging pathogen in the Mediterranean area and is neuroinvasive in its most severe form. Basic knowledge on TOSV biology is limited. We conducted a systematic review on travel-related infections to estimate the TOSV incubation period. We estimated the incubation period at 12.1 days.

Toscana virus (TOSV) is an arthropodborne virus transmitted to humans through a bite from an infected sand fly (*1*). An RNA virus, it belongs to the genus *Phlebovirus*, species *Sandfly fever Naples phlebovirus* (family *Phenuiviridae*, order *Bunyavirales*) (*2*). TOSV infections are endemic to the Mediterranean basin and are considered frequent even though they are neglected (*3*). TOSV can be neuroinvasive and is a major cause of meningitis and encephalitis during summer months in areas to which it is endemic (*4*). However, most infections are asymptomatic or produce mild symptoms (*5*). Thus, TOSV cases are massively underestimated and unreported. Cases are mainly diagnosed by reverse transcription PCR in cerebrospinal fluid, blood, and, rarely, urine or by detecting virus-specific IgM or IgG (*6*). A total of 3 different TOSV lineages (A, B, and C) have been identified, but no clear evidence of a link between clinical manifestation and lineages exists (*7*).

In this study, we considered the incubation period (IP) of an infectious disease as the delay between infection and symptom onset; this definition differs from the latent period, which is defined as the time from infection to infectiousness. For arthropodborne viruses, the infectious bite represents the date of infection (*8*). The potential period of exposure is represented by the length of stay in the country of infection before symptom onset. We therefore focused on imported cases.

Determining the IP is primordial for disease surveillance, outbreak investigation, public health interventions, infectious disease control, and modeling (*9*). However, IP estimates are often unsourced, imprecise, and based on limited evidence, as illustrated by the heterogeneous values proposed (Appendix Table 1, https://wwwnc.cdc.gov/EID/article/27/12/20-3172-App1.pdf). In this context, we conducted a systematic review of symptomatic travel-related neuroinvasive forms of TOSV to provide an evidence-based estimate of the IP.

The Study

We used PubMed and ISI Web of Knowledge search engines with no restriction on language and the phrase "Toscana AND virus AND (case report OR case-report OR travel* OR import*)." We conducted a systematic search on ProMed and Google Scholar, as well as cross-reference checking. The inclusion criteria were laboratory-documented acute TOSV infection, indication of a travel-related infection in a TOSV-endemic area, and number of days between travel return and symptom onset. Two reviewers screened titles, abstracts, and full-text articles independently.

We extracted clinic and biologic elements from neuroinvasive TOSV case reports. For each patient, data related to the duration of travel and the time of symptom onset, gender, age, country in which case was reported, and country of infection were reported.

To estimate the IP, we used censored time-to-event models (*10*). Interval-censored observations related to travel duration represented the exposure time. Absence of a departure date was treated as left-censored data, whereas onset of illness during the travel period was considered right-censored. We performed data analysis by using R with the icenReg

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package (R Foundation for Statistical Computing, https://www.r-project.org). We defined the data distribution with 4 parametric models (log-normal, log-logistic, Gamma, and Weibull). To determine the best model for our distribution, we calculated the Akaike information criterion. We used the nonparametric log-rank test from the interval package to assess the effect of age and gender as covariates. To check the result stability, we performed an additional Bayesian approach, fitting with the Weibull distribution.

Regarding imported case reports, 142 documents were identified on PubMed and 133 on Web of Knowledge. We removed 79 duplicates and excluded 118 records after screening titles and abstracts. A total of 42 articles were eligible after full-text reading. We then selected 22 documents for data extraction. A total of 24 cases were selected (Appendix Table 2, Figure). All travel-associated cases fulfilling the inclusion criteria were neuroinvasive; these cases were diagnosed in a non–TOSV-endemic area after a stay in a proven TOSV-endemic area (Figure 1).

We selected Weibull distribution because it presented the lower Akaike information criterion (Figure 2; Appendix Table 3). The median IP for neuroinvasive forms is estimated to be 12.1 (95% CI 10.2–14.4) days. In 5% of neuroinvasive cases, symptoms will develop by 6.8 (95% CI 3.8–9.9) days after an infectious bite; symptoms will develop in 95% of cases by 16.8 (95% CI 13.9–21.7) days after the infectious bite. We found no evidence of age or gender effect on the length of the IP (p value >0.05 by log-rank test). By using Bayesian analysis, we found an IP of 12.1 (95% CI 9.9–14.4) days (data not shown; results same as Figure 2).

Conclusions

In the literature, the IP values of TOSV are often heterogeneous, unsourced, or without evidence and therefore do not constitute a valid estimate for clinical or infection control decisions. Our literature review identified 24 neurologic cases of TOSV infection. Some travel durations were reported approximately in case reports and were not included in the analysis. All the data used were based on severe neurologic forms of the disease, which required hospitalization soon after the exposure period.

We estimated the median IP of TOSV at 12 (95% CI 10.2–14.4) days. Considering the delay from infection to symptom onset, this value is greater than that for most other arboviruses (*11*). Our estimate of the IP is evidence-based and relies on data from well-characterized cases. However, cases that cause mildersymptoms, as opposed to neuroinvasive forms of the disease, might have a shorter IP (similar to

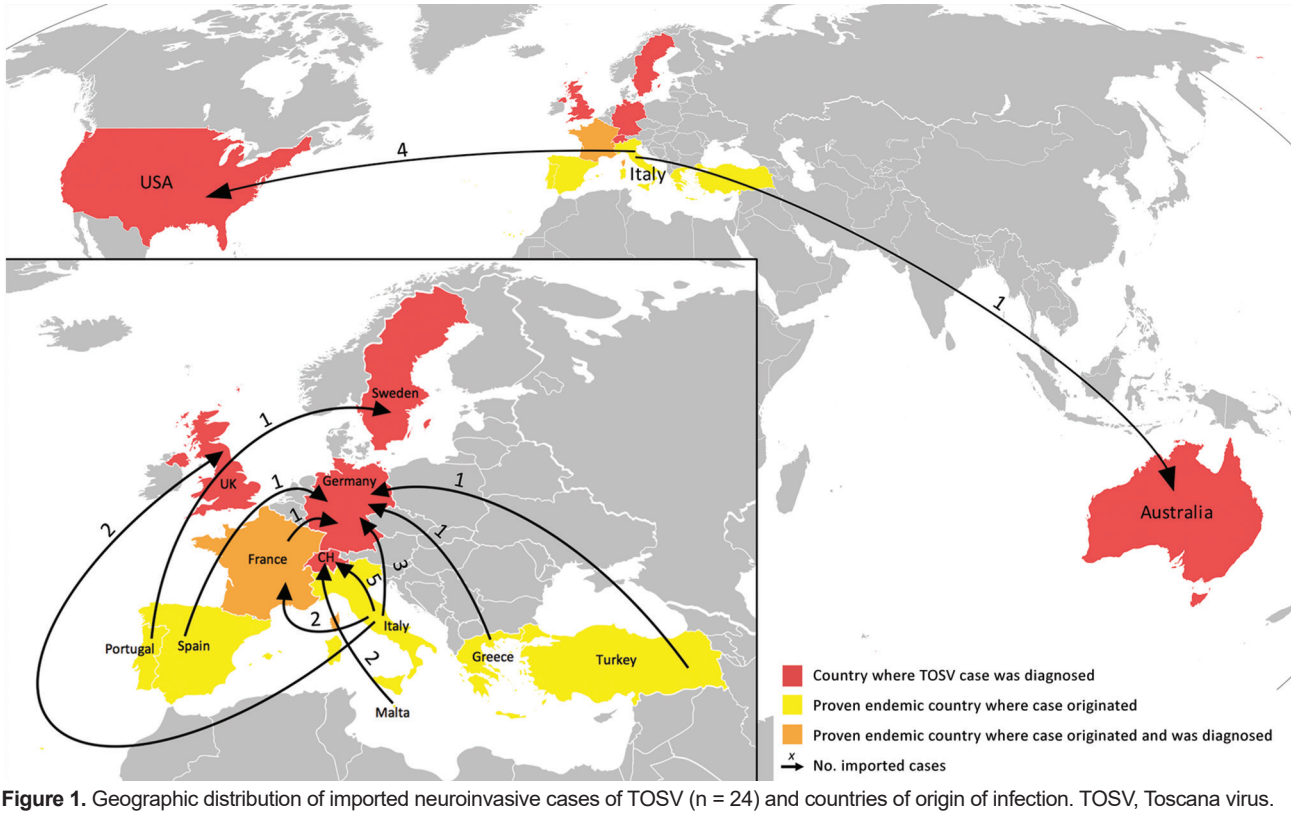


Figure 1. Geographic distribution of imported neuroinvasive cases of TOSV (n = 24) and countries of origin of infection. TOSV, Toscana virus.

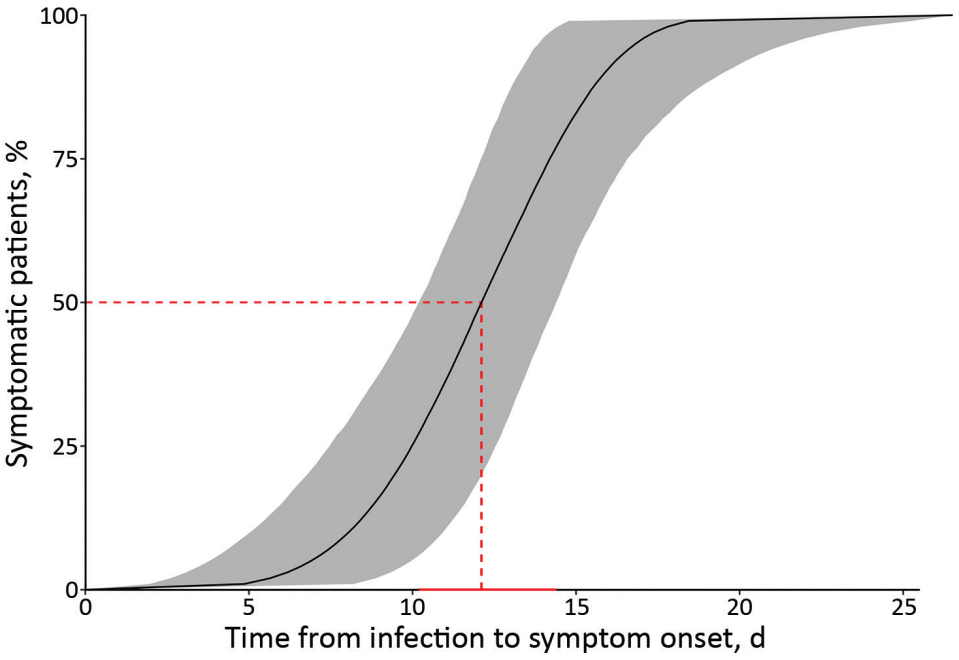


Figure 2. Cumulative percentage of Toscana virus cases manifesting with neurologic symptoms by a given day under the estimates for the Weibull parametric distribution (n = 24). Red dashed line represents the median estimation of the incubation period. Solid red horizontal line represents the 95% CI of the median. Gray shading indicates the 95% CI of the values.

other arboviruses). Other symptoms associated with paucisymptomatic forms of TOSV might not have been described yet and should be further investigated to improve case definition and diagnosis.

We also cannot exclude infections by other sandfly fever Naples phleboviruses because of cross-reaction risk in serologic analyses due to their close genetic relationships (*12*). However, the incidence in the population of other genetically similar phleboviruses is lower than TOSV, and TOSV remains the most common cause of neuroinvasive symptoms (*3*). Knowledge of TOSV genotypes and their aptitude to cause different clinical forms is limited (*12*). Analyzing this hypothesis was not possible because of the limited amount of available data. In addition to the genotype, other parameters may influence the IP, such as viral strain, patient's immune status, or viremia (*9*). The amount of virus transmitted during bites (viral load) could also influence the IP and should be further investigated.

In addition, all other cases were diagnosed in countries or regions to which TOSV is not endemic (United States, United Kingdom, Sweden, Germany, Switzerland, Australia, and France). These imported cases represent a risk for emergence in these areas when vectors are established (*13*), as has been observed for other vectorborne diseases (*14*). Moreover, sand flies are known to spread in countries or regions to which TOSV is not endemic (*15*).

Currently, information on TOSV infections is lacking (*12*). Precise definitions of the IP should provide more information on the disease epidemiology and on its development in the human host. Moreover, because the IP is a key parameter for disease modeling (*9*), it would improve our understanding of the disease transmission dynamics. More reports of travel-related cases and standardization of data collection with reliable information (e.g., location and duration of the trips and precise dates of symptom onset) are clearly needed. The IP estimation will be improved with addition of new data.

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ii. Diagnostic, treatment and prevention

Diagnosis of *Phlebovirus* can be realized with different techniques such as: virus isolation in vitro on mammalian, bird and sand fly cell lines and molecular reverse transcription–polymerase chain reaction (RTqPCR) techniques, with primers on the S segment (129). *Phlebovirus* diagnosis is also made by detection of specific IgG and/or IgM antibody groups via enzyme-linked immunosorbent assay (ELISA) and indirect fluorescent antibody (IFA) assay (96). However, as these serological techniques are more prone to cross-reactions with genetically close viral strains, a viral neutralization assay is necessary to identify the strain (130). To date, human vaccines against sandfly-borne phleboviruses are still under development (131).

Interferons are proteins capable of interfering with viral replication in cells by inducing the synthesis of new mRNAs and proteins, thereby help the host immune system to fight infection (132,133). An interferon-inducing antiviral medication, Ribavirin, has been identified as a candidate to treat infections caused by viruses of the Bunyaviridae family being effective in inhibiting these viral infections in animal models (134). Besides, another protein, bovine Lactoferrin, was able to inhibit the replication of TOSV *in vitro* (135).

To control sandfly-borne diseases, it is important to reduce sand fly population densities and to decrease the host-vector contact in areas where people are at high risk. Personal protective measures such as repellents and insecticide-treated bed nets are recommended in endemic areas (98). However, as with leishmaniasis, vector control is limited by the difficulties inherent in a non-aquatic, land-breeding vector that is not susceptible to large-scale insecticide treatment programs (136).

4. Interactions between sand fly, *Leishmania* and *Phlebovirus*

a. Co-circulation

These pathogens co-circulate in insect vectors and/or vertebrate organisms. **Table 1** shows some examples of *Phlebovirus* and *Leishmania* species that co-circulate in the Mediterranean basin. The simultaneous detection in space and time of *Phlebovirus* and *Leishmania* is evidence of shared possible reservoir and vector between the two microorganisms (137).

Table 1. *Phlebovirus* and *Leishmania* co-circulating in the Mediterranean area. Phleboviruses involved: Toscana virus (TOSV) lineages A, B and C, Massilia virus (MASV), Sandfly fever Sicilian virus (SFSV), Sandfly fever Naples virus (SFNV), Alcube virus (ACBV), Salehabad virus (SALV), Punique virus (PUNV). *Le*. for *Leishmania*, *Ph*. for *Phlebotomus*.

Location	Pathogens involved		Hosts	Reference
Croatia	TOSV (B)	TOSV (C)	*Ph. neglectus*	(138)
France	MASV	*Le. infantum*	*Ph. perniciosus*	(139)
Italy	TOSV	7 other phleboviruses	*Ph. perniciosus & Ph. perfiliewi*	(140)
Italy	TOSV	*Le. infantum*	*Ph. perniciosus & Ph. perfiliewi*	(137)
Morocco	SFSV	SFNV	Human*	(101)
Portugal	MASV	ACBV	*Ph. perniciosus*	(141)
Portugal	TOSV	SFSV	Human*	(142)
Tunisia	TOSV	PUNV	Human*	(143)
Tunisia	TOSV (A)	*Le. infantum*	*Ph. perniciosus & Ph. perfiliewi*	(144)
Turkey	SFSV	SFNV & SALV	*Ph. tobbi & Ph. perfiliewi*	(145)

* Antibody detection in sera

b. Co-infection

As some *Phlebovirus* share the same vector as *Leishmania* parasites, they could play a crucial role as co-infecting pathogens. Many cases of co-infection have been reported in several countries and studied by experimental infections (**Table 2**). In cases of co-infections, it is possible for one pathogen to affect pathogenicity or transmission of the other. For example, HIV/*Leishmania* co-infections contribute to increased incidence of leishmaniasis due to the patient's immunosuppression (146). Experimental co-infection in mice have demonstrated that TOSV can affect the severity of *Leishmania guyanensis*, by increasing both lesion size and parasite load through a type I interferon-dependent mechanism (147). In addition, it is well established that the presence of other RNA viruses in the host, such as the *Leishmania* RNA virus (LRV), can influence the severity and recovery of leishmaniasis (148). Co-infections have been shown in pools of field-collected sand flies, but never at the individual level. Until now, no study has experimentally examined the impact of *Phlebovirus/Leishmania* co-infection in sand flies.

Table 2. RNA viruses and *Leishmania* co-infections detected in sand fly vectors, mammal hosts or by experimental infections (EI). Viruses involved: Toscana virus (TOSV), Human immunodeficiency virus (HIV), *Leishmania* RNA virus (LRV), Sandfly fever Sicilian virus (SFSV), West Nile virus (WNV). *Le*. for *Leishmania*, *Ph*. for *Phlebotomus*.

Location	Pathogens involved		Host	Reference
Cyprus	TOSV	*Le. infantum*	*Ph. tobbi*	(149)
France	HIV	*Leishmania spp.*	Human*	(150)
France	TOSV	*Le. infantum*	Human*	(151)
Iran	LRV	*Leishmania spp.*	Human*	(152)
Morocco	TOSV	*Le. tropica*	*Ph. sergenti*	(153)
Portugal	SFSV	*Le. infantum*	Dog*	(154)
Turkey	TOSV	*Le. infantum*	Dog*	(155)
Turkey	TOSV	WNV	Human*	(156)
EI	TOSV	SFSV	Dog*	(157)
EI	LRV	*Leishmania spp.*	Hamster	(148)
EI	SFSV	*Le. major*	Mice	(84)
EI	*Phlebovirus*	*Le. amazonensis*	Mice	(158)
EI	TOSV	*Le. guyanensis*	Mice	(147)

* Antibody detection in sera

OBJECTIVES

Leishmania parasites have been studied for a long time and are becoming well known. The study of the interactions between *Leishmania* and their sand fly vectors provides a better understanding of the mechanisms of parasite development and gives important information on vector competence that could help to identify novel ways to fight leishmaniasis. However, sandfly-borne phleboviruses infections remain neglected and few data are available on their natural cycle. In addition, interactions between the different pathogens transmitted by sand flies have not been much studied. The different pathogens could interact within the sand flies or the hosts and influence the infection process. Recent studies have highlighted the co-circulation of *Phlebovirus* and *Leishmania* species. As these pathogens share sand fly vectors, co-infection is suspected, especially for TOSV and *Le. infantum* parasite, both transmitted by the vector *Ph. perniciosus* in the Mediterranean region.

In this context, the main objective of this thesis is to study pathogen-vector interactions to better understand the impact on vector biology and transmission dynamics. This thesis is divided into three chapters, the first deals with the vector *Ph. perniciosus*, the second with the parasite *Le. infantum* and the third with the TOSV.

CHAPTER 1

Phlebotomus perniciosus rearing for experimental infection

This thesis section focuses on the establishment of sand fly rearing for the purpose of experimental infections. The objective of this work is to give a chronology of the colony set-up from the beginning to the experimental infections, through optimization of rearing methods already described. Several parameters of the development cycle of *Ph. perniciosus* have been studied and described.

CHAPTER 2

Dynamics of *Leishmania infantum* infection and impact on vector biology

The second part of this thesis is dedicated to the impact of *Le. infantum* infection on the life history traits of its vector *Ph. perniciosus*. It is well known that pathogen infection can have a cost and affect the fitness of its vector. In this section, we investigate whether infection with *Le. infantum*, by different parasite concentration, can have an impact on the survival and/or

fecundity of *Ph. perniciosus* under experimental conditions. We also investigate whether the dynamics of infection in the vector are affected by the amount of parasite ingested.

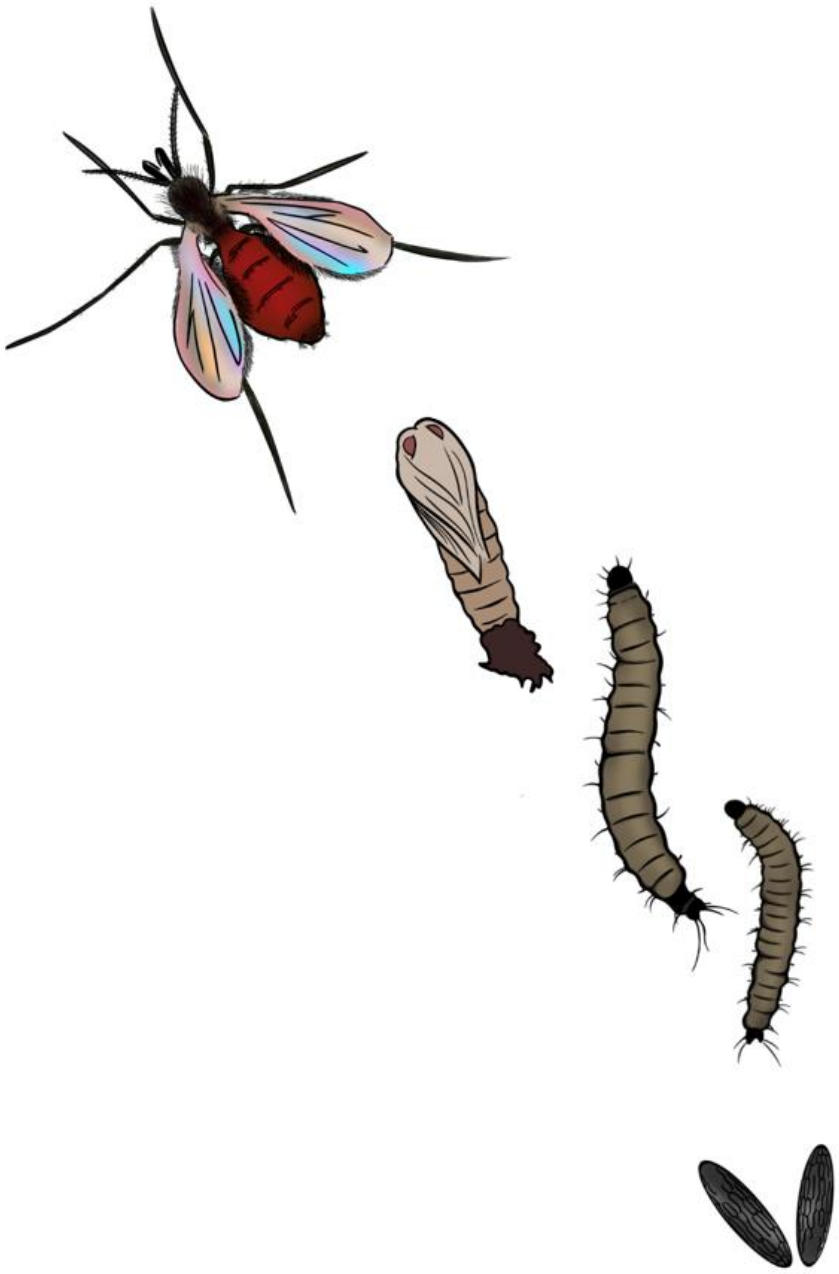
CHAPTER 3

Toscana virus infection dynamics, persistence and impact on vector biology

In the third part, we focused on the TOSV ecology and its interactions with its vector *Ph. perniciosus*. We measured TOSV persistence in two different environments in order to estimate the potential epidemiological risks. In this chapter, the infection dynamics of TOSV within the vector were described and the limiting infection dose was estimated according to three different virus doses. We localized in which sand fly body parts the TOSV replicates and from when it could be transmitted (estimation of the extrinsic incubation period). Secondly, we studied the impact of TOSV infection on life history traits of *Ph. perniciosus*, such as survival and/or fecundity.

CHAPTER 1

Phlebotomus perniciosus rearing for experimental infection



The establishment and maintenance of sand fly colonies is essential for the experimental study of their biology, physiology, behavior, vectorial competence and capacity. The presence of such colonies allows also the development of vector control methods, particularly for insecticide assays. The colonies are essential to study the natural cycles and transmission dynamics of *Leishmania* and *Phlebovirus*.

Worldwide, there are very few sand fly rearing facilities compared to other insect vectors. We set up a colony of two species of the genus *Phlebotomus* in our laboratory in Montpellier (French National Research Institute for Sustainable Development) at the beginning of my thesis. The establishment of *Ph. perniciosus* and *Ph. papatasi* colonies was carried out with Dr. Jorian Prudhomme under the valuable advices of Dr. Mireille Killick Kendrick following previously described protocols (34,159).

As this thesis focuses on the interactions between *Leishmania* and *Phleboviruses* of the Mediterranean basin with their sand fly vector, we decided to work with the *Ph. perniciosus* species. All the experiments of this thesis were carried out with this vector, common to *Le. infantum* and TOSV. We set up the experimental infections with *Le. infantum* in the first part, and with TOSV in the second part of my thesis. The protocol for the experimental infection is the same for both pathogens (See detailed protocol in Appendix 1). The objective of this work was to give a chronology of the colony set-up from the beginning of sand fly rearing to the experimental infections, through the optimization of the rearing methods already described. All the details of this study are described in: "Jorian Prudhomme*, Lison Laroche*, Anh Nguyễn Hoàng Quân, Justine Fournier, Idris Mhaidi, Aloïs Berard, Mireille Killick-Kendrick and Anne-Laure Bañuls. Establishment and maintenance of two sand fly colonies (*Phlebotomus perniciosus* and *Phlebotomus papatasi*) for experimental infections. Submitted to the journal *Parasite*".



Who is *Phlebotomus perniciosus*?

Described from the island of Malta by R. Newstead in 1911, *Ph. perniciosus* occupies a wide geographical range covering the mid-European and Mediterranean regions. If global warming provides suitable environmental conditions, populations of *Ph. perniciosus* should be able to disperse in Northwestern Europe. In temperate region, this species is active between March and October, with abundance peaks in July and September. Paradoxically, for a species with a wide distribution, *Ph. perniciosus* does not rise to higher altitudes. Due to its endophilic behavior during host seeking, it is mainly found inside houses, near stables and hutches. The hosts are mainly domestic mammals, such as dogs. *Ph. perniciosus* is the main vector of *Le. infantum* and TOSV in the Mediterranean basin.



Article 3

Establishment and maintenance of two sand fly colonies (*Phlebotomus perniciosus* and *Phlebotomus papatasi*) to implement experimental infections

Jorian Prudhomme*, **Lison Laroche***, Anh Nguyễn Hoàng Quân, Justine Fournier, Idris Mhaidi, Aloïs Berhard, Mireille Killick-Kendrick, Anne-Laure Bañuls

**these authors contributed equally to the work*

Submitted to the Journal Parasite

In order to study sand fly-*Leishmania*-*Phlebovirus* interactions we set up a sand fly colony in our laboratory. The chronology of the colony establishment has been described in the following article, in order to give information on the establishment and optimization of the rearing conditions. Several parameters of the life history traits of *Ph. perniciosus* have been studied and described. We have also added recommendations on how to facilitate the rearing procedures in order to carry out experimental infections.

Parasite

Establishment and maintenance of two sand fly colonies (Phlebotomus perniciosus and Phlebotomus papatasi) to implement experimental infections

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Abstract:	Sand flies are vectors of Leishmania and Phlebovirus, and these pathogens remain a public health problem in many countries. Sand fly colonies for experimental infection are essential to conduct research on these vector and the pathogens they transmit. However, there are few sand fly colonies in the world. In 2016, around 100 colonies, with 33 different species, were found worldwide. Methods to establish and maintain sand fly colonies have been well described in the literature. However, there are few colonies of Phlebotomus perniciosus and Phlebotomus papatasi although they are important vectors. In order to encourage the creation of new colonies, we provide a timeline for researchers who want to implement colonies of Ph. perniciosus and Ph. papatasi. In this study, we established colonies of both species to test and optimize their breeding conditions. For Ph. perniciosus, we reached a stable production of 800 individuals per week, one year after the start of the colony. For Ph. papatasi, a stable production of 400 individuals per week was reached 11 months after establishing the colony. Moreover, we successfully performed experimental infections and insecticide resistance tests, 10 and 11 months after establishing Ph. perniciosus and Ph. papatasi colonies, respectively. In conclusion, our data on these two species showed that colonies could be established without difficulty with 1000-1500 eggs. It is possible to reach a stable weekly production of 800 individuals one year after the start of the colony. We also describe colony settings and breeding recommendations to facilitate this procedure for other laboratories.
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Establishment and maintenance of two sand fly colonies (*Phlebotomus perniciosus* and *Phlebotomus papatasi*) to implement experimental infections

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Abstract

Sand flies are vectors of Leishmania and Phlebovirus, and these pathogens remain a public health problem in many countries. Sand fly colonies for experimental infection are essential to conduct research on these vector and the pathogens they transmit. However, there are few sand fly colonies in the world. In 2016, around 100 colonies, with 33 different species, were found worldwide.

Methods to establish and maintain sand fly colonies have been well described in the literature. However, there are few colonies of *Phlebotomus perniciosus* and *Phlebotomus papatasi* although they are important vectors.

In order to encourage the creation of new colonies, we provide a timeline for researchers who want to implement colonies of *Ph. perniciosus* and *Ph. papatasi*. In this study, we established colonies of both species to test and optimize their breeding conditions.

For *Ph. perniciosus*, we reached a stable production of 800 individuals per week, one year after the start of the colony. For *Ph. papatasi*, a stable production of 400 individuals per week was reached 11 months after establishing the colony. Moreover, we successfully performed experimental infections and insecticide resistance tests, 10 and 11 months after establishing *Ph. perniciosus* and *Ph. papatasi* colonies, respectively.

In conclusion, our data on these two species showed that colonies could be established without difficulty with 1000-1500 eggs. It is possible to reach a stable weekly production of 800 individuals one year after the start of the colony. We also describe colony settings and breeding recommendations to facilitate this procedure for other laboratories.

Keywords: Sand flies, *Phlebotomus*, insect rearing, life cycle, experimental infection.

Établissement et entretien de deux colonies de phlébotomes (*Phlebotomus perniciosus* et *Phlebotomus papatasi*) pour la mise en place d'infections expérimentales

Résumé

Les phlébotomes sont des vecteurs de *Leishmania* et de *Phlebovirus*, des agents pathogènes qui restent un problème de santé publique dans de nombreux pays. Les colonies de phlébotome sont essentielles pour mener des recherches sur ces vecteurs et les agents pathogènes qu'ils transmettent. Cependant, il existe peu de colonies dans le monde. En 2016, environ 100 colonies, avec 33 espèces différentes, ont été recensé.

Les méthodes pour établir et maintenir des colonies ont été bien décrites dans la littérature. Cependant, il existe peu de colonies de *Phlebotomus perniciosus* et *Phlebotomus papatasi* alors qu'elles sont des espèces vectrices importantes.

Afin d'encourager la création de colonies de ces deux espèces, nous fournissons un échéancier pour les chercheurs voulant en mettre en place. Dans cette étude, nous avons établi ces colonies pour tester et optimiser leurs conditions d'élevage.

Pour *Ph. perniciosus*, nous avons atteint une production stable de 800 individus par semaine, un an après le début de la colonie. Pour *Ph. papatasi*, une production stable de 400 individus par semaine a été atteinte 11 mois après l'établissement de la colonie. De plus, nous avons réalisé des infections expérimentales et des tests de résistance aux insecticides, 10 et 11 mois après l'établissement des colonies de *Ph. perniciosus* et *Ph. papatasi*, respectivement.

En conclusion, nos données ont montré que les colonies peuvent être établies sans difficulté avec 1000-1500 œufs. Il est possible d'atteindre une production hebdomadaire stable de 800 individus un an après le début de la colonie. Nous décrivons également les paramètres de la colonie et les recommandations d'élevage afin de faciliter cette procédure pour d'autres laboratoires.

Introduction

Sand flies are small hematophagous dipteran insects. There are more than 900 species of sand flies, and among these 98 are potential vectors of diseases [1]. They are vectors of *Leishmania* and *Phlebovirus*, and both these pathogens remain a public health problem in many countries [2].

Leishmania can cause different clinical forms in humans, from asymptomatic or benign cutaneous forms, to visceral forms which are lethal without treatment. About 20 *Leishmania* species are pathogenic to humans [3]. For example, *Leishmania infantum* is endemic around the Mediterranean basin and can cause cutaneous or visceral forms. The number of leishmaniasis cases and their distribution areas are still on the rise and many reports show that this disease is far from being eradicated [2, 4]. Moreover, there is a re-emergence in old foci and emergence in new foci [5, 6].

As for the genus *Phlebovirus,* it comprises about 70 viruses, including the Toscana virus, responsible for human meningoencephalitis in the Mediterranean region. This virus is the main cause of aseptic meningitis during the warm season in Spain, Italy and Southern France [7]. Both pathogens, *Leishmania* and *Phlebovirus*, co-circulate in the Mediterranean basin [8].

Many physical and behavioral characteristics of these insects are still unknown (*e.g.*, inter and intraspecific vectorial capacity, poorly known food preferences). Sand fly colonies for experimental infection are essential to conduct research on these vector insects and the pathogens they transmit. However, there are only few sand fly colonies in the world. In 2016, only around 100 colonies, with 33 different species, were found worldwide [9].

The objective of our study was to establish *Phlebotomus perniciosus* and *Ph. papatasi* colonies. *Phlebotomus perniciosus* is the main vector of *Le. infantum* in Southwestern Europe [10]. *Phlebotomus papatasi* is one of the major vectors of *Leishmania major* in the Old World [11]. Methods to establish and maintain sand fly colonies have been well described in the literature [12-14]. However, there are few colonies of these two species although they are easy to breed and important vectors.

In order to encourage the creation of new colonies of these sand flies, we provide a timeline for researchers who want to implement sand fly colonies of the species *Ph. perniciosus* and *Ph. papatasi*. In this study, in 2020, we established, in IRD Vectopôle (Montpellier, France), colonies of both species to test and optimize their breeding conditions.

Materials and methods

Ethical statement

Rabbit blood draws performed in the context of this study were approved by the Animal Care and Use Committee named “Comité d’Ethique pour l’Expérimentation Animale Languedoc Roussillon n°36” under protocol number 2018022712203932. Rabbits, coming from the animal facility at IRD, were not subjected to anesthesia, analgesia or sacrifice. The chicks come from the Experimental Infectiology Platform of Nouzilly (INRAE Centre Val de Loire, France). They were killed according to the Directive 2010/63/EU (Appendix IV) appropriate to the species. The chicks were dead before the use of tissue samples for experimental infection, in accordance with article R214-89 of the French “Code rural et de la pêche maritime”, Section 6.

Starting colonies

We received sand fly eggs in November and March 2020 for *Ph. perniciosus* and *Ph. papatasi*, respectively. To start the sand fly colonies, we used eggs from well-adapted laboratory colonies of *Ph. perniciosus* (Laboratorio de Entomología Médica, Instituto de Salud Carlos III, Madrid, Spain) and *Ph. papatasi* (Department of Parasitology, Charles University, Prague, Czech Republic). The eggs were shipped in rearing pots by our collaborators Pr. Ricardo Molina and Pr. Petr Volf. Our colonies were started, in IRD Vectopôle (Montpellier, France), with approximatively 1,500 and 1,000 eggs from *Ph. papatasi* and *Ph. perniciosus*, respectively.

Recommendations

To start a colony, we recommend to use eggs when the species are available from other laboratories for the following reasons: 1) transportation is easier, 2) this method does not require blood feeding and, 3) it makes it possible to collect data from the first generation. However, it is also possible to use individuals collected from the field [12].

Sand fly rearing

Sand flies were maintained under standard conditions (26 ± 1°C, 80% relative humidity, 14h/10h light-dark cycle). For colony maintenance, we used protocol described by Volf [12], Lawyer [13] and Molina [14], with some modifications described below.

Adults were maintained in large fabric-net holding cage (30x30x30cm) and fed with 50% glucose solution (protocol in Supp. Table S1). Blood feeding was done with glass feeders, filled with rabbit blood and covered with chicken skin membranes. Five-days-old chicks were used as they present a thin skin which allows efficient blood feeding (protocol in Supp. Table S2). The blood in the feeders was maintained at a 37°C constant temperature by a water bath with external circulation. Feeding was performed for five-six hours with a regular manual blood mix.

After 24 hours, blood-fed females were transferred in clear plastic containers filled with 1cm layer of Paris plaster. These pots were stored in plastic boxes containing humidified sand [12]. After hatching, larvae were fed with a matured mixture of 40% rabbit feces, 40% rabbit chow, and 20% mice chow (protocol in Supp. Table S3).

We performed blood feeding once a week for *Ph. papatasi*. For *Ph. perniciosus*, three blood feedings per week were done for the first generation (colony amplification), once a week for the second generation (due to COVID-19 lockdown measures), twice a week from the third generation to seventh generation (colony amplification), and once a week from the seventh generation onwards (colony routine maintenance).

Recommendations

Using glass feeders allows longer blood feeding and consequently increases blood-fed female numbers. To facilitate feeding, stimulate females every 30 minutes by breathing on the cage. Moreover, if the purpose of the colony is to perform experimental infections, the same protocol can be used in BSL2 or BSL3 laboratories, with the exception of stimulation by breathing [15]. During experimental infection, adding males (10%) will improve the engorgement rate [12].

We recommend three blood feedings a week for the two first generations (colony amplification) and then once a week (routine maintenance).

Monitoring of sand fly colonies

We monitored the breeding data daily: mortality rate, mean blood feeding rate, development time, average generation time and productivity of the colony. Ranges of minimum developmental times were obtained by observing the first appearance of each stage. The recording of these parameters was done every day for the first generation, twice a week for the second generation (due to COVID-19 sanitary restrictions) and then four times a week (routine maintenance). The goal was to achieve the minimum of care without impacting the colony.

We used these parameters to determine the duration of each development stage duration, colony productivity and acclimatization.

Recommendations

In the early stages of the colony, it is imperative to come every day to check the survival rate, as well as to identify and solve possible problems as soon as they appear. A daily check of the larvae also prevents an overly rapid fungi proliferation which can be lethal for the larvae.

For standardized data collection and tracking, we recommend the use of a detailed file available in Supp. Table S4. The follow-up of these parameters makes it possible to plan experiments and to check for any extended generation time (*i.e.*, the first sign of an ailing colony) [12].

Use of sand fly colony

Colony productivity determination makes it possible to define when and how many individuals are available for experiments. In our case, we performed insecticide resistance tests [16] on *Ph. papatasi* and experimental infections [15] on *Ph. perniciosus*. Indeed, the setting up of sand fly colonies has the following objectives: 1) to ensure the continuation of the colony and 2) to conduct suitable experiments (experimental infections or insecticide resistance testing).

Recommendations

The colony size has to be monitored and it is essential to determine the number of individuals that can be sacrificed for the experiments. Indeed, to maintain the colony, no more than a third of its size should be taken [13]. Females under three to four days old do not feed and females aged more than ten days have a lower survival rate after blood feeding [12]. We used females aged from four to eight days (females produced over the week). As such, it is important to know the weekly production but also how much time is necessary for production to meet the needs of the laboratory.

Statistical analysis

Statistical analyses of developmental times in different generations were performed using a nonparametric Kruskal-Wallis test followed by a post-hoc test (*i.e.*, Mann-Whitney tests with Bonferroni correction), using R software version 3.1.0 [17].

Results and discussion

Colony establishment

The colony is currently in its eighth generation for *Ph. papatasi,* and in its tenth generation for *Ph. perniciosus*. The number of individuals in the colonies according to time is detailed in Figure 1.

We were not able to take care of the sand flies every day during the COVID-19 lockdown period (March to May 2020), which led to a proliferation of fungi in the rearing pots and affected the mass production of sand flies. In addition, mites (Figure 2) invaded our colonies. These insects can feed on eggs, which also influences mass production.

At the end of COVID-19 confinement measures, we managed to overcome the fungus problem. On the other hand, mite infestations are almost impossible to avoid, we are currently testing several processes to eliminate them (*e.g.*, daily removal of dead individuals, ovipots checked daily, mite removal) [13].

The establishment of the colonies and the survival rate over two years and several generations show that the membrane feeding system is viable and has multiple advantages:

- it avoids the use of live animals;
- it does not require trained personnel;
- it allows the choice of blood;
- it avoids colony adaptation to alternative blood, which can be a long process and can take several generations [12].

Colony productivity

Table 1 shows the productivity by generation. An average of all generations can be estimated. In total, 5,284 *Ph. papatasi* females and 10,651 *Ph. perniciosus* females were put in rearing pots. To date, the number of emerged adults was 24,773 and 38,435 and the mean number of females was 9.9 and 6.9 for *Ph. papatasi* and *Ph. perniciosus*, respectively.

We observed a decrease of productivity after the first generation for *Ph. perniciosus*. Among the many factors that can be responsible for this decrease, two explanations seem more likely. First, fungi were present in the rearing pots in the second generation, affecting larvae mobility and feeding behavior. Second, the colony may have needed to adapt to our insectary (*e.g.*, climatic condition, manipulation). However, we observed an increase in productivity of *Ph. perniciosus* females at the fourth generation, which could be the result of a good adaptation to the insectary. However, there was a decrease at the seventh generation probably due to the female number per pot. Indeed, a high density of females can generate stress and lead to a decrease of eggs production [13].

With a 50/50 sex ratio, the mean percentage of blood-fed females during blood meals is higher for *Ph. papatasi* (23% [1.93; 58.61]) than for *Ph. perniciosus* (21% [1.59; 61.36]).

For *Ph. papatasi* and *Ph. perniciosus*, 29% and 17% of females died before laying eggs, respectively. However, these results are probably underestimated because it was not possible to determine if all the females laid eggs after the first egg appearance. These data also highlighted that our *Ph. perniciosus* colony adapted well whereas our *Ph. papatasi* colony needed time to adapt.

Colony weekly production

For *Ph. perniciosus*, we were able to conduct six experimental infections with 1951 females, with the first one performed 10 months after the establishment of the colony. We reached a stable weekly production of at least 800 individuals one year after the start of the colony. For *Ph. papatasi*, we used almost 6,000 individuals for insecticide resistance tests, with a first experimentation performed 11 months after the establishment of the colony. A stable weekly production of at least 400 individuals was reached 11 months after the start of the colony (Figure 3).

Generation time

Mean periods for preoviposition, hatching, development of larvae and pupae, and for the total developmental time per generation are reported in Table 2. The total developmental time, from blood meal to the next generation of adult emergence, is 49.9 days [48.4; 51.1] and 47.4 days [43.7; 52.0], for *Ph. papatasi* and *Ph. perniciosus*, respectively. The data presented in this work are similar to the data found in the literature [12, 13, 18, 19].

Hatching period and developmental times of larvae and pupae were not significantly different among the generations for the two species (p-value = 0.9808 and 0.9999 for *Ph. papatasi* and *Ph. perniciosus*, respectively).

As expected [13], we observed semi-asynchronous emergence. The adults emerged over an average period of 38.5 [18; 34.9] and 61.9 [41.3; 73.1] days calculated on all generations for *Ph. perniciosus* and *Ph. papatasi*, respectively.

To date, we observe a lower number of larval diapauses for *Ph. perniciosus* than for *Ph. papatasi*, which could be the result of the better adaptation of *Ph. perniciosus* to our insectary [18].

Acknowledgments

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Conflicts of interest

The authors have no conflicts of interest to declare.

Author contributions

J.P. and L.L. established the colonies. J.P., L.L., A.N.H.Q., J.F., I.M. and A.B. maintained the colonies. J.P., L.L, A.N.H.Q. analyzed the data and wrote the manuscript with the support of A-L.B. and M.K.K. Finally, J.P., L.L., A.N.H.Q., M.K.K, J.F., I.M., A.B. and A-L.B. read, amended and approved the final version of the manuscript.

Tables

Table 1. Number of adults produced by females. G: generation; Female no.: number of blood-fed females; No. pot: number of breeding pots used; Mean female: mean number of females by breeding pot; No. adult: number of emerged adults; No. adult / female: mean number of emerged adults by female.

Species	G	Female no.	No. pot	Mean female	No. adult	No. adult / female
Phlebotomus papatasi	1	198	13	15	2056	7.83
	2	375	11	34	3235	8.59
	3	273	10	27	2954	9.64
	4	407	9	45	5131	13.93
	5	1385	14	99	NA*	NA*
	6	1336	12	111	NA*	NA*
	7	1177	12	98	NA*	NA*
	8	NA*	NA*	NA*	NA*	NA*
Phlebotomus perniciosus	1	148	20	7	1635	9.01
	2	273	13	21	1121	4.39
	3	301	16	19	1640	4.59
	4	374	12	31	3617	9.55
	5	782	16	46	4819	10.08
	6	1584	20	79	8460	6.52
	7	2196	20	110	5822	3.93
	8	1831	13	141	8185	NA*
	9	1847	15	136	NA*	NA*
	10	NA*	NA*	NA*	NA*	NA*

NA* Data not available (these generations are still in production).

238 **Table 2.** Mean times between female blood meal and oviposition and mean times of development of the immature
239 stages by species. G: Generation; Ovi: Oviposition.

Species	Average development time in days								
	G	Ovi	Egg-L1	L1-L2	L2-L3	L3-L4	L4-pupae	Pupae-adult	Total
Phlebotomus papatasi	1	8.53 (±4.31)	8.92 (±2.81)	7.18 (±1.34)	7.27 (±1.54)	7.36 (±3.62)	5.55 (±2.43)	7.91 (±1.50)	50.46 (±4.40)
	2	6.45 (±0.89)	9.45 (±1.16)	6.45 (±2.06)	6.09 (±1.50)	5.45 (±1.56)	7.54 (±1.67)	9.64 (±1.92)	51.09 (±2.23)
	3	6.9 (±1.76	8.30 (±1.35)	7.22 (±1.87)	6.22 (±1.40)	6.22 (±1.75)	6.33 (±1.94)	9.11 (±2.28)	50.56 (±3.17)
	4	5.67 (±0.67)	7.67 (±0.94)	7.00 (±1.41)	5.56 (±1.64)	5.44 (±1.43)	9.33 (±4.11)	7.78 (±1.87)	48.44 (±3.24)
	5	4.64 (±1.04)	8.14 (±1.41)	6.21 (±1.42)	5.71 (±1.58)	6.07 (±2.25)	9.86 (±4.32)	8.5 (±2.16)	49.14 (±4.83)
	6	5.17 (±1.25)	7.75 (±1.41)	6.58 (±1.57)	3.67 (±1.64)	7.42 (±1.13)	9.08 (±1.85)	8.92 (±2.94)	48.58 (±2.95)
	7	4.50 (±1.12)	9.70* (±NA*)	7.00* (±NA*)	7.00* (±NA*)	7.00* (±NA*)	9.00* (±NA*)	7.40* (±NA*)	50.80* (±NA*)
	8	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*
Phlebotomus perniciosus	1	7.56 (±2.57)	7.80 (±2.86)	4.40 (±1.31)	4.93 (±2.59)	3.93 (±1.34)	8.79 (±2.76)	11.57 (±1.18)	49.29 (±2.34)
	2	7.69 (±1.26)	9.69 (±3.17)	5.54 (±1.74)	4.69 (±2.13)	3.15 (±1.41)	6.75 (±1.96)	9.92 (±1.38)	45.92 (±2.32)
	3	7.81 (±3.81)	8.80 (±2.34)	5.00 (±1.83)	5.40 (±2.03)	3.87 (±1.36)	6.00 (±2.66)	9.00 (±2.16)	47.13 (±10.17)
	4	6.00 (±1.41)	8.00 (±1.96)	6.25 (±1.09)	4.33 (±0.85)	3.42 (±0.95)	6.75 (±2.24)	9.83 (±1.52)	44.58 (±2.75)
	5	4.82 (±1.10)	7.18 (±1.25)	6.47 (±1.29)	4.77 (±1.35)	3.06 (±0.87)	6.41 (±2.06)	11.00 (±3.11)	43.71 (±3.71)
	6	4.15 (±0.48)	8.45 (±1.36)	6.15 (±1.15)	5.55 (±1.36)	3.70 (±1.35)	6.95 (±1.80)	12.95 (±4.60)	47.90 (±6.48)
	7	5.20 (±1.60)	7.50 (±1.03)	5.75 (±1.87)	5.10 (±1.58)	4.80 (±1.4)	7.55 (±2.71)	16.10 (±4.82)	52.00 (±5.06)
	8	4.62 (±0.92)	8.08 (±1.90)	6.85 (±1.41)	4.54 (±1.45)	3.69 (±1.26)	7.69 (±1.98)	13.23 (3.70)	48.69 (±4.01)
	9	4.27 (±1.18)	8.93 (±1.65)	5.60 (±1.50)	4.67 (±1.25)	4.20 (±1.64)	10.13 (±2.42)	9.85* (±NA*)	47.77* (NA*)
	10	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*

240 NA* Data not available (these generations are still in production).

Figure legends

Figure 1. Number of individuals present, dead and transferred to the colony per day for *Phlebotomus perniciosus* (A) and *Phlebotomus papatasi* (B).

Figure 2. Phorid mites found in the colony. A. Dorsal aspect; B. Lateral aspect. (Photos by N. Rahola).

Figure 3. Number of individuals produced by week for *Phlebotomus perniciosus* (A) and *Phlebotomus papatasi* (B).

Supp. Table S1. Detailed protocol for 50% glucose solution preparation.

Supp. Table S2. Detailed protocol for sterile dissection of chicken skins.

Supp. Table S3. Detailed protocol for larval food preparation.

Supp. Table S4. Detailed file on standardized data collection and tracking of sand fly colonies.

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Figure 1

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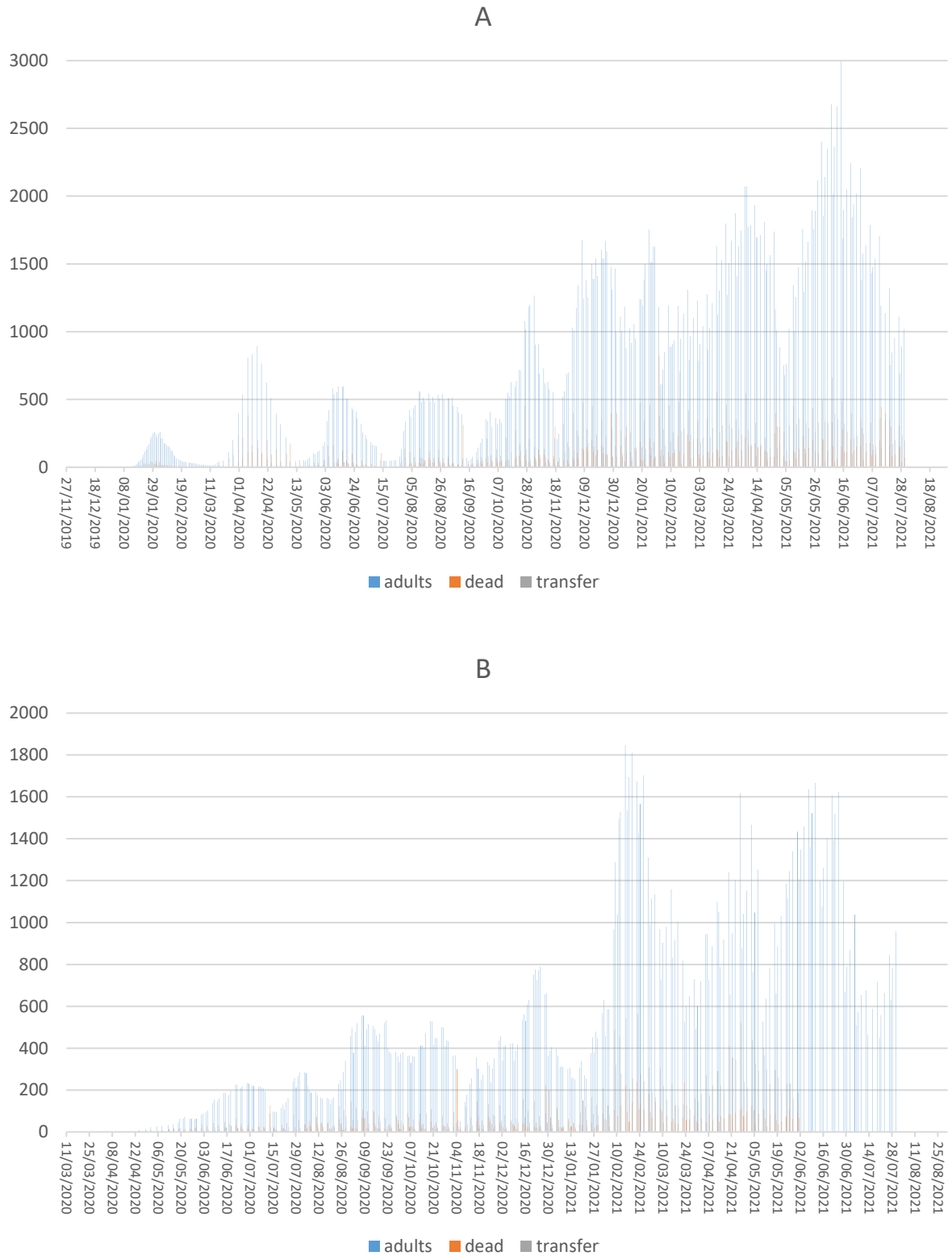


Figure 2

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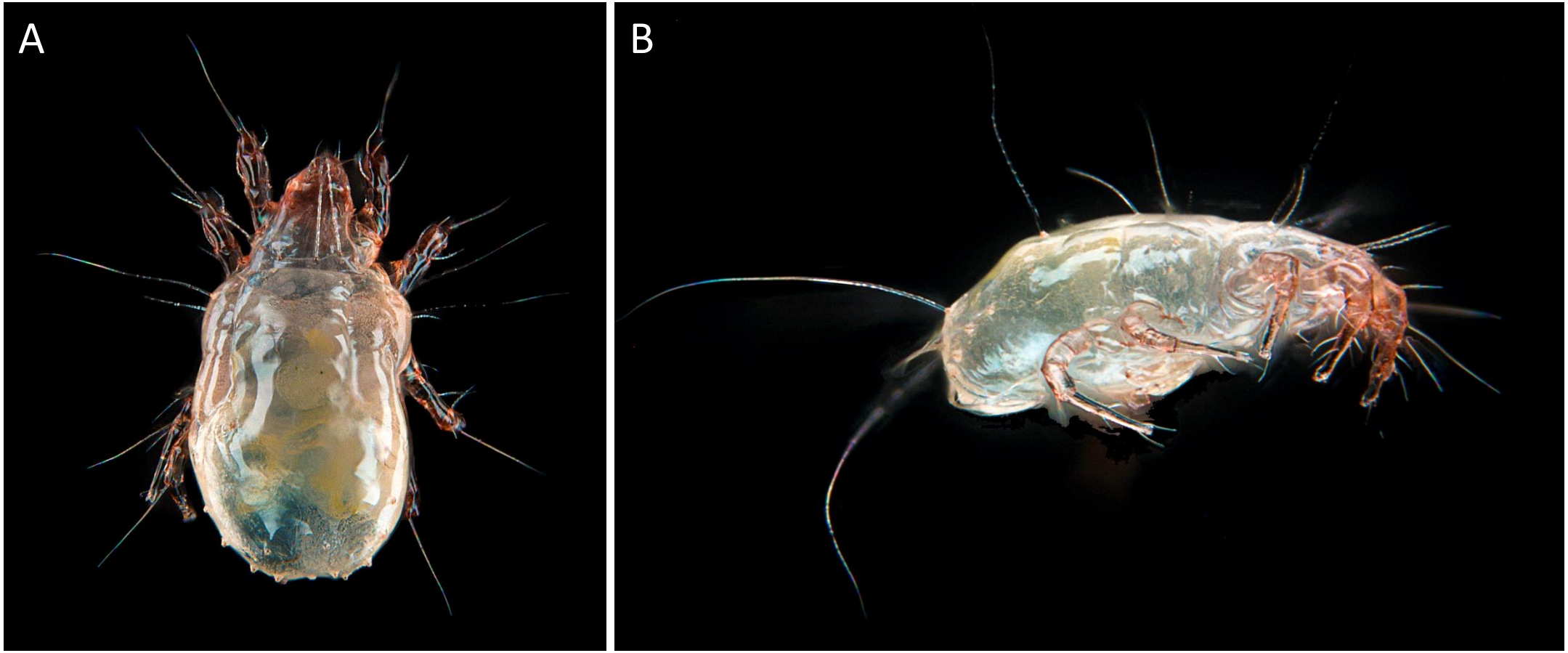
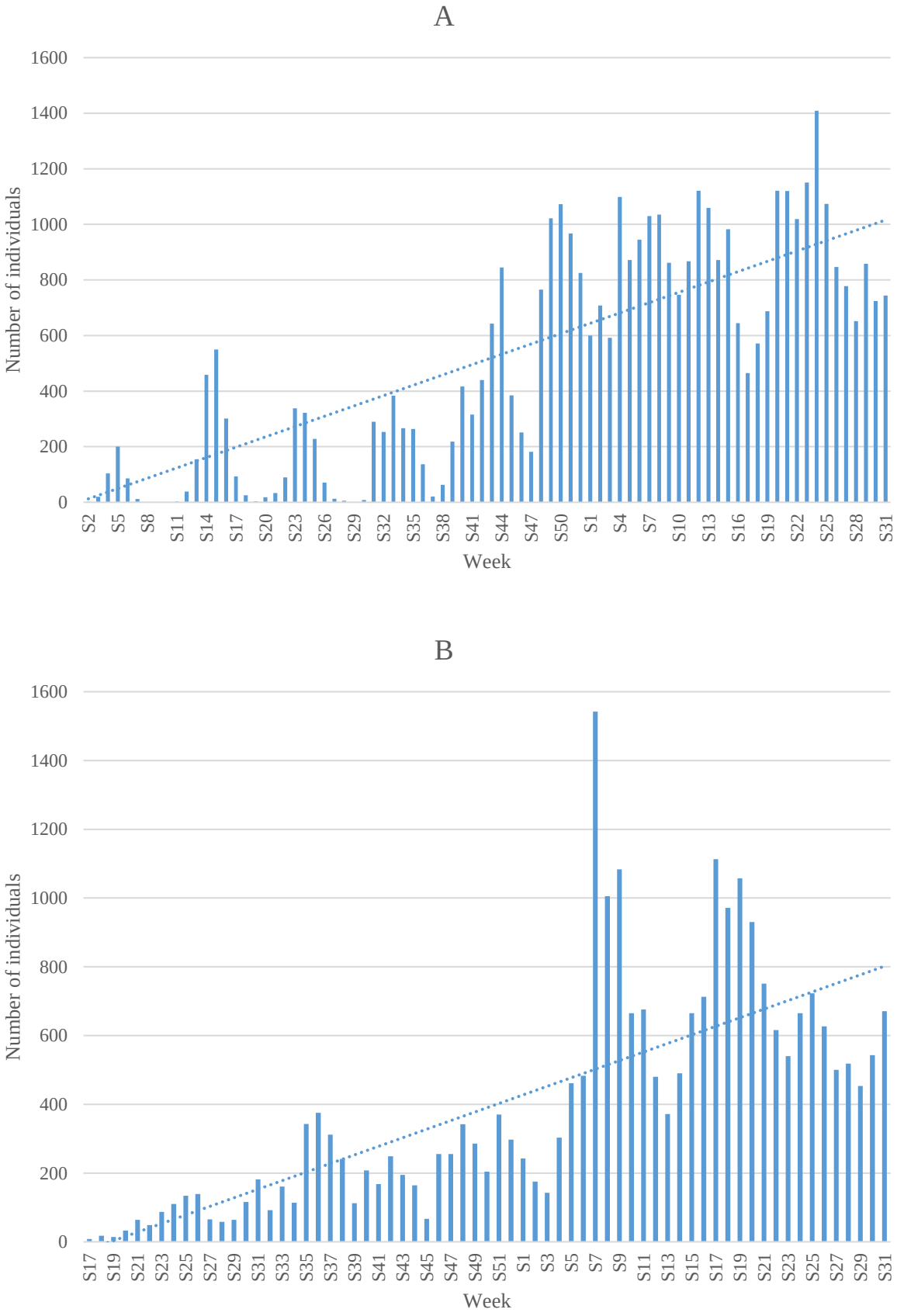


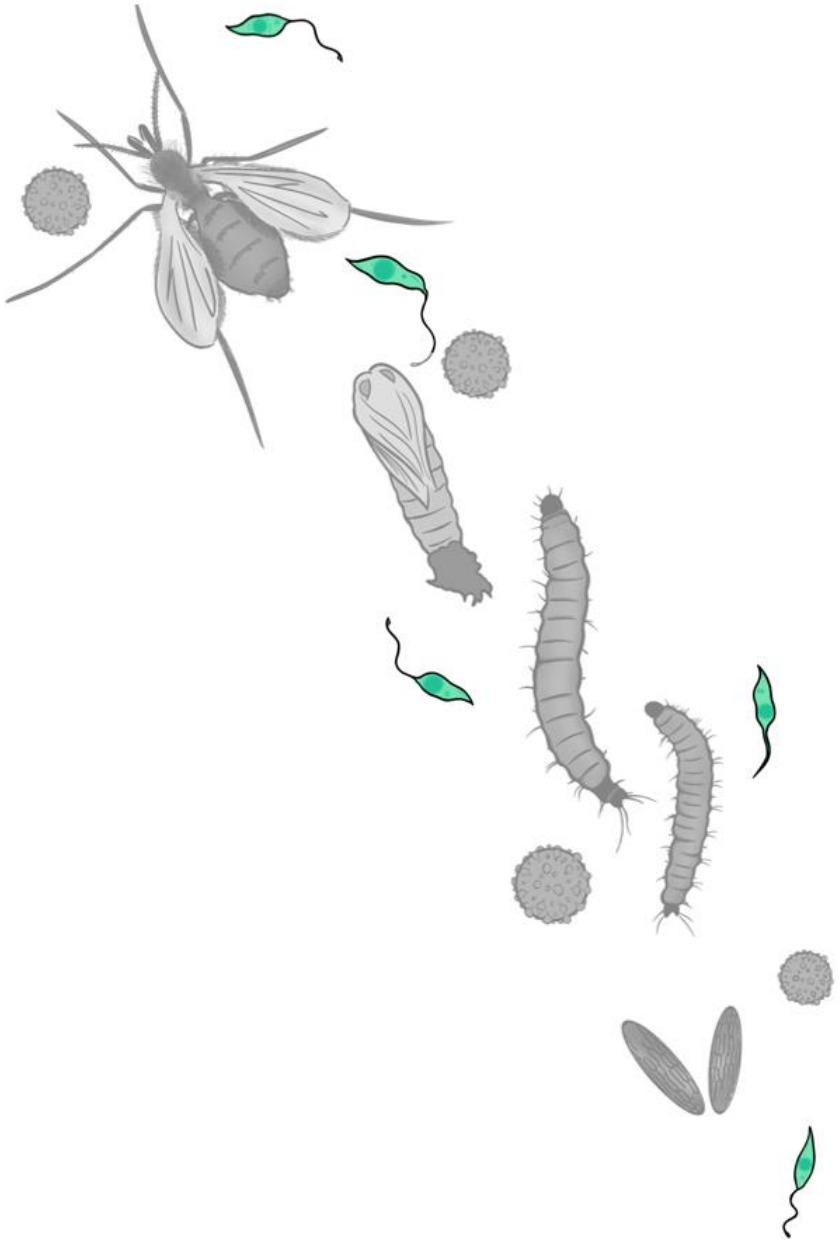
Figure 3

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CHAPTER 2

Dynamics of *Leishmania infantum* infection and impact on vector biology



Worldwide, *Leishmania* parasites are of significant public health importance, especially in developing countries (43). Treatments used have serious side effects, parasites are increasingly developing drug resistance, and there is currently no human vaccine (74,160). There is a need for more research on this parasitic disease, especially studies on the parasite interactions with its vectors, to better understand transmission dynamics and anticipate the spread of leishmaniasis. New approaches to reduce the transmission of vector-borne diseases, such as transmission-blocking vector vaccines, have resulted from experimental studies on parasite-vector interactions (161). Although several publications have focused on these studies, few have examined the infection impact on the sand fly life history traits (78–80). Further information on *Leishmania* is detailed in the Introduction part of the thesis.

In this chapter, we study the interactions between the parasite *Le. infantum* and its vector *Ph. perniciosus* under experimental conditions. The objectives were to determine the infection dynamics in the vector and the infection impact on vector life history traits. In a first part, we set up experimental infections with different parasite concentrations using a green-labelled strain of *Le. infantum*, in order to follow the development of the parasite in real time in the vector. As pathogen infection can have a cost on the vector fitness (162,163), we then studied the impact of *Le. infantum* infection on the survival and fecundity of *Ph. perniciosus*.



Who is *Leishmania infantum*?

Historically, the species *Le. infantum* was first described and identified by Charles Nicolle in 1908 at the Pasteur Institute in Tunis. Human leishmaniasis due to *Le. infantum* occurs sporadically around the Mediterranean area. The disease mainly affects children, hence the name "infantum", and immunocompromised adults. The domestic dog is the main reservoir of *Le. infantum* and the main vectors, in France, are *Ph. perniciosus* and *Ph. ariasi.* This thesis aims to study experimentally the interactions between *Leishmania/Phlebovirus* and the sand fly vector. Thus, we have chosen the model *Le. infantum*, which co-circulates with the TOSV in the Mediterranean basin and shares the same vector, *Ph. perniciosus*.



1. Methodology

a. *Leishmania* culture

We used the reference strain *Le. infantum* (MHOM/MA/67/ITMAP263) originally isolated from the bone marrow of a human patient in Morocco and cloned by micromanipulation. Promastigote forms were maintained in incubator at 26°C by weekly transfers in HEPES (1M)-buffered RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% hygromycin 100X. Maintenance in *in vitro* culture leads to a progressive loss of infectivity, which rapidly generates a bias towards the experimental data (164). Thus, as recommended by Moreira *et al*. (2012) (164), we used for experimental infections only parasites that have been subjected to a full life cycle (transformation into amastigote) with less than 20 passages after defrosting. *Leishmania* can be stored frozen in cryotubes containing RPMI medium with 5% cryopreservative DMSO for six months at -80°C and for more than two years in liquid nitrogen.

b. Green Fluorescent Protein-Tagged *Leishmania infantum*

The Green Fluorescent Protein (GFP) tagged parasites were produced by Dr. Baptiste Vergnes (MIVEGEC, IRD-Montpellier). GFP was integrated within the 18S ribosomal DNA locus with the use of the pLEXSY-hyg2 system (Jena Bioscience) following a previously described methodology (165). This fluorescent protein is a good indicator of parasite metabolic activity, well expressed during the parasite multiplication phases (166). GFP-tagged *Leishmania* parasites are easily visible after dissection of infected sand flies via fluorescence microscopy (EVOS™ M5000) (**Figure 11**).

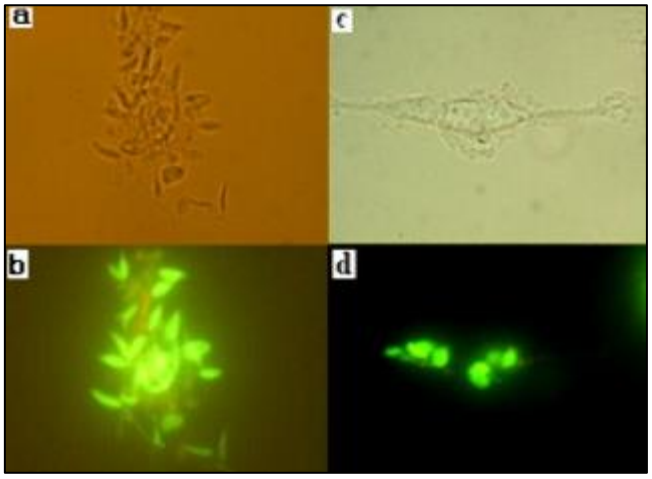


Figure 11. Detection of GFP by fluorescence microscopy; these images show GFP expression in transfected *Leishmania infantum* promastigotes (without (a) and with fluorescence light (b)) and intracellular GFP-expressing amastigotes (without (c) and with fluorescence light (d)) in human macrophages 48h after infection (pictures from Bolhassani *et al*., 2011 (165))

c. Experimental infections

Experimental infections were carried out, as described in the Appendix 1, using parasites in the promastigote from logarithmic phase cultures (four days after the last passage). As different parasite concentrations were needed, parasite count was realized before resuspending them in heat-inactivated rabbit blood. The LUNA-FL™ fluorescent cell counter counts only live parasites and gives an accurate parasite numbers per milliliter. Depending on the number of sand flies available in the colony, 100 to 200 females aged from five to nine days were used per group.

The first experiment aimed to follow over time the development of *Le. infantum* in *Ph. perniciosus*, according to two parasite concentrations: 10^3 and 10^6 promastigotes/ml (**Figure 12**). Two, eight and 11 days after infection, 10 females were killed and stored at -80°C for *Leishmania* detection and quantification by qPCR and 30 females were dissected for *Leishmania* localization determination by fluorescence microscopy. These numbers varied according to blood-feeding rates and female survival.

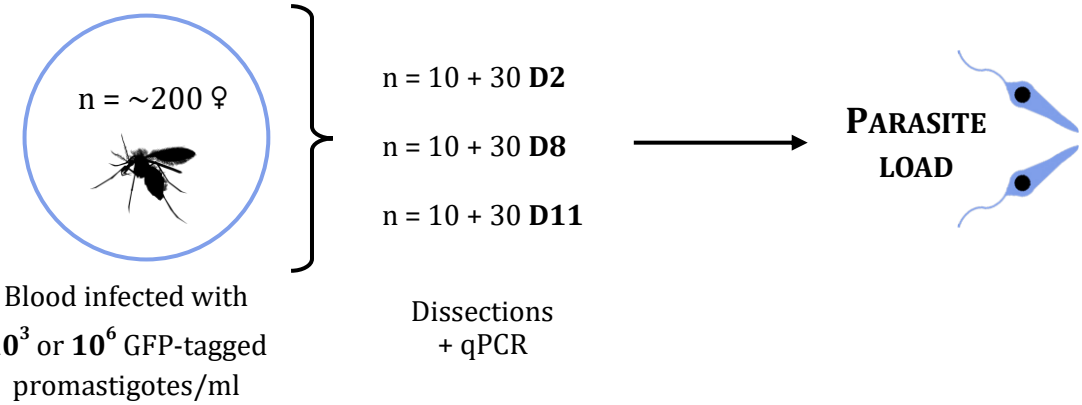


Figure 12. Experiment methodology for the study of *Leishmania infantum* infection dynamics in its vector *Phlebotomus perniciosus* according to two parasite concentrations (10^3 and 10^6 promastigotes/ml)

The second experiment aimed to determine the infection impact on sand fly life history traits, according to three parasite concentrations: 10^3, 10^5 and 10^6 promastigotes/ml (**Figure 13**). Different parameters were monitored: female survival, oviposition time, hatching time, number of eggs and first stage larvae. Every day, dead females were individually collected and stored at -80°C for *Leishmania* detection and quantification by qPCR.

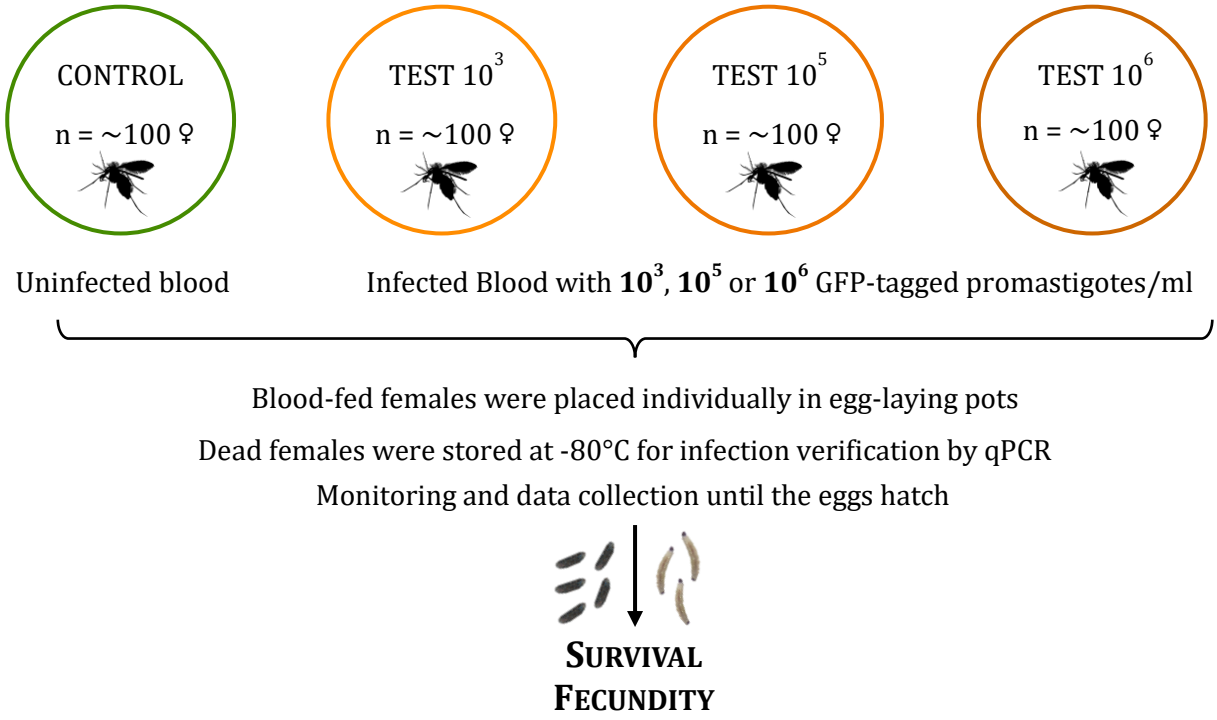


Figure 13. Experiment methodology for the study of the *Leishmania infantum* infection impact on its vector *Phlebotomus perniciosus* biology according to three different parasite concentrations (10^3, 10^5 and 10^6 promastigotes/ml)

d. Sand fly dissections

The midgut of infected sand flies was dissected individually on a glass slide with a drop of sterile saline buffer using fine forceps under a binocular microscope. Microscope and dissection tools were sterilized before every dissection. We checked for the presence of *Leishmania* at two, eight and 11 days post infection (dpi), in the abdominal midgut, thoracic midgut and stomodeal valve (**Figure 14**).

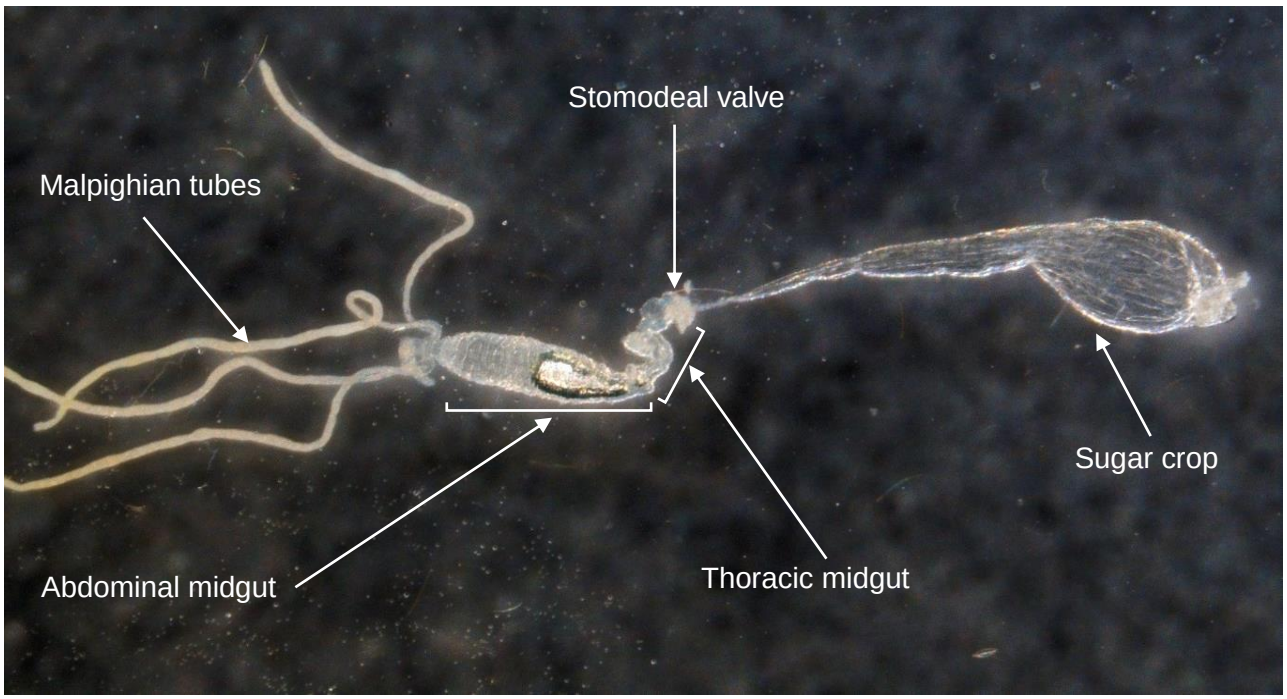


Figure 14. Dissected midgut of a female *Phlebotomus perniciosus* (Pictures by Dr. Jorian Prudhomme & Nil Rahola)

e. Quantification: DNA extraction and qPCR

i. DNA extraction

Sand fly DNA was extracted from entire individual and *Leishmania* DNA from promastigotes in culture. Parasites were collected in the logarithmic phase of growth, counted and washed with saline buffer (PBS). After centrifugation at 6000xg for 10 minutes, the dry pellet of promastigotes was stored at -80°C or used directly for extraction. Individual sand fly bodies or *Leishmania* pellets were crushed with a sterile piston into 1.5ml tubes with 300µl of CTAB solution. For complete lysis, 10µl of proteinase K and 0.6µl of β-mercaptoethanol were added, and the samples were placed at 60°C for 2 hours. After the addition of 300µl of chloroform, the homogenates were slowly mixed by inversion and then centrifuged for 5min at 13,000×g at 4°C. In the collected supernatants, DNA was precipitated by the addition of 600µl of cold isopropanol, then centrifuged for 20min at 13,000xg at 4°C. The liquid was removed and 1ml of cold 70% ethanol was added. After 5min of centrifugation at 13,000xg at 4°C, the liquid was removed and the pellets were dried. The DNA pellets were eluted in 20µl of pure water. DNA quantification and purity were determined by spectrophotometry with a NanoDrop™ and the samples stored at -20°C until use.

ii. qPCR

The *Le. infantum meta1* gene was used (forward primer 5'-GTAAACCGCAAGCTCTCTGG-3' and reverse primer 5'-CGAGCTTGATGGTGTGTGTT-3'). *Meta1* is a single copy gene that plays a role in metacyclogenesis and virulence. Therefore, it can be used to quantify both the absolute parasite number in the sand fly (DNA) and the absolute number of metacyclic promastigote (RNA). All qPCR were performed on a LightCycler® 96 (Roche). The total volume of each reaction was 10µL containing 1µL of DNA, 2µL of EvaGreen® qPCR mix, 0.3µL of each primer (at 10µmol/µL), and 6.4µL of pure water. The qPCR protocol included a 15 minutes pre-incubation step at 95°C followed by 45 cycles of amplification at 95°C (15s), 60°C (20s) and 72°C (20s). Negative controls were added with DNA-free reactions and with uninfected sand fly DNA. A positive control was also run with the DNA of 10^3 promastigotes/µl. Standard curves were realized to determine the parasite concentrations of each sample.

2. Results

a. *Leishmania infantum* infection dynamics and localization in its vector according to different parasite concentration

Due to a low number of sand flies available in the colony, a maximum of 295 females was available for a first experimental infection with 10^3 promastigotes/ml and 82 females for a second infection with 10^6 promastigotes/ml. In total, 70% and 77% of the females took blood infected with 10^3 and 10^6 promastigotes/ml, respectively. Due to a daily mortality of 10%, we could only dissect eight and 10 females for 10^3 and 10^6 infections, respectively. For qPCR analysis, females were collected at two (n=30), eight (n=26) and 11 (n=15) dpi for the 10^3 infection and at two (n=10) and eight (n=13) dpi for the 10^6 infection. For both experimental infections, *Leishmania* were observed in all dissected females, in the abdominal midgut two dpi, in the abdominal midgut and stomodeal valve eight dpi, in the thoracic midgut and around the stomodeal valve 11 dpi (**Figure 15**). We observed lower fluorescence of GFP-tagged *Leishmania* at 11 dpi.

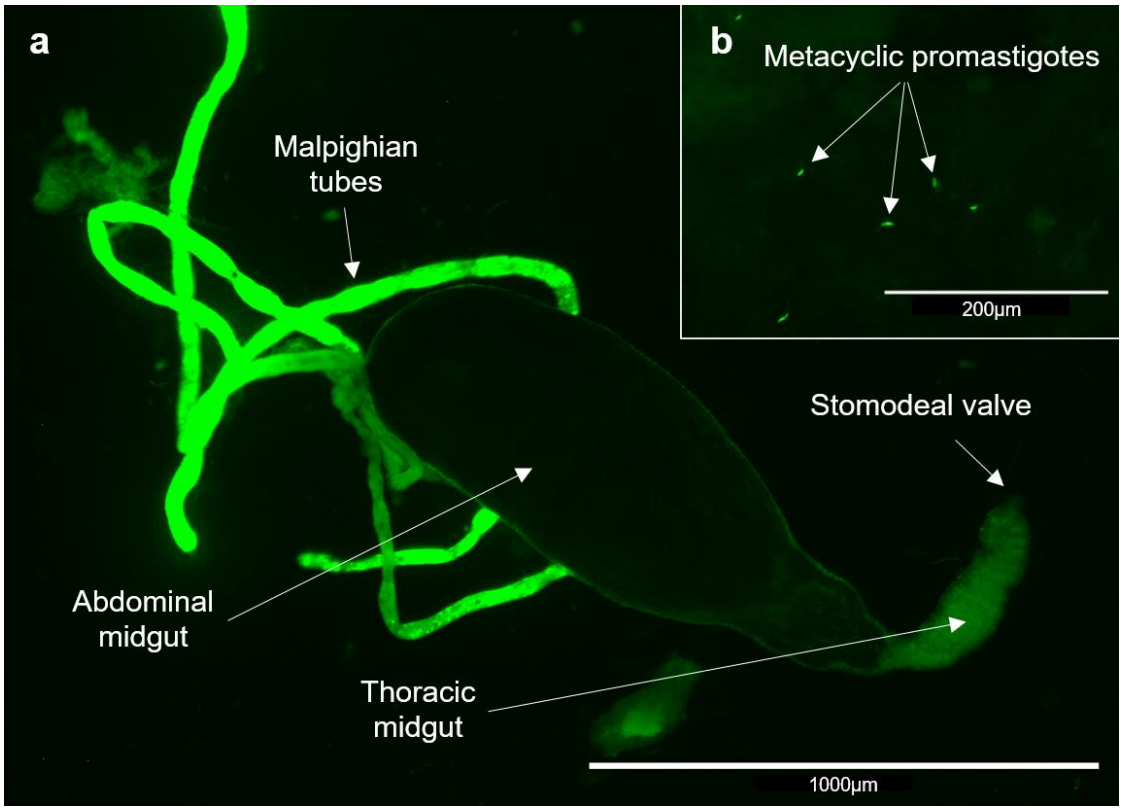


Figure 15. *Leishmania infantum* metacyclic promastigotes observed in *Phlebotomus perniciosus* midgut 11 days post-infection. a) Entire sand fly midgut; b) Focus on the stomodeal valve environment with promastigotes (potentially metacyclic) forms

For *Leishmania* DNA/RNA quantification, it was not possible to test all samples due to issues with the qPCR optimization (*Leishmania* detection). Indeed, we were able to construct standard curves with the *meta1* primers, but we were not able to amplify *Leishmania* in infected sand flies "test" samples with 10^6 promastigotes/ml. These samples came from a previous experiment performed by Dr. Jorian Prudhomme in 2018 (167); we chose to perform the optimization of the qPCR protocol with these samples rather than ours, due to the low number of sand flies available in the colony at the beginning of the thesis.

Only the quantification of *Leishmania* in our samples infected with 10^6 promastigotes/ml could be performed. To ensure that this was not a problem of DNA degradation or a complete absence of *Leishmania* in the samples, we tried another set of previously described primer set (168), under the same qPCR conditions. These primers target the kinetoplast DNA (forward, 5′-CCTATTTTACACCAACCCCCAGT-3′; reverse, 5′-GGGTAGGGGCGTTC TGCGAAA-3′). In total, 80%

of the sand flies analyzed two dpi (n = 10) and 69% of the sand flies analyzed eight dpi (n = 13) were positive by qPCR. For the positive samples, the average number was 249.8 *Leishmania* per sand fly at two dpi and 5161.3 *Leishmania* per sand fly at eight dpi. However, there were no significant differences in the mean number of *Leishmania* per sand fly at two and eight dpi (Kruskal-Wallis test, $P = 0.7308$).

b. *Leishmania infantum* infection impact on *Phlebotomus perniciosus* biology

As the colony was not yet productive enough, only 145 females were available, which were divided into four study groups: uninfected control (n=39), infected groups with 10^3 (n=35), 10^5 (n=34) and 10^6 promastigotes/ml (n=37). **Table 3** shows the different life history trait data collected during 30 days of monitoring until the last hatching. We tested the significance of the *Leishmania* infection on each life history traits using general linear models (GLM) through Rstudio software version 2022.07.1 - © 2009–2022 (169).

Table 3. Summary of the different *Phlebotomus perniciosus* life history traits studied. BF: Blood-Fed, BF*: Blood-Feeding, No: Number of. Means are indicated with their standard error.

	No. ♀ initial	**No. ♀ BF**	**BF* rate (%)**	**Survival time (days)**	**No. eggs**	**Oviposition time (days)**	**No. larvae**	**Hatching time (days)**
Control	39	28	72	5.7 ± 0.5	27.8 ± 5.1	6.9 ± 0.5	19.4 ± 4.9	6.0 ± 0.2
Test 10^3	35	12	35	5.6 ± 0.8	39.4 ± 7.7	6.4 ± 0.8	30.2 ± 12.6	5.6 ± 0.3
Test 10^5	34	18	53	5.4 ± 0.8	41.3 ± 7.7	7.3 ± 1.3	25.0 ± 9.8	6.0 ± 0.6
Test 10^6	37	24	65	4.7 ± 0.6	48.3 ± 5.2	5.8 ± 0.4	30.6 ± 7.0	5.6 ± 0.2

The blood feeding rate was different between the groups and very low (35%) for the group infected with 10^3 *Leishmania*/ml. We were constrained to deprive the females of sugar meal only a couple of hours before the experimental infection. Thus, not being sufficiently starved, some females did not take a blood meal. There was no significant difference between the survival rate and the oviposition time of infected (tests 10^3, 10^5 and 10^6) and uninfected (control) females (data not shown). Similarly, there was no significant difference between the number of larvae produced and the egg hatching times of infected and uninfected females (data not shown). However, there was a significant difference in the number of eggs laid between uninfected (27.8 ± 5.1 eggs) and infected females (48.3 ± 5.2 eggs) with 10^6 *Leishmania*/ml (Lm

test, estimate 20+\-7, *P* = 0.0123). We observed that the higher the concentration of *Leishmania* they have ingested, more they lay eggs (**Figure 16**).

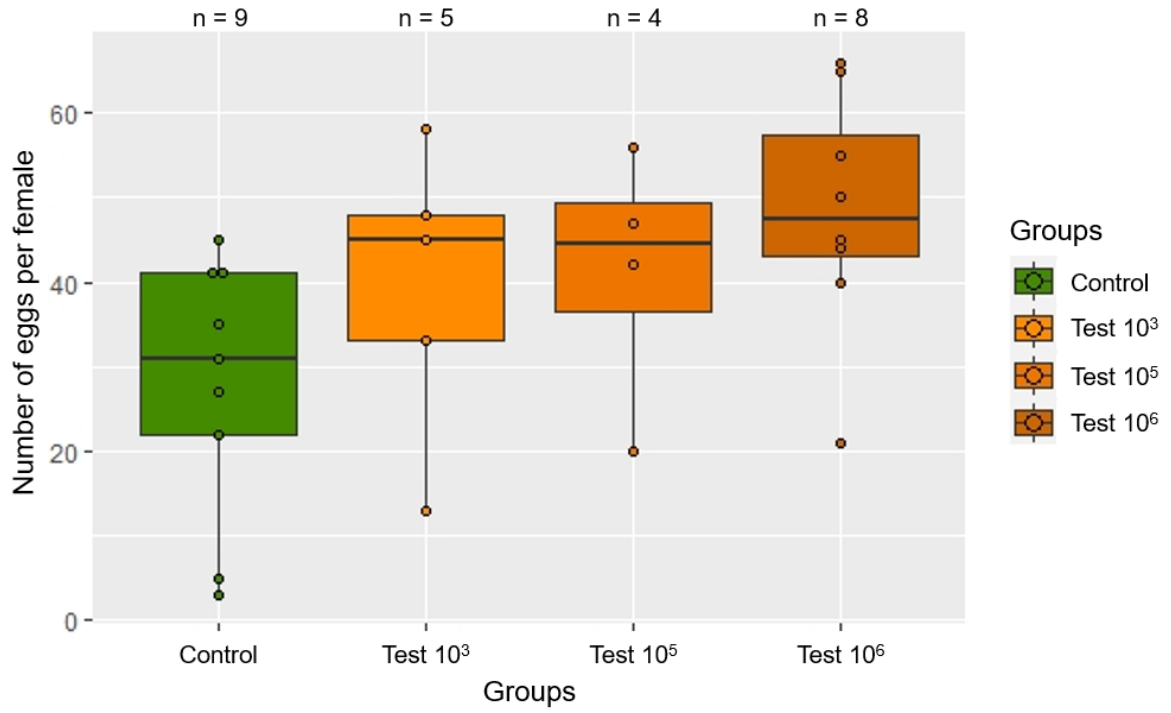


Figure 16. Effects of *Leishmania infantum* infection on the number of eggs laid by *Phlebotomus perniciosus* according to four different conditions: control (uninfected females), infected tests 10^3, 10^5 and 10^6 promastigotes/ml

3. Discussion

We explored how *Le. infantum* affects *Ph. perniciosus*. These experiments highlighted the infection impact on the vector life history traits. The question of parasite virulence in insect vectors is of great medical importance. Some studies focused on parasite manipulation and their successful transmission by their vector. These studies led, for example, to parasite-resistant vectors, as for *Plasmodium* parasites, and to the development of vaccines that block malaria transmission by the vector (170). The infection cost for the vector will influence the transmission efficiency and the competitive ability of parasite resistant and non-resistant vectors. Therefore, information on the parasite impact on their vectors can improve knowledge of vectorial biology and more specifically the vectorial competence.

The experimental studies provided information on the establishment of *Leishmania* parasite life cycle in the sand fly midgut (50). It is well known that two days after the infective blood meal, the parasites escape the deteriorating peritrophic matrix and anchor on the abdominal midgut. After eight days, *Leishmania* are found in the thoracic midgut and around the stomodeal valve. After 11 days, the infective stages, metacyclic promastigotes, are mainly located around the stomodeal valve and are adapted for successful transmission to the mammalian host (171). Anchoring to the midgut is a crucial event in the parasite life cycle and one of the factors that determine the sand fly vectorial competence. Here, we studied the infection kinetics of *Le. infantum* in *Ph. perniciosus* with two different concentrations: 10^3 and 10^6 promastigotes/ml. We tried to determine the limit infectious dose using low concentration of *Leishmania*. According to our results, we observed the same dynamics of *Leishmania* development in the sand fly as previously demonstrated in the literature. These results were found for the high infectious dose (10^6 promastigotes/ml) as well as for the low infectious dose (10^3 promastigotes/ml). We also wanted to try with a medium dose of 10^5 promastigotes/ml, but this was not possible due to an insufficient number of sand fly available. In addition, qPCR analyses for sand flies infected with the highest concentration of parasites (10^6) showed that on average 250 parasites/female were found at two dpi, and 5161 parasites/female were found at eight dpi. Theoretically, *Ph. perniciosus* females ingest 0.5µl of blood (172), so approximately 500 parasites in a blood meal infected with 10^6 parasites/ml. In our experiment, at two dpi, about 50% of the parasites are still present in the midgut after sand fly defecation. The fact that the parasites escaped the peritrophic matrix deterioration to anchor in the vector midgut and multiply, between two and eight dpi, determines the vectorial competence of *Ph. perniciosus*. Therefore, this methodology can be used in the future for vector competence analysis.

Usually, in vector-pathogen systems, the infectious dose is one of the strongest determinants of the infection probability by the pathogen (173). The EIP, duration of the parasite's development that starts with the ingestion of infective *Leishmania* and ends with the invasion of the stomodeal valve when the sand fly becomes infectious, can be influenced by many factors such as the initial parasite infective dose ingested (174). In our experience, it seems that the EIP duration is not affected by the infectious dose, as the parasites localization in the vector over time is the same at 10^3 as at 10^6 promastigotes/ml. Although qPCR analyses could not be performed for sand flies infected with the low concentration of parasites, our preliminary results suggest that the kinetics of infection are similar regardless of the initial dose of *Leishmania* in the blood meal.

Infection could have an impact on other parameters of the vector biology, as previously demonstrated for other vectors. For example, *Plasmodium* parasites high-density infection significantly reduced *Anopheles stephensi* lifespan (175). For some *Lutzomyia* sand flies, infection with *Plasmodium mexicanum* had no impact on survival, but the infected sand flies produced fewer eggs (176). Consequently, we investigated if *Leishmania* infection at low (10^3), medium (10^5) and high (10^6) concentration have an impact on the vector survival or fecundity. Our results showed that there was no infection impact on the survival time of infected sand flies (regardless of concentration) compared to uninfected sand flies. However, the lifespan of sand flies is shorter in laboratory. Experimental blood feeding reduces the likelihood of female survival, as egg laying causes stress and exhaustion, which can lead to death (177). In our experiment, the uninfected sand flies lived under the same conditions as the infected sand flies, and all survived less than six days on average. Moreover, in our previous experimental infection (*Leishmania* infection dynamics) we observed that females, not having the possibility to lay eggs, stayed alive for at least 11 days. As it is possible that infection has an effect on lifespan, it would be interesting to repeat this experiment by preventing the females from laying eggs in order to measure their survival over a longer period. Furthermore, we did not observe any impact of the infection on oviposition time, number of first instar larvae and hatching time. In contrast, sand flies infected with the highest concentration of parasites (10^6) produced significantly more eggs. The parasite infection at high concentration could induce a female terminal investment. The terminal investment hypothesis proposes that individuals facing a significant threat to their survival, and thus a reduced residual reproductive value due to a shorter lifespan, spend more energy to current reproduction rather than to other life history traits in order to maximize reproductive output (178). Parasite overload could be a threat to survival, as mentioned above for *Plasmodium*-infected mosquitoes or also for *Lutzomyia longipalpis* sand flies infected with *Le. infantum* that suffered a reduction in life expectancy, fertility and fecundity (179). In our case, there was no infection impact on survival but only on vector fecundity. Indeed, this difference could be due to the species studied and could also vary according to the pathogen-vector couple. Our study provides further insight into the effect of *Leishmania* infection on the sand fly biology.

To summarize, the infection dynamics of *Le. infantum* in *Ph. perniciosus* is the same at 10^6 and 10^3 parasites/ml. This means that, in a population, despite a low parasite load in the hosts, leishmaniasis can be transmitted as well as when the host parasite load is high. This could be one of the explanations for the low-noise persistence of leishmaniasis in some areas, as in the case of Morocco (180). Then, we explore the impact of the parasite load on the vector life history

traits. The high dose infection of *Le. infantum* had an impact on the *Ph. perniciosus* fecundity. Laying more eggs suggests that the highly infected females would increase the local density of sand flies in natural condition. Since vector capacity is influenced by vector population density, the risk of transmission may be increased in this case (181).

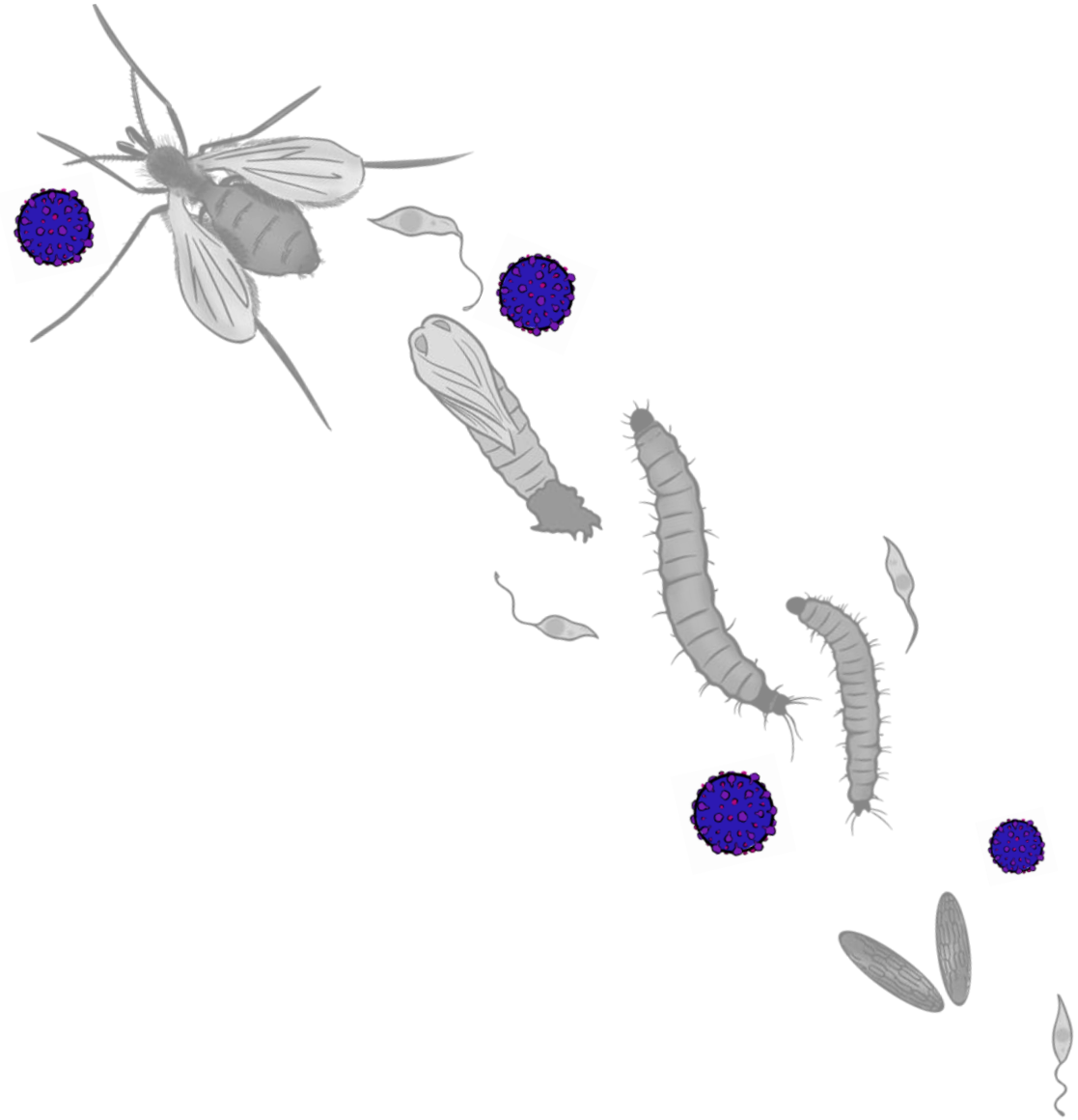
Our study results are only preliminary as it took a long time to set up and maintain the sand fly colony productively. The first experimental infection was only possible one year after the colony's establishment. As explained earlier (Chapter 1), it is not possible to take more than a third of the colony size to avoid the decline of the production. Therefore, most of the experiments with *Leishmania* were carried out with few sand flies. It would be, thus, necessary to replicate these experiments.

Finally, the qPCR protocol would be optimized in order to analyze samples from females infected with 10^3 promastigotes/ml and to determine the prevalence of infectious forms (metacyclic promastigotes) out of the total number of parasites over time. Because the *meta1* primers did not amplify any *Leishmania* DNA and the primers targeting the kinetoplast DNA do not differentiate the infectious stages, we would work with other primers. The establishment of a multiplex RT-qPCR should allow the quantification of both infectious and non-infectious stages using the *sherp* gene, which is over-expressed in the metacyclic stage, and the *ssrRNA* gene, which is consistently expressed in all parasite stages. The *sherp* transcript has sufficient specificity and sensitivity to discriminate and quantify infectious forms of *Le. infantum* (182). The abundance determination of the *ssrRNA* and *sherp* transcripts would allow to establish standard curves for the quantification of parasites and metacyclic forms.

Moreover, as the thesis is not only focused on the study of *Leishmania*-vector interactions (already well known), but also *Phlebovirus*-vector interactions (still very little studied), sand flies were prioritized for the *Phlebovirus* experiments during the second year of my thesis.

CHAPTER 3

Toscana virus infection dynamics, persistence and impact on vector biology



Vector-borne disease epidemics are influenced by intrinsic (*e.g.* vector competence, life-cycle traits) and extrinsic (*e.g.* climate change, globalization) factors that affect virus activity and vector biology. Pathogens can affect both vertebrate hosts and insect and significantly alter virus transmission patterns. Studies on vector-virus interactions provide a better understanding of disease transmission dynamics. Although phleboviruses continue to be described worldwide, they are still neglected and need to be further studied. Due to its neurotropic characteristic, TOSV is currently an important sand fly-borne phlebovirus in terms of public health. Furthermore, due to environmental and climatic changes, the geographical distribution of TOSV is likely to expand at the same time as the sand fly vectors. To better understand the dynamics of transmission, in this chapter we explore the interactions between TOSV and the vector *Ph. perniciosus* under experimental conditions.

In a first part we measured the persistence of viral infectivity in the blood and sugar meals of sand fly, in order to estimate the potential epidemiological risk. This study is described in the article: "Lison Laroche, Nazli Ayhan, Rémi Charrel, Anne-Laure Bañuls, and Jorian Prudhomme. Persistence of Toscana virus in sugar and blood meals of phlebotomine sand flies: epidemiological and experimental consequences. *Under revision in Scientific reports*".

In a second part, we determined the infection kinetics with different TOSV doses, estimated the extrinsic incubation period, and measured the impacts of infection on the life history traits (survival, development time, fecundity) of the infected vector. This study has improved our knowledge on the TOSV ecology and its transmission pathways; it is presented in the article: "Lison Laroche, Nazli Ayhan, Rémi Charrel, Anne-Laure Bañuls and Jorian Prudhomme. Intra-sand fly dynamics of Toscana virus and impact on *Phlebotomus perniciosus* life-history traits. *In Preparation*".



Who is Toscana virus?

Toscana virus (TOSV) is a tri-segmented, enveloped RNA arbovirus that belongs to the genus *Phlebovirus*, family Phenuiviridae and order Bunyavirales. It was first isolated in 1971 in Monte Argentario (Grosseto, Tuscany) from the sand flies *Ph. perniciosus* and *Ph. perfiliewi*. TOSV was only recognized as a causative agent of neurological disease in humans in 1983, when it was first isolated from a young woman with lymphocytic meningitis. This virus frequently infects humans during the warm season in areas where sand flies are present, mainly in the Mediterranean region. Infections are usually asymptomatic, however TOSV can cause high fever, severe headache, and aseptic meningitis with a benign course, followed by a medium length convalescence. Currently, there is no treatment or vaccine for the prevention of TOSV infections. Since humans or animals are considered dead-end hosts with no significant role in the natural cycle of TOSV, sand flies could be the reservoirs. Thus, we have chosen the model TOSV, which co-circulates with *Leishmania infantum* parasites in the Mediterranean basin and which share the same vector, *Ph. perniciosus*.



Article 4

Persistence of Toscana virus in sugar and blood meals of phlebotomine sand flies: epidemiological and experimental consequences

Lison Laroche, Nazli Ayhan, Rémi Charrel, Anne-Laure Bañuls, Jorian Prudhomme

Under revision in Scientific Reports

It is a common believe that enveloped and RNA genome viruses, such as TOSV, have limited stability in the environment outside the host. We therefore study its persistence in sugar meal to have a complete view of the TOSV biological and epidemiological cycle and in blood for methodological considerations. The blood meal of *Ph. perniciosus* in the colony we set up in our laboratory lasts six hours, we wanted, thus, to check the viability/infectivity of TOSV during this period. Regarding the TOSV persistence in sugar, since sand flies of both sexes can be infected by TOSV and they both feed mainly on sugar juice in nature, it is thus essential to check the viral persistence in the sugar meal. The persistent infectivity/viability of TOSV in both of these environments may have important epidemiological consequences and raises new hypotheses about its natural cycle. Our study has contributed to a better understanding of phleboviruses persistence and transmission.

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Persistence of Toscana virus in sugar and blood meals of phlebotomine sand flies: epidemiological and experimental consequences

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Abstract

Many virological studies have tested the persistence of enveloped RNA viruses in various environmental and laboratory conditions and shown their short-term persistence. In this article, we analyzed Toscana virus (TOSV) infectivity, a pathogenic sandfly-borne phlebovirus, in two different conditions: in the sugar meal and blood meal of sand flies. Our results showed that TOSV RNA was detectable up to 15 days in sugar solution at 26°C and up to 6 hours in blood at 37°C. Moreover, TOSV remains infective for 7 days in sugar solution and for minimum 6 hours in rabbit blood. TOSV has shown persistent infectivity/viability under different conditions, which can lead to important epidemiological consequences and raises new hypotheses about its natural cycle.

Introduction

Toscana virus (TOSV) is an enveloped, protein encapsidated, tri-segmented RNA virus that belongs to the *Phlebovirus* genus (family *Phenuiviridae*, order Bunyavirales)[1]. It is an arthropod-borne virus transmitted to humans through the bite of infected female sand flies[2]. The geographical distribution of TOSV is highly dependent on this vector's presence and abundance[1]. Human cases are observed during the warm season, with a peak during summer related to a high vector activity[3]. TOSV infections are endemic in the Mediterranean basin and considered frequent during the warm season even though they remain neglected[4]. Most infections are believed to be asymptomatic or mildly symptomatic even though the exact proportion remains unknown[5]. TOSV has a particular neurotropism and is a major cause of meningitis and encephalitis in endemic areas[3]. Few symptomatic neuroinvasive cases have been reported, with an estimated incubation period of 12.1 days[6].

Until now, little is known about the natural cycle of TOSV and its transmission ways. Since there is no concrete evidence that vertebrate host species are reservoirs, it has been hypothesized that vector sand fly species can be the primary reservoir of TOSV[1,7]. Transovarial and venereal transmissions have been observed experimentally with a low infection rate in *Phlebotomus perniciosus*, the main identified vector of TOSV throughout the Mediterranean basin[3,8]. These transmission pathways are probably not efficient enough to maintain TOSV in the wild. Alternative transmission routes should exist although they remain unknown. For example, in aphids, the horizontal transmission of insect densoviruses was demonstrated through honeydew sugar meals[9].

As both male and female sand flies feed mainly on plant nectar and honeydew[10], it is important to explore the possibility of TOSV transmission between individuals during sugar meals. Only one study using Massilia virus, another phlebovirus genetically close to TOSV, has evidenced the possible transmission through this pathway[7]. Enveloped and RNA genome viruses have limited stability in the environment outside the host[11,12]. However, it is essential to determine arbovirus persistence/viability in different conditions to better understand the strategies employed by arboviruses to persist in the natural

environment[13]. In this study, we assessed TOSV stability and infectivity in sugar meals and in blood meals, sequentially, to estimate epidemiological consequences.

Results

TOSV persistence in blood

After six hours at 37°C in heat-inactivated rabbit blood, the quantity of TOSV ranged from 8.3 to 8.8 $\log_{10}$ RNA copies/ml (Fig. 1, A). There was no decrease in the quantity of virus RNA in blood over time. TOSV infectivity decreased less than 0.5 log over time after infection (Fig. 1, B). All samples were positive for cytopathic effects. TOSV remains infectious for at least six hours in blood at 37°C.

Toscana virus persistence in sugar

TOSV quantity ranged from 8.2 to 8.4 $\log_{10}$ RNA copies/ml in sugar solution over the first six hours and up to 15 days post infection (Fig. 2). There was no decrease in the quantity of virus genome in the sugar solution over 15 days.

The infectivity experiments showed that TOSV titration ranged from 6.3 to 6.5 log10 $TCID_{50}$/ml during the first six hours and began to decrease one log per day until day seven post infection (Fig. 3). TOSV remains infectious during seven days post infection.

Discussion

The persistence time of a pathogen in the environment can strongly affect disease dynamics[14]. Several studies on the persistence of arboviruses in different systems (humans, animals, vector insects), *in vitro* (cell line cultures) or according to environmental conditions (e.g., temperature, pH, light) provided additional information on the virus natural cycle[15–21] (22–28). A recent study demonstrated the persistence of the Chikungunya virus in liquid environments (packed red blood cells, plasma, and CHIKV-spiked water) in the dark[20]. Pezzi, et al. [20] provided new insights into the main factors responsible for the stability of enveloped RNA viruses outside their hosts.

In this study, we first explored TOSV persistence in rabbit blood, which is essential information for standardized experimental infection studies. The aim was to check whether sand flies took the same amount of virus at the beginning than at the end of the blood feeding. Viral RNA was detectable at a stable amount for up to six hours in blood at 37°C and TOSV remained infective throughout this period. Thus, sand flies feeding at the end of the blood feeding took the same quantity of virus as those feeding at the beginning. This justifies the exposure to blood for six hours as is the case in standard sand fly colony maintenance[22]. This result was essential to implement experimental infection protocols, as most sand flies start to take their blood meal within a few hours after the beginning of the exposure[22]. This is not the case for all hematophagous insects reared in laboratories. For example, for *Aedes* or *Anopheles*

mosquitoes, an artificial blood feeding of 20–30 min is sufficient to obtain 90% of engorged females[23]. Since the blood may clot over time, it is difficult to estimate whether TOSV infectivity remains stable after six hours[24]. It is however essential to determine virus persistence in human blood as well to estimate the transfusion contamination risks. The risk of TOSV transmission to virus-naive persons by blood transfusions and organ transplant is suspected but still needs to be assessed[25]. The characteristics required for transmission by transfusion have been described for many infectious disease pathogens, including virus presence in the blood of asymptomatic donors and virus persistence in blood during storage[26]. For example, blood transfusion is a likely route of Zika virus transmission as its RNA has been shown to persist in the red blood cells of asymptomatic blood donors for several months[27,28]. Arbovirus RNA can be present in human saliva, semen, urine and faeces, as in the case of the Zika virus[12], and TOSV which was found in urine and semen[29,30]. Other possible means of transmission of TOSV need to be explored.

In general, viruses can be excreted from infected hosts into the environment. Once viruses are outside their cellular hosts and in the environment, they have the potential to persist in order to reach other hosts again[31]. Thus, we sought to evaluate the persistence of TOSV infectivity in sugar meals, which are considered as the main energy source of sand flies. We show that the TOSV genome remains stable in sugar solution: viral RNA was detectable at a constant level for up to 15 days. In addition, the infection stability assay confirmed that TOSV infectivity lasts up to seven days. The assay also suggests that TOSV could be transmitted from infected to uninfected sand flies during sugar meals. An experimental study showed that another *Phlebovirus* (Massilia virus) could be transmitted between sand flies either by co-feeding or by an infected sugar source such as plant sap[7]. By investigating the transmission route of phleboviruses, they showed that both sand fly genders became infected through the sugar meal containing virus. However, in this study, only RNA viral loads were measured and not the titers in $TCID_{50,}$ which are necessary for tittering infectious virus. Other arboviruses of the *Flaviviridae* family have been shown to be expectorated by infected mosquitoes during the sugar feeding process[32]. The indirect transmission of pathogens through another source of sweet meals (e.g., fruit) has been demonstrated for the Marburg and Ebola viruses between fruit-eating mammalian hosts. Studies showed that these infectious viruses could persist up to 6 hours on different fruits[33,34]. Consequently, the hypothesis of TOSV horizontal transmission between sand flies via a sugar meal remains to be explored, as this transmission pathway could play an important role in the natural cycle of *Phlebovirus*. Furthermore, it would be necessary to test TOSV infectivity/viability under different temperatures and UV-light exposures, as they might be important factors under natural conditions. Finally, the transmission of TOSV via a sugar solution could represent an alternative for laboratories unable to carry out experimental infections with infected blood.

In conclusion, this study assessed TOSV infectivity in two environments, sugar and blood. The amount of viral RNA remained constant over a long period of time and no viral replication occurred. Although TOSV is an enveloped RNA virus, known for its limited stability in the environment outside the host, our study

clearly demonstrates its persistence in different environments. In general, long-term viral persistence increases indirect transmission risks for hosts or arthropod vectors[35].

Methods

Ethical statement

Rabbit blood draws performed in the context of this study were approved by the Animal Care and Use Committee named “Comité d’Ethique pour l’Expérimentation Animale Languedoc Roussillon n°36” under protocol number 2018022712203932. Rabbits, coming from the animal facility at the French National Research Institute for Sustainable Development, were not subjected to anesthesia, analgesia or sacrifice. The chicks come from the Experimental Infectiology Platform of Nouzilly (INRAE Centre Val de Loire, France). They were killed according to the Directive 2010/63/EU (Appendix IV) appropriate to the species. The chicks were dead before the use of tissue samples for experimental infection, in accordance with article R214-89 of the French Code rural et de la pêche maritime, Section 6.

Preparation and storage of TOSV aliquot

Lyophilised TOSV aliquots (strain MRS2010, lineage B) were provided by the UVE laboratory (Unité des Virus Emergents, Marseille, France). Vero E6 (African green monkey kidney) cells were grown in monolayers in Minimum Essential Medium (MEM, Gibco™) complemented with 7% heat-inactivated Fetal Bovine Serum (FBS, Eurobio Scientific), 1% L-glutamine (Gibco™) and 1% penicillin-streptomycin (Gibco™) at 37°C with 5% CO2. Stock of TOSV was obtained by dissolving the lyophilisates in sterilized pure water. Vero E6 cells were infected with 0.1 multiplicity of infection (MOI) and supernatant media were harvested on day 5 post infection. TOSV stocks at a concentration of 4.2x106 50% tissue culture infective doses ($TCID_{50}$/ml) were aliquoted in 2ml cryotubes and stored at -80°C.

TOSV persistence in blood

The experiment was performed under the standard conditions for sand fly experimental infections (i.e., 26°C, 80% relative humidity and 1% luminosity)[36]. A glass feeder, covered with chicken skin, was filled with heat-inactivated rabbit blood infected with 10^6 $TCID_{50}$/ml of TOSV. The glass feeder was connected to a water bath with external circulation to maintain blood at a constant temperature of 37°C. As in standard experimental infections, this system was maintained during six hours[22]. In order to quantify TOSV genome stability and infectivity kinetics, 150µl of infected blood was collected every 30 minutes (after blood homogenization) (Figure 4, A) and stored in cryotubes at -80°C for subsequent titration.

TOSV persistence in sugar solution

The experiment was performed by infecting a sugar solution with 10^6 $TCID_{50}$/ml of TOSV, according to the standard conditions for sand fly experimental infections, during 15 days. The sugar solution was the same as the one used for the maintenance of sand fly colonies: 50% organic brown sugar in sterile distilled water prepared as described previously[37,38]. The infected sugar solution was collected (150µl) every hour during six hours on the first day and once a day until day 15 (Figure 4, B) in order to quantify TOSV genome stability and infectivity kinetics. The solution was stored in cryotubes at -80°C for subsequent titration.

Kinetics of TOSV genome stability

The viral loads of blood and sugar samples were analyzed by RT-qPCR. A total of 200µl was used for nucleic acid extraction with the QIAcube® (QIAGEN) machine and the Virus Extraction Mini Kit (QIAGEN). The RT-qPCR assays were performed with a SuperScript® III Platinum® One-Step RT-qPCR Kit with ROX (Invitrogen - Thermo Fisher Scientific) on a QuantStudio 12K Flex thermocycler (ThermoFisher). A volume of 5µl of RNA was added to 20µl of mix containing 12.5µl of 2X Reaction Mix, 0.5µl of Superscript III RT/Platinum Taq Mix, and 10µM of STOS primers and probes[39]. Negative (pure water) and positive controls (at $4.81x10^4$ RNA in vitro transcribed copies/µl as described by Beckert and Masquida [40]) were included in each RT-qPCR run. Samples with a Ct value < 40 were considered positive.

Kinetics of TOSV infectivity

RT-qPCR can be used as a surrogate to measure viral RNA copies. However, the results need to be correlated to $TCID_{50}$ data which are the only measures that inform on the capacity of transmission and infection of the virus. TOSV titers in blood and sugar samples were determined by end-point dilution assay. Tenfold dilutions were used to infect confluent Vero E6 cells in a 96-wells plate in MEM (5% FBS, 1% penicillin-streptomycin, 1% L-Glutamine, 3% amphotericin (Gibco™) at 37°C in 5% CO_2. Wells were classified as positive (cytopathic effect) *versus* negative (no cytopathic effect) at five days post infection. $TCID_{50}$/ml was calculated according to the Reed and Muench [41].

Declarations

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Author contributions

L.L and J.P. conceptualized the study. L.L. realized the experiments with the support of N.A. and J.P. L.L analyzed the data and wrote the manuscript with the support of N.A., R.C., A-L. B. and J.P. Finally, L.L., N.A., R.C., A-L. B. and J.P. read, amended and approved the final version of the manuscript.

Data availability statement

All resources used in this article are provided and all the analyses are detailed allowing the assessment or verification of the manuscript's findings.

Competing Interests Statement

The authors have no conflicts of interest to declare.

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Figures

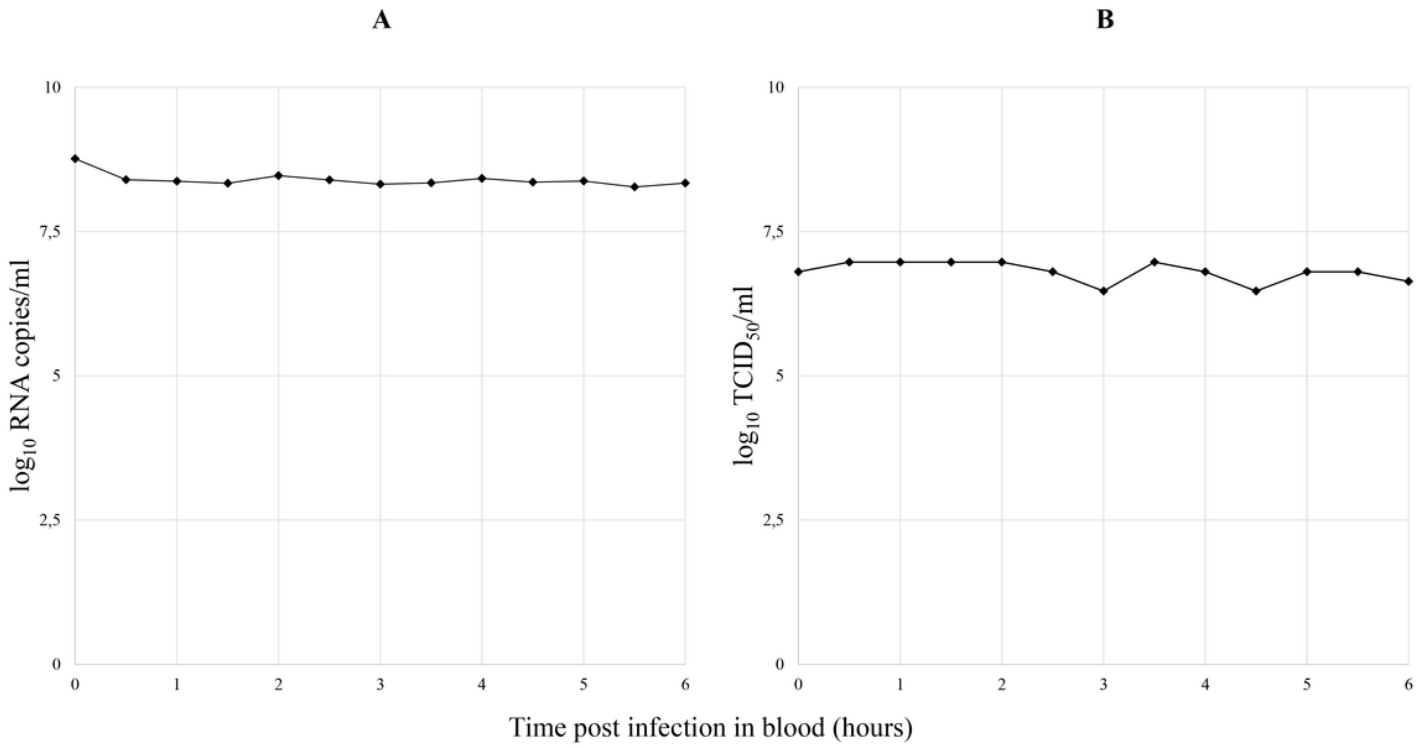


Figure 1

(**A**) Toscana virus RNA load (log_{10} copies/ml) and (**B**) TOSV infectivity ($TCID_{50}$/ml) in blood over time.

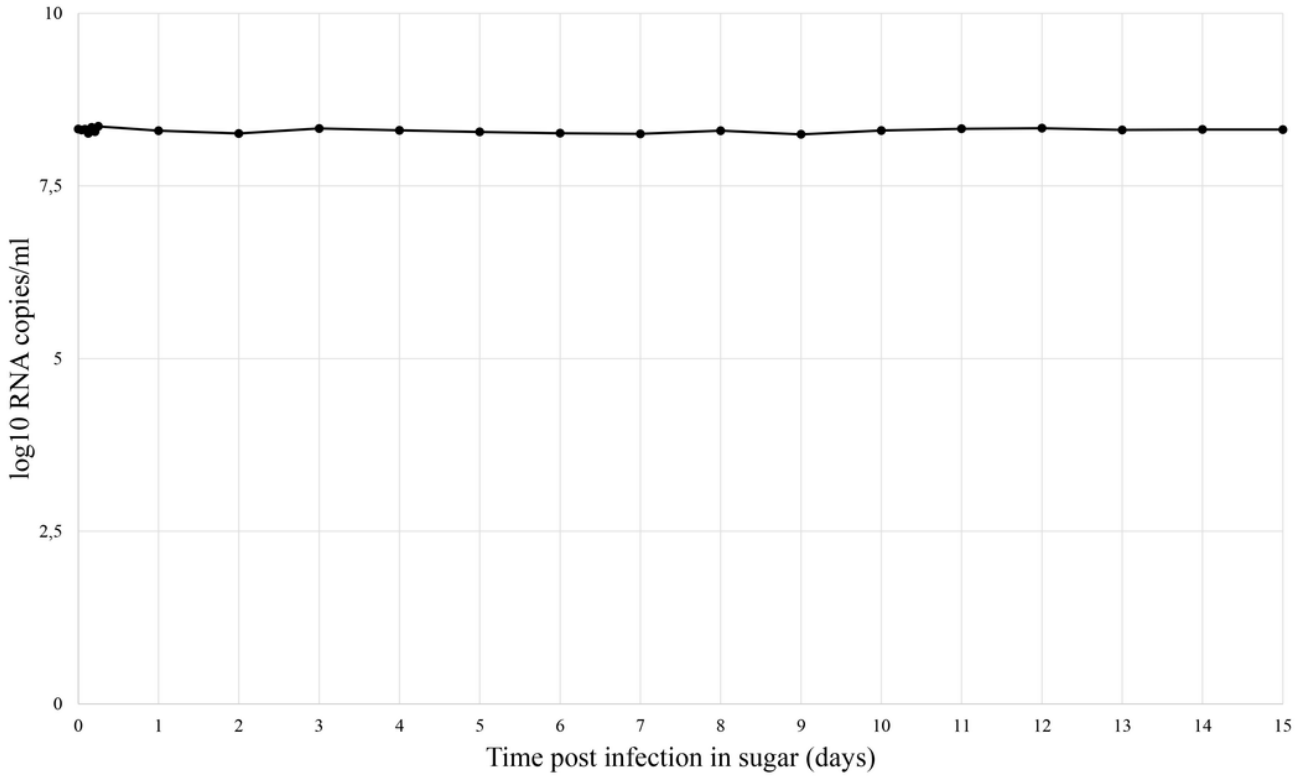


Figure 2

Toscana virus RNA load (log_{10} copies/ml) in sugar solution over time post infection.

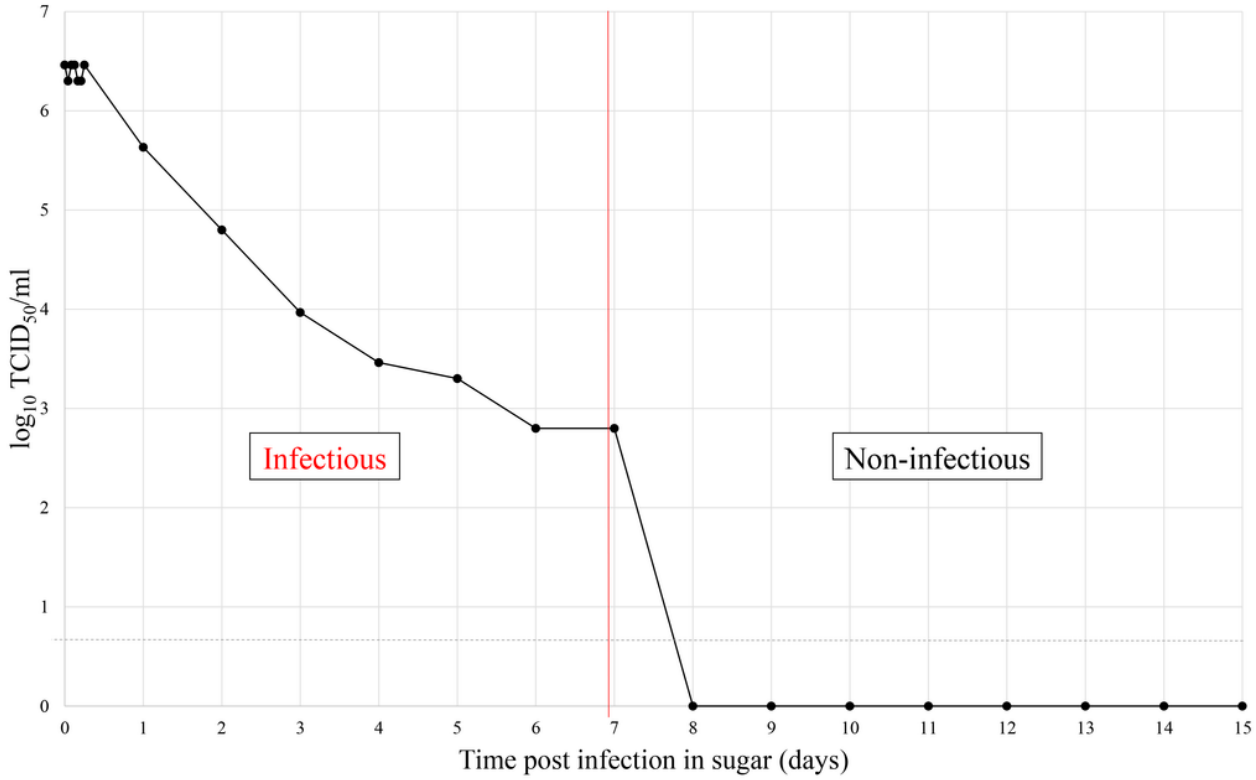


Figure 3

Toscana virus infectivity ($TCID_{50}$/ml) in the sugar solution over time post infection. The red vertical line represents the boundary between infectious and non-infectious samples. The grey dashed horizontal line corresponds to the limit of detection of infectivity (0.8 $\log_{10}$ $TCID_{50}$/ml).

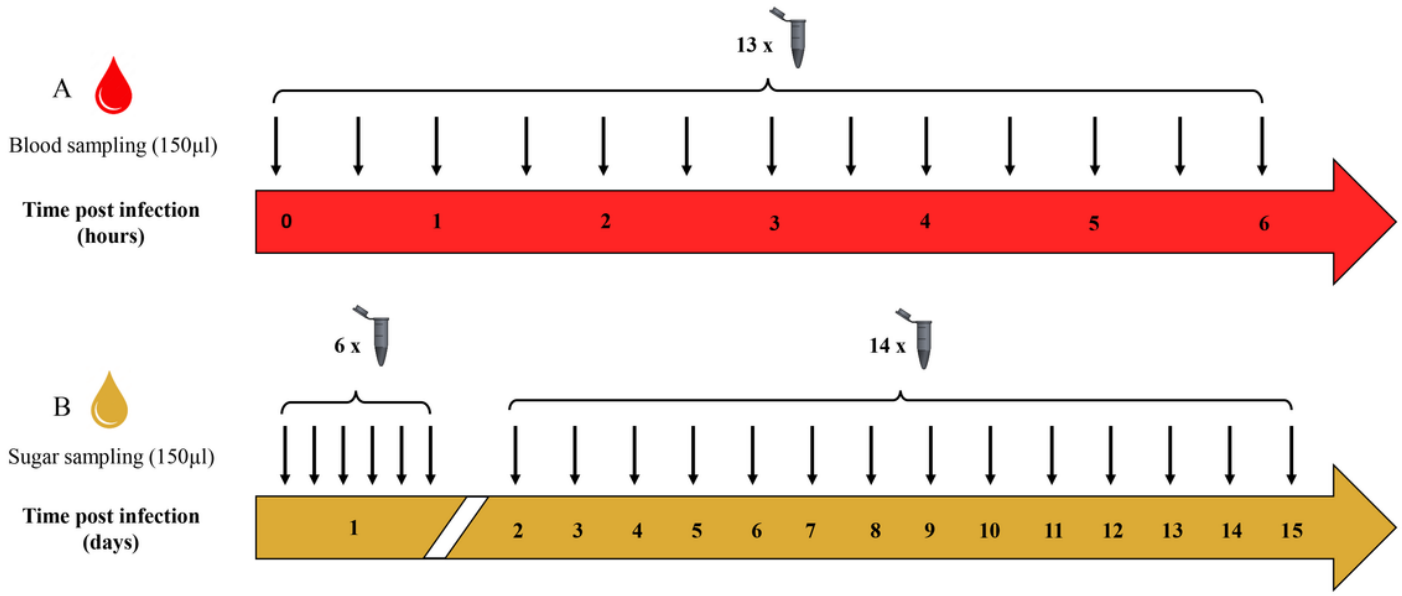


Figure 4

Sampling method for the assessment of TOSV persistence (A) in rabbit blood at 37°C and (B) in sugar solution at 26°C.

Article 5

Intra-sand fly dynamics of Toscana virus and impact on *Phlebotomus perniciosus* life-history traits

Lison Laroche, Nazli Ayhan, Rémi Charrel, Anne-Laure Bañuls, Jorian Prudhomme

To be submitted

TOSV is considered the most common cause of summer viral meningitis in central Italy and France, and is responsible for potentially severe neurological cases. However, very few studies on its transmission dynamics were realized. The main sand fly vectors of TOSV have been identified, but the viral development cycle remains poorly known. Although numerous field studies have demonstrated the presence of TOSV in *Ph. perniciosus* populations, very few experimental studies have yet assessed the competence and vectorial capacity of this species. In order to improve the knowledge on this model, we studied the TOSV infection dynamics in *Ph. perniciosus* over time and the infection impact on vector life history traits. This study confirmed the vectorial competence of *Ph. perniciosus* and gave a first outline of the TOSV development cycle and its impact on the vector while raising new hypotheses on the transmission dynamics.

Title: Intra-sand fly dynamics of Toscana virus and impact on *Phlebotomus perniciosus* life-history traits

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Conflict of interest: None.

Abstract

Toscana virus (TOSV) is considered the most frequent cause of summer viral meningitis in central Italy and France, and is responsible for severe neurological cases. In the Mediterranean basin, TOSV is transmitted by hematophagous sand flies of the genus *Phlebotomus*. Currently, very few studies on the transmission dynamics, the competence and vectorial capacity of the major TOSV vector, *Ph. perniciosus*, have been conducted. The main vectors of TOSV have been identified, but the viral development cycle remains poorly understood. To improve the knowledge of this model, we studied the dynamics of TOSV infection in *Ph. perniciosus* over time and the impact of infection on the vector's life history traits. Female sand flies were infected with TOSV while feeding on blood under experimental conditions. We observed systemic dissemination approximately three days after infection, which could lead to a shorter extrinsic incubation period. In addition, we showed an impact of infection on hatching time, which is longer for eggs from infected females. This study confirmed the vector competence of *Ph. perniciosus* and provided the first insight into the developmental cycle of TOSV and its impact on the vector while raising new hypotheses on transmission dynamics.

Keywords: *Phlebovirus*, Toscana virus, sand fly, infection dynamics, experimental infection, transmission

Introduction

Toscana virus (TOSV) is an enveloped, protein encapsidated, tri-segmented RNA virus that belongs to the *Phlebovirus* genus, *Phenuiviridae* family and Bunyavirales order. It was first isolated in 1971 from *Phlebotomus perniciosus* and *Phlebotomus perfiliewi* in central Italy (1). TOSV is endemic in the Mediterranean basin, where 250 million people are exposed to this virus. TOSV infections are becoming more frequent, although this virus remains neglected due to the lack of diagnostic tools (2). Cases are usually not reported, as commonly observed with arthropod-borne viruses (arboviruses), most TOSV infections are asymptomatic or produce mild symptoms (3).

This virus has a particular neurotropism and is a major cause of meningitis and encephalitis in endemic areas (4). Human cases are observed during the warm season, with a peak during the warmer months in relation to vector activity (3). Three lineages (A, B and C) of TOSV circulate in the Mediterranean area, but, up to date, no difference in pathogenicity has been demonstrated. This sandfly-borne phlebovirus is transmitted to humans through a bite of an infected female sand fly (5). There are currently four sand fly species known to be vectors of TOSV: *Ph. perniciosus*, *Ph. perfiliewi*, *Phlebotomus sergenti* and *Phlebotomus neglectus*. The geographical distribution of TOSV, highly dependent on sand fly presence and abundance, is expanding and it is possible that other sand fly species have a role in its maintenance and transmission (6). Until now, little is known about the TOSV natural cycle and transmission. Transovarial and venereal transmissions have been observed experimentally in its main vector, *Ph. perniciosus* (4). Transmission from infected to uninfected sand flies during sugar feeding may represent an important part of the natural cycle. Indeed, this transmission was shown experimentally with Massilia virus, another *Phlebovirus* genetically close to TOSV (7). In recent decades, arboviruses have become the most important cause of death and disability worldwide, requiring a better understanding of their transmission dynamics (8). Arbovirus

epidemics are influenced by intrinsic (vector competence, virus strain, host susceptibility, etc.) and extrinsic (temperature, rainfall, human activity, etc.) factors that can affect vector biology (9,10). These factors vary in time and space, which can influence the infection and transmission dynamics of arboviruses such as TOSV (11). There are many experimental studies on the arbovirus infection dynamics in mosquitoes, such as Zika or Dengue viruses (12–14). However, only few studies have been performed on the *Phlebovirus* infection dynamics in sand flies (15–17). Because of its neuroinvasive nature, TOSV is currently the most important sandfly-borne phlebovirus for public health, and its natural cycle needs to be further studied (18). In order to study the impact of TOSV infection on *Ph. perniciosus* biology and the potential effect on transmission, we performed experimental infections with the following objectives; (i) confirm that *Ph. perniciosus* is a competent vector for TOSV lineage B strain, (ii) determine the infection dynamics of TOSV in this vector, (iii) explore if there is an effect of TOSV dose on the viral infection kinetics in the vector, and (iv) describe the impacts of the TOSV infection on the life history traits (survival, developmental time, fecundity) of the infected vector.

Methods

1. Sand fly rearing

In order to realize experimental studies, we established in our laboratory (IRD Vectopôle, Montpellier, France**)** a *Ph. perniciosus* (from Pr. Ricardo Molina, Laboratorio de Entomología Médica, Instituto de Salud Carlos III, Madrid, Spain) colony according to protocols already well described (19–21) with few modifications (22). Sand fly colonies are maintained in climate chamber under standard conditions (26 ± 1°C, 80% relative humidity (rh), 14h: 10h light: dark cycle). Adults are fed with cotton pads soaked in 50% organic sugar solution. Blood meal is realized once a week with glass feeders filled with rabbit blood maintained at 37°C and covered

with a 3-day-old chick skin membrane. Feeding is performed for six hours with a regular manual blood mix to avoid clotting. After 24h, blood-fed females are transferred to plastic pots filled with a 1cm layer of plaster of Paris. These pots are stored in plastic boxes containing moistened sand. After hatching, larvae are fed with a macerated mixture of 40% rabbit excrement, 40% rabbit food and 20% mouse food. Newly emerged adults are manually transferred to rearing cages or in small cage for experimental infection.

2. Toscana virus isolates

Lyophilized TOSV aliquots (strain MRS2010, lineage B) were provided by the laboratory UVE (Unité des Virus Emergents, Marseille, France). Vero E6 cells were grown in monolayers in Minimum Essential Medium (MEM, Gibco™) complemented with 7% heat-inactivated Fetal Bovin Serum (FBS, Eurobio Scientific), 1% L-glutamine (Gibco™) and 1% penicillin-streptomycin (Gibco™) at 37 °C with 5% CO_2. Stock of TOSV was obtained by dissolving the lyophilisates in pure water and used for infecting Vero E6 cells at a multiplicity of infection (MOI) of 0.1 after 5 days of post infection. TOSV stocks at a concentration of 4.2 x 10^6 50% tissue culture infective dose ($TCID_{50}$/ml) were aliquoted and stored at -80°C.

3. Sand fly experimental infection

Experimental infections of females *Ph. perniciosus* with TOSV were performed in enhanced biosafety level 3 (BSL-3) laboratories (IRD Vectopôle, Montpellier, France). Twenty-four hours prior to the experiment, 5 to 9-day-old females from the colony were starved by deprivation of sugar meal. A couple of hours before infection, approximately 100 females were transferred into a feeding pot and stored in the BSL-3 climate chamber under standard

conditions (26 ± 1°C, 80% rh) to acclimatize. Approximately 10% males were added to mate and stimulate females to take blood (19). Glass feeders were fill of heat-inactivated rabbit blood and covered with a chick skin membrane. For infected batches, TOSV was added to the blood at a final concentration of 10^2 $TCID_{50}$/ml (dose1), 10^4 $TCID_{50}$/ml (dose2) or 10^6 $TCID_{50}$/ml (dose3). For control batch (control), the same volume of viral culture medium, without virus, was added. The complete system was installed in the climate chamber where the feeders were connected to a 37°C circulating water bath and fixed to the feeding pots (**Figure 1**). Feeding was performed for six hours, reducing the brightness, with a regular manual blood mix to avoid clotting. As engorged females are very fragile, it is recommended to handle them after the first 24 hours of digestion (19). Thus, they were sorted on ice the day after blood meal. Males and non-engorged females were killed and removed. All the experiments were replicated twice.

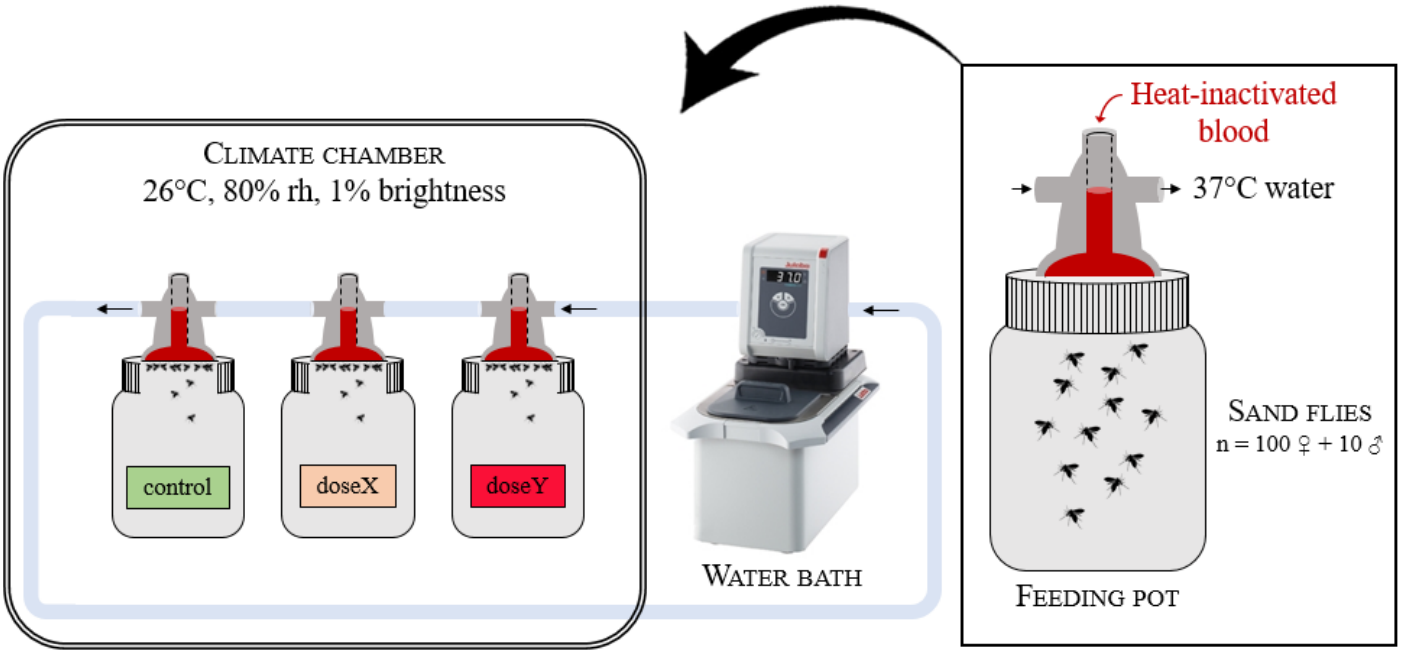


Figure 1. Blood feeding system in the BSL-3 laboratory. rh: relative humidity.

a. Infection dynamics

Engorged females were placed by 30 in cardboard boxes in the climate chamber with a daily intake of cotton pads soaked in 50% organic sugar solution. Three groups were set up for this experiment, infected with 10^2 $TCID_{50}$/ml, 10^4 $TCID_{50}$/ml and 10^6 $TCID_{50}$/ml of TOSV. Between 10 to 30 individuals were dissected in three parts: head, body (thorax and abdomen), wings and legs, every day for 15 days (**Figure 2**), and stored at -80°C for further TOSV detection and quantification analysis. In order to detect virus released by the female, the sugar cotton pads were collected daily, and stored at -80°C. They were analyzed with the same RTqPCR protocol as the sand fly samples.

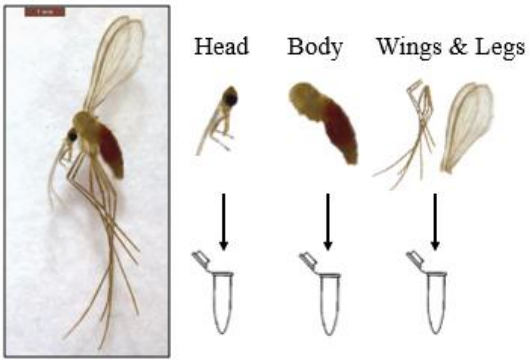


Figure 2. Dissection of infected females in three parts: head, body, wings and legs for Toscana virus detection (Photo by J. Prudhomme)

b. Impact of infection on life-history traits

For life-history trait measurement, engorged females were individually dispatched in small egg-laying pots filled with a 1cm layer of plaster of Paris, pooled by group in plastic boxes containing moistened sand, placed in the climate chamber. A pool of sand flies was set aside in a cardboard box for TOSV infection verification by RT-qPCR. Three groups were set up for this experiment, one control with uninfected individuals, and two tests of individuals infected

147 with 10^4 $TCID_{50}$/ml (dose2) and with 10^6 $TCID_{50}$/ml (dose3) of TOSV. Life history traits were
148 measured daily for each female individually: death date, oviposition date, number of eggs laid,
149 egg-hatching date and number of larvae. Immature stages were only monitored up to first instar
150 larva, due to potential cannibalism between larvae (23).

151 For the female survival, measurement was performed with a different batch of females that did
152 not have access to an egg-laying pot, and were therefore unable to lay eggs. Indeed, blood-fed
153 females die less quickly without the stress egg-laying induced by the rearing conditions (22).
154 After blood feeding, engorged females were transferred to cardboard boxes with a maximum
155 of 30 individuals per box in the climate chamber and maintained under standard conditions.
156 Five blood-fed females were killed, dissected and stored at -80°C for RTqPCR analysis directly
157 after oral infection to measure the initial amount of virus ingested. The survival time of two
158 female groups, fed with healthy blood (control) and infected blood with high dose of TOSV
159 (10^6 $TCID_{50}$/ml), was measured every day until death of all the individuals. To prevent the
160 development of fungal infection which can affect the survival of the sand flies, dead females
161 were removed and the sugar source was changed every day.

162

163 4. Toscana virus detection

164 Viral load was analyzed by RT-qPCR. Entire female or each part (head, body, wings and legs)
165 were individually crushed in 300µl of MEM (enriched with 1% penicillin–streptomycin, 1%
166 (200mM) l-glutamine, 1% kanamycin, and 3% amphotericin B (Gibco™)). Sand fly tissues
167 were homogenized by a Tissuelyser II (Qiagen) using 3mm tungsten carbide beads (Qiagen),
168 and centrifuged at 10000 RPM for 5min. A volume of 200µl homogenate supernatant was used
169 for nucleic acid extraction with the QIAcube® (Qiagen) machine and with Virus Extraction
170 Mini Kit (Qiagen). The RT-qPCR assay were performed with a SuperScript® III Platinum®

One-Step RT-qPCR Kit with ROX (Invitrogen - Thermo Fisher Scientific) on a QuantStudio 12K Flex thermocycler (ThermoFisher). A volume of 5µl of RNA was added to 20µl of mix containing 12.5µl of 2X Reaction Mix, 0.5µl of Superscript III RT/Platinum Taq Mix, and primers and probes STOS at 10µM (24). Negative (pure water) and positive controls (at 4.81 x 10^4 RNA in vitro transcribed copies/ml as described by Beckert & Masquida (25)) were included in each RT-qPCR run; samples with Ct value < 40 were considered positive.

TOSV titers were determined by end-point dilution assay. Tenfold dilutions were used to infect confluent Vero E6 cells in a 96-wells plate in MEM (5% FBS, 1% penicillin-streptomycin, 1% kanamycin, 3% amphotericin B (Gibco™)) at 37°C and 5% CO_2. Wells were classified as positive (cytopathic effect) *versus* negative (no cytopathic effect) five days post infection and $TCID_{50}$/ml was calculated according to the Reed-Muench method (26).

5. Statistical analysis

Data analysis were carried out with R through Rstudio software version 2022.07.1 - © 2009–2022 (27). Survival was examined with the Kaplan Meier product limit estimator, and a log-rank test was used to compare survival distributions between uninfected and TOSV-infected sand flies, using the packages survival (28) and survminer (29). The significance of the TOSV doses (10^4 and 10^6 $TCID_{50}$/ml) and the effect of time post-infection on the infection dynamics was tested by general linear models (GLM). Probabilities of systemic infection according to the TOSV dose or/and time post-infection were estimated with a logistic model. Median systemic infection doses were calculated based on logistic regression parameter estimates. Figures 5 and 7 were made using the package ggplot2 (30).

Results

Toscana virus infection dynamics

A total of 1836 females were used for TOSV infection dynamics experiments. Respectively, 20% (n=73), 68% (n=761) and 77% (n=335) of females fed on blood infected with TOSV dose1, 2 and 3. Due to the natural daily mortality of 10-20%, it was not possible to dissect all blood-fed females. To be able to have enough samples until 15 days post infection (dpi) females were dissected every second days. However, for dose2 infection monitoring was extended to 17 and 21 dpi as more blood-fed females were available. The dissected number of females are described in **Table 1**.

Table 1. Number of *Phlebotomus perniciosus* dissected per day for Toscana virus infection dose of 10^2, 10^4 and 10^6 $TCID_{50}$/ml.

	Day post infection								
	3	**5**	**7**	**9**	**11**	**13**	**15**	**17**	**21**
10^2 $TCID_{50}$/ml	10	5	5	6	5	5	2	Ø	Ø
10^4 $TCID_{50}$/ml	20	47	39	46	52	55	45	26	30
10^6 $TCID_{50}$/ml	20	20	20	20	10	10	4	Ø	Ø

At dose1 (lowest dose), TOSV was detected in the bodies of 2 out of 10 females at three dpi and 1 out of 5 females at seven dpi. No virus was found in heads or wings and legs after infection with dose1. For the dose2 infection, we observed in female bodies amounts higher than 10^4 TOSV RNA copies/ml at three dpi. This amount increased then to 10^6 TOSV RNA copies/ml at 11 dpi, indicating viral replication (**Figure 3**). The viral load in the heads, wings and legs was lower, but started to increase from four to five dpi. There was a stabilization of the viral load in the wings and legs from nine dpi, remaining between 10^2 and 10^3 RNA copies/ml until 15 dpi. In individuals infected by the dose2, viral load continued to increase to reach $2x10^6$ (body), $8x10^4$ (head) and $2x10^4$ (wings and legs) RNA copies/ml up to 21 days (data not shown).

For the infection with dose3, we observed RNA copies in all sand fly parts (head, body, wings and legs) until 15 dpi (**Figure 4**). The viral load increased from more than 10^5 to 10^6 RNA copies/ml from three to 13 dpi. The viral load started to increase in the head, wings and legs from four to five dpi, but with a lower RNA copy number. We tested the significance of the TOSV dose (10^4 and 10^6 $TCID_{50}$/ml) and the effect of time post-infection on the infection dynamics, using GLM. Data from dose1 infection was not included in the analysis due to low sample size. The amount of virus significantly affected the infection dynamics in sand fly (P = 0.0002). Infection rates increased with the dose of TOSV infection, with a significant effect of post-infection time (P = 0.0314).

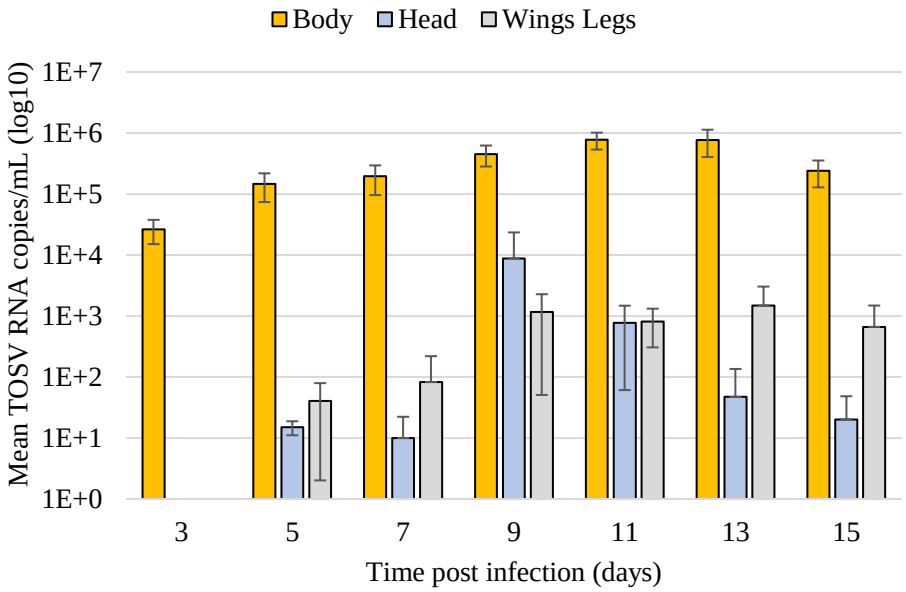


Figure 3. Average amount of viral RNA copies/ml (log10) in body, head, wings and legs of infected females with 10^4 $TCID_{50}$/ml of Toscana virus over time.

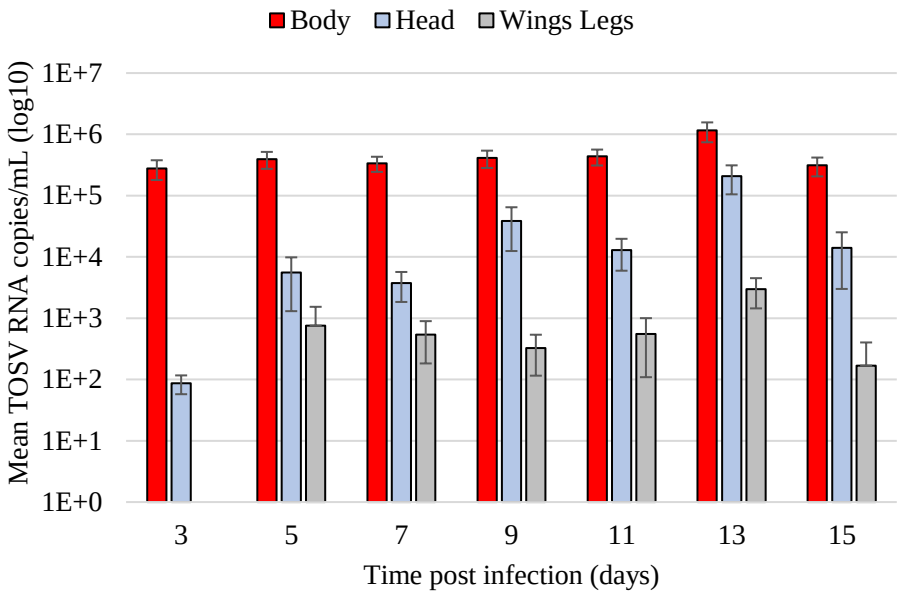


Figure 4. Average amount of viral RNA copies/ml (log10) in body, head, wings and legs of infected females with 10^6 $TCID_{50}$/ml of Toscana virus over time.

Systemic infection prevalences were measured by counting the sand fly number with an infected head over the number with an infected body. The within-host dynamics of systemic TOSV infection were represented for the two virus doses by fitting a non-linear regression model (**Figure 5**). The prevalences of systemic infection did not saturate at 100% for either TOSV dose. The estimated time of 50% systemic infection was approximately five (SE: 0.1) and three (SE: 0.3) days for sand flies infected with 10^4 and 10^6 $TCID_{50}$/ml of TOSV, respectively. To determine the virus infectivity in the different sand fly samples, TOSV titers of bodies, heads, wings and legs were determined by an end-point dilution assay. Of the 84 samples analyzed, seven sand fly bodies infected with dose3 were positive: at 1, 3, 5, 7, 9, 11 and 14 dpi (data not shown).

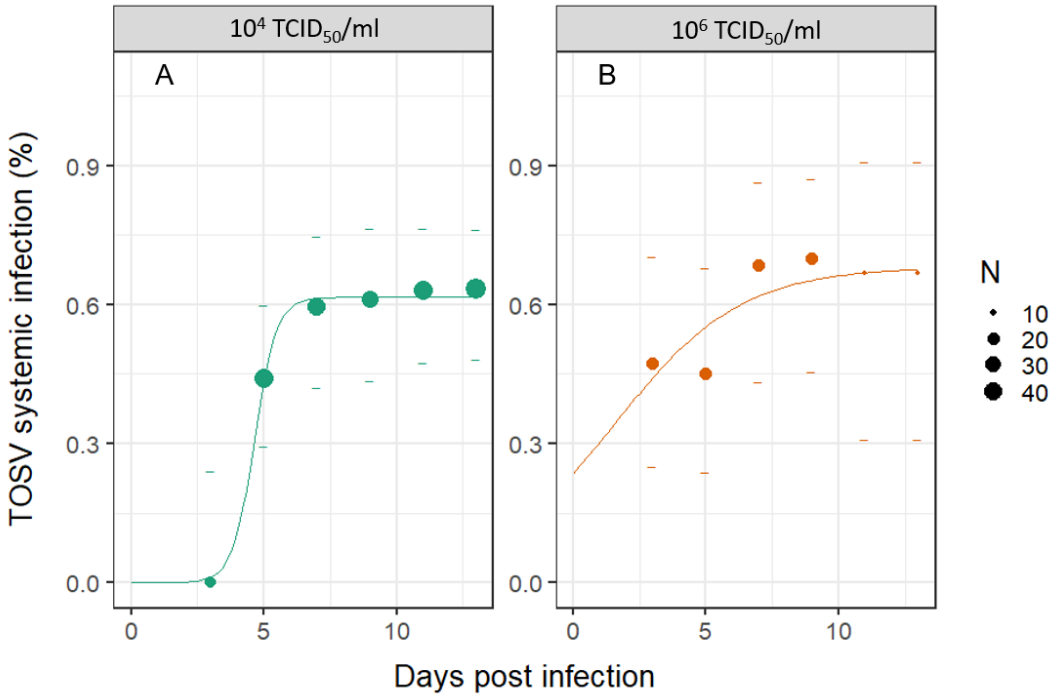


Figure 5. Systemic infection dynamics for *Ph. perniciosus* infected with 10^4 $TCID_{50}$/ml (A) and 10^6 $TCID_{50}$/ml (B). Cumulative prevalence of systemic (disseminated) infections over time post Toscana virus infection are represented as point, dots size indicate the number of samples. Dashes represent the 95% confidence intervals of prevalence. The fitted values obtained with a non-linear regression model are represented by a line.

In the sugar cotton pads, an average of 10^3 (log10) RNA copies/ml of TOSV was detected in samples collected at two dpi (30 females pool). At six (27 females pool) and eight (20 females pool) dpi, viral particles were detected in the sugar samples at an average of 10^3 and 10^4 (log10) RNA copies/ml, respectively (data not shown).

Toscana virus impact on vector life history traits

For the survival experiment, a total of 140 and 98 sand flies were used for the control (uninfected females) and the test (infected females with dose3), respectively. Blood-fed females were killed and dissected directly after oral infection. They ingested an average of $2.5x10^5$ TOSV RNA copies/ml. According to the Kaplan-Meier method, the survival distributions were not significantly different between the two groups (log-rank test, $P = 0.92$). The survival probabilities were the same for uninfected and infected individuals (**Figure 6**). The last infected live female was killed at 45 dpi, dissected and analyzed by RTqPCR for TOSV detection. This data was considered censored for the survival analysis. An amount of $2.2x10^4$ TOSV RNA copies/ml was found in the body, while the head, wings and legs were negative.

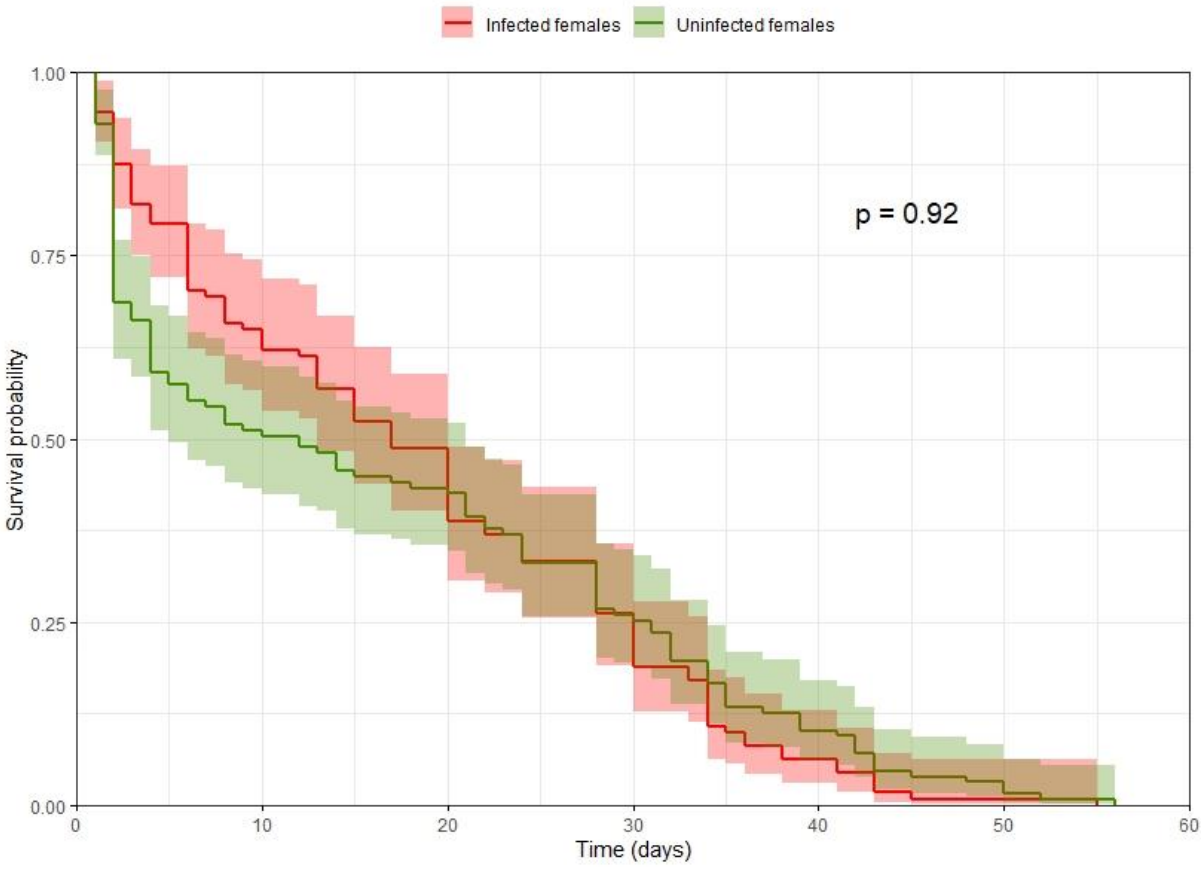
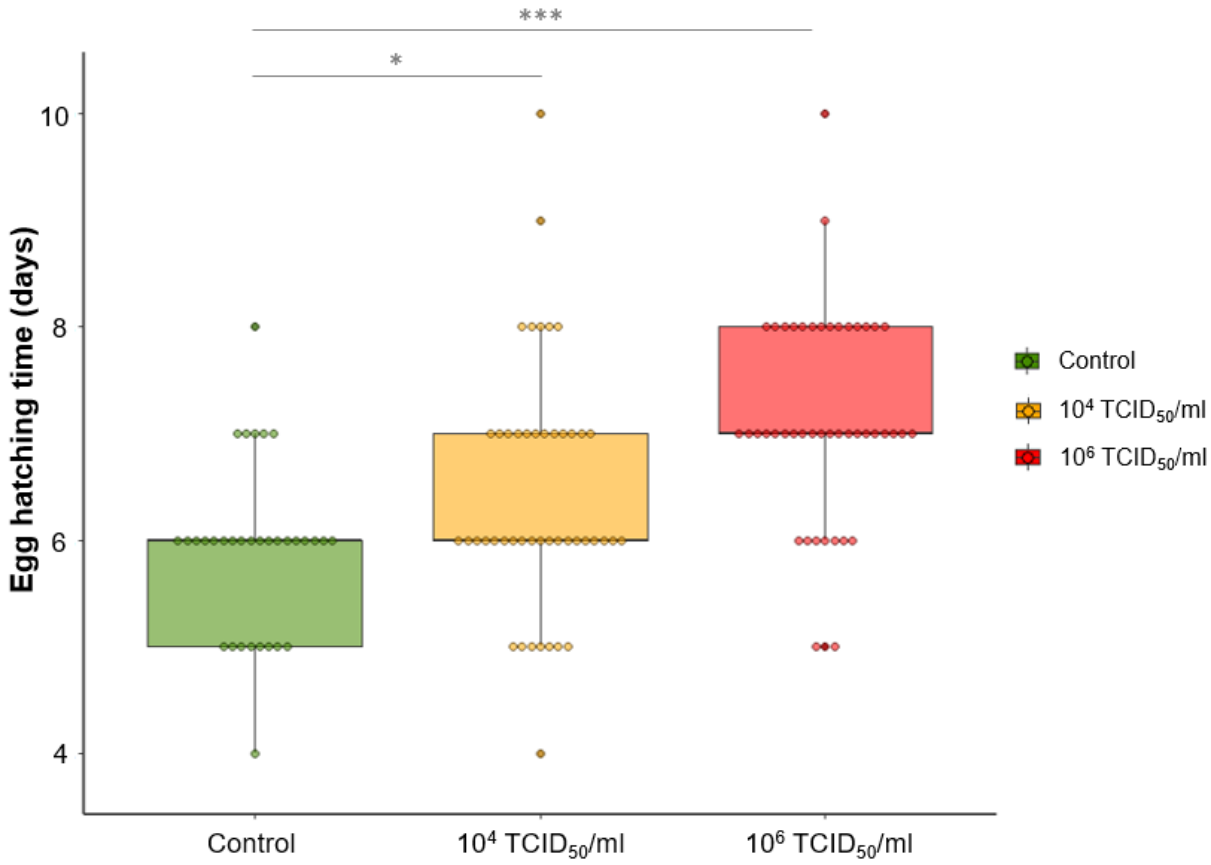


Figure 6. Kaplan-Meier survival estimates for infected in red (n=98) and uninfected in green (n=140) sand flies with Toscana virus with 95% confidence intervals

A total of 543 females were used from the colony for the life history trait experiments. The blood-feeding rates were relatively low for each group (50%). A total of 85 (control), 94 (dose2) and 96 (dose3) individuals were placed in individual laying pots. The **Table 2** shows the different life history trait data collected during 35 days of monitoring until the last hatching. Among all the parameters studied, only hatching times of test groups dose2 (GLM test, P = 0.0247) and dose3 (GLM test, P = 5.61e-07) were significantly different from the control group. The average eggs hatching time were 5.9 ± 0.1 for the control, 6.4 ± 0.2 and 7.1 ± 0.1 for dose2 and for dose3 groups, respectively. Hatching times tend to increase with the dose of TOSV ingested (**Figure 7**).

Table 2. Summary of the experiments with the different life history traits of *Phlebotomus perniciosus* studied. BF: Blood-Fed, BF*: Blood-Feeding, No: Number of. Means are indicated with their standard error.

	No. ♀ initial	**No. ♀ BF**	**BF* rate (%)**	**Survival time (days)**	**No. eggs**	**Oviposition time (days)**	**No. larvae**	**Hatching time (days)**
Control	170	85	50	7.6 ± 0.4	29.0 ± 2.2	6.0 ± 0.4	21.8 ± 2.0	5.9 ± 0.1
Test 10^4	182	94	52	9.2 ± 0.3	32.2 ± 2.0	7.9 ± 0.4	21.1 ± 1.8	6.4 ± 0.2
Test 10^6	191	96	50	8.6 ± 0.4	27.9 ± 2.1	7.2 ± 0.3	19.5 ± 1.6	7.1 ± 0.1



285

286 **Figure 7.** Boxplot representing the Toscana virus infection impact on *Phlebotomus*
287 *perniciosus* egg hatching time. Dots represent the average hatching time of eggs from one
288 female. In green, the control group of uninfected females; in yellow, the test group of females
289 infected with 10^4 $TCID_{50}$/ml; in red, the test group of females infected with 10^6 $TCID_{50}$/ml.
290 The stars are the result of the GLM-based comparison.

291

292

Discussion

Even though TOSV, an emerging sandfly-borne phlebovirus in the Mediterranean basin, is considered the most common cause of summer viral meningitis in central Italy and France, and is responsible for potentially severe neurological cases, very few studies on its transmission dynamics have been conducted (4). The main sand fly vectors of TOSV have been identified, but the viral development cycle remains unknown (31). Although numerous field studies have demonstrated the presence of TOSV in *Ph. perniciosus* populations, very few experimental studies have yet assessed this species vectorial competence and capacity (17,32,33). In this context, we studied the dynamics of TOSV infection in *Ph. perniciosus* and the impact of this infection on the vector life history traits.

First, we experimentally explored the infection dynamics in the vector with three different doses of virus. Our study revealed that sand fly infection rate increases depending on the viral dose in the infectious blood meal. A systemic infection is considered to occur when virus particles disseminate from the midgut cells to the vector haemocoel (34). During infection with a low (10^2 $TCID_{50}$/ml) TOSV dose, virus particles did not disseminate through the vector over time, and only a few body samples were tested positive prior to the sand fly defecation. However, for sand flies infected with medium (10^4 $TCID_{50}$/ml) and high (10^6 $TCID_{50}$/ml) TOSV dose, the virus disseminates over the secondary tissues and the infection persisted until at least 15 days. Thus, in our experiment, the minimum infectious dose for TOSV systemic infection in *Ph. perniciosus* was 10^4 $TCID_{50}$/ml. For another arbovirus model, a study shown that doses as low as $10^{3.9}$ plaque-forming units per ml (representing ~10^4 $TCID_{50}$/ml) of an Asian genotype Chikungunya virus strain can lead to transmission by *Aedes albopictus* (35). Viremia levels observed in *Phlebovirus* infections in human or non-human vertebrates, in the wild or in the laboratory, are known to be low and transient, usually requiring ingestion of a large amount of virus by the vectors (17,36–38). In one of the few experimental infection studies of *Ph.*

perniciosus with TOSV, despite high doses of infection (greater than 10^6 $TCID_{50}$/ml), the prevalence of infected sand flies decreased over time, indicating that a larger quantity of virus must be ingested to infect the sand fly (17). In contrast, we have shown that even when ingesting a medium dose of virus (10^4 $TCID_{50}$/ml), the sand fly is able to become infected until at least 21 days and probably transmit the virus. It is possible that this difference between their study and ours is due to the TOSV lineage, which could have an impact on vectorial competence and infection dynamics.

Then, we investigated whether the sand fly is able to transmit TOSV. In laboratory, females die before the second blood meal (22); therefore, it was not possible to measure transmission during a second artificial blood meal. Preliminary data revealed virus presence in the sugar cotton pads on which sand flies infected with dose3 fed. More specifically, the results showed the presence of TOSV in the cottons collected at two, six and eight dpi at 10^3, 10^3 and 10^4 RNA copies/ml, respectively. To predigest the sugar, sand flies release a small amount of saliva (39). Even if the presence of viral particles in the cotton pads at two dpi can be explained by viral residues (contamination between individuals, defecation, blood residues in mouthparts, etc.), at four and six dpi, the presence of virus can be suspected in the salivary glands. We can therefore hypothesize that TOSV reached the salivary glands of *Ph. perniciosus* at about six dpi after infection. The average extrinsic incubation period (EIP) of a *Phlebovirus*, *i.e.* the time between ingestion of the virus and the sand fly transmission capacity (presence of the virus in the salivary glands), is at least one week (40). For example, EIP duration of the *Phlebovirus* Rift Valley Fever virus is at least 14 days after infection (41). Based on our preliminary results, we hypothesize that TOSV EIP in *Ph. perniciosus* is approximately six days, which would be shorter than other Phleboviruses.

For many arboviruses, the viremia peak of symptomatic infections in human is estimated to be around 10^6 $TCID_{50}$/ml (42,43). Nevertheless, asymptomatic human cases can reach viremia

levels as high as the lowest estimates for symptomatic cases (44). To our knowledge, no study has reported the viral load of TOSV in asymptomatic patients. Because sand flies can become long-term infected with a lower (10^4 $TCID_{50}$/ml) dose than the viremia peak dose, they may become infected during most of the patient's viremic period, symptomatic or not. Many factors probably influence the EIP length including environmental conditions (*e.g.* temperature), vector immune system, vector lifespan, and, as demonstrated here, the initial dose of virus ingested by the vector (45–47). To accurately estimate the TOSV EIP in the vector, it is therefore essential to reproduce the sugar cotton pads experiment with a larger number of samples and to explore other parameters influencing the EIP as mentioned above. This parameter is essential since pathogens with a shorter EIP lead to an earlier transmission after infection (48).

In the second part of this study, we experimentally explored the impact of TOSV infection on the vector biology. We wanted first to know if high-dose TOSV infection could have an impact on the vector survival. According to our results, infected sand flies had the same lifespan as uninfected ones. If there is no change in sand fly survival, this means that the virus will be able to be transmitted throughout the vector life during these multiple meals of blood (for females) and sugar (for males and females) and through multiple oviposition. Knowing that the average lifespan of a female is two months, this increases the virus transmission (49). Then, we assessed the impact of two TOSV infection doses (10^4 and 10^6 $TCID_{50}$/ml) on different life-history traits of *Ph. perniciosus*. The main result is the impact on hatching time, which is longer for eggs of infected females. Knowing that transovarial transmission of TOSV exists in *Ph. perniciosus*, we can hypothesize that infected sand flies will emerge later, which may lead to a delay in the risk period for TOSV transmission (50). This delay should be taken into account in the application of vector control measures.

From this study, we confirmed that *Ph. perniciosus* is a competent vector for TOSV even at a lower dose in the blood meal (10^4 $TCID_{50}$/ml) than the virus dose during viremia peak in

mammals (10^6 $TCID_{50}$/ml). All our results suggest that the transmission of TOSV is rapid and that the epidemic risk in endemic areas must be considered. These experimental results provide a better understanding of virus maintenance in sand fly populations and the natural cycle of TOSV, while raising new hypotheses on transmission dynamics.

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GENERAL DISCUSSION

This work highlights the importance of studying vector-pathogen interactions in order to better understand transmission dynamics and prevent the spread of vector-borne diseases. Research on insect-pathogen interactions allows to understand the processes that determine the insect ability to transmit human or animal pathogens. This thesis confirms the need to take into consideration vector, pathogen, host and environment in an infectious system in order to prevent emergences in a sustainable manner, which are central concerns in a One Health framework. Our objective was to experimentally investigate the dynamics of *Le. infantum* and TOSV in *Ph. perniciosus* and their impact on the sand fly biology. Based on these data, ecological and epidemiological hypotheses are discussed.

The sand fly colony

In order to carry out these experimental studies, it was necessary to set up a sand fly colony in our laboratory. This rearing was set up, at the beginning of my thesis, with Dr. Prudhomme using *Ph. perniciosus* eggs from the colony of Dr. Molina. We chose the species *Ph. perniciosus* because it is the major vector common to *Le. infantum* and TOSV in the Mediterranean basin. Nevertheless, other sand fly species should also be studied, such as *Ph. papatasi* or *Ph. tobbi* (111). As we could not take more than one third of the colony size to perform experiments, and as the sand fly production was limited due to the laboratory capacities (rearing room size, manpower limited to two persons), we had to perform our experiments according to the production fluctuations. The first experimental infection could only take place one year after the colony set up and due to the production fluctuation the sand fly population available for our experiments was not constant. Even during periods of high production, we encountered constraints and difficulties. During the Covid-19 crisis, the BSL-3 laboratory was not accessible for a long period (during the 1st PhD year) and it was also closed a couple of months for renovations (during the 2nd PhD year). In addition, the rabbits used for blood production suffered from a parasitic infection which stopped the blood accessibility during the treatment period. Indeed, the presence of drugs in the rabbit's blood could kill sand flies. As described previously, it is necessary to underline the importance of cleanliness and sterility of equipment used in sand fly rearing (34,159). As several studies have already mentioned, maintaining a sand fly colony requires time, care and patience. In the first three years, we have

optimized some of the rearing techniques (See Chapter 1). But many methods still need to be optimized, such as the use of chick skin as an artificial membrane for the blood meal. Indeed, working with chick skins is very time-consuming. Protocol modifications are possible, such as the use of Parafilm®, but it did not work with our sand fly species.

In addition, we had a mite invasion, following the introduction of a new species (*Ph. papatasi*), into our sand fly colony. These mites of the genus *Tyrophagus* may play a role in the loss of colony productivity (183). We tried many different time-consuming techniques to eliminate them (34), but this was unsuccessful. However, we were still able to maintain the colony production. The *Ph. perniciosus* rearing, once optimized and productive, allowed us to realize the experimental infections necessary for our study. We used a total of 4343 sand flies to perform three experimental infections with *Le. infantum* and seven with Toscana virus. The **Table 4** summarizes the different experiments carried out with their duration.

Table 4. Summary of the experimental infections realized during the thesis. The quantities represent the number of promastigotes/ml used for *Le. infantum* experiments and the $TCID_{50}$/ml for TOSV. LHT: Life History Traits.

Objectives of the experiment	Duration (days)	No. sand flies	Pathogen
Experimental infections set-up	31	103	*Leishmania infantum*
Impact on LHT, infection at 10^3 10^5 & 10^6	29	145	*Leishmania infantum*
Infection dynamics at 10^3	11	295	*Leishmania infantum*
Infection dynamics at 10^6	15	435	Toscana virus
Impact on LHT, infection at 10^6	43	257	Toscana virus
Infection dynamics at 10^2 & 10^4	15	716	Toscana virus
Impact on survival, blood infection at 10^6	45	380	Toscana virus
Impact on survival, sugar infection at 10^6	65	676	Toscana virus
Infection dynamics at 10^4 & 10^6	25	730	Toscana virus
Impact on LHT, infection at 10^6	35	606	Toscana virus
TOTAL	**314**	**4343**	

Sand fly colonies are not as common as mosquito colonies, partly because studies on sand flies are less frequent than those on mosquitoes, but also because the rearing conditions are more standardized and optimized for mosquitoes. For example, the mosquito larval stages are aquatic and generally fed on commercial fish food (184). Whereas sand fly larvae are terrestrial and require to be fed with a special food that we have to make ourselves, using organic matter and fungi that have grown on rabbit faeces (34). Sand fly breeding sites are still not well known. More studies on this subject would allow to determine the most frequent fungi species in the

diet and to standardize the larval food (185). Many other techniques need to be optimized, such as artificial blood meals to avoid the use of chicks, as described previously. Despite the presence of these issues, we manage to implement sand fly colonies experimental infection. We highlight the importance of developing sand fly rearing methods as they are indispensable for studying the development cycles and transmission dynamics of sand fly-borne pathogens and answer to the following question: *What is the impact of vector-pathogen interactions on transmission dynamics and vector biology?*

Vector and pathogen life cycles

Sand fly colony monitoring allowed us to obtain accurate life history traits follow-up of *Ph. perniciosus*. We were able to collect data from each developmental stage over several generations (186). This step was necessary to estimate the experimental infection dates and the sand fly number available. Moreover, the life history traits data obtained were similar with those of uninfected individuals, used as controls in the BSL-3 laboratory experiments. Sand flies are vectors of microorganisms that can cause infectious diseases in vertebrates. Thus, the question is: these microorganisms can thus have a harmful impact on their hosts (*e.g.* mammals), but what is the impact on their host vector? Many studies have focused on this question. Multiplication in the vector provides at least two advantages for pathogen transmission, (*i*) the amount of initial inoculum is amplified in the vector (*ii*) and the persistence throughout the vector lifespan is assured (187). This highlights the importance of vector life history traits in vector capacity equation (188). However, virus or parasite replication in the vector can have a cost on its fitness by reducing its lifespan or fecundity. An excessive cost on the vector would be counterproductive and would risk reducing the chances of pathogen transmission (189). It was demonstrated that high *Leishmania* infection leads to a reduction of the sand fly longevity (66). A study showed a negative impact of the parasite *Le. infantum* on the vector *Phlebotomus langeroni* fecundity, with females laying fewer eggs (67). As infection of some vectors (*e.g.* malaria in mosquitoes) can reduce reproductive fitness leading to the production of fewer eggs (190), we decided to examine the potential effects of *Leishmania* on sand fly fecundity. Surprisingly, we observed an increase in the egg number laid by *Ph. perniciosus* infected with *Le. infantum*. The increased oviposition of infected sand flies may be a form of terminal investment, as organisms with low chances of survival devote their energy to reproduction before dying (191). When studying the infection dynamics of *Le. infantum*, our results were consistent with the cycles previously well

described in the literature, since the parasites reached the stomatal valve at eight dpi. However, we demonstrated for the first time that a small inoculum of *Leishmania* developed in the sand fly as quickly as a large inoculum. These preliminary results, with very few samples analyzed need to be confirmed, but could have an impact on the dynamics of transmission, and deserve further study. Indeed, this could be one of the explanations for the low-noise persistence of leishmaniasis is some areas.

As *Leishmania* are more widely studied than *Phlebovirus*, we also investigated whether there is an impact of viral infection on sand flies. Researchers have hypothesized that horizontally transmitted arboviruses generally induce significant adaptation costs in mosquitoes (192). For example, it has been shown that infection of female *Aedes aegypti* with DENV-2 significantly reduces the vector longevity and fecundity. In our study we did not observe any impact of TOSV infection on the survival of *Ph. perniciosus* (following blood or sugar meals). However as previously described (See Chapter 2), experimental blood feeding reduces the female survival. Therefore, it would be necessary to repeat this experiment by preventing the females from laying eggs in order to measure their survival over a longer period. Moreover, the study was only carried out on the first sand fly generation (F1). Since TOSV is vertically transmitted in *Ph. perniciosus*, it would be interesting to measure the survival rate of subsequent generations that may be affected (114). However, our results revealed an impact of TOSV on the hatching time of eggs laid by infected females. These eggs hatched later. Insect hatching times can be affected by environmental conditions such as temperature or humidity and hatching rate by microorganisms infection such as *Wolbachia* (193–197). Nevertheless, to our knowledge, no study has measured the influence of microorganism infection on insect hatching times.

Only few studies have looked at the development cycle of TOSV (114). In this thesis, we describe for the first time the infection kinetics of TOSV in *Ph. perniciosus*. According to our results, half of the sand flies can have a systemic infection after three days on a high infected blood meal (10^6 $TCID_{50}$/ml), and after five days when taking a less infected blood meal (10^4 $TCID_{50}$/ml) (**Figure 17**). We estimated the TOSV extrinsic incubation period (EIP) at six days under 26°C, using a protocol adapted from a study on *Plasmodium falciparum* parasites in mosquito vectors (198). As some infected vectors spit virus during their sugar meal (199,200), we investigated the virus expectorated into the sugar by sand flies when they feed. We showed that *Ph. perniciosus* is able to transmit TOSV six days after an infectious blood meal.

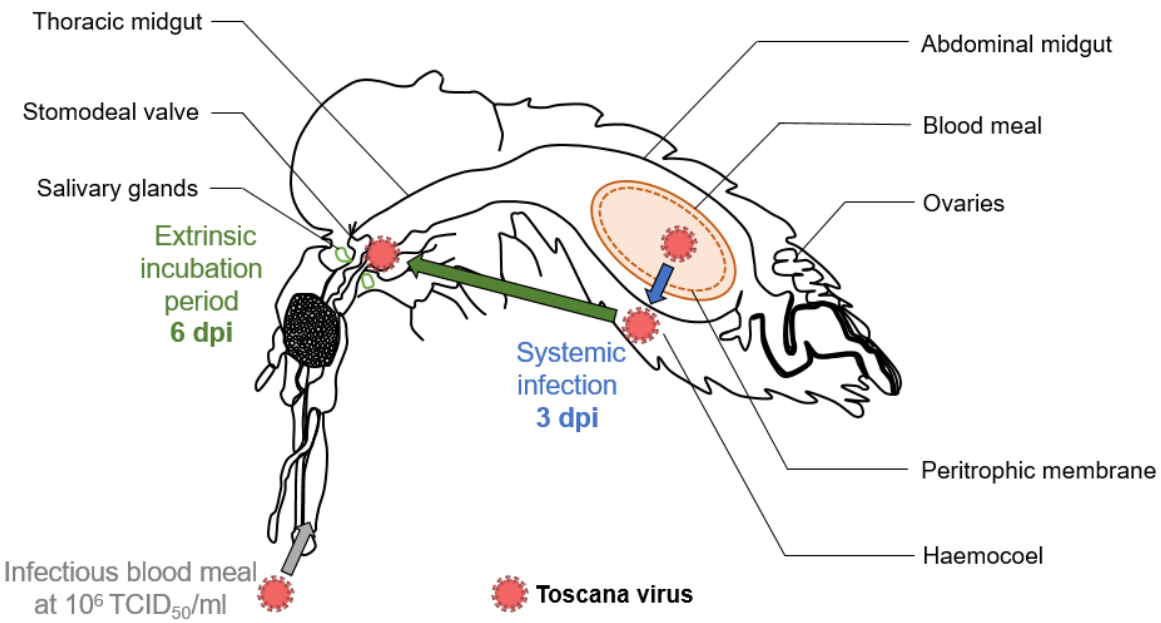


Figure 17. Toscana virus infection dynamics in *Phlebotomus perniciosus*. Grey arrow represents infection via a blood meal with 10^6 $TCID_{50}$/ml TOSV on a viremic host, the blue arrow represents the time necessary for the virus to reach secondary tissues (systemic infection) and the green arrow represents the time necessary for the sand fly to become infectious and transmit TOSV during a second bite (extrinsic incubation period). Dpi: days post infection.

Unlike other arboviruses, TOSV develops rapidly in its vector. The EIP is approximately 10 days for Zika and Dengue viruses at 26°C, and at least 14 days for another *Phlebovirus*, the RFVF (201–203). However, as explained in Chapter 3, the EIP is influenced by many factors (*e.g.* environmental temperature or vector microbiota) and it would be interesting to determine the impact of these factors on the TOSV EIP. We have described here part of the TOSV natural cycle in the vector, but there is still much to learn. Arboviruses are obligate intracellular pathogens that exploit the host's cellular machinery in order to replicate (204). Currently, we do not know in which sand fly organs TOSV replicates. It is possible that the virus replicates in the salivary glands since we detected it in the sugar meal, but it could also replicate in the genital organs or in the eggs (due to transovarial and venereal transmission). We estimated as well the TOSV intrinsic incubation period (IIP), time between a human being infected and the onset of symptoms, at 12 days for neuroinvasive infections (127). IIP is different from the latency period, which is the period between infection (via an infective sand fly bite) and the onset of infectiousness (205). The IIP is an important determinant of the dynamics of human diseases

and can be used for a differential diagnosis, for example, for a traveler returning from TOSV endemic area (206).

Thus, this thesis work has demonstrated the impact of pathogen infection on the sand fly life cycle, studied the dynamics of infection and described for the first time the development cycle of TOSV in the vector. The question is now how the transmission dynamics of vector-borne pathogens could be influenced by these parameters?

Epidemiological risks

Arthropod-borne pathogens have a horizontal transmission cycle that alternates between vertebrate hosts and hematophagous arthropod vectors (207). This alternating cycle imposes unique constraints on the pathogen evolution, as they are forced to adapt to different hosts simultaneously. Many studies focus on the trade-offs that shape pathogen replication dynamics in vertebrate hosts, and how these dynamics influence transmission to vectors, but few focus on theses dynamics in the vector (208,209). Our study highlighted the impact of pathogen infection on the sand fly life cycle and more specifically on fecundity.

The impact of *Leishmania* in the oviposition rate of *Ph. perniciosus* may influence the vector population densities and thus, the vectorial capacity. If infected females lay more eggs, as we have shown, the sand fly population is likely to increase, leading to a greater local epidemiological risk. This behavior could be explained by the terminal investment theory. However, a study showed that infection with a RNA virus in a mycophagous insect could induce adult females to produce more eggs (210). They suggested that this phenomenon could be linked to a mutual interaction between the vector and the pathogen (211). It is possible that this kind of interaction exist between *Le. infantum* and *Ph. perniciosus* which may lead to an enhancement in pathogen transmission by improving the fitness of the vector, as already demonstrated between the vector *Ixodes scapularis* and the pathogen *Anaplasma phagocytophilum* (212). Further studies are necessary to assess these two hypotheses.

According to our results on the infection kinetics of *Le. infantum*, sand flies become infected as quickly when feeding on a high or low infected dose. However, as we do not yet have the results of parasite quantification in sand flies, we do not know whether the infected vector with a low inoculum will have a sufficient parasite load to transmit. If this parasite load is high,

this could partially explain the low-noise transmission of *Le. infantum* in some endemic areas (180).

Concerning TOSV infection dynamics we obtained a first overview of the cycle in the vector and also several hypotheses can be posed regarding the different transmission pathways (**Figure 18).**

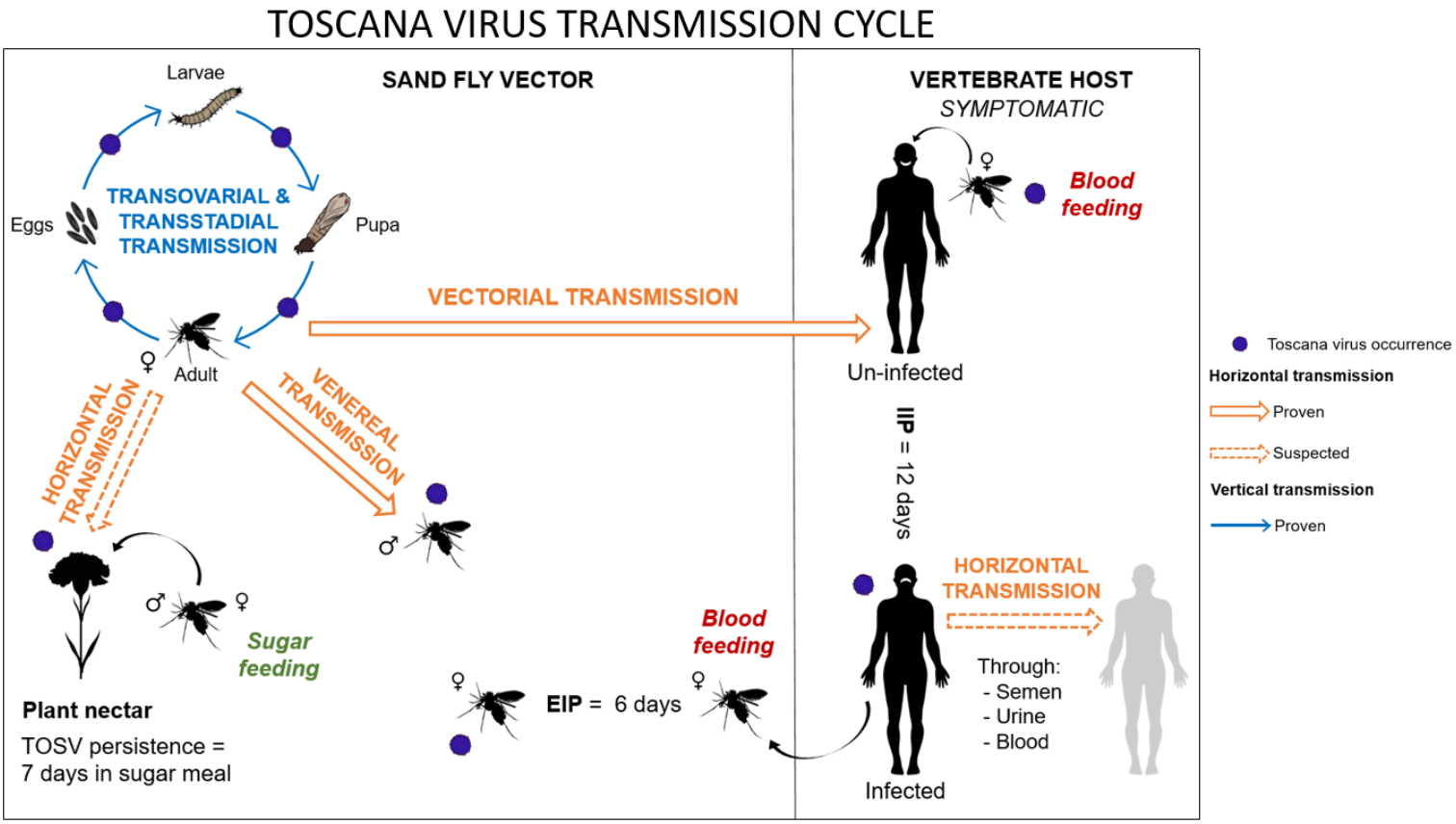


Figure 18. Toscana virus transmission cycle with proven and suspected transmission pathways. EIP: Extrinsic incubation period, IIP: Intrinsic incubation period.

Transovarial and transstadial transmissions of TOSV were already described more than 35 years ago and have not been studied again since then (213). Venereal transmission of TOSV has been demonstrated experimentally from transovarially infected males of *Ph. perniciosus* to uninfected virgin females (113). Vectorial transmission has already been proven and here we confirmed the vectorial competence of *Ph. perniciosus* for TOSV (lineage B). In addition, we suspected two other horizontal transmissions illustrated in **Figure 18**, one on the vector side and one on the vertebrate host side. We have shown that the sand fly releases virus particles during sugar feeding and that TOSV persists and remains infective in sugar juice for up to seven days at 26°C (Chapter 3). We have not yet analyzed the infection dynamics in the vector following

an infected sugar meal. We only measured their survival over time (data not shown) and concluded that there was no impact of TOSV via sugar juice on the survival of infected males and females. However, we assume that TOSV could replicate and disseminate in the vector after a sugar meal. Indeed, during the sugar meal, the sugar juice (plant nectar, honeydew) is first stored in an organ called "crop" as a food reserve before being evacuated into the vector's gut where digestion takes place (214). The high concentration of carbohydrates and the acidic pH in the crop could facilitate the development of microorganisms like TOSV (215). In addition, horizontal transmission through sugar has already been demonstrated experimentally with Massilia virus, another *Phlebovirus* genetically close to TOSV (200). Thus, transmission from infected sand flies to uninfected sand flies during sugar feeding could represent an important part of TOSV natural cycle.

Then, we demonstrated experimentally that TOSV could be persistent and infectious in heat-inactivated mammalian blood for at least 6 hours. The risk of TOSV transmission to virus-naive individuals through human blood transfusions and organ transplants has already been reported (216). We can thus assume a risk of infection through blood transfusion. Horizontal transmission between vertebrate hosts could occur through blood but also via contact with seminal fluid or urine in which TOSV has already been detected infectious in seminal fluid and as nucleic acid in urine in symptomatic patients. (217,218).

The variety of transmission routes increases the chances of TOSV circulation. However, TOSV infection rate in sand fly populations is very low, 0.05% in Spain for example (138,219). And so far, there is no evidence for a vertebrate species acting as reservoir or amplifying host in the natural cycle of TOSV (111). Despite all these transmission pathways, how it is possible that TOSV continues to circulate at low level in endemic area?

Many human, animal and plant pathogens are transmitted both vertically and horizontally, and horizontal transmission is usually necessary to maintain them (220). Vertical transmission exerts a natural selection on pathogens to be less harmful to their host (221). Since host survival will contribute to pathogen multiplication, by being less virulent, pathogens will have higher fitness (220). Indeed, we did not observe any impact of TOSV on vector survival. A study showed that the percentage of transovarially infected *Ph. perniciosus* with TOSV did not remain constant but tended to decrease in subsequent generations (114). It is possible that the vector immune system affects the viral infection over time (222). Vertical transmission alone would therefore not be able to maintain TOSV, but in combination with horizontal transmission it could allow the

maintenance of TOSV circulation in sand fly populations. Moreover, in absence of viremic vertebrate, the venereal transmission provides alternative method for virus amplification in the vector population (113).

In addition, the **encounter filter** between pathogens and vertebrate hosts could also influence the TOSV low-level circulation, such as food preferences or environment sharing. Assessing the relative strength of this filter is a fundamental step in determining the mechanisms that drive the distribution of a pathogens between hosts (223). It is possible that the majority of TOSV infected sand flies does not encounter hosts (that can be infected) frequently enough. Or, viremic vertebrate hosts are not located close to areas with a high density of sand fly vectors. Vertebrates are not good reservoir as they present little or no TOSV amplification. Therefore, studies have suggested that the reservoir could be the vector (111). This hypothesis could also explain the low prevalence of TOSV in natural population. Indeed, due to their small size, we can assume that vectors are less efficient reservoir than mammals. However, it is important not to neglect the possible emergence of TOSV variants more adapted to other hosts which would favor transmission. It is therefore important to understand the factors that modulate host distribution, as changes in these factors alter the dynamics of transmission.

We highlight the importance of considering vector-pathogen interactions in a One Health strategy to assess transmission risks. After studying these interactions, we raised further questions. Given that TOSV and *Le. infantum* co-circulate in natural populations, what might be the interactions between these two pathogens within the vector? And what impact could these interactions have on transmission dynamics?

Co-infections

The data obtained in this work can be used as a basis for studying the impact of co-infection on the two pathogen, *Leishmania*/TOSV, transmission and vector biology. Pathogens co-circulation is common and can lead to co-infection within vector and host populations (224). *Phlebovirus* and *Leishmania*, have common vectors and hosts, co-circulate in several regions of the world and co-infection cases have already been described (149–156). Co-infections can occur simultaneously or sequentially and can involve different interactions. Firstly, there may be **competitive interactions** between the two pathogens which may lead to the suppression of one or both pathogens. Most of the interactions reported during co-infection in mosquito cell lines are competitive (225,226). The co-infection frequency, in host or vector, in nature could

then depend on the competition between pathogens for access to a resource. Secondly, there may be **cooperative interactions** in which the pathogens share resources; or the action of one pathogen on the vector immune system may result in the enhanced propagation of the other pathogen. In experimental co-infection in mice, it has been shown that *Le. guyanensis* infection is exacerbated by the action of TOSV on the mouse immune system (147). Specific RNA viruses shared by *Leishmania* vectors or reservoirs can enhance and maintain the activation of RNA sensors in host cells, leading to an exacerbation of parasitic infection (158). It is of interest to study antiviral pathways during co-infection, given their role as modulators of protozoan infections. Finally, **coexistence** can occur when the interactions do not significantly alter the fitness and transmission of either pathogen (227,228). Furthermore, during *Leishmania* infection, the parasite's manipulation of the sand fly leads it to take several sequential blood meals for efficient transmission (229). Through this mechanism, several hosts can be infected thus increasing the probability of a successful infection. In contrast, in TOSV infection, the virus does not change the feeding behavior of the sand fly and a single infectious blood meal is sufficient for vertical and horizontal transmission. Thus, by biting only one host, the probability of infection by TOSV is lower than for *Le. infantum*. Although this probability increases with the combination of vertical and horizontal transmission, it appears to be less important than for *Le. infantum*. These parasites being more complex organisms may have undergone a longer evolution and thus be better adapted to their hosts (230). Co-infection of the sand fly with *Le. infantum* and TOSV could then boost TOSV transmission, due to changes in the infected sand fly behavior (66).

As it is possible that one pathogen can promote the epidemiological cycle of the other, it is necessary to take both pathogens into account when implementing control strategies through a One Health perspective (231). Indeed, it is necessary to define the interaction (coexistence, competitive or cooperative interactions) between parasite and arbovirus to understand the transmission cycle in the vectors but also in the hosts.

CONCLUSION

Along this thesis, we set up a sand fly colony and optimized rearing techniques to realize experimental infections. The reliability and robustness of the colony allowed us to perform several experimental infections and to study the interactions between the vector *Ph. perniciosus* and *Le. infantum* or TOSV. We observed the impact of these infections on the vector life cycle and increase our knowledge on the pathogen natural cycles. The cycle of *Le. infantum* is well known, however, TOSV natural cycle remains poorly studied. Our work gives estimations of the EIP of TOSV in the vector and the IIP in neuroinvasive human cases. These periods are important determinants of the temporal dynamics of TOSV transmission and are therefore essential for clinical diagnosis, investigation of epidemics, implementation of prevention and control programs, and mathematical modelling of TOSV transmission. To better know this transmission, we discussed the effects that environmental persistence of TOSV might have on host-pathogen dynamics. We made several hypotheses on the different TOSV transmission pathways and tried to understand how circulation is maintained in natural populations of vertebrate hosts and vectors. Finally, this study significantly improves our understanding of infection and transmission dynamics of two sand fly-borne pathogens, and these findings can now be incorporated into both epidemiological and experimental models of infection. This work opens up new opportunities of research on (i) the interactions between sand flies, *Leishmania* and TOSV, (ii) the impact on these two pathogens infection on the sand fly biology and (iii) the consequences of these impact on their transmission.

PERSPECTIVES

The work realized during this thesis has provided new insights into the understanding of sand fly-*Leishmania*/*Phlebovirus* interactions. However, there are still issues to be clarified. Due to fluctuations in sand fly production, the number of individuals available for experimental infections was limited. Therefore, as a first step, it would be necessary to reproduce the *Le. infantum* experiments with larger numbers of sand flies to confirm our results on the female fecundity. Moreover, it would be interesting to repeat this experiment with other *Leishmania* strains and other sand fly species: for example, with *Le. major* and *Ph. papatasi*, a species colony we have also set up during my thesis. In addition, qPCR optimization has to be implemented in order to detect the proportion of parasite infectious forms in the sand fly over time.

The experiments with TOSV have significantly improved our knowledge of the infection dynamics in the vector, but further studies are needed. We have confirmed the vectorial competence of *Ph. perniciosus* for TOSV (B lineage) with both high and low viral doses in the blood meals. *Ph. papatasi* are naturally infected with TOSV, but it would be interesting to investigate their competence through different virus doses (232). Infection dynamics analysis could also be measured for the other lineages of TOSV: lineage A (already isolated) and lineage C (need to be isolated) (138). We estimated TOSV EIP in *Ph. perniciosus* and showed the influence of the virus dose taken by the sand fly upon this period. Other factors may influence EIP (201). We could thus measure the effect of temperature or the vector microbiota on TOSV EIP. We detected the presence of *Wolbachia* in the microbiota of sand flies from our colonies (data not shown). As previously described, these bacteria can have an impact on vector competence (233), it would be interesting to reproduce our experiments by treating the sand flies to make them *Wolbachia*-negative and to compare infection kinetics. We estimated the TOSV EIP by determining the time when the sand fly spits viral RNA into the sugar meal. To confirm this result, it will be necessary to reproduce this experiment with more samples and to verify the TOSV infectivity by $TCID_{50}$ titration. An alternative to our method would be sand fly salivation and TOSV quantification in saliva directly (234).

We know that TOSV can be transmitted vertically, but the exact part of the female genitalia it replicates still need to be explored. In general, we do not have the precise locations of viral replication in the vector. This is why we collaborated with Dr. Ratinier Maxime, Dr. Arnaud Frédérick and Thiesson Adrien (IVPC laboratory, Lyon University) to work with a fluorescent TOSV strain developed by reverse genetics. We succeeded in setting up this strain by

inserting a red fluorescent protein in the S segment of TOSV lineage B, as previously done for TOSV lineage A (235). We will try to infect *Ph. perniciosus* with this strain to test its virulence and to localize the virus under the microscope on freshly dissected sand flies, as we already do with GFP-tagged *Le. infantum*. TOSV labelling will also allow to localize the virus during a sugar meal infection and determine whether it passes directly from the crop to the haemolymph, or whether it passes first through the sand fly gut. We measured the survival of sand flies infected with TOSV through a sugar meal (no significant impact), but the kinetics of infection through a sugar meal remains to be done.

The next step will be to carry out experimental co-infection of *Ph. perniciosus* with *Le. infantum* and TOSV. We have already established protocols for simultaneous and sequential infections. The objective will be to observe the infection dynamics and possible competitive, mutualistic or neutral interactions between the two pathogens within the vector. Having GFP-tagged *Le. infantum* and RFP-tagged TOSV will allow us to distinguish them in the vector using the fluorescence microscope.

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Published

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- Ayhan Nazli*, Prudhomme Jorian*, **Laroche Lison**, Bañuls Anne-Laure, Charrel Rémi (2020). Broader geographical distribution of Toscana virus in the Mediterranean region suggests the existence of larger varieties of sand fly vectors. *Microorganisms*, 8(1), 114.

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- **Lison Laroche**, Nazli Ayhan, Rémi Charrel, Anne-Laure Bañuls, Jorian Prudhomme (2022). Persistence of Toscana virus in sugar and blood meals of phlebotomine sand flies: epidemiological and experimental consequences. *Under revision in Scientific reports.*
- Jorian Prudhomme*, **Lison Laroche***, Anh Nguyễn Hoàng Quân, Justine Fournier, Idris Mhaidi, Aloïs Berhard, Mireille Killick-Kendrick, Anne-Laure Bañuls (2022). Establishment and maintenance of two sand fly colonies (*Phlebotomus perniciosus* and *Phlebotomus papatasi*) for implementation of experimental infections. *Submitted to the journal Parasite*

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- **Lison Laroche**, Nazli Ayhan, Rémi Charrel, Anne-Laure Bañuls, Jorian Prudhomme. Intra-sand fly dynamics of Toscana virus and impact on *Phlebotomus perniciosus* life-history traits. *In Preparation*

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LIST OF COMMUNICATIONS

ESOVE 2022 – 22nd International Congress, Sofia, Bulgaria. Oral presentation: "Sand fly and Toscana virus: impact on vector life-history traits using experimental infection and potential effects on transmission." **Award** for the best PhD oral presentation.

Symposium "Behaviour of Arthropods Disease Vectors" 2022 – International conference at Montpellier University. Poster presentation: "Experimental infection of *Phlebotomus perniciosus* with Toscana virus: viral infection dynamics and impact on life history traits"

Agapiades & PhDays 2022 – Conference of IRD and CIRAD Montpellier PhD students. Oral presentation: "Experimental infection of *Phlebotomus perniciosus* with Toscana virus: impact on life history traits and transmission"

MEEGID XV 2021 – International Congress online. Oral presentation: "Incubation Period for Neuroinvasive Toscana Virus Infections"

PhDays 2021 – Conference of IRD Montpellier PhD students. Oral presentation: "Determination of the incubation period for neuroinvasive cases of Toscana virus infection"

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LIST OF ABBREVIATIONS

ACBV	Alcube virus
ARBV	Arbia virus
BSL-3	Biosecurity level 3 laboratory
CL	Cutaneous leishmaniasis
DNA	Deoxyribonucleic acid
EIP	Extrinsic incubation period
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
GFP	Green fluorescent protein
HIV	Human immunodeficiency virus
IFA	Indirect fluorescent antibody
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IIP	Intrinsic incubation period
LHT	Life history traits
LRV	*Leishmania* RNA virus
MALDI-TOF	Matrix assisted laser desorption ionization time of flight
MASV	Massilia virus
MCL	Muco-cutaneous leishmaniasis
NS	Nonstructural protein
PBS	Phosphate-buffered saline
PUNV	Punique virus
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RNAi	RNA interference
RTqPCR	Reverse transcriptase quantitative polymerase chain reaction
RVFV	Rift Valley fever virus
SALV	Salehabad virus
SFNV	Sandfly fever Naples virus
SFSV	Sandfly fever Sicilian virus
TOSV	Toscana virus
VL	Visceral leishmaniasis
WNV	West Nile virus

APPENDICES

Appendix 1. Experimental infection protocol

Experimental infections of females *Ph. perniciosus* with parasites or viruses were performed in enhanced biosafety level 3 (BSL-3) containment laboratories (IRD Vectopôle, Montpellier, France). Twenty-four hours prior to the experiment, 5 to 9-day-old females from the colony were starved by deprivation of sugar meal. A few hours before infection, approximately 100 females were transferred into a feeding pot and stored in the BSL-3 climate chamber under standard conditions (26 ± 1°C, 80% rh, 2% brightness) to acclimatize. Approximately 10% males were added to excite females to take blood and to continue mating. Glass feeders were fill of heat-inactivated rabbit blood and covered with a chick skin membrane (**Figure 1**). For infected batches (TEST1, TEST2), a volume of parasite or virus is added to the blood at a precise final concentration. For control batch (CTRL), the same volume of parasite or viral culture medium is added, without the pathogen agent. The complete system is installed in the climate chamber where the feeders were connected to a 37°C circulating water bath and fixed to the feeding pots. Feeding is performed for six hours, reducing the brightness to 1%, with a regular manual blood mix to avoid clotting. As engorged females are very sensitive, it is recommended to handle them after the first 24 hours of digestion. Thus, they were sorted on ice the day after the artificial blood meal and transferred into the climate chamber. Males and non-engorged females were killed and removed.

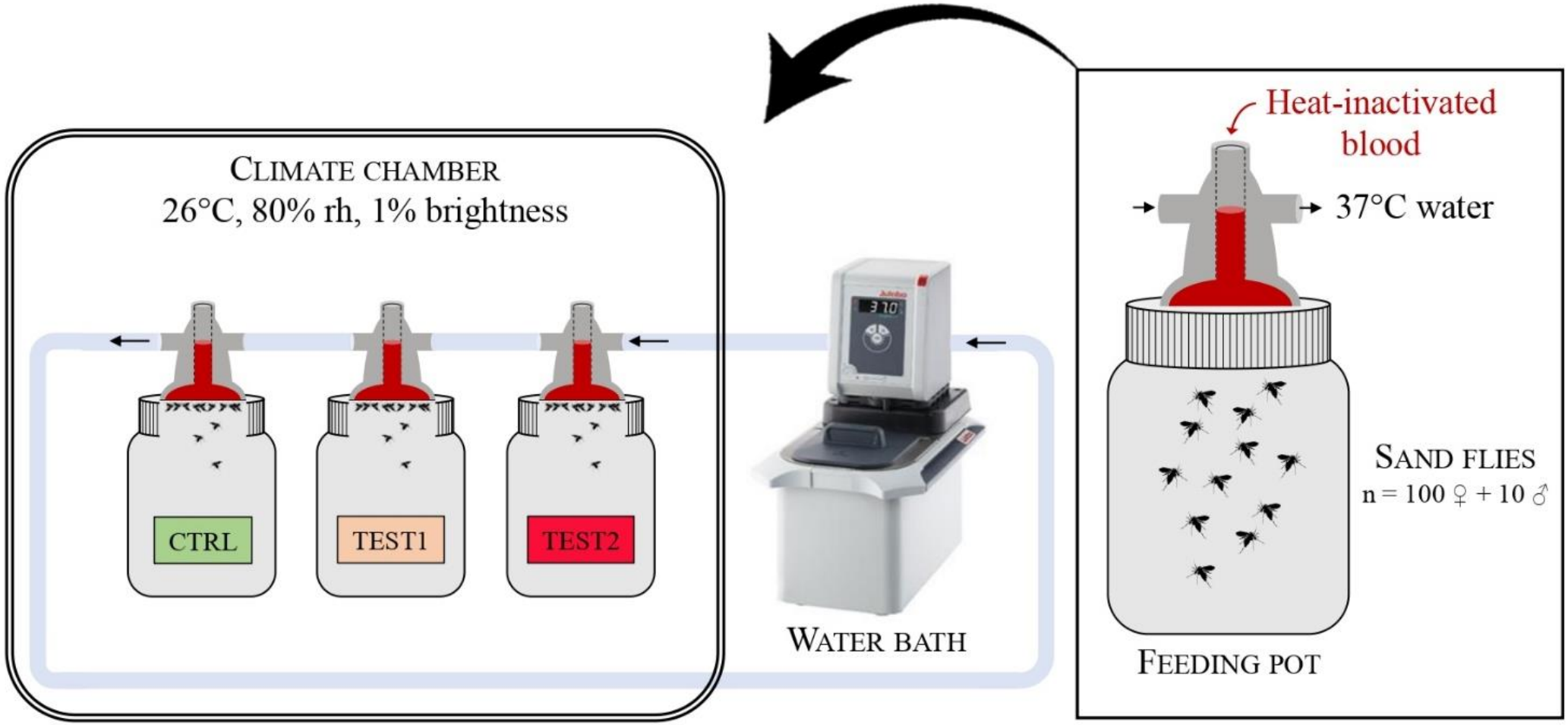


Figure 1. Blood feeding system of female sand flies in a BSL-3 laboratory.

ABSTRACT

Sand flies, present in all intertropical regions, are dipteran insects belonging to the Psychodidae family and the Phlebotominae subfamily. About a hundred species have been identified as vectors, of which 40% are proven vectors. These species are involved in the transmission of several pathogens such as *Leishmania* and *Phlebovirus*. *Leishmania* are Kinetoplastidae protozoa of the Trypanosomatidae family belonging to the genus *Leishmania* responsible for parasitic diseases. They infect a wide range of vertebrate hosts, including humans. For example, *Leishmania infantum* is a species that circulates in the Mediterranean region and can cause severe visceral forms, lethal if left untreated. Viruses of the genus *Phlebovirus* belong to the Phenuiviridae family and the Bunyavirales order. Some *Phlebovirus* are associated with human diseases, such as Toscana virus (TOSV) which present a neurotropic activity. The parasite *Le. infantum* and TOSV co-circulate in the Mediterranean basin and share some vector species, such as *Phlebotomus perniciosus*. Studies have shown co-infections in mammalian hosts, but none have been able to confirm this in sand fly natural populations. The biological interactions between TOSV and sand flies and between these two pathogens in host and vector are still poorly understood.

In this context, the main objective of this thesis project is to study, through an experimental approach, the interactions of these two pathogens with their vector to better understand the impact on their transmission and on the sand fly biology. This thesis is divided into three chapters. The first chapter deals with the sand fly model, the colony establishment and maintenance in our laboratory, and the development of experimental infections. The second chapter studies the interactions between the parasite *Le. infantum* and the vector *Ph. perniciosus*. These experiments show the infection impact on one of the sand fly life history traits: infected females lay significantly more eggs. The third chapter explores the natural cycle of TOSV in *Ph. perniciosus*. The study of infection dynamics in *Ph. perniciosus* reveals that, depending on the TOSV dose ingested by the vector, systemic infection occurs between three and five days. In addition, TOSV infection resulted in an increase of egg hatching time. Therefore, the results demonstrate that both pathogens have an impact on the vector biology and the infection conditions can have consequences on their transmission.

This thesis provides fundamental knowledge on the biology of vector-pathogen interactions in the sand fly-*Phlebovirus*-*Leishmania* model. The results represent a solid and essential baseline for the study of *Ph. perniciosus* co-infection by these two pathogens. In perspective, the study of their interactions within the vector will allow to determine whether the presence of one can modulate the infectious potential of the other and its transmission to the host.

RESUME

Les phlébotomes, présents dans toutes les régions intertropicales, sont des insectes diptères appartenant à la famille des Psychodidae et à la sous-famille des Phlebotominae. Une centaine d'espèces sont identifiées comme vecteurs dont 40% sont des vecteurs confirmés. Ces espèces sont impliquées dans la transmission de différents agents pathogènes tels que les leishmanies et les *Phlebovirus*. Les leishmanies sont des protozoaires Kinetoplastidae de la famille des Trypanosomatidae appartenant au genre *Leishmania* responsables de maladies parasitaires. Elles parasitent un large panel d'hôtes vertébrés, dont l'homme. Par exemple, *Leishmania infantum* est une espèce qui sévit sur le pourtour méditerranéen et qui peut provoquer des formes viscérales graves, mortelles en absence de traitement. Les virus du genre *Phlebovirus* appartiennent à la famille des Phenuiviridae et à l'ordre des Bunyavirales. Certains *Phlebovirus* sont associés à des pathologies humaines, tel que le virus Toscana (TOSV) qui présente une activité neurotrope. Le TOSV et le parasite *Le. infantum* co-circulent dans le bassin méditerranéen et partagent certaines espèces vectrices, telles que *Phlebotomus perniciosus*. Des études ont mis en évidence des co-infections chez des hôtes mammifères, mais aucune n'a pu le confirmer chez le phlébotome en population naturelle. Les interactions biologiques entre le TOSV et le phlébotome et entre ces deux agents pathogènes chez l'hôte et le vecteur sont encore méconnues.

Dans ce contexte, l'objectif principal de ce projet de thèse est d'étudier, par une approche expérimentale, les interactions de ces deux agents pathogènes avec leur vecteur afin de mieux comprendre l'impact sur leur transmission et sur la biologie des phlébotomes. Cette thèse est divisée en trois chapitres. Le premier est consacré au modèle phlébotome, à la mise en place et au maintien de l'élevage dans notre laboratoire, ainsi qu'au développement des infections expérimentales. Le second chapitre étudie les interactions entre le parasite *Le. infantum* et le vecteur *Ph. perniciosus*. Ces expériences ont mis en évidence l'impact de l'infection sur un des traits d'histoire de vie du phlébotome : les femelles infectées pondent significativement plus d'œufs. Le troisième chapitre explore le cycle naturel du TOSV chez *Ph. perniciosus*. L'étude de la dynamique d'infection chez *Ph. perniciosus* révèle que, selon la dose de TOSV ingérée par le vecteur, l'infection systémique survient entre trois et cinq jours. De plus, l'infection par le TOSV entraine une augmentation du temps d'éclosion des œufs des femelles. Ainsi, les résultats démontrent que ces deux agents pathogènes ont un impact sur la biologie du vecteur et que les conditions d'infection peuvent avoir des conséquences sur leur transmission.

Cette thèse apporte des connaissances fondamentales sur la biologie des interactions vecteur-pathogène sur le modèle phlébotome-phlébovirus-leishmanies. Les résultats constituent une base solide et indispensable pour l'étude de la co-infection de *Ph. perniciosus* par ces deux agents pathogènes. En perspectives, l'étude de leurs interactions au sein du vecteur permettrait de déterminer si la présence de l'un peut moduler le potentiel infectieux de l'autre et sa transmission chez l'hôte.

RESUME LONG

Les maladies à transmission vectorielle sont causées par des agents pathogènes (c'est-à-dire des parasites, des virus, des bactéries) qui sont inoculés et propagés par des vecteurs. Elles sont responsables de plus de 17 % des maladies infectieuses et causent plus de 700 000 décès chaque année dans le monde. Les vecteurs sont des arthropodes hématophages qui transmettent activement (mécaniquement ou biologiquement) un agent pathogène d'un vertébré à un autre. Il s'agit principalement d'insectes et d'acariens hématophages, comme les phlébotomes, les moustiques ou les tiques. L'épidémiologie des maladies à transmission vectorielle dépend de plusieurs facteurs : la distribution des vecteurs, la capacité et la compétence vectorielle ; l'infectivité de l'agent pathogène, sa spécificité d'hôte et sa résistance aux médicaments ; les activités humaines et les conditions environnementales (par exemple le climat). Les interactions vecteur-hôte-pathogène peuvent être modulées par ces facteurs, il est donc nécessaire de les comprendre pour prévenir la propagation des maladies vectorielles.

Cette thèse se concentre sur les interactions vecteur-pathogène entre phlébotome, *Leishmania* et *Phlebovirus*. Les phlébotomes sont distribués dans la plupart des régions intertropicales du monde et sont responsables de la transmission d'agents pathogènes. Ce sont de petits insectes appartenant à l'ordre des diptères, au sous-ordre des nématocères, à la famille des Psychodidae et à la sous-famille des Phlebotominae. Sur les quelques 1000 espèces identifiées et décrites dans le monde, au moins 98 ont été définies comme des vecteurs potentiels ou avérés. Les adultes se nourrissent de jus sucrés mais seules les femelles prennent des repas de sang nécessaires pour la maturation de leurs œufs. C'est par ce comportement hématophage que les phlébotomes peuvent affecter la santé humaine et contaminer les animaux domestiques

et sauvages en transmettant des agents pathogènes. La présence de phlébotomes vecteurs est considérée comme un facteur de risque pour l'émergence de maladies transmises par ces derniers. Par conséquent, leur présence et toute modification dans leur distribution sont à prendre en compte pour déterminer le changement potentiel de la distribution ou de l'expansion de ces maladies. Dans ce contexte, l'objectif principal de cette thèse est d'étudier les interactions vecteur-pathogène pour mieux comprendre l'impact sur la biologie du vecteur et la dynamique d'infection et de transmission. Cette thèse est divisée en trois chapitres, le premier sur le vecteur *Phlebotomus perniciosus*, le second sur le parasite *Leishmania infantum* et le troisième sur le virus Toscana.

Dans un premier chapitre nous décrivons la mise en place d'un élevage de phlébotomes dans le but de réaliser des infections expérimentales. En effet, l'établissement et le maintien de colonies de phlébotomes sont essentiels pour l'étude expérimentale de leur biologie, physiologie, comportement, compétence et capacité vectorielle. La présence de telles colonies permet également le développement de méthodes de contrôle des vecteurs, notamment pour les essais d'insecticides. Les colonies sont essentielles pour étudier les cycles naturels et la dynamique d'infection des parasites *Leishmania* et des *Phlebovirus*. Dans le monde, il existe très peu d'élevages de phlébotomes par rapport aux autres insectes vecteurs. Au début de ma thèse, nous avons mis en place des colonies pour deux espèces du genre *Phlebotomus* dans notre laboratoire à Montpellier (Institut de Recherche pour le Développement). Nous avons décidé de travailler avec l'espèce *Ph. perniciosus*, vecteur commun du parasite *Le. infantum* et du virus Toscana. L'objectif de ce travail était de donner une chronologie pour la mise en place d'une colonie depuis le début de l'élevage jusqu'à la mise en place des infections expérimentales, en passant par l'optimisation des méthodes d'élevage déjà décrites. De plus, plusieurs paramètres du cycle de développement de *Ph. perniciosus* ont été étudiés et décrits dans ce chapitre.

Dans un second chapitre, nous nous sommes intéressés au parasite *Leishmania*, transmis par les phlébotomes et responsables de la leishmaniose, une maladie qui peut être mortelle en l'absence de traitement. Les parasites *Leishmania* ont une répercussion importante en matière de santé publique dans le monde entier, notamment dans les pays en développement. Les traitements utilisés ont des effets secondaires graves, les parasites développent de plus en plus de résistance à ces derniers et il n'existe actuellement aucun vaccin humain. Il est nécessaire d'intensifier les recherches sur cette maladie, en particulier les études sur les interactions entre les parasites et leurs vecteurs, afin de mieux comprendre la dynamique de transmission et d'anticiper la propagation de la leishmaniose. De nouvelles approches pour réduire la transmission des maladies vectorielles, telles que les vaccins anti-vecteurs bloquant la transmission, ont été rendue possible par des études expérimentales sur les interactions vecteur-parasite. Bien que plusieurs publications aient porté sur ces aspects, peu d'entre elles ont examiné l'impact de l'infection sur les traits d'histoire de vie des phlébotomes. Les informations sur l'impact des parasites sur leurs vecteurs peuvent améliorer la connaissance de la biologie du vecteur et plus particulièrement la compétence vectorielle. Ainsi, nous avons exploré comment *Le. infantum* affecte la biologie de *Ph. perniciosus.* Nous avons observé une augmentation du nombre d'œufs pondus par les femelles *Ph. perniciosus* infectées par *Le. infantum*. L'augmentation de la ponte pourrait être une forme d'investissement terminal, les organismes ayant de faibles chances de survie consacrant leur énergie à la reproduction avant de mourir. Lors de l'étude de la dynamique d'infection de *Le. infantum* chez le vecteur, nos résultats étaient conformes aux cycles précédemment bien décrits dans la littérature, puisque les parasites ont atteint la valve stomodéale à huit jours post-infection. Cependant, nous avons démontré pour la première fois qu'un petit inoculum de *Leishmania* se développe chez le phlébotome aussi rapidement qu'un grand inoculum. Ces résultats préliminaires, malgré une

observation sur peu d'échantillons, pourraient entrainer un impact sur la dynamique de transmission.

Dans un troisième et dernier chapitre, nous nous sommes intéressés aux virus du genre *Phlebovirus*, également transmis par les phlébotomes, et responsables de maladies infectieuses en expansion dans le bassin méditerranéen. Ces virus à ARN enveloppés et tri-segmentés appartiennent à la famille des Phenuiviridae dans l'ordre des Bunyavirales. Nous avons travaillé avec le virus Toscana, responsable de méningites et méningo-encéphalites dans le bassin méditerranéen. En raison de son caractère neurotrope, le virus Toscana est actuellement le phlébovirus le plus préoccupant en termes de santé publique. En outre, en raison des changements globaux, la distribution géographique de ce virus est susceptible de s'étendre en adéquation avec les phlébotomes vecteurs. Pour mieux comprendre la dynamique de transmission, nous explorons dans des conditions expérimentales les interactions entre le virus Toscana et le vecteur *Ph. perniciosus*. Dans une première partie, nous avons mesuré la persistance de l'infectivité virale dans les repas sanguins et sucrés du phlébotome, afin d'estimer le risque épidémiologique et la pertinence de la méthodologie d'infection expérimentale. Nos résultats ont montré que l'ARN du virus Toscana était détectable à une quantité stable jusqu'à 15 jours dans une solution sucrée à 26°C et jusqu'à six heures dans du sang à 37°C. De plus, le virus Toscana reste infectieux pendant sept jours dans une solution sucrée et jusqu'à six heures dans le sang. Le virus a montré une infectivité/viabilité persistante dans différentes conditions, ce qui peut avoir des conséquences épidémiologiques non négligeables et soulève de nouvelles hypothèses sur son cycle naturel. Dans une seconde partie, nous avons déterminé la cinétique d'infection avec différentes doses de virus Toscana, estimé la période d'incubation extrinsèque à environ six jours, et mesuré les impacts de l'infection sur les traits d'histoire de vie (survie, temps de développement, fécondité) du vecteur infecté. De plus, nous avons montré que 50%

des phlébotomes infectés par 10^6 $TCID_{50}$/ml de virus avaient une infection systémique au bout de trois jours seulement. Cette étude a permis d'améliorer nos connaissances sur l'écologie du virus Toscana et ses voies de transmission.

Ainsi, ce travail souligne la pertinence d'étudier les interactions vecteur-pathogène afin de mieux comprendre les dynamiques d'infection et de transmission et de prévenir la propagation des maladies vectorielles. La recherche sur les interactions insecte-pathogène permet de comprendre les processus qui déterminent la capacité de l'insecte à transmettre des pathogènes humains ou animaux. Cette thèse confirme la nécessité de prendre en compte le vecteur, l'agent pathogène, l'hôte et l'environnement dans un système infectieux afin de prévenir les émergences de manière durable, qui sont des préoccupations centrales dans une perspective One Health.