Development of *Leishmania orientalis* in the sand fly *Lutzomyia longipalpis* and the midge *Culicoides soronensis*

Wetpisit Chanmol[a], Narissara Jariyapan[b]*, Pradya Somboon[b], Michelle D. Bates[c] and Paul A. Bates[c]

[a]*Graduate PhD Degree Program in Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand*

[b]*Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand*

[c]*Division of Biomedical and Life Sciences, Faculty of Health and Medicine, Lancaster University, UK*

***Corresponding author. Tel.: +66819381855**

E-mail address: wetpisitchanmol@gmail.com (W. Chanmol)
njariyapan@gmail.com (N. Jariyapan)
pradya.somboon@cmu.ac.th (P. Somboon)
m.bates@lancaster.ac.uk (M.D. Bates)
p.bates@lancaster.ac.uk (P.A. Bates)

ABSTRACT

Leishmania (*Mundinia*) *orientalis* is a new species causing human leishmaniasis in Thailand whose natural vector is unknown. *L. orientalis* infections in sand flies and/or midges under laboratory conditions have not been previously investigated. In this study, the development of *L. orientalis* in two experimental vectors, *Lutzomyia longipalpis* sand flies and *Culicoides sonorensis* midges was investigated for the first time using light microscopy, scanning electron microscopy, and histological examination. The results showed that *L. orientalis* was unable to establish infection in *Lu. longipalpis*. No parasites were found in the sand fly gut 4 days post-infected blood meal (PIBM). In contrast, the parasite successfully established infection in *C. sonorensis*. The parasites differentiated from amastigotes to procyclic promastigotes in the abdominal midgut (AMG) on day 1 PIBM. On day 2 PIBM, nectomonad promastigotes were observed in the AMG and

migrated to the thoracic midgut (TMG). Leptomonad promastigotes appeared at the TMG on day 3 PIBM. Clusters of leptomonad promastigotes and metacyclic promastigotes colonized around the stomodeal valve with the accumulation of a promastigote secretory gel-like material from day 3 PIBM onwards. Haptomonad-like promastigotes were observed from day 5 PIBM, and the proportion of metacyclic promastigotes reached 23% on day 7 PIBM. The results suggest that biting midges or unusual sand flies might be vectors of *L. orientalis*.

Keywords: Leishmania orientalis, *Mundinia*, *Lutzomyia longipalpis*, *Culicoides sonorensis*, Leishmaniasis, Thailand

1. Introduction

Leishmaniasis, a vector-borne disease, has been reported all over the world, especially in tropical and sub-tropical areas. Among the 54 known species of *Leishmania* parasites, 21 species have been reported as human pathogens, mostly belonging to the subgenera *Leishmania* (*Leishmania*) and *Leishmania* (*Viannia*) (Akhoundi et al., 2017). However, parasites in the new subgenus *Leishmania* (*Mundinia*) (Espinosa et al., 2018) are becoming increasingly important to human health. Three species of parasites in the subgenus *Mundinia* have been reported to infect humans, these being *Leishmania martiniquensis, Leishmania* "Ghana strain", and *Leishmania orientalis* (previously called "*Leishmania siamensis*") (Pothirat et al., 2014; Chiewchanvit et al., 2015; Kwakye-Nuako et al., 2015; Jariyapan et al., 2018). The two other known species are *Leishmania enrietti*, found in guinea pigs (*Cavia porcellus*), and *Leishmania macropodum* (previously called "*Leishmania* sp. AM-2004"), found in red kangaroos and other macropods (Rose et al., 2004; Dougall et al., 2011; Barratt et al., 2017).

Sand flies in the genera *Phlebotomus* and *Lutzomyia* are the proven vectors of leishmaniasis in the Old World and the New World, respectively (Maroli et al., 2013). However, although *Culicoides* midge spp. are currently not considered to be vectors of *Leishmania* parasites, *Leishmania* DNA has been found in several species. In Tunisia, Slama et al. (2014) have detected *Leishmania infantum* DNA in wild caught *Culicoides*

spp., *Leishmania braziliensis* DNA has been detected in *Culicoides ignacioi*, *Culicoides insignis*, and *Culicoides foxi*; and *Leishmania amazonensis* DNA in *Culicoides filariferus* and *Culicoides flavivenula* (Rebêlo et al., 2016). Further, *L. macropodum* parasites have been isolated from field-collected *Forcipomyia* midges in Australia (Dougall et al., 2011). These findings have led to investigations of the vector competence of midges for *Leishmania* under laboratory conditions. Development of *Leishmania infantum* in a laboratory colony of *Culicoides nubeculosus* was investigated, but it was found that *L. infantum* could not complete its development in the midgut of *C. nubeculosus* (Seblova et al., 2012). In contrast, Seblova et al. (2015) demonstrated that *L. enriettii* parasites are capable of developing to the late stage of infection in *Culicoides sonorensis*, and that this species of midge could become infected by feeding on *L. enriettii*-infected domestic guinea pigs (*Cavia porcellus*). However, *L. enriettii* parasites were unable to develop to maturity in the usually permissive sand fly vector, *Lutzomyia longipalpis.* In *C. sonorensis*, *L. macropodum* also developed early stage infections at high rates with moderate infections (100-1,000 promastigotes/gut) (Seblova et al., 2015). The findings have suggested the possibility that biting midges may be capable of infection with *Leishmania* parasites belonging to the members of the subgenus *Mundinia*, and could participate in the transmission of leishmaniasis.

L. orientalis is a new species causing leishmaniasis among Thai patients (Jariyapan et al., 2018). A recent study has shown that "*L. siamensis*" DNA has been detected in one female sand fly, *Sergentomyia iyengari* (Siripattanapipong et al., 2018). However, development of *L. orientalis* infection and the vector competence of sand flies and midges for *L. orientalis* have never been investigated and determined under laboratory conditions. Using wild caught midges from infected areas to investigate the development of *Leishmania* parasites is not currently feasible, since no information is available on likely sand fly or midge vectors or colonized insects from Thailand. Therefore, investigation of the development of *L. orientalis* in two experimental vectors, *Lu. longipalpis* and *C. sonorensis*, was performed in this study.

2. Materials and methods

2.1. Parasite strain

L. orientalis parasites (MHOM/TH/2014/LSCM4) were used in this study (Jariyapan et al., 2018). Axenic amastigotes were cultured in Grace's insect medium (Life Technology-Gibco, Grand Island, NY, USA) supplemented with FCS 20%, 2% human urine, 1% BME vitamins (Sigma-Aldrich, St Louis, MO, USA), and 25 µg/ml gentamicin sulfate (Sigma-Aldrich, St Louis, MO, USA), pH 5.5 at 35°C (Chanmol et al., 2019).

2.2. Vectors

Lu. longipalpis (Jacobina colony) was maintained at Lancaster University, UK under standard conditions (Modi and Tesh, 1983). Females from a colony of *C. sonorensis* were sent to Lancaster University from the Pirbright Institute, UK. All insects were kept under controlled temperature 26°C, humidity > 80% and photo period 8 h light/ 16 h darkness and fed on a diet consisting of autoclaved 70% w/v sucrose solution on cotton wool *ad libitum* before exposure to feeding.

2.3. Membrane feeding on infected blood

All infection experiments were performed at Lancaster University, UK. In each experiment, approximately 500 *Lu. longipalpis* or *C. sonorensis* females (both 4-5 days old) were fed through a chick-skin membrane on sheep blood containing $5x10^6$ *L. orientalis* axenic amastigotes/ml via a Hemotek membrane feeder (Discovery workshops, UK) for 1 h. After 1 h post-infected blood meal (PIBM), fully engorged females were separated and maintained at 26°C. The infected flies were dissected for 7 consecutive days PIBM and at least 10 flies were dissected per day. The localization and intensity of *Leishmania* infection in guts were evaluated *in situ* under a light microscope, by scoring the proportion of flies with low (<100 parasites/gut), moderate (100-1,000 parasites/gut) or heavy (>1,000 parasites/gut) infections (Seblova et al. 2015) and parasite location was recorded as abdominal midgut (AMG), thoracic midgut (TMG), and stomodeal valve (SV). All experiments were performed in triplicate.

2.4 Light microscopy (LM)

Smears from midguts of *Lu. longipalpis* or *C. sonorensis* (1-7 days PIBM) infected with *L. orientalis* were fixed with methanol, stained with 5% Giemsa solution, examined under a light microscope (Olympus America Inc., USA) with an oil-immersion objective and measured using Olympus CX41. Body length, flagellar length and body width of parasites were measured for determination of morphological forms according to the criteria of Chanmol et al. (2019). The following morphological forms were distinguished: (i) procyclic promastigote: body length 8.0-11.5 µm and flagellar length < body length; (ii) nectomonad promastigote: body length ≥ 12.5 µm and flagellar length varied (iii) leptomonad promastigote: body length 8.0-11.5 µm and flagellum ≥ body length; (iv) metacyclic promastigote: body length <11.5 µm and flagellar length ≥ two times body length. A minimum of 200 parasites was examined and classified at each time point. In addition, infected flies were fixed with 4% paraformaldehyde-PBS for histology and midguts of infected flies were fixed with 2.5% glutaraldehyde in 0.1 cacodylate buffer (pH 7.2) for scanning electron microscopy (SEM).

2.5 Scanning electron microscopy

For SEM, midguts were fixed with 2.5% glutaraldehyde in 0.1 cacodylate buffer (pH 7.2) for a few days at 4 °C. After washing with the same buffer, the cells were dehydrated in a graded series of ethanol (50%, 70%, 90%, 95% for 10 min each and then twice with 100% ethanol for 30 min each). After that, the specimens were placed in acetone for 2 h, followed by critical point drying in liquid CO_2 and coated with gold particles in a sputter-coating apparatus. The gold-coated preparations were examined under a scanning electron microscope (JEOL JSM- 5910LV, JEOL Ltd., Japan), at 25-30 kV. To observe the development of the parasites in each day PIBM, some fixed samples were fractured before being coated with gold, while others were gently opened and the contents were washed out with phosphate buffer saline before the fixation.

2.6 Histological examination

The infected flies collected on each day PIBM were fixed in 4% paraformaldehyde-PBS for 1 week and subsequently embedded in paraffin. Sections (5 mm) were cut on a microtome (Zeiss Hyrax M25) and stained with Hematoxylin-Eosin (HE). Photomicrographs were taken on an image-capturing microscope (Olympus CX41, Olympus America Inc., USA).

3. Results

3.1. Infection of L. orientalis in Lu. longipalpis

Lutzomyia longipalpis females were experimentally infected with *L. orientalis* axenic amastigotes. A high infection rate was obtained on days 1 and 2 post-infected blood meal (PIBM) corresponding to an average of 76.7% and 83.3%, respectively (Fig. 1). All parasites were located in the AMG. However, following defecation of blood meal remnants in the majority of flies, the infection rate was reduced to 10% on day 3 PIBM. The only parasite stages observed in *Lu. longipalpis* were procyclic promastigotes (Fig. 2), the next developmental stage, nectomonad promastigotes, or any other stage were not seen. On days 4-7 PIBM, no parasites were found in *Lu. longipalpis.*

3.2. Infection of L. orientalis in C. sonorensis

The infection of *C. sonorensis* by *L. orientalis* axenic amastigotes was performed using the same procedures as described above. In these experiments, approximately 40-50% of fully blood-engorged female midges were obtained from each population after feeding on the infected-blood meal. An infection rate of 100% was observed on day 1 PIBM, no uninfected blood-fed midges were found in the three experiments performed (Fig. 3). On day 2 PIBM due to defecation, the infection rate was slightly reduced to 93.94%, and thereafter the rate of infection gradually decreased day by day to reach 21% by day 7 PIBM, coinciding with a decrease of infection intensities in the midges

(Fig. 3). Nevertheless, some infected midges were observed through to the end of the observation period, in contrast to what was observed with *Lu. longipalpis*.

3.3 Development of L. orientalis in C. sonorensis

The development of the parasite in *C. sonorensis* was investigated throughout the seven consecutive days of infection observed. On day 1 PIBM, the *L. orientalis* axenic amastigotes had mainly (86.5%) transformed into procyclic promastigotes (Fig.4). All of these parasites were localized in the AMG (Fig. 5), with morphology typical of procyclic promastigotes, possessing a relatively short cell body with small flagellum (Fig. 6A). On day 2 PIBM, during which defecation of blood remnants occurred, the majority of parasites were still found in the AMG, but some had spread forward to the TMG and backwards into the pylorus (Fig. 5). The parasites had also undergone morphological change, with the majority (74%) having transformed into nectomonad promastigotes (Fig. 4, 6B). The remaining parasite population was procyclic promastigotes (26%) on day 2 PIBM. On day 3 PIBM, the overall population had migrated further forward, with the parasites more or less evenly distributed between the AMG and TMG, a small number remained in the pylorus (Fig5). The population was still dominated by nectomonad promastigotes (66%), exhibiting typical morphology (Fig. 6C), but the first significant numbers of leptomonad promastigotes (14%) were observed (Fig. 4). In addition, in some midges the colonization of the SV and presence of some metacyclic promastigotes were observed on day 3 PIBM. From days 4-7 PIBM, (Fig. 4, 7), the proportion of nectomonad promastigotes decreased, whilst both leptomonad and metacyclic promastigotes increased (Fig. 4), these being in the SV region (Fig. 6D, E), exhibiting characteristic morphology (Fig. 6F, 6G). During this period, the infections that remained became increasingly concentrated in anterior regions of the midge gut, such that by days 6 and 7 PIBM the only infections found were in the SV (Fig. 5). Clusters of leptomonad promastigotes and metacyclic promastigotes around the opening of the SV appeared to be embedded in some material under SEM (Fig. 6E), which had the gelatinous appearance of PSG under light microscopy (Fig. 7A, C). In addition, although they were not significantly represented in the counted populations, probably because they remained attached to the gut, forms

resembling haptomonad promastigotes were seen by SEM (Fig. 6H) and in histological sections of the SV (Fig. 7B). Haptomonad forms attach to cuticle-lined parts of the gut via their flagella. Finally, over days 5-7 PIBM the proportion of metacyclic promastigotes in the midge parasite populations gradually increased, and reached 23% by day 7 PIBM.

4. Discussion

The life cycle of *Leishmania* spp. in their proven vectors, female phlebotomine sand flies, has been studied in a variety of vector-parasite combinations (Dostalova and Volf, 2012). This work led to the description of five main promastigote forms of *Leishmania* spp. in their sand fly vectors: procyclic promastigotes, nectomonad promastigotes, leptomonad promastigotes, metacyclic promastigotes, and haptomonad promastigotes. Of these, procyclic promastigotes and leptomonad promastigotes are multiplicative forms of the parasites in the sand fly gut, and the developmental cycle of the parasites concludes with metacyclogenesis, the process of differentiation of leptomonad promastigotes to highly infective metacyclic promastigotes (Gossage et al., 2003; Bates, 2007; Dostálová and Volf, 2012). Haptomonad promastigotes use hemi-desmososme like structures in their flagella to attach to cuticle-lined parts of the gut. Recently, Serafim et al. (2018) have demonstrated that metacyclic promastigotes can dedifferentiate in the sand fly gut into new leptomonad-like replicative stages called retroleptomonad promastigotes upon ingestion of additional blood meals. These retroleptomonad promastigotes multiply and then rapidly redifferentiate into metacyclic promastigotes, increasing in the number of the infective stage parasites and enhancing infectiousness (Bates, 2018).

In their sand fly vectors, various factors have been identified that influence the successful establishment and transmission of *Leishmania* parasites, including midgut proteolytic enzymes, navigation of the peritrophic matrix (PM) barrier, midgut epithelium attachment, differentiation of parasites, colonization at the stomodeal valve, and PSG synthesis (Bates, 2007; Dostálová and Volf, 2012). In the early phase of the infection in sand flies, secretion of digestive enzymes is induced by ingestion of the blood meal and a chitinous PM is formed (Pruzinova et al., 2015). Increased protease activities (trypsin and chymotrypsin-like enzymes) were detected at 6 h post blood meal

(PBM) and the highest levels have been observed at 18-48 h PBM depending on the species of sand fly (Dillon and Lane, 1993; Telleria et al., 2010). Several studies have shown that activities of digestive enzymes affect *Leishmania* development and reduce parasite burdens in the midgut of sand flies on the first day after blood feeding (Pimenta et al., 1997; Schlein and Jacobson, 1998; Rogers et al., 2002). During blood digestion, procyclic promastigotes proliferate and differentiate to nectomonad promastigotes inside the endoperitrophic space surrounded by the PM. Nectomonad promastigotes escape from the blood bolus by migrating through the breaking down PM that surrounds the blood meal, and then attach to the midgut epithelium microvilli to mitigate against being expelled from the midgut by defecation. The mechanism of attachment to the gut epithelium depends on the parasite-vector pair (Wilson et al., 2010; Jecna et al., 2013). Binding to midgut microvilli of nectomonad promastigotes is mediated by lipophosphoglycan (LPG) and other parasite expressed glycoconjugates depending on the *Leishmania* species (McConville et al., 1992; Dostálová and Volf, 2012), and gut-associated lectins on the midgut epithelium expressed in each sand fly species (Kamhawi et al., 2004; Myskova et al 2016). Following defecation, established infections undergo further developmental processes culminating in metacyclogenesis (Pimenta et al., 1997; Dostálová and Volf, 2012).

In this study, *L. orientalis* was unable to develop to the infective stage inside *Lu. longipalpis*, despite high infection rates on day 1 and 2 PIBM, and successfully transforming into procyclic promastigotes. By day 3 PIBM the infection rate was very low, and from day 4 PIBM onwards no parasites were observed in any dissected flies. These results show that *L. orientalis* is unable to establish in *Lu. longipalpis*. This sand fly species is generally permissive to most species of *Leishmania*, supporting their development through to metacyclic promastigotes, but similar results to those described here were also obtained with two other *Mundinia* species, *L. enriettii* and *L. macropodum* (Seblova et al., 2015). The loss of infections observed here might be due to inability to bind to the gut and defecation, but probably not to the existence of a PM barrier, as in *Lu. longipalpis* the PM starts to disintegrate from 48 h after ingestion (Secundino et al., 2005). At the end of blood meal digestion, around 72 h, the size of the *Lu. longipalpis* midgut is very similar to an unfed midgut. Interestingly, no nectomonad promastigotes were observed in the infected *Lu. longipalpis*, in agreement with previous

observations that differentiation of procyclic promastigotes to nectomonad promastigotes is required for establishment of *Leishmania* in sand flies.

In the infected *C. sonorensis,* although blood remnant defecation occurred rapidly (day 2) it did not cause significant parasite clearance and the infection rate was still high day 2-3 PIBM. Nectomonad promastigotes were found in the AMG and TMG on day 2 PIBM as the population migrated forward to the anterior midgut. Given the failure of *L. orientalis* to become established in *Lu. longipalpis* but succeed in *C. sonorensis*, these results indicate that *L. orientalis* nectomonad promastigotes must have different surface glycoconjugates such as LPG or other molecules compared most other *Leishmania*, so the promastigotes were unable to interact with the gut microvilli of *Lu. longipalpis* but were able to bind the gut microvilli of *C. sonorensis*. Characterisation of the surface glycoconjugates of *L. orientalis* might provide useful information for identifying the vector(s) of *L. orientalis* in nature.

On days 4-7 PIBM, *L. orientalis* metacylic promastigotes were found at the TMG and SV of the midges indicating the parasites were able to generate potentially transmissible infections. Similar results were observed by infection of *C. sonorensis* with *L. enriettii* and *L. macropodum* (Seblova et al., 2015). In addition to the presence of metacyclic promastigotes in the anterior midgut of infected vectors, the generation of a PSG plug is an important factor for their successful transmission (Rogers et al., 2002; Rogers et al., 2004; Bates, 2007). PSG is produced by leptomonad promastigotes in the late phase of infection, with both metacyclic and leptomonad promastigotes packed in filamentous proteophosphoglycans, producing a "blocked fly" that forces the infected sand flies to regurgitate infective stages before they can take another blood meal (Rogers et al., 2002; Rogers et al., 2004). In the current study, LM, SEM, and histological examination of the *L. orientalis* infected midguts of the *C. sonorensis* midges revealed dense parasite clusters in a gel-like material similar to PSG. Further, the SV region was colonized by leptomonad and metacyclic promastigotes. These findings confirmed successful infection and development of *L. orientalis* in the *C. sonorensis* midges. Interestingly, PSG-like material was also found in *Forcipomyia* midges infected with *L. macropodum* in Australia (Dougall et al., 2011). Presumably, PSG found in midges could play the same role as in sand flies in facilitating the transmission of *Leishmania* parasites to mammalian hosts during biting for blood meals.

The presence of haptomonad promastigotes is also characteristic of transmissible infections. Definitive identification of these forms was not possible in the current study, as transmission electron microscopy is required to demonstrate the presence of hemi-desmosomal attachment, however, forms very similar to haptomonad promastigiotes were also observed.

The role of sand flies as vectors of *L. orientalis* in Thailand is unclear. Several surveys of sand fly species have been conducted in the affected areas in different parts of Thailand. More than 27 species of the four genera of sand flies including *Sergentomyia*, *Phlebotomus*, *Idiophlebotomus*, and *Chinius* have been identified (Apiwathsorn et al., 2011). Also, DNA of *L. martiniquensis* has been detected in *Sergentomyia gemmea* (Kanjanopas et al., 2013) and *Sergentomyia barraudi* (Chusri et al. 2014). However, dissections demonstrating metacyclic promastigotes at the SV of the sand flies were not reported in those studies. Our results show that *L. orientalis* could be present in *Lu. longipalpis* for up to three days PIBM but could not produce transmissible infections. Therefore, the presence of *Leishmania* DNA alone cannot be reliably used to indicate the identity of potential vectors of the parasite. Dissection for metacyclic promastigotes at the SV of suspected vectors is a prerequisite for the identification of the real vector of transmission of *Leishmania* parasites in nature (Seblova et al. 2012). Our findings support the hypothesis that biting midges could be natural vectors of the parasites belonging to the members of the subgenus *Mundinia*.

5. Conclusion

L. orientalis was able to establish infection in *C. sonorensis* midges but not *Lu. longipalpis* sand flies. Metacyclic promastigotes were found colonized at the SV and mixed with leptomonad promastigotes producing PSG-like material. The results suggest that the vector(s) of *L. orientalis* in Thailand might be biting midge(s) or unusual sand fly(s). The current study provides an important advance in understanding the biology of the new *Leishmania* species *L. orientalis*, and prepares the way for further study on its transmission in nature.

Authors' contributions

NJ conceived the idea and designed the study. WC, NJ, MDB and PS performed the data collection, analysis and interpretation of this manuscript. NJ and PB led the writing and revision of this manuscript. All authors read and approved the final version of the manuscript.

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Conflicts of interest

All authors declare that they have no conflict of interest.

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506
507 **FIGURE LEGENDS**

Fig. 1. Experimental infection of *Lu. longipalpis* sand flies with *L. orientalis.* Intensities of infection were estimated as light (<100 promastigotes/gut), moderate (100-1,000 promastigotes/gut) or heavy (>1,000 promastigotes/gut). Numbers above each bar indicate the number of dissected female sand flies. The results are the average from three independent experiments.

Fig. 2. Giemsa-stained smear showing procyclic promastigotes (rosette form) in the AMG of the sand fly *Lu. longipalpis* on day 2 PIBM.

Fig. 3. Experimental infection of *C. sonorensis* with *L. orientalis.* Intensities of infection were estimated as light (<100 promastigotes/gut), moderate (100-1,000 promastigotes/gut) or heavy (>1,000 promastigotes/gut). Numbers above each bar indicate the number of dissected females. The results are the average from three independent experiments.

Fig. 4. Development of *L. orientalis* in *C. sonorensis* midges. After experimental infection with *L. orientalis* axenic amastigotes, parasites transformed into procyclic promastigotes from day 1 PIBM and followed by increasing of other developmental forms. Nectomonad promastigotes peaked on day 2 PIBM and the increase of leptomonad promastigotes was found on day 4 PIBM. Metacyclic promastigotes were detected from day 3 PIBM and gradually increase through the end of the experiment (day 7). The results are the average from three independent experiments.

Fig. 5. Localization of *L. orientalis* promastigotes inside midgut of *C. sonorensis.* AMG is abdominal midgut; TMG is thoracic midgut; SV is stomoseal valve. The results are the average from three independent experiments.

Fig. 6 Representative SEM micrographs and LM image of *L. orientalis* in the midgut of *C. sonorensis*. (A). SEM micrograph of early procyclic promastigotes in the midgut on day 1 PIBM. (B). LM image of procyclic promastigotes in the midgut on day 2 PIBM. (C). SEM micrograph of nectomonad promastigotes in the midgut on day 3 PIBM. (D)

SEM micrograph of an intact stomodeal valve on day 4 PIBM showing promastigotes inside (arrow). (E) SEM micrograph of clusters of leptomonad promastigotes and metacyclic promastigotes embedded in gel-like materials inside stomodeal valve on day 4 PIBM. (F). Higher magnification of a representative image of clusters of leptomonad promastigotes (asterisks) and metacyclic promastigotes (arrows) at stomodeal valve on day 4 PIBM. (G) SEM micrograph of a metacyclic promastigote in the thoracic midgut on day 5 PIBM. (H) Higher magnification of a representative SEM micrograph of haptomonad-like promastigotes in the midgut on day 5 PIBM.

Fig. 7. Midguts dissected from *C. sonorensis* females with infection of *L. orientalis* colonizing the stomodeal valve (SV). (A). LM image showing a cluster of leptomonad promastigotes and metacyclic promastigotes (arrowheads) and promastigote secretory gel-like material (asterisks) extruding from the stomodeal valve. (B). Histological examination of midgut tissue section indicating numerous of *L. orientalis* promastigotes (arrowheads) attached to the SV and lined up around the midgut opening. (C). A cluster of leptomonad promastigotes and metacyclic promastigotes and promastigote secretory gel-like material (asterisks) stained with Giemsa's stain.