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. 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. Here, we show that the prototypic smORF-containing gene *mille-pattes/polished-rice/tarsalless (mlpt/pri/tal)* 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

Tarsal-less, gut formation, haem digestion, ecdysone, hormone, development, cell, differentiation.

Introduction:

Chagas disease was originally described in the American continent in the first decade of the 20th century as a disease caused by the flagellated parasite *Trypanosoma cruzi* [1]. Even when acute infection resolves, patients can undergo chronic disease, mainly characterised by cardiomyopathy and arrhythmias. This anthropozoonosis has spread through migration through America and many challenges remain unresolved [2], particularly regarding the control of triatomine vectors [3].

The subfamily Triatominae contains the main species responsible for the parasite transmission, particularly from the genera *Panstrongylus* (Berg, 1879), *Triatoma* (Laporte, 1832) and *Rhodnius* (Stål, 1859) [4]. *Rhodnius prolixus* seminal studies of Sir Vicent Wigglesworth pioneered the field of insect physiology [5] and in the past years, the publication of *R. prolixus* genome and the establishment of functional genomics tools *e.g* transcriptomes, *in situ* hybridization and RNA interference re-established this species as a great model system to study the physiology and the vector-parasite interaction [6-10].

In the past ten years, comparative evolutionary and functional analysis provided evidence that genes containing small open reading frames (smORFs or sORFs, < 100 amino acids) constitute an unexplored and important part of metazoan genomes [11, 12]. Analysis from the insect model species *Drosophila melanogaster* showed over 400 genes containing smORFs actively transcribed [13]. These smORF-containing genes are often transcribed from large mRNA precursors and can be polycistronic, *e.g.* more than one peptide can be encoded from a single mRNA.

mille-pattes (mlpt) was the first polycistronic gene containing smORFs identified in the beetle *Tribolium castaneum* [14]. *mlpt* and its homologs *polished rice (pri)* or *tarsal-less(tal)* play major roles during the embryogenesis of beetles, fruit flies, wasps and bugs [15-17]. An important role of *mlpt/pri/tal* in the differentiation of epithelia such as trichomes, gut precursors and tarsus was also described [16, 18, 19]. Mechanistically, the conserved peptides of 10-13 amino acids encoded by the smORFs in the *mlpt* gene are involved in the control of N-terminal processing of the Shavenbaby transcription factor [19-22]. Recently, multiple roles of the single ortholog of *mlpt* in *R. prolixus* during embryogenesis and the emergence of a new hemiptera-specific peptide were described [17], although other functions of this gene in the kissing remain unknown.

Importantly, mRNA levels of *mlpt/pri/tal* were shown to be modulated by ecdysone in *Drosophila melanogaster* [20], even though a clear evidence for a role of this gene in larval

or nymphal development and moult in other insect species is still lacking. Thus, in the present manuscript the postembryonic role of the prototypic smORF-containing gene *mlpt* was investigated in the kissing bug *R. prolixus (Rp).* Overall, this is the first report of the requirement of a smORF-containing gene for vector-parasite interaction and for many aspects of insect development and physiology.

Methods:

Rhodnius prolixus laboratory stock*:*

R. prolixus bugs were kept in an incubator at 28 °C and 70 – 80% relative humidity. Different life cycle stages (eggs, nymphs and adult insects) were kept separately in plastic jars. Insects were fed through controlled exposure to rabbits. Blood ingestion occurred through bites in the rabbit's ears, as previously described [23]. This study was approved by the Committee on Ethics in the Use of Animals (**CEUA)** from the Health Sciences Center (CCS), Federal University of Rio de Janeiro (UFRJ), under reference code MAC032.

Trypanosoma cruzi cell culture:

Epimastigote forms of *Trypanosoma cruzi* were maintained in LIT medium (Liver infusion tryptose - 0.4% sodium chloride (NaCl), 0.04% chloride potassium (KCl), 0.8% dibasic sodium phosphate (Na2HPO4), 0.2% glucose, 0.5% tryptose (Difco) and 0.5% liver infusion (Difco) [24]. The pH was adjusted to 7.2 and then the medium was sterilized by filtration (0.22 µM pore membrane). After filtration, 0.0025% sterile hemin (Sigma-Aldrich) was added to the medium. Then the medium was supplemented with 10% Fetal Calf Serum (FCS; Cultilab), with 10,000 U / L and 10 mg / L of penicillin and streptomycin, respectively, and with 200 µg / mL of Geneticin (G418 - Sigma-Aldrich; only for clone Dm28c-GFP). The number of viable cells was determined by hemocytometer counts of motile cells. Epimastigotes grown to mid logarithmic phase with more than 90% viable cells were harvested for infection assays.

Feeding of experimental groups:

Nymphs challenged with ds*mlpt* and *dsGFP* were fed by three distinct methods: (1) females were fed directly into the rabbit's ear; (2) females were fed in an artificial feeder at 37 °C with rabbit blood collected with a heparinized syringe; and (3) female nymphs were

fed in an artificial feeder at 37 °C with rabbit blood collected with a heparinized syringe plus *T. cruzi*.

For infection assays, rabbit blood was centrifuged during 10 min at 2000 g and 24 °C to separate plasma from erythrocytes. Then, the plasma was heat inactivated at 56 °C for 3 hours, while erythrocytes were resuspended and washed three times in phosphate-buffered saline, before being reunited with inactivated plasma. Simultaneously, *T. cruzi* epimastigotes were centrifuged during 10 min at 400 g and 16 °C and washed twice in phosphate-buffered saline. Parasites were then added to the recomposed blood to the concentration of 1×10^7 cells/mL and this mixture was applied to the artificial feeder.

Extraction of total RNA and synthesis of complementary cDNA:

Total RNA from fat body, intestine and salivary glands of R. prolixus was extracted at 1, 6 and 16 days after blood feeding, while for *T. cruzi* infected triatomines RNA was extracted at 7 and 18 days after blood feeding. The RNA extraction was done using the TrizolTM reagent (Invitrogen, Cat. No. 15596026). concentration was estimated by spectrophotometry at 260 nm and purity was determined by 260/280 and 260/230 ratios, using a Nanodrop 2000 spectrophotometer (ThermoFisher Scientific). cDNA synthesis was carried with High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Cat. No. 4368814), following manufacturer's instructions. Primers sequences of the constitutive gene *Rp-eEF-1*, *Rp-mlpt* and *Rp-EcR* have been previously described [17, 25]. The Green Fluorescent Protein (GFP) cDNA sequence was used as a template for the synthesis of an unrelated dsRNA using the following primers Fwd. taatacgactcactatagggACGTAAACGGCCACAAGTTCAGCGTGTC and Rev. taatacgactcactatagggTCACGAACTCCAGCAGGACCATGTGATC. Lower case letters refer to the T7 adaptor sequence.

RT-qPCR amplifications were performed using *SYBR® Green PCR Master Mix* (Applied Biosystems) on a *StepOnePlus*™ thermocycler (Applied Biosystems, CA, USA). All assays were performed with at least three technical replicates and a control without cDNA template. Threshold cycles (Cq) were determined by default thermocycler parameters. Amplification efficiencies for each primer pair were determined from standard curves obtained by linear regression of Cqs from RT-qPCR reactions using 10-fold serial dilutions of fat body, midgut, salivary gland cDNA. Efficiencies (€) were calculated applying the formula $E = 100 \times (10^{-1/ac} - 1)$, in which “ac” corresponds to angular coefficients of standard

curves obtained by linear regression. Relative expression was accessed with the comparative $2^{-\Delta\Delta Cq}$ method [26], using *eEF-1* as reference of invariant expression, as previously determined by Majerowicz et al. [25] and Berni et al. [27].

Gene knockdown via RNA interference:

RNAi was performed as previously described [27]. Double stranded RNAs were generated with Ambion T7 Megascript Kit (Cat. No. AM1334), after insertion of T7 promoter sites at the end of PCR products. 4 µg of dsRNA were injected for each target gene, and silencing e□ciency was accessed by the analysis of relative expression through RT-qPCR, using the comparative $2^{-\Delta\Delta Cq}$ method normalized with the endogenous reference gene *eEF-1*.

Immunofluorescence microscopy:

For immunofluorescence staining, insects were dissected and tissues were collected and fixed at room temperature with 4% paraformaldehyde (PFA) for 1 day, followed by staining with phalloidin conjugated to Alexa Fluor 488 (1: 100, SIGMA) and DAPI (1: 500, SIGMA) for 30 min in phosphate-buffered saline with tween-20 1% (v/v) as previously described [27]. Images were acquired using a Leica M205 stereoscopic microscope.

Hemozoin extraction:

Hemozoin was extracted from the midgut of *R. prolixus*, as previously described [28]. Final sediments were solubilized in 0.1 M NaOH and heme amount determined at 400 nm in a UVmini-1240 spectrophotometer (SHIMADSU).

Native hemoglobin polyacrylamide gel electrophoresis (PAGE):

This technique was adapted from Laemmli [29]. Sample proteins were quantified by BCA method, and separated by PAGE in TRIS-glycine running buffer (pH 8.3), at 25 mA and 100 V. Gels were then placed in trichloroacetic acid 12.5% (m:v) for 30 minutes and washed in distilled water for 30 minutes at room temperature. Finally, gels were incubated in 2 mM DMB (3,3'-dimethylbenzidine) and 0.1 M sodium citrate buffer, pH 4.5, followed by addition of hydrogen peroxide 30%.

T. cruzi quantification in *R. prolixus* hindgut:

Infected insets were dissected and hindgut samples were homogenized in 100 µL of phosphate-buffered saline. The number of parasites was determined by counting in a Neubauer haemocytometer in an Olympus optical microscope. *T. cruzi* epimastigotes were differentiated from metacyclic trypomastigotes based on their characteristic morphologies and motility patterns. Intermediate stages were considered as epimastigotes.

Statistical Analysis:

For normally distributed data, comparisons between means of two independent groups were done with an unpaired t-test, and multiple comparisons were done with one-way analysis of variance (ANOVA), followed by the Tukey post-test. For nonparametric data, multiple comparisons were done with the Kruskal-Wallis test, followed by Dunn's post-test, and pairwise comparisons were done with the Mann-Whitney test. Results are expressed as the group mean $\pm$ S.E. Significance was considered whenever $p < 0.05$. Graphpad Prism software (v. 6.0) was used for statistical analysis.

Results and Discussion:

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 (Svb) 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 [30]. 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 [20], 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 [5]. 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 [31]. 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 [32]. Since *Rp-mlpt* is essential for moulting during nymphal stages, we hypothesized that it could 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 [32, 33]. 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 [34]. 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 [13] and are insufficiently investigated subjects in the studies of evolution of new genes [35], 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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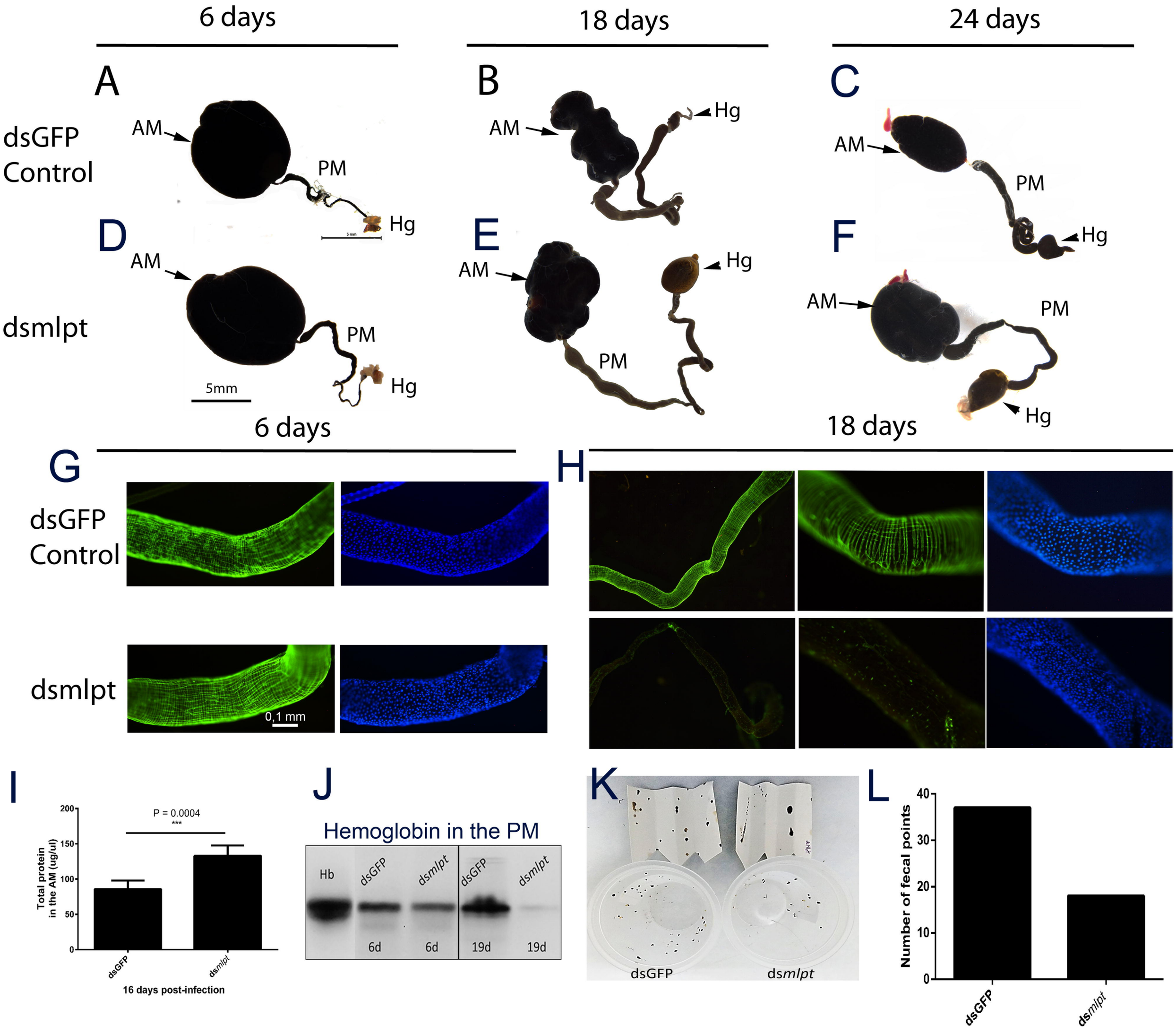
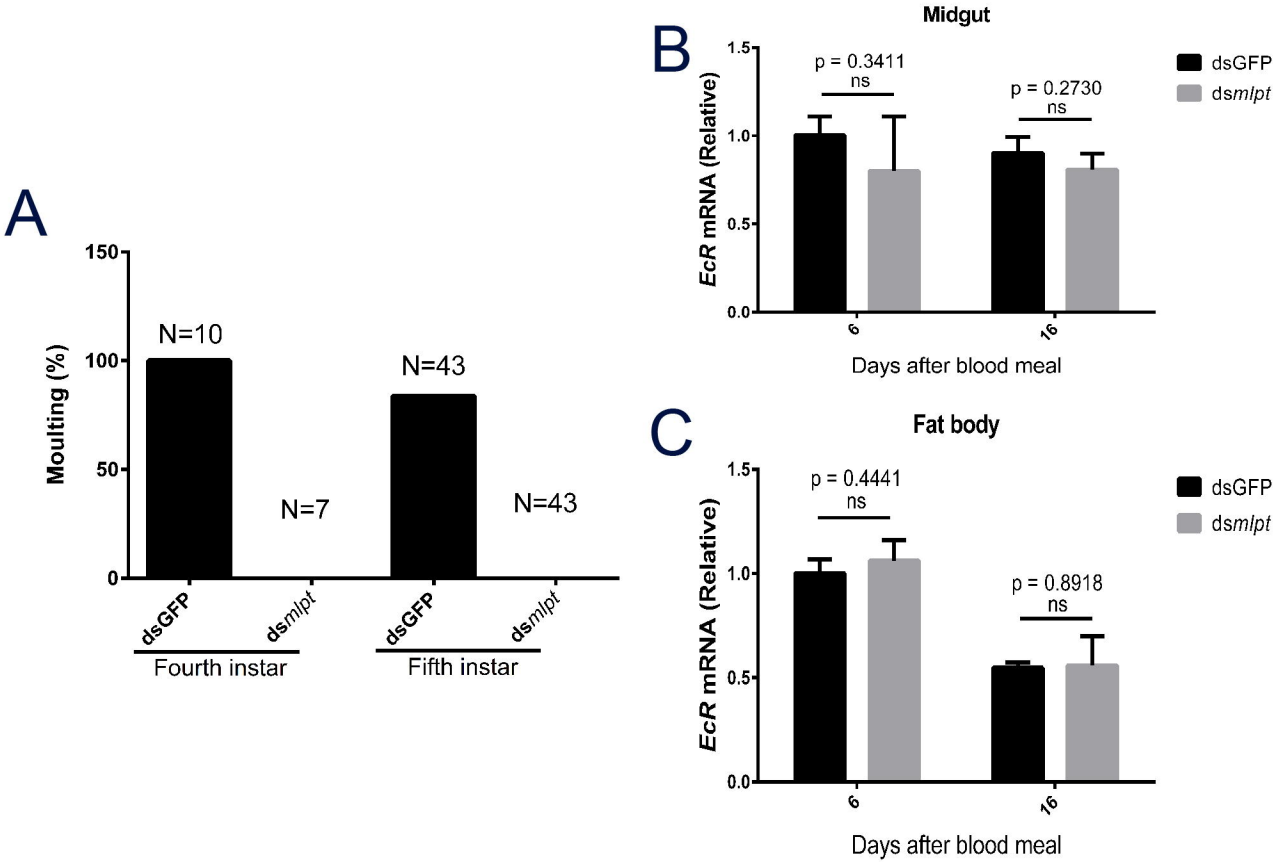
Figure Legends:

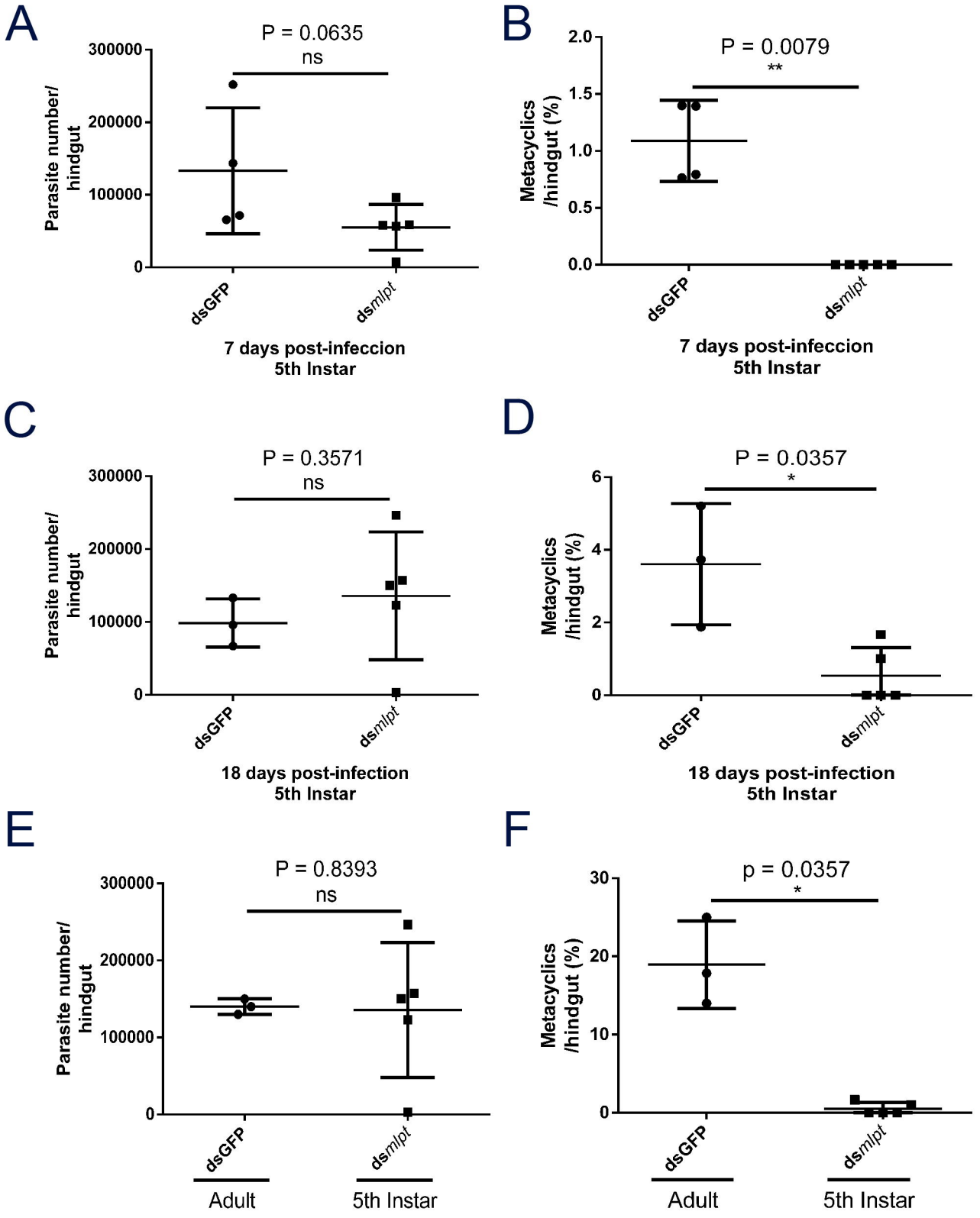
Figure 1: ***mlpt* knockdown slows blood digestion and reduces defecation in fifth instar of *Rhodnius prolixus.***
(A-F) Gut morphology of *dsGFP* (control) and *dsmlpt* RNAi (*dsmlpt*) 6, 18 and 24 days after blood feeding (DAF). An increase in the size of the anterior midgut (arrows) and hindgut (arrowhead) 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.


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