Title: The smORF-containing gene *mille-pattes* is required for moulting and *Trypanosoma cruzi* metacyclogenesis in the Chagas disease vector *Rhodnius prolixus*

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Summary

Chagas disease is estimated to affect 8 million people worldwide and is responsible for approximately 10,000 deaths in Latin America every year [1]. Control of the triatomine bugs that transmit the flagellated parasite *Trypanosoma cruzi* has been the most successful strategy to avoid disease spread. Genes containing small open reading frames (smORFs, < 100 amino acids) constitute a putative reservoir of new vector control targets, since hundreds of these genes are present in insect genomes [2]. Here, we show that the prototypic smORF-containing gene *mille-pattes/polished-rice/tarsalless (mlpt/pri/tal)* [3-6] is essential for postembryonic development of the kissing bug *Rhodnius prolixus* and for *T. cruzi* metacyclogenesis during the nymphal stages. Injection of double-stranded RNA against *mlpt* (*Rp-dsmlpt*) during the nymphal stages leads to a plethora of phenotypes, which impair postembryonic development. First, fourth or fifth stage nymphs injected with *Rp-dsmlpt* do not moult even in the presence of the ecdysone receptor (EcR) mRNA. Second, *Rp-dsmlpt* nymphs have defects in gut morphology, delayed haemoglobin digestion, and decreased defecation volume compared with those of the control nymphs. Third, *Rp-mlpt* knockdown inhibits *T. cruzi* differentiation to the trypomastigote infective stage (metacyclogenesis) inside the *R. prolixus* gut. Overall, our study is the first to provide evidence that a smORF-containing gene regulates vector physiology and parasitic cycle thus enabling the development of novel molecular strategies to eliminate Chagas disease transmission.

Keywords

tarsalless, gut formation, haem digestion, ecdysone, hormone, development, cell, differentiation.

Results:

***mlpt* knockdown alters blood digestion in *Rhodnius prolixus* fifth instar nymphs**

Polished rice (Pri) peptides are encoded by the polycistronic *mlpt/pri/tal* gene and are required for embryogenesis, trichome development and tarsus formation in various insect species [3-7]. The mRNA levels of *Drosophila melanogaster pri* are modulated by ecdysone by posttranslational control of the Shavenbaby transcription factor [8-11], although clear evidence for a role of Pri/Mlpt in larval or nymphal moult is lacking. Thus, the expression and function of *mlpt* during postembryonic development of *R. prolixus* were investigated. *Rp-mlpt* is expressed during all nymphal stages and is differentially regulated after blood feeding in several organs in adulthood (Sup. Fig. 1). During the fifth nymphal stage, *Rp-mlpt* expression is modulated in the midgut and fat body depending on the feeding status similar to the regulation observed in adult insects (Sup. Fig. 2). To determine the role of *Rp-mlpt*, unfed fifth stage nymphs were injected with double-stranded RNA (dsRNA) to knockdown the expression of the gene. The overall morphology of the gut and salivary glands was not altered six days after blood feeding (DAF) (Fig. 1A,D, Sup. Fig. 3). Importantly, the weight was similar in the control and *dsmlpt*-injected nymphae, indicating that blood ingestion is not affected by *mlpt* knockdown (Sup. Fig. 4). Interestingly, remarkable morphological changes were observed 18 and 24 DAF (Fig. 1 B-C and E-F). Anterior midgut (AM) and hindgut (Hg) were enlarged in the *Rp-dsmlpt* RNAi-treated group (abbreviated as *Rp-dsmlpt*) compared with those in the controls (Fig. 1B-C and E-F). A decrease in the peristaltic movements of the anterior midgut in *Rp-dsmlpt* was detected at 16 DAF (Sup. Video 1 and 2), suggesting that the blood trafficking was reduced after RNAi treatment. Filamentous actin staining was not affected 6 DAF but was considerably disrupted at 18 DAF in the posterior midgut (PM) of *Rp-dsmlpt* (Fig. 1G,H) in agreement with the previously reported role of Pri peptides in epidermal differentiation [12]. Additionally, higher levels of total protein in AM of *Rp-dsmlpt*

confirm blood accumulation in the anterior compartment compared with that in nymphs injected with *dsGFP* (Fig. 1I). Comparison of the haemoglobin content in the midgut indicated that the levels in *Rp-dsmlpt* were lower than that in *Rp-dsGFP* nymphs at 19 DAF (Fig. 1J). Finally, defecation was considerably decreased in *Rp-dsmlpt* (Fig. 1K-L) and correlated with altered morphology of the hindgut (Fig. 1C,F). Overall, our data demonstrate severe digestive defects in *Rp-dsmlpt* nymphs when compared with *dsGFP* controls. Thus, *R. prolixus mlpt* gene is essential for proper blood trafficking and digestion during nymphal development.

***mlpt* knockdown blocks ecdysis**

To investigate the role of *mlpt* in *R. prolixus* life cycle, *Rp-dsmlpt* was injected in fourth and fifth instar animals and the impacts on moulting to the next stage were analysed by comparison with *dsGFP*-injected control insects. Remarkably, nymphs injected with *Rp-mlpt* dsRNA were unable to moult from the fourth to the fifth instar or from the fifth nymphal stage to the adulthood (Fig. 2A), indicating that *mlpt* is critically important for moulting in *R. prolixus* life cycle. Since previous studies in *D. melanogaster* revealed that Pri activation is modulated by ecdysone [8], the changes in the expression of the ecdysone receptor (EcR) in the midgut and fat body were assayed after *Rp-mlpt* knockdown (Fig. 2B and 2C). Expression of *Rp-EcR* was similar in the controls and *Rp-mlpt* knockdown suggesting that *Rp-mlpt* acts at another level of the ecdysone cascade or that this gene regulates moult by an alternative mechanism.

***mlpt* is essential for *T. cruzi* metacyclogenesis during nymphal development**

The kissing bug *R. prolixus* was the key experimental model in initial seminal studies in the field of insect physiology by Sir Vincent Wigglesworth [13]. The interaction between *R. prolixus* blood-sucking hemiptera with the flagellated parasite *T. cruzi* during nymphal stages is a well-characterized relationship, involving digestive physiology of the vector and proliferation and differentiation of the parasite [14]. Hormone ecdysone is essential for the synchronic differentiation from proliferative epimastigotes to the trypomastigote infective stage, which is a process dependent on the microenvironmental conditions [15]. Since *Rp-mlpt* is essential for moulting during nymphal stages, we hypothesized that it can modulate *T. cruzi* proliferation and metacyclogenesis along the insect digestive tract. Parasite burden and differentiation status were analysed 7 and 18 DAF. At 7 DAF, only an initial reduction in the total number of parasites in the hindgut was detected in the *Rp-dsmlpt* nymphs compared with *dsGFP* (Fig. 3A); however, a complete absence of metacyclic trypomastigotes was observed (Fig. 3B). As expected, at 18 DAF some control fifth instar *dsGFP* nymphs started ecdysis to the adult stage, while *Rp-dsmlpt* nymphs did not undergo ecdysis even at 30 DAF. As observed at 7 DAF, the total number of parasites in the hindgut of *Rp-dsmlpt* nymphs at 18 DAF was similar to that in the *dsGFP* nymphs (Fig. 3C). However, the percentage of metacyclic trypomastigotes in the hindgut of *Rp-dsmlpt* nymphs was remarkably reduced compared with that in the control *dsGFP* nymphs (Fig. 3C-D) and adult insects (Fig. 3E-F). These data suggest that *Rp-mlpt* is essential for synchronisation of the vector and parasite development. It is well known that neuroendocrine manipulations can influence vector and parasite development, and ecdysone plays a particularly important role in these processes [15, 16]. Comparison of the digestive tract of *Rp-dsmlpt* nymphs infected with *T. cruzi* with uninfected *Rp-dsmlpt* triatomines showed similar physiological alterations in digestion in both groups (Sup. Fig. 5). We hypothesized that a reduction in ingested blood flow from AM to PM after *Rp-dsmlpt* impairs parasite development. Biochemical studies demonstrated that

globin fragments derived from haemoglobin degradation by proteolytic attack in the posterior midgut are important modulators of *T. cruzi* transformation from epimastigotes into metacyclic trypomastigotes [17]. Thus, a decrease in haemoglobin digestion in the *Rp-dsmlpt* group (Fig. 1J) may limit the availability of this differentiation inductor for the parasite. The effect of *Rp-mlpt* knockdown on *T. cruzi* differentiation is specific to the nymphal developmental stages, since similar experiments in the adult *T. cruzi* insects did not influence total numbers of the parasites or the number of infective trypomastigote forms (Sup. Fig. 6).

Overall, our results demonstrate that the *R. prolixus* smORF-containing gene *mlpt* is essential for digestive physiology, moulting and *T. cruzi* metacyclogenesis during vector development. Since hundreds of genes containing smORFs are present in the insect genomes [2] and are insufficiently investigated subjects in the studies of evolution of new genes [18], our results open up new possibilities in the search for new targets for vector control.

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Author Contributions

C.A., F.M., B.R., and J.L.N-S., conceptualization of ideas, conducting research and investigation, writing and original draft preparation; L. R. and S. O., conducting research and investigation; C.L, funding acquisition; R. N.-d-F, conceptualization ideas, conducting research and investigation, writing and original draft preparation, funding acquisition.

Declaration of Interests

"The authors declare no competing interests."

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Figure Legends:

Figure 1: ***mlpt* knockdown slows blood digestion and reduces defecation in fifth instar of *Rhodnius prolixus.***
(A-F) Gut morphology of *dsGFP* (control) and *dsmlpt* RNAi (*dsmlpt*) 6, 18 and 24 days after blood feeding (DAF). An increase in the size of the anterior midgut (arrows) and hindgut (arrowhead) is observed in *dsmlpt* RNAi 18 and 24 DAF compared with those in the *dsGFP* control. (G,H) Nuclear DAPI (blue) and filamentous actin (phalloidin-Alexa 488; green) fluorescent staining of *dsmlpt* and *dsGFP* posterior midgut. Phalloidin and DAPI staining patterns at 6 DAF were similar in the *dsmlpt* and *dsGFP* midguts, and nuclear disorganization and irregular phalloidin staining are visible at 18 DAF in *dsmlpt* RNAi compared with *dsGFP*. (I) Total protein levels are higher in the anterior midgut of *dsmlpt* nymphs at 16 DAF when compared with that in the control. (J) Haemoglobin content is reduced in the posterior midgut in the *dsmlpt* RNAi groups compared with that in the control, particularly at 19 DAF. (K,L) Defecation is considerably reduced after *dsmlpt* injection compared with that in the control according to the number of faecal points deposited in the breeding container.

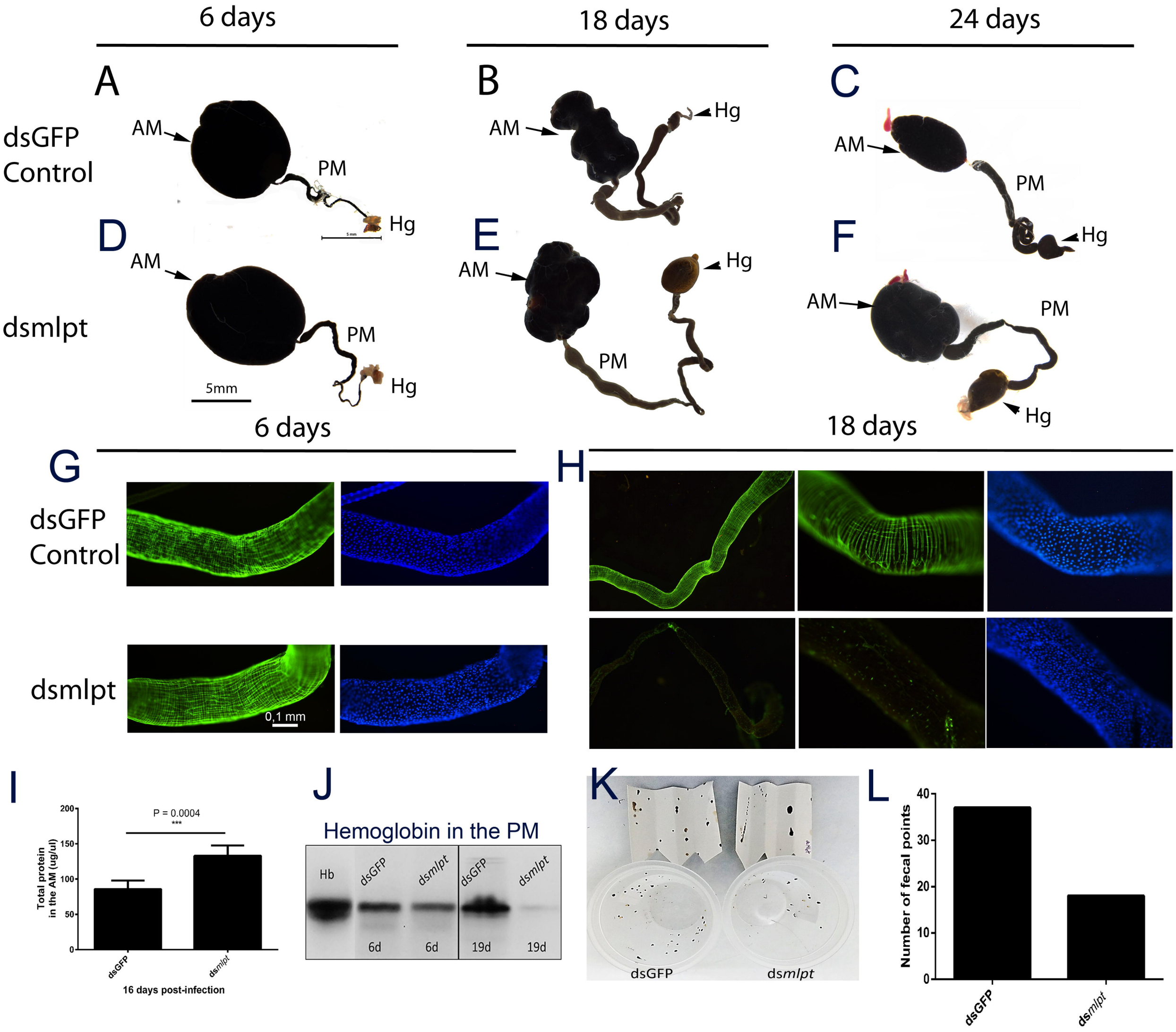
Figure 2: ***mlpt* knockdown blocks ecdysis, but not at the ecdysone receptor (*EcR*) level.** (A) Moulting after ds*GFP* or *dsmlpt* dsRNA injection during fourth or fifth instar. Ecdysis is abolished in both instars after *dsmlpt* injection. (B,C) Expression of EcR mRNA after blood meal in the control (*dsGFP*) and *dsmlpt* RNAi-treatment groups in the fat body and in the midgut.

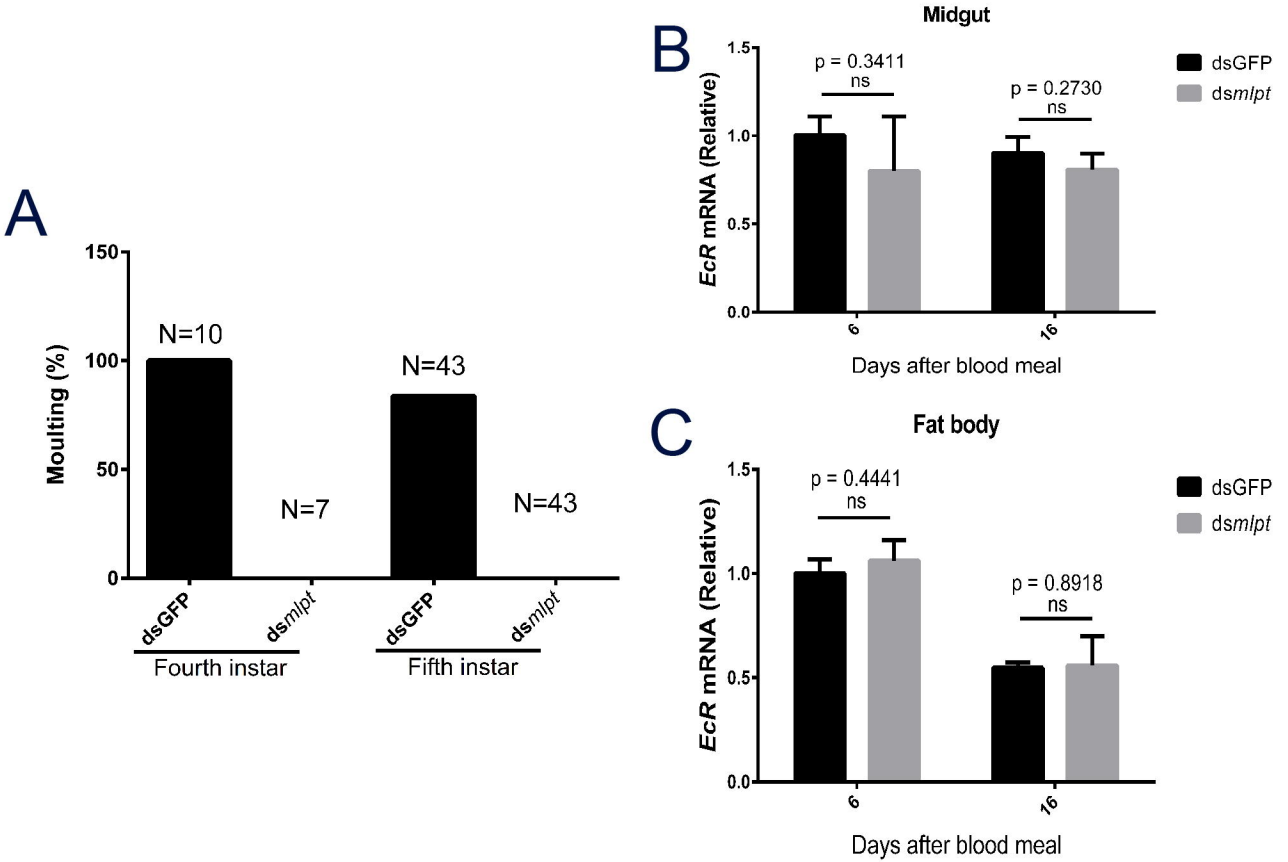
Figure 3: ***mlpt* is essential for *T. cruzi* metacyclogenesis during nymphal stages.** (A) Total number of parasites and (B) percentage of metacyclic trypomastigotes in the hindgut of the *Rp-dsmlpt* and control *dsGFP* fifth instar nymphs at 7 DAF. A significant reduction in the total number of metacyclic trypomastigotes was observed in *Rp-dsmlpt* at 7 DAF. (C-F) 18 DAF. (C) Total number of parasites and (D) percentage of metacyclic trypomastigotes in the hindgut of the *Rp-dsmlpt* and control *dsGFP* fifth instar nymphs at 18 DAF. (E) Total number of parasites and (F) percentage of metacyclic trypomastigotes in the hindgut of adults after ecdysis and *Rp-dsmlpt* fifth instar insects at 18 DAF. The total numbers of parasites were not significantly different between the control (*dsGFP*) and *Rp-dsmlpt* groups; however, the percentage of metacyclic trypomastigotes is significantly reduced, as specifically indicated by comparison of the adults and nymphs. Differences in the percentage of metacyclic

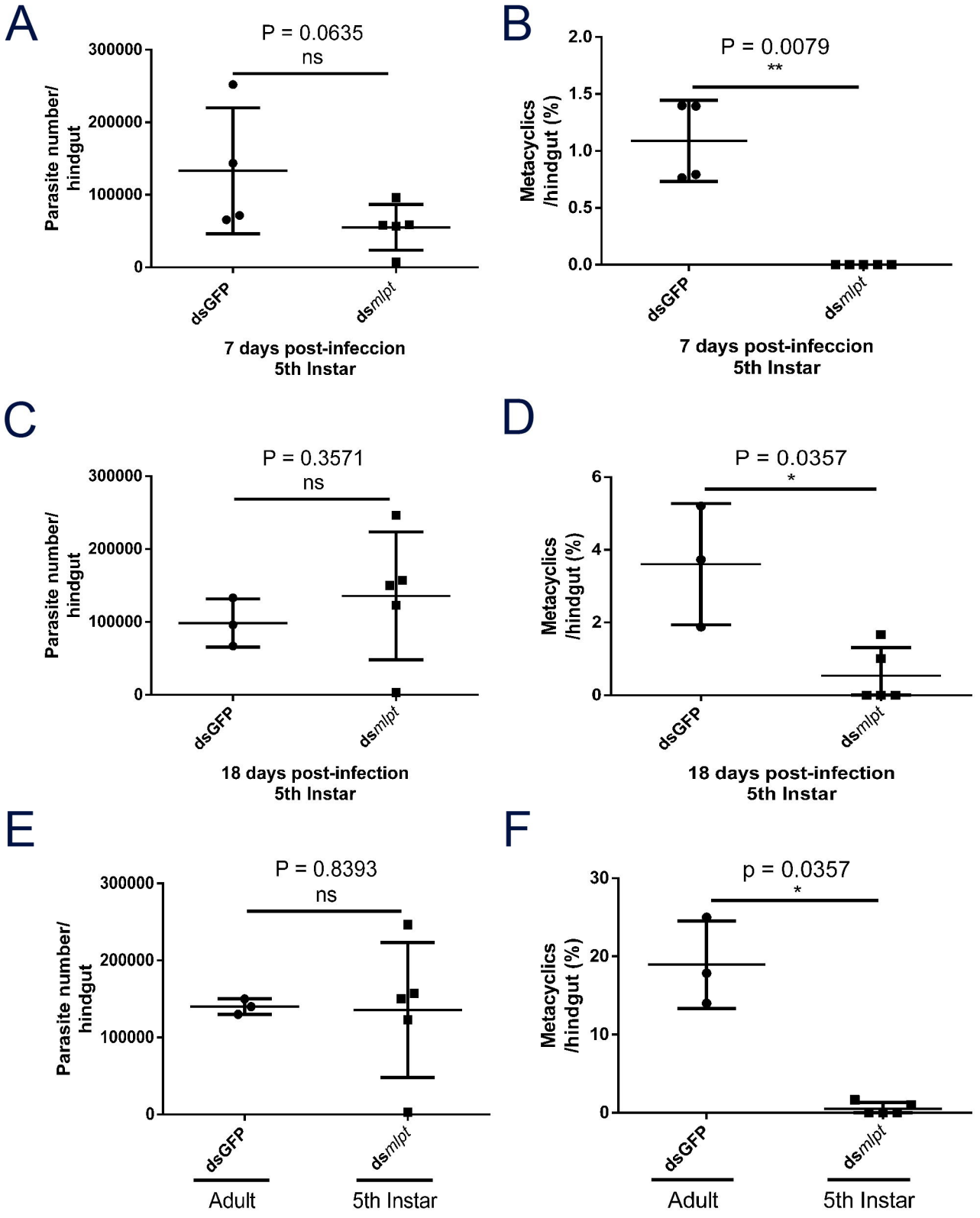
trypomastigotes were not observed when *T. cruzi* infection was performed in adulthood (See also Sup. Fig. 6). Data are presented as the mean ± SEM.

STAR METHODS

Detailed methods and Supplementary Figures are described in the supplementary file.


6 days
18 days
24 days
A
B
C
dsGFP
Control
AM
PM
Hg
5 mm
D
E
F
dsmlpt
5mm
6 days
18 days
G
H
dsGFP
Control
dsmlpt
0,1 mm
I
P = 0.0004
***
Total protein in the AM (ug/ul)
200
150
100
50
0
dsGFP
dsmlpt
16 days post-infection
J
Hemoglobin in the PM
Hb
dsGFP
dsmlpt
dsGFP
dsmlpt
6d
6d
19d
19d
K
dsGFP
dsmlpt
L
Number of fecal points
40
30
20
10
0
dsGFP
dsmlpt


A
Moulting (%)
150
100
50
0
N=10
N=7
N=43
N=43
dsGFP
dsmlpt
dsGFP
dsmlpt
Fourth instar
Fifth instar
B
Midgut
EcR mRNA (Relative)
1.5
1.0
0.5
0.0
p = 0.3411
ns
p = 0.2730
ns
dsGFP
dsmlpt
6
16
Days after blood meal
C
Fat body
EcR mRNA (Relative)
1.5
1.0
0.5
0.0
p = 0.4441
ns
p = 0.8918
ns
dsGFP
dsmlpt
6
16
Days after blood meal


A
P = 0.0635
ns
Parasite number/ hindgut
300000
200000
100000
0
dsGFP
dsmlpt
7 days post-infeccion
5th Instar
B
P = 0.0079
**
Metacyclics /hindgut (%)
2.0
1.5
1.0
0.5
0.0
dsGFP
dsmlpt
7 days post-infeccion
5th Instar
C
P = 0.3571
ns
Parasite number/ hindgut
300000
200000
100000
0
dsGFP
dsmlpt
18 days post-infection
5th Instar
D
P = 0.0357
*
Metacyclics /hindgut (%)
6
4
2
0
dsGFP
dsmlpt
18 days post-infection
5th Instar
E
P = 0.8393
ns
Parasite number/ hindgut
300000
200000
100000
0
dsGFP
dsmlpt
Adult
5th Instar
F
p = 0.0357
*
Metacyclics /hindgut (%)
30
20
10
0
dsGFP
dsmlpt
Adult
5th Instar