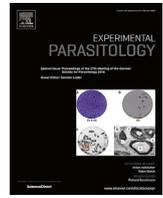




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Structured illumination microscopy reveals actin I localization in discreet foci in *Plasmodium berghei* gametocytes



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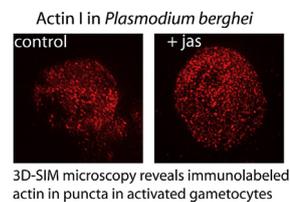
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HIGHLIGHTS

- Actin I was detected in discreet ~130 nm foci in *P. berghei* gametocytes.
- Treatment with jasplakinolide did not modify the length of the structures.
- Sedimentation of actin I at 100 000 × g confirms filamentous actin in gametocytes.

GRAPHICAL ABSTRACT



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ABSTRACT

Actin has important roles in *Plasmodium* parasites but its exact function in different life stages is not yet fully elucidated. Here we report the localization of ubiquitous actin I in gametocytes of the rodent model parasite *P. berghei*. Using an antibody specifically recognizing F-actin and deconvolution microscopy we detected actin I in a punctate pattern in gametocytes. 3D-Structured Illumination Microscopy which allows sub-diffraction limit imaging resolved the signal into structures of less than 130 nm length. A portion of actin I was soluble, but the protein was also found complexed in a stabilized form which could only be completely solubilized by treatment with SDS. An additional population of actin was pelleted at 100 000 × g, consistent with F-actin. Our results suggest that actin in this non-motile form of the parasite is present in short filaments cross-linked to other structures in a cytoskeleton.

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1. Introduction

Plasmodium parasites have a complex life cycle alternating between motile, invasive forms and non-motile stages. While the asexual replicative blood stage parasites are responsible for the

pathogenesis of the parasite, the sexual stages are essential for transmission to the mosquito. Sexually differentiated male and female gametocytes develop in red blood cells in the vertebrate host, transforming into mature gametes only when they are taken up in a blood meal by a mosquito. Gametogenesis is a rapid process taking roughly 10–15 min. It involves escape from the red blood cell by both males and females, whereupon the male undergoes three mitotic divisions and assembly of 8 axonemes, finally resulting in the formation of 8 flagellar gametes. These move rapidly and fuse with the female gamete to form the zygote, which

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after roughly 20 h has developed into the ookinete. Gametogenesis can also be achieved *in vitro* by lowering the temperature and diluting the blood-containing gametocytes in a defined cell culture medium; this process is hereafter referred to as activation.

Plasmodium has two actin isoforms. Actin I is the major form found in all life stages, while actin II is specifically required during the sexual stages (Andreadaki et al., 2014; Angrisano et al., 2012a; Deligianni et al., 2011). Actin I is an essential protein and the gene cannot be genetically disrupted. It has been shown to be crucial for gliding motility of the motile and invasive stages (Angrisano et al., 2012b; Baum et al., 2006; Baum et al., 2008; Siden-Kiamos et al., 2012), but evidence is accumulating for other important functions in the asexual blood stages. These include haemoglobin uptake and endocytosis (Lazarus et al., 2008; Smythe et al., 2008), ring stage morphology (Gruring et al., 2011), and gene repositioning in the nucleus (Zhang et al., 2011). The molecular function of the second isoform is not yet understood.

We have previously reported the presence of actin I (PBANKA_1459300) in male gametocytes of the rodent model parasite *P. berghei* (Deligianni et al., 2011), using a monoclonal antibody recognizing specifically actin I of the *Plasmodium* parasite (Siden-Kiamos et al., 2012). The role of actin in this stage remains unknown but recent studies suggest that actin I plays an important role in gametocyte remodelling in *P. falciparum* (Hliscs et al., 2015). In the human parasite actin I accumulates at the ends of the elongating gametocyte and appears to contribute to the morphogenesis of the crescent-shaped gametocytes of *P. falciparum*.

In *P. falciparum*, which form elongated gametocytes over a period of about 12 days, short-term treatment with agents such as cytochalasin D or jasplakinolide that disrupt or stabilize actin filaments, had little effect (Hliscs et al., 2015). However long-term treatment is associated with disruption of the normal mitochondrial organization and decreased gametocyte viability (Hliscs et al., 2015). The gametocytogenesis phase is more difficult to access in *P. berghei* but treatment with cytochalasin D and jasplakinolide had no effect on gametogenesis following activation *in vitro* (Deligianni et al., 2011). This suggests that the function of actin I in gametocytes does not require rapid filament turnover, or that filaments are stabilized and thus not accessible to the drugs.

Here, we extend our previous analysis and investigate actin I in *P. berghei* gametocytes. We find that actin I is present in short capped filaments, complexed to cytoskeletal structures.

2. Materials and methods

2.1. Ethics statement

All animal work was carried out in strict compliance with the Greek Presidential Decree 56/2013 implementing the EU Directive 2010/63/EE on the protection of animals used for scientific purposes. The protocol has been approved by Committee for Evaluation of Research Protocols at FORTH (protocol number 6740/08/10/2014 to Inga Siden-Kiamos).

2.2. Parasites and parasitology methods

The parasites used in the study were *P. berghei* WT strain ANKA 2.34 and strain 15cy1A (Vlachou et al., 2004), expressing GFP in zygote, retorts and ookinetes. Parasites were maintained in Theiler's Original mice or Swiss mice. Gametocytes were obtained from mice infected with the WT strain and treated for two consecutive days with an anti-folate drug to eliminate asexual parasites (Rodriguez et al., 2002). Parasites were monitored on Giemsa stained blood smears.

2.3. Immunofluorescence assay

Samples were activated by dilution in a five-fold volume of RPMI1640 supplemented with L-glutamine, 25 mM HEPES, 2 g/L NaHCO₃, 10% FBS, 50 μM xanthurenic acid, pH8.0 and incubating the samples for 10 min at 19 °C. In treated samples 250 nM jasplakinolide was added to the RPMI medium. The samples were fixed in 4% paraformaldehyde, permeabilized in 0.2% Triton X-100, and labeled with the ACT_{16–30} antibody diluted in PBS supplemented with 3% BSA overnight at 4 °C. After washing with PBS the secondary antibody anti-rabbit antibody conjugated with Alexa 568 was added and the sample incubated 1 h at RT, followed by labeling with TER-119 antibody for 1 h. DNA was stained with DAPI and the samples were mounted in Vectashield. The gametocytes were viewed in a Zeiss Axioskop 2 plus microscope fitted with fitted with an Axiovert CCD camera (Zeiss). Alternatively, as indicated, a DeltaVision Elite deconvolution system (Applied Precision) was used. Z-stacks (0.15–0.2 μm steps) were deconvolved using the default settings in the SoftWoRx 5.0 acquisition software. 3D-Structured Illumination Microscopy (3D-SIM) was implemented on a DeltaVision OMX V4 Blaze™ (Applied Precision). Samples were excited using 488 and 568 lasers and imaged using 528/48 nm, 608/37 nm and with a 60× oil immersion lens (1.42 NA) as previously described (McMillan et al., 2013). Images were analyzed with ImageJ software (<http://rsbweb.nih.gov/ij>).

2.4. Solubilization of actin and fractionation of cells

For solubilization experiments gametocytes purified by Nycodenz gradient centrifugation were resuspended in PBS with 1 mM PMSF and sonicated on ice, followed by centrifugation at 15 000×g for 15 min. The pellet was extracted with 1% SDS or 4 M urea at room temperature, or 1% Triton X-100 or 100 mM Na₂CO₃ on ice for 1 h followed by centrifugation at 15 000×g, 15 min. The samples were loaded on SDS-PAGE and analyzed by Western blot. For fractionation studies gametocytes were either non-treated or treated with jasplakinolide for 10 min and then lysed in F-actin buffer (5 mM Tris, pH 8.0; 50 mM KCl; 2 mM MgCl₂; 0.2 mM CaCl₂; 1 mM ATP; 0.5 mM DTT, and 1% Triton X-100) for 30 min. The samples were then pelleted at 700×g for 10 min, the supernatant was collected and centrifuged at 15 000×g for 10 min after which the soluble fraction was subjected to ultra-high speed centrifugation at 100 000 g for 1 h. The pellet and supernatant samples were processed for SDS-PAGE and analyzed by Western blot. The blots were probed with the monoclonal anti-*Dictyostelium* actin antibody specific for parasite actin and an antiserum directed against *P. berghei* enolase was used as a control. The secondary anti-mouse antibody was conjugated with horse radish peroxidase. The signal was detected using the SuperSignal West Pico solution (Pierce Biotechnology).

2.5. Antibodies

The ACT_{16–30} antibody and the monoclonal anti-*Dictyostelium* actin antibody (obtained from Developmental Studies Hybridoma Bank) have been described previously (Siden-Kiamos et al., 2012). TER-119 conjugated to Alexa-488 was purchased from Biozol. For the enolase antiserum part of the *P. berghei* enolase gene (PBANKA_1214300) was amplified by PCR using primers cgcggatccGGCTATATGTAGAGCAGGTGCGGC cggaaattcTGCATTCTTTCACTTCTGCATGGTGCTC (*Bam*HI and *Eco*RI restriction sites underlined). The fragment corresponded to amino acids 123 to 418 and was inserted into pRSETB (Thermo Fisher Scientific) expression vector. The recombinant protein containing a Hisx6 tag was expressed in *E. coli* BL21(DE3)pLysS. The protein was purified on Ni-

NTA beads under denaturing conditions and BALB/C mice were immunized. The antiserum discriminates between parasite and mosquito enolase (Supplementary Fig. S1).

2.6. High content screening assay of ookinete development

Infected blood samples were diluted in RPMI and immediately added to 24 well plates which contained the drugs at the different concentrations tested. After 24 h incubation at 19 °C the samples were transferred to 96 well dishes for measurements in the Operetta high content screening system from Perkin Elmer. Images were obtained with an excitation filter at 480–520 nm, an emission filter at 500–550 nm and scanning 300 fields/well. Data was analyzed with the Harmony 4.1 software using the morphological criteria length, width, circularity and area. The algorithm was trained to separate elongated ookinetes, spherical zygotes and optical noise (debris). The EC50 was calculated as (number of ookinetes/(number of ookinetes + number zygotes)) against concentration of the drug (nM).

3. Results and discussion

3.1. Actin I is localized in discreet puncta in *P. berghei* gametocytes

An antibody against an actin I peptide, here called anti-ACT_{16–30} (Siden-Kiamos et al., 2012) has previously been characterized. This antibody detected actin I, but not actin II, in Western blot experiments of *E. coli* expressed proteins and labeled rod-like structures in *P. berghei* ookinetes, one of the motile stages of the life cycle. The latter result indicated that anti-ACT_{16–30} specifically detects F-actin (Siden-Kiamos et al., 2012). Here, we used this antibody to label actin I in gametocytes of *P. berghei*. Wide field fluorescence microscopy of the immunolabeled gametocytes revealed actin in a diffuse pattern in the cell with no obvious localization to particular structures (Fig. 1A). The information available from conventional wide field microscopy is limited due to the signal from out-of-focus features. We thus imaged actin I in activated gametocytes using deconvolution microscopy, which revealed a non-homogeneous pattern (Fig. 1Ba,b). Filament stabilization, using jasplakinolide,

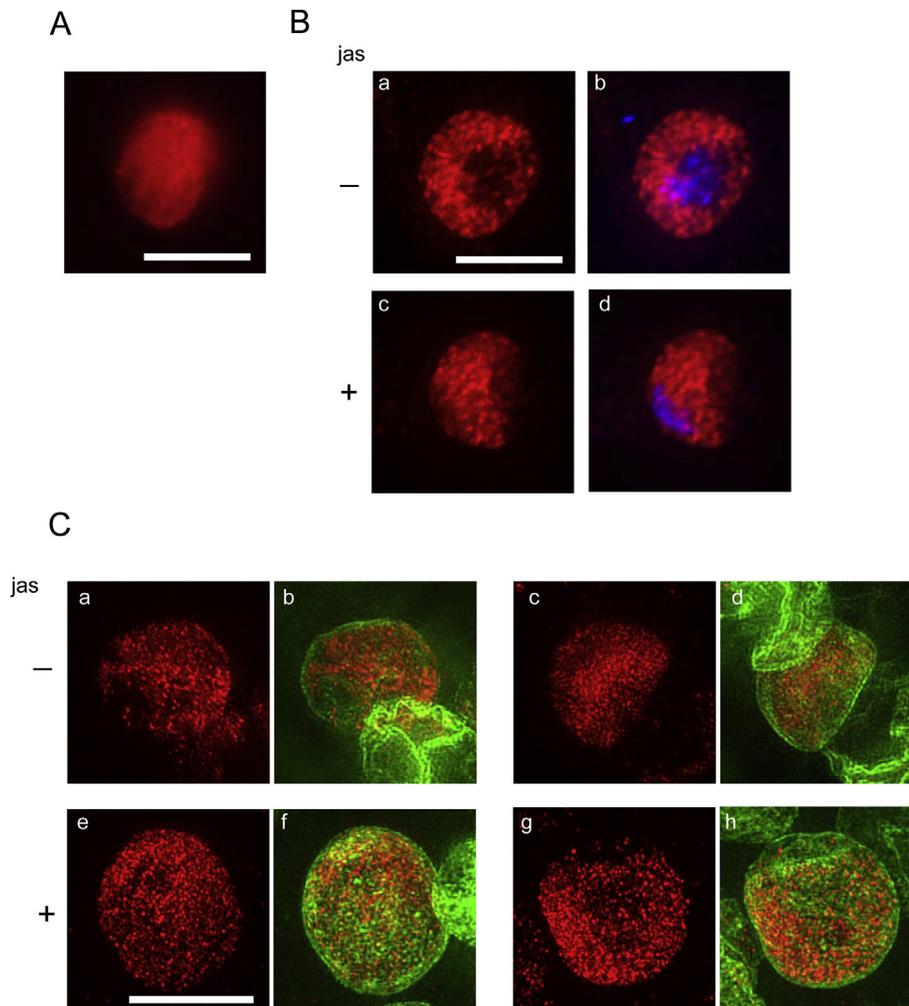


Fig. 1. Actin I is present as very short filaments in *P. berghei* gametocytes. A. Epifluorescence microscopy of a gametocyte shows actin I in a diffuse pattern with no obvious structure. B. Deconvolution microscopy reveal actin (red) in dot-like structures in non-treated (a,b) as well as jasplakinolide treated cells (c,d). DNA was stained with DAPI (blue). C. The punctate pattern is more clearly revealed by 3D-SIM microscopy of untreated (a–d) and jasplakinolide (jas) treated activated gametocytes (e–g). Actin label is in red. The red blood cell membrane was stained with the TER-119 antibody (green); it is still largely intact (b,d,f,g). Two cells of each sample are shown. Scale bars, 5 μm. See also Supplementary Movie S1 (corresponding to gametocyte in panel a) and Supplementary Movie S2 (corresponding to gametocyte in panel e). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

has previously been shown to generate F-actin at the apical ends of *Plasmodium* merozoites (Angrisano et al., 2012a) and ookinetes (Angrisano et al., 2012b; Mizuno et al., 2002; Siden-Kiamos et al., 2006) as well as of *T. gondii* tachyzoites (Shaw and Tilney, 1999). By contrast treatment of activated *P. berghei* gametocytes with jasplakinolide had no discernible effect (Fig. 1B,c,d). To improve resolution we used 3D-Structured Illumination Microscopy (3D-SIM), providing an 8-fold increase in volume resolution (Hanssen et al., 2010) (Fig. 1C, movie S1,S2). At this higher resolution the dotted pattern was resolved into small point-like structures in non-treated activated gametocytes (Fig. 1C,a-d, movie S1) as well as jasplakinolide-treated (Fig. 1C,e-g, movie S2). This suggests that actin is present as short stabilized assemblies with a maximum length of ~130 nm.

Supplementary video related to this article can be found at <http://dx.doi.org/10.1016/j.exppara.2017.08.001>.

3.2. Actin I is present as globular and filamentous actin in gametocytes

To examine the physical organization of actin I in the gametocytes we fractionated cell extracts. Gametocytes were lysed by sonication followed by extraction of the pellet and Western blotting (Fig. 2A and B). In these experiments actin was identified by a monoclonal antibody which is specific for parasite actin, and does not cross-react with mouse actin. The cells were completely lysed by sonication as shown using an antibody directed against soluble glycolytic enolase. In contrast, actin I was found in both pellet and

supernatant fractions after centrifugation at 15 000×g. Extraction of the pellet with 1% SDS was required to fully solubilize the protein, while treatment with 6 M urea and 1% Triton X-100 resulted in partial solubilization; extraction with NaHCO₃ did not lead to any solubilization. We interpret these data that some actin I is complexed to other structural elements in the gametocyte. We used differential centrifugation to further investigate the physical state of actin I. A pellet was obtained after sedimentation at 100 000×g, consistent with the presence of cytoplasmically located F-actin. Attempts to increase the F-actin fraction by treatment with jasplakinolide were not successful.

3.3. Jasplakinolide treatment does not interfere with gametocyte development

The above results suggest that the short filaments in the gametocytes are not accessible to jasplakinolide. We next wanted to determine whether jasplakinolide treatment of gametocyte has any effect on the further development of gametocytes to ookinetes. To this end we used an assay for high content imaging. The assay employs the *P. berghei* strain 15cy1A which expresses GFP in zygotes, retorts and ookinetes ((Vlachou et al., 2004), Deligianni, unpublished data). Infected blood was added to ookinete medium containing the drugs to be tested. After 24 h the samples were analyzed in the Operetta high content screening system. The results revealed that jasplakinolide at concentrations ≤500 nM had no effect on the development of gametocytes to ookinetes (Fig. 3A). As a control the drug atovaquone was tested in parallel and as

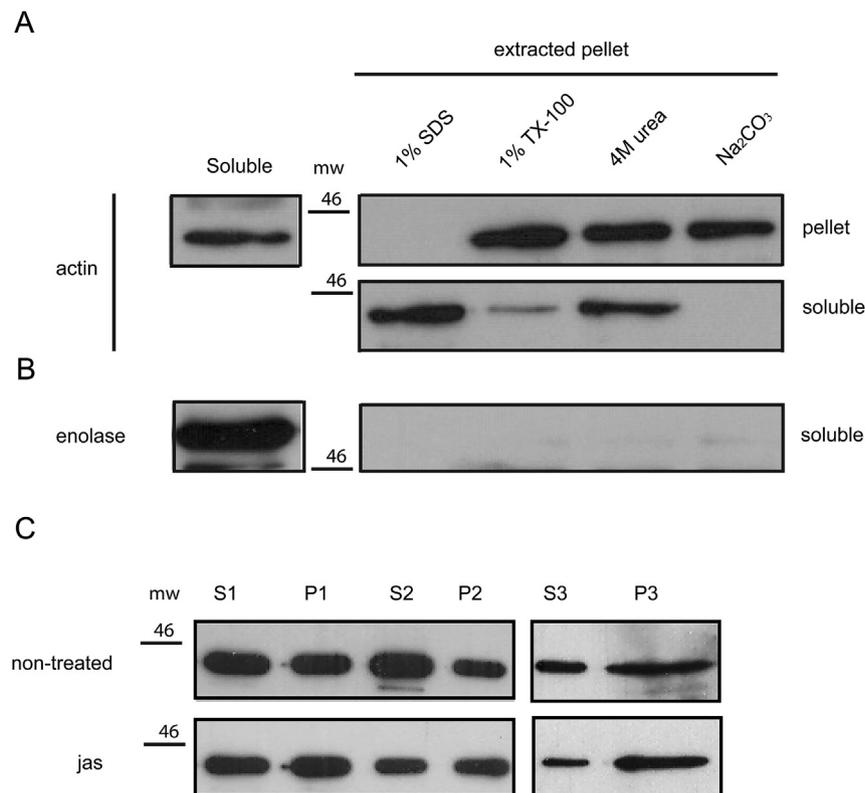


Fig. 2. Cell fractionation reveals globular and filamentous actin I in gametocytes. A,B. Gametocytes were lysed by sonication and insoluble material pelleted at 15 000×g for 10 min. The pellet was extracted with 1% SDS, 4 M urea, 1% Triton X-100 or 100 mM Na₂CO₃ followed by centrifugation at 15 000×g, 15 min. A. A parasite-specific anti-actin antibody was used to detect actin I. B. An antiserum directed against enolase was used to verify that cytoplasmic proteins were solubilized. C. Western blot analysis of extracts from non-treated gametocytes (top) and gametocytes treated with jasplakinolide (bottom) for 10 min. The samples were lysed in F-actin, followed by differential centrifugation. S1, P1 = low speed supernatant and pellet (700×g for 10min), S2, P2 = supernatant and pellet at 15 000×g (10 min), S3, P3 = ultra-high speed supernatant and pellet at 100 000 g (1 h). The same antibody as in A was used for detection of actin I. mw, molecular weight marker (kDa).

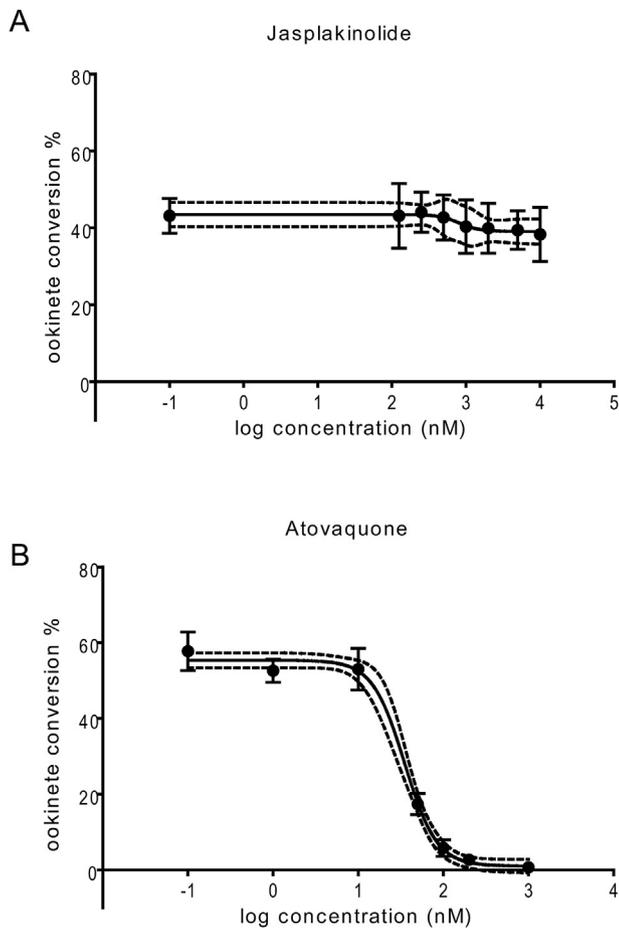


Fig. 3. No effect of jasplakinolide treatment on gametocyte to oocyst development. A high content screening assay using the Operetta platform was employed to determine the inhibitory effect of jasplakinolide on oocyst formation. A. Oocyst formation in the presence of jasplakinolide. No inhibition was detected using concentrations up to 500 nM. Oocyst formation in the presence of atovaquone. EC50 was determined to 35.6 nM.

expected found to be inhibitory at nanomolar concentrations (Fig. 3B).

4. Conclusions

Taken together these data show that actin I in *P. berghei* gametocytes is present in short filaments and complexed to other structures, although unpolymerized soluble actin is also present. In this stage of the parasite, jasplakinolide, which acts by allowing formation of actin filaments by spontaneous induction of nucleation sites (Bubb et al., 2000), appears unable to promote extension of these short actin I filaments. This is in contrast to treatment of oocysts with the drug, which results in the formation of long filament-like structures (Angrisano et al., 2012a, 2012b; Siden-Kiamos et al., 2012). Jasplakinolide also stabilizes actin I *in vitro*, leading to formation of long filaments (Vahokoski et al., 2014). Our results therefore suggest that actin I forms short capped filaments that cannot be extended.

A recent study of *P. falciparum* gametocytes found actin to be localized in filament-like bundles closely associated with the microtubules (Hliscs et al., 2015). *P. berghei* gametocytes lack microtubules and this may be the reason why actin I in the rodent parasite is present as short filaments. However, both the localization pattern and the fact that actin I is found complexed to other

structures suggest that actin I in this parasite is also part of a cytoskeleton.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.exppara.2017.08.001>.

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