

Characterization of *Cystoisospora suis* sexual stages in vitro

Anna Sophia Feix

Veterinaermedizinische Universitaet Wien

Teresa Cruz-Bustos (✉ Teresa.Cruz-Bustos@vetmeduni.ac.at)

Veterinaermedizinische Universitaet Wien

Bärbel Ruttkowski

Veterinaermedizinische Universitaet Wien

Anja Joachim

Veterinaermedizinische Universitaet Wien

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Abstract

Background: The porcine coccidium *Cystoisospora suis* is characterized by a complex life cycle during which asexual multiplication is followed by sexual development with two morphologically distinct cell types, the micro- and macrogametes. Genes related to the sexual stages and cell cycle progression were previously identified in related Apicomplexa. Here, the sexual stages of *C. suis* were characterized *in vitro* morphologically and for temporal expression changes of the mentioned genes to gain insight into this poorly known phase of coccidian development. **Methods:** Sexual stages of *C. suis* (micro- and macrogametes) developing *in vitro* in porcine intestinal epithelial cells were examined by light and electron microscopy. The transcriptional levels of genes related to merozoite multiplication and sexual development were evaluated by quantitative real-time PCR at different time points of cultivation. Dynein light chain and male gamete fusion factor HAP2 expression is presumably restricted to microgametes. Tyrosin-rich proteins are a part of the wall-forming bodies and oocyst wall proteins. The RAD51/Dmc1 protein plays a role during the cell division process, and Nima-related protein kinases regulate the cell cycle and are associated with male or female Plasmodium gametocytes. Transcription levels were compared for parasites in culture supernatants at 6–9 doc (day of cultivation) and intracellular parasites at 6–15 doc. **Results:** Sexual stage of *C. suis* could be found during 8–11 doc *in vitro*. Microgametes had a spherical body (3.0–5.0 μm) and two flagella (10.8–12.3 μm). Macrogametes were spherical with a diameter of 11.5–13.0 μm . Merozoite gene transcription peaked on 10 doc and then declined. Genes related to the sexual stages and cell cycle showed an upregulation with a peak on 13 doc, after which they declined. **Conclusions:** The present work linked gene expression changes to the detailed morphological description of *C. suis* sexual development *in vitro*, including fertilization, meiosis and oocyst formation *in vitro*. Following this process at the cellular and molecular level will elucidate details on potential bottlenecks of development of *C. suis* (and coccidian parasites in general) which could be exploited as novel targets for control.

Background

Cystoisospora suis (syn. *Isospora suis* BIESTER AND MURRAY 1934) is a coccidian parasite of swine which causes diarrhea and reduced weight gain in suckling piglets, mostly in the first three weeks of life, and leads to unthriftiness at weaning, considerably impairing animal health and productivity [1–4]. As with all coccidian parasites, the life cycle of *C. suis* is characterized by asexual multiplication (sporogony, merogony) followed by sexual development with two morphologically distinct cell types, the micro- and macrogametes which presumably fuse to form a zygote from which the oocyst arises [5, 6].

Previous studies have shown that the development of *C. suis in vitro* is comparable with the life cycle *in vivo*, however the development of sexual stages *in vitro* is delayed [7]. This makes it possible to observe, harvest and examine sexual stages in the short time frame in which they occur. Macrogametes of Coccidia form without division and are large and immobile, while microgamonts divide several times to form microgametes, consisting of a small body and flagella which are used to move quickly in search of macrogametes [8].

After fertilization by a microgamete the wall-forming bodies of the macrogamete fuse and form the oocyst wall, as seen in other Coccidia [9, 10]. In the coccidian parasite *Eimeria* two types of wall-forming bodies can be found, an electron dense form, giving rise to the outer layer of the oocyst, and sponge-like bodies that fuse to form the inner layer of the oocyst wall [9].

Transcriptomic and proteomic analyses carried out in *Eimeria* and *Toxoplasma* revealed different genes coding for proteins related to the sexual stages [11, 12]. The two main protein families participating in oocyst wall formation in coccidia are tyrosine-rich proteins called GAM [13] and cysteine-rich proteins OWPs, first described in detail in

Cryptosporidium [14, 15]. GAM proteins are found in the wall-forming bodies type 1. Proteases break down GAM proteins into tyrosine-rich peptides, which are then oxidatively crosslinked by peroxidases and incorporated into the oocyst wall in dityrosine bonds [16, 17]. GAM proteins are the most studied proteins of the oocyst wall; they were developed as antigens for transmission blocking vaccines targeting the gametocyte-specific proteins GAM56, GAM82 and GAM22 of *Eimeria* [13, 18–20]. Cysteine-rich OWPs have also been described in *Toxoplasma gondii* [21]. They are found in the wall-forming bodies type 2. The cysteine residues form disulfide bridges responsible for the stabilization and formation of the oocyst and sporocyst walls, conferring additional strength and rigidity [22]. Proteins with important roles in apicomplexan male sexual stages are involved in axoneme and flagella assembly and construction [23], DNA replication [24], microgamete budding from microgamonts [25] and gamete fusion [26]. Upregulated expression of genes coding for tubulins, dyneins, radial spokes, basal body family proteins, a centrin family protein, kinesins, enkurin related-protein, HAP2 and intraflagellar transport proteins was observed by RNA Seq analysis of *Eimeria tenella* sexual stages [27]. HAP2 is essential for the fusion of gametes surface membrane and subsequent fertilization [27], and It has been proposed as a possible candidate for a transmission blocking vaccine in apicomplexan parasites [28, 29].

The process of fertilization in Coccidia is still poorly understood and has not been visualized yet, but it is commonly assumed that, that following fusion of the micro- and macrogamete, the apicomplexan zygote develops into an unsporulated oocyst. The oocysts are excreted to the environment and the development continues, and meiosis and mitosis result in infectious haploid sporozoites [17, 30]. Among the genes thought to be involved in these processes are the meiotic recombination *Rad51* and *Dmc1*, *protein kinase*, *Aurora* and *Nima* genes, *Cyclin dependent kinase* and *Polo* genes [15, 31, 32].

In this study we aimed to provide a first characterization of *C. suis* sexual stages *in vitro* by comparing the morphology of the stages and the transcriptional profiles of selected conserved genes related to the sexual development of apicomplexan parasites. We hypothesized (a) that all developmental stages of *C. suis* occur *in vitro* and are comparable to those *in vivo* experiments, although the *in vitro* development of the parasite takes longer compared to infections of piglets, and (b) that during sexual development, transcription of genes related to this life cycle phase is increased in *C. suis* similar to other Coccidia and *Plasmodium*.

Methods

***Cystoisospora suis* oocyst collection**

Cystoisospora suis oocysts (strain Wien 1) were obtain from experimental infected suckling piglets. Piglets were raised with the sow in the animal facilities of the Institute of Parasitology, University of Veterinary Medicine Vienna, Austria. Infection of piglets, oocyst collection, oocyst isolation, oocyst purification and excystation were performed as described previously [7]. In deviation to the original protocol, after NaOCl treatment and washing, the oocysts were vortexed three times for 45 sec with Precellys® glass beads (Pqlab, Erlangen, Germany) in 2% sodium taurocholate hydrate (Sigma-Aldrich, St. Louis, USA) in DMEM/Ham's F-12 culture medium (Gibco, Thermo Fisher Scientific, Waltham, USA).

***In vitro* culture and parasite harvest**

Intestinal porcine epithelial cells (IPEC-1, ACC 705, Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Leibniz, Germany) [33–35] were used as host cells *in vitro* (seeded 4×10^5 per well in a six well plate), in a DMEM/Ham's F-12 medium (Gibco) with 5% fetal calf serum (FCS, Gibco) and 100 U/ml penicillin and 0.1

mg/ml streptomycin (PAA, Pasching, Austria) at 37°C in 5% CO₂. After 24 h of cell growth IPEC-1 were infected with 5x10³ sporozoites released from excysted oocysts and incubated further at 40°C. Free sexual stages were harvested by collecting culture medium supernatant daily at 9–11 days of cultivation (doc). The collected stages were washed with phosphate buffered saline (PBS; Gibco; 144.0 mg/L KH₂PO₄, 9000.0 mg/L NaCl, 795.0 mg/L Na₂HPO₄·7H₂O) and purified using a Percoll® (GE Healthcare, Uppsala, Sweden) density gradient with layers at 80%, 40% and 30%, and the sample on top. The gradient was centrifuged at 600 ´ g for 10 min at 20°C in a Mega Star 3.0R swing bucket centrifuge (VWR International, Leuven, Belgium). Both acceleration and deceleration were at the lowest possible setting. For the sampling of intracellular sexual stages, adhering host cells and parasites were incubated with Accutase® (Thermo Fisher Scientific, Waltham, USA) for 30 min and the detached material was washed twice with PBS and pelleted by centrifugation at 600 ´ g for 10 min. The numbers of sexual stages were estimated in the cell culture chambers and counted in a Neubauer-counting chamber at each given time point, and the mean numbers of sexual stages per well were calculated.

Light and electron microscopy

Digital microphotographs of all *in vitro* *C. suis* stages, but especially isolated sexual stages, both live and fixed in 100% EtOH, were taken with an Olympus IX71 inverse microscope (Olympus, Shinjuku, Japan) or a Zeiss Imager Z2 microscope (Zeiss, Jena, Germany) at 400 ´ and 600 ´ magnification and measured (n=50 per stage) with Zeiss ZEN lite software (Zeiss).

For scanning electron microscopy sample preparation cover slips were washed in 100% EtOH and coated with 0.1% poly-D-lysine (Merck Millipore, Burlington, USA) on which the isolated sexual stages were left to settle for 1 h at 36°C in PBS. Afterwards the parasites were fixed for 3 min on the cover slip using 2.5% glutaraldehyde in PBS. The samples were washed twice in PBS for 15 min. Post-fixation was performed with 1% osmium tetroxide for 3 min. The coverslips were dehydrated in an ascending alcohol series from 30-100% EtOH for 3 min each. Thereafter the samples were critical point dried in a Leica CPD 300 (Leica Microsystems, Wetzlar, Deutschland). The dried samples were mounted on metal stubs and gold sputtered for 80 sec with a JEOL JFC-2300HR (JEOL GmbH, Freising, Germany). All scanning electron microscopy work was performed at the Core Facility Cell Imaging and Ultrastructure Research, University of Vienna-member of the Vienna Life-Science Instruments (VLSI). Sexual stages were photographed with a JEOL IT 300 scanning electron microscope (JEOL) and measured with Zeiss ZEN lite software (Zeiss).

Transcription levels at different time points of *C. suis* development *in vitro*

Quantitative real-time PCR (qPCR) was used to quantify the transcripts levels of four genes related to sexual stages, four genes related to cellular division and meiosis and one related to the merozoite stage of *C. suis* at different time points of development *in vitro*.

Total RNA was isolated from infected cell cultures using an RNeasy® mini kit (Qiagen, Hilden, Germany) and treated with RNase-free DNase (Qiagen) according to the manufacturer's instructions to remove any DNA contamination. Total RNA was quantified using a NanoDrop® 2000 (Thermo Fischer Scientific, Waltham, MA, USA). cDNA synthesis was accomplished using the iScript cDNA synthesis kit (Bio-Rad, Hercules, California, USA).

The nucleotides sequences for genes linked with sexual development in Apicomplexa (Table 1) were searched using the Basic Search Alignment Tool (BLAST) in the genomic resource database ToxoDB (<https://toxodb.org/toxo/>). Alignment analysis and percentage of identities were performed using Clustal omega (<https://www.ebi.ac.uk/Tools/msa/clustalo>).

Transcription levels were assessed daily from 6–15 doc. Quantitative PCR amplification of cDNA was carried out on a Mx3000P thermal cycler (Agilent Technologies, Santa Clara, CA, USA). The primers for gene amplification are listed in Table 2. Reaction mixtures contained 2.5 µl of sample DNA (100 ng/µl), 5 µl of SsoAdvanced™ Universal Probes Supermix (Bio-Rad) and 1.3 µl of nuclease-free water with primers and probes at a final concentration of 500 and 200 nM, respectively. Activation of polymerase was performed at 95°C for 2 min, followed by 50 cycles of 95°C for 15 sec, 60°C for 30 sec. Each sample was run in triplicate and the complete experiment was performed in two separate biological replicates. The qPCR results were normalized against each of the two reference genes, namely glyceraldehyde-3-phosphate and actin. Average gene expression relative to the endogenous control for each sample was calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

All values are expressed as means ± standard error of the mean (SEM). Significant differences among groups were compared using the Student's t-test or one-way ANOVA with multiple comparisons. Differences were considered statistically significant at $P < 0.05$. All statistical analyses were conducted using GraphPad® Prism 8.2 (GraphPad Software, San Diego, CA).

Results And Discussion

Morphology

Sporozoite-infected cell cultures are a suitable model for producing all stages of *C. suis* *in vitro*. Free merozoites can be found in appreciable numbers from day 6 doc. Sexual stages occur in appreciable numbers between 8-11 doc and are mainly found outside the host cell. Early sexual stages were first detected at 8 doc. First gamonts could be located from 9 doc onwards, whereas first macrogametes and motile microgametes could be found a day later (Fig. 1). We estimated that the ratio of gamonts : early sexual stages was about 1 : 2. The first oocysts appeared 11 doc until 13 doc.

The sexual development of Coccidia, including *C. suis*, is not well characterized [8]. Although it is presumed that sexual stages are crucial in parasite development, little is known about their morphology, especially *in vitro* [6]. As the life cycle of *C. suis* takes longer *in vitro* (about 11 days) than *in vivo* (5 days) [4], it is possible to collect samples of every stage of *C. suis* development, hence making sexual stages available for further research.

Early sexual stages (immature gamonts) varied in form and size but their length was generally 15.6 ± 0.5 µm (n=50) and their width 11.6 ± 0.4 µm (n=50, Fig. 2a). Both micro- and macrogamonts were subspherical and had very similar diameter, however microgamonts were between 16.8 ± 0.9 µm (n=50), whereas macrogamonts were 16.6 ± 1.1 (n=50) in diameter (Table 1). In light microscopy, unstained microgamonts were recognized by their large vacuole and motile microgametes inside (Fig. 2b, c). Both micro- and macrogamonts are often found in close proximity to each other (Fig. 2b, 2d, Additional file 1), and the egress of microgametes from microgamonts could be observed during a four-hour time frame. Each microgamont contained between 30-40 microgametes.

Scanning electron microscopy observations show that microgametes consisted of a small, spherical (3.5 ± 0.5 µm, n=50) body with two opposing flagella of 11.2 ± 0.5 µm length (n=50, Fig. 3a), which enabled the quick movement of the microgamete in search for a macrogamete. Macrogametes on the other hand were immobile, spherical with a smooth surface and had a diameter of 12.1 ± 0.5 µm (n=50, Fig. 3b, Table 1).

Our study provides the first detailed *in vitro* characterization of sexual stages of *C. suis*. Previous *in vivo* studies already described immature micro- and macrogamonts in tissue sections of the small intestine [5, 36]. Early sexual stages are described as ovoid to elongate and smaller in size than those *in vitro* (immature microgamonts: 11.8 x 8.4 µm and immature macrogamonts: 9.4 x 6.5 µm) while the shape and size of mature micro- and macrogamonts are comparable to early sexual stages [6]. Other closely related coccidian species show similarities morphologies of sexual stages [37, 38]; however microgametes of *T. gondii* and *Eimeria* [17] have flagellae on the posterior end, whereas in *C. suis* they are clearly positioned on opposite sides, which might also affect their movement on the search for a macrogamete. With an *in vitro* system that allows for the collection and examination of mature sexual stages of *C. suis*, further studies on their properties and the fertilization process in Coccidia will be possible.

Genes linked to sexual stages

Identification of genes linked to the sexual stages is crucial to understand the developmental biology and the fertilization process of organisms with sexual development, including apicomplexan protozoa. Here, we analyzed eight of the genes with highest upregulated transcripts in microgametes, macrogametes and oocysts based on previous studies in Apicomplexa [11, 12, 27, 29, 31, 39–42]. To identify genes or their orthologues related to the sexual development in *C. suis*, we used the ToxoDB parasite database and determined four genes related to sexual stages and four genes related to meiosis and cellular division (see Table 2).

For microgametes, the orthologues of dynein light chain 1 (*DLC1*) and the male gamete fusion factor (*HAP2*), *CsDLC1* (CSUI_000751) and *CsHAP2* (CSUI_000472), clustered with the respective genes from closely related coccidian parasites. Sequence analyses revealed identities greater than 60% with *T. gondii* and *Neospora caninum*, signifying the close relationship of *C. suis* with these two species [42], and more than 36% with *Eimeria necatrix* (Table 2).

Two of the most highly transcribed genes found in macrogametes code for proteins involved in the formation of the oocyst wall. *CsOWP1* (CSUI_006207) has more than 65% of identity with that of *T. gondii* (Table 2). Genome analysis of *C. suis* and *T. gondii* failed to identify orthologues for the GAM56 protein of *Eimeria* but revealed three low molecular weight hypothetical proteins possessing both a leader peptide and tyrosine-rich sequences. CSUI_001473 is an orthologue with 54% of homology with a gene coding for a tyrosine rich protein in *Toxoplasma* (TGME49_037080). Two of these three genes (TGME49_037080 and TGME49_087250) have peak expression levels in oocysts and the encoded proteins are incorporated as a part of the oocyst wall [40].

Among the genes with high expression in oocysts of *Toxoplasma* and *Cryptosporidium* [15, 40] we determined one coding for the meiotic recombination Rad51/Dmc1-like protein, and the orthologue in *C. suis*, CSUI_004539, had a 93% similarity to the gene of *Toxoplasma* (TGARI_272900) (Table 3).

Protein kinases have a role not only for signaling, but also during the transition states of cells [43]. Nima (“never in mitosis-gene A”)-related kinases of *Plasmodium* and *Toxoplasma* are involved in post-fertilization processes and in the meiosis [32]. We found three orthologues for *Nima* genes, one of them specific for male gametocytes, *Nima1* (CSUI_004317) and two of them for female gametocytes, *Nima2* and *Nima4*, (CSUI_003099 and CSUI_000744) and all three showed more than 40% of homology with the respective genes of *Toxoplasma* (Table 3).

The expression profiles of one gene related to asexual stages (merozoites) of *C. suis* (CSUI_005805 [44]), the four genes related to sexual stages and the four genes related to cellular division and meiosis were examined at different time points of cultivation *in vitro* in extracellular and intracellular parasites.

To evaluate the development of merozoites during cultivation and to test the suitability of the qPCR analysis for the detection of temporal changes in gene transcription, we included the uncharacterized merozoite-specific gene CSUI_005805. The level of the transcription for merozoites increased until it reached the maximum around 9–10 doc and then declined (Fig. 4). In previous studies, this gene showed a higher transcription in merozoites compared to sporozoites and in the present study, it showed higher levels during merozoite development compared to sexual development and oocyst formation. The transcriptional level increased throughout the entire merogony, indicating that this protein might be important for the establishment and/or growth of merozoites inside the host cell [44].

Transcript levels of genes related to sexual development in extracellular parasites were compared with 6 doc as a reference for merozoites released into the medium and 9–10 doc for extracellular gamonts/gametes. The transcription levels were eight to nine times higher in gamonts/gametes compared to merozoites (Fig. 5a), which agrees with the high level of upregulation demonstrated in RNA-seq analysis of sexual stages of *Eimeria* spp. [11].

Transcription levels of intracellular parasites were compared daily at 6–15 doc. As a reference point, we used day 10 because on that day first mature gametes were seen *in vitro*. The microgametes related genes transcripts reach peaks on 13–14 doc and the increase was 10– to 90–fold time higher compared to merozoites at 6 doc (Fig. 6) which is in agreement with results for RNA seq comparing merozoites from third-generation merozoites and gamonts of *Eimeria* [11]. The levels for *DLC1* and *HAP2* (Fig. 6a, c, d) transcripts on 13 and 14 doc were 30– and 60–fold higher, respectively, compared to day 6 with no detectable expression. Dynein proteins are part of the flagellum of microgametes and form part of the microtubule motor [45]. They are also involved in mitosis and meiosis, and are the major constituents of mitotic spindles, which are used to pull eukaryotic chromosomes apart [46]. As judged from the gene upregulation, these processes take place in *C. suis* *in vitro* between 9 and 15 doc. However, before that time point no transcription of *DLC1* could be measured and the expression of *DLC1* in Apicomplexa seems to be restricted to microgametes as described earlier [11].

In addition, we observed an upregulation of the gene expression of the fusogen *HAP2* during 9–13 doc followed by a decrease (Fig. 6a, d). Its expression is also restricted to male gametes and its function in gamete fusion and during fertilization is extensively described for plants and unicellular eukaryotes [26, 47]. The results in *C. suis* correlate with previous reports on *Eimeria* and *Toxoplasma*. *HAP2* is found in microgametes and unsporulated oocysts but not in sporulated oocysts or sporozoites [26, 27, 40, 48, 49]. Moreover, transcription of *HAP2* was increased during the enteric development of *T. gondii* in the intestine of cats. *HAP2* knockout parasites failed to fertilize and produce oocysts *in vivo*, and this supports the hypothesis that interfering with the fertilization process can be utilized in a transmission-blocking vaccine [29]. As for *DLC1*, expression seems to be restricted to microgametes.

Oocyst wall formation is a hallmark of coccidian development, and *OWP* and *GAM*-encoded proteins have previously been characterized in *Eimeria* and *Toxoplasma* as well as *Cryptosporidium* as constituents of the oocyst wall [29, 50, 51].

The *GAM* proteins EmGam56 and EmGam82 were identified as antigens that conferred protection against different species of chicken *Eimeria* due to their conserved nature. A subunit vaccine for immunization was previously developed from *E. maxima* gamont proteins and commercialized for the prevention of coccidiosis [9, 52].

No homologues for *GAM* genes were found in the *C. suis* genome or in the *Toxoplasma* database. However, oocysts of *C. suis* display the characteristic autofluorescence similar to other coccidia [17, 53] which is likely due to the dityrosine bonds formed between tyrosine-rich proteins present in the oocyst wall [11, 51]. A search in the proteome of *Toxoplasma* for predicted proteins with sequences rich in tyrosine identified five hypothetical proteins, and three of them presented highest levels of expression in the oocyst wall proteomic fraction [31] and were upregulated in oocyst

transcriptomes compared to tachyzoites and bradyzoites [40]. We identified and analyzed an orthologue of one of them, TyRP. Gene transcripts of both proteins presumably involved in oocyst wall formation, *OWP1* and *TyRP*, were upregulated until 13 doc and then steeply declined (Fig. 6b, e, f). While *TyRP* transcription could only be detected from 8 doc (Fig. Fig. 6b, f), low transcription levels for *OWP1* were already found from 6 doc (Fig. 6b, e). indicating that merozoites present at this time point are probably already committed to a further development into macrogamonts, as described for *E. tenella* [17, 27].

In extracellular parasites, the RNA transcription of *Rad51/Dmc1-like* showed similar levels of transcription in parasites from 6 and 9–10 doc (Fig. 5b). In intracellular stages of *C. suis*, the RNA transcription levels of *Rad51/Dmc1-like* were rather constant from 6–12 doc except for an increase 13–14 doc (Fig. 7a, c) which probably corresponds to meiosis during the formation of the oocyst, since the *RAD51/Dmc1-like* gene codes for a protein of the Rad51 family which assists in repair of DNA double strand breaks during mitosis, while the two recombinases, Rad51 and Dmc1, facilitate the recombination between homologous chromosomes during meiosis [54].

Nima-related kinases (Nek or NRK) represent a conserved family of serine/threonine kinases implicated in the regulation of distinct cellular events [47]. Neks have important roles in the maintenance of centrosome function and structure, mitotic microtubule organization, and the regulation of axonemal microtubule in cilia and flagella. Nima1 is an orthologue of Nek2 in humans which is involved in the maintenance of centrosome structures and mitotic microtubule organization, thus playing a role during mitosis. In *Toxoplasma*, Nima1 is essential for centrosome splitting, proper formation of daughter cell budding and faithful segregation of genetic material. A point mutation in a conserved portion of the gene causes a severe mitotic defect [48]. In *Plasmodium*, Nima1 is found in replicative forms of the parasites, in asexual and sexual stages, with a role in mitosis, and specifically in microgametes. In extracellular parasites, Nima1 showed almost double the expression levels in gamonts compared to parasites at 6 doc, and Nima2 and Nima4 were almost exclusively transcribed in gamonts/gametes (Fig. 5b). The mRNA levels in intracellular parasites found for Nima1 were significantly higher on 6 and 14 doc compared to 10 doc (Fig. 7 b, d). Our results correlate with the presence of Nima1 in asexual stages and sexual stages [49]. Due to the simultaneous presence of both in cell culture an upregulation in sexual stages could not be observed. By contrast, Nima2 and Nima4 were practically absent before 9 doc when the first mature gamonts occurred and peaked at 13 respectively 14 doc (Fig. 7b, e, f). In *Plasmodium*, Nima2 and Nima4 are only found in female gametocytes and the two encoded proteins are necessary for completion of the sexual cycle. In Nek2 knockout parasites premeiotic DNA replication is dysregulated and the parasites do not develop ookinetes, suggesting that the principle role of Nek2 is during DNA replication preceding the meiosis Nek 4 does not appear to be required for gametocytogenesis but is essential for premeiotic DNA replication in the zygote, consistent with the cell cycle related function [55]. As these two *Nima* genes were expressed in parallel with the occurrence of gamonts and unsporulated oocysts *in vitro* we assume that the encoded proteins have similar roles during the development of *C. suis*.

Conclusion

Although sexual stages of Coccidia have previously been addressed for intervention in *T. gondii* infections, the lack of models for detailed studies on the involved stages *in vitro* has been highly prohibitive for more detailed research. In the present study, we could demonstrate mature gamonts, gametes and oocysts of *C. suis in vitro* in a defined time frame as well as a correlation of size and form of stages *in vitro* with those found *in vivo*. We also identified genes linked to the developmental and cell cycle progression of *C. suis in vitro*. We defined the demonstration of sexual stages *in vitro*, their time-limited occurrence and the gene expression of stage-specific genes. It was previously demonstrated in other Coccidia, *Eimeria* and *Toxoplasma*, that interfering with fertilisation can block transmission of

this parasite, providing a novel tool for intervention strategies and a hint at a potential developmental bottleneck in the life cycle of *C. suis*.

Abbreviations

BLAST: basic local alignment search tool; DLC1: dynein light chain type 1 protein; GAM: sexual (macrogamete/macrogametocyte) stage proteins (wall-forming proteins, wfp); HAP2: male gamete fusion factor hapless-2; IPEC: Intestinal porcine epithelial cells; Nima: “never in mitosis-A”-related kinases; Nek / NRK: Nima-related kinases; OWP1: oocyst wall protein 1; qRT-PCR: quantitative real time polymerase chain reaction; Rad51: DNA repair protein 51; TyRP: tyrosine rich protein.

Declarations

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Ethics statement

All procedures in this study involving experimental animals were approved by the Austrian Federal Ministry of Science and the Ethics Committee of the University of Veterinary Medicine Vienna in strict accordance with the Austrian Animal Protection law (BMWF-68.204/0059-V/3b/2018). All efforts were made to minimize the numbers of animal used for *C. suis* oocyst generation.

Consent for publication

Not applicable

Availability of data and materials

All data and materials of the experiments described here are included in the manuscript and its additional file.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

AJ designed the study. BR provided oocysts, sporozoites and merozoites and was responsible for the maintenance of the cell culture. ASF isolated and prepared the stages from *in vitro* cultures and performed all morphological analyses. TCB processed and analyzed all genetic experiments. ASF and TCB drafted the manuscript. All authors read and approved of the final version of the manuscript.

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Tables

Table 1. Overview of mean, standard deviation, variance and range of *C. suis* sexual stages in vitro, n=50

values in μm	early sexual stages		microgamonts	macrogamonts	microgametes		macrogametes
	width	length	diameter	diameter	body	tail	diameter
mean	11.6	15.6	16.8	16.6	3.5	11.2	12.1
standard deviation	0.4	0.5	0.9	1.1	0.5	0.5	0.5
variance	0.2	0.2	0.9	1.3	0.3	0.3	0.2
minimum	11.0	15.0	15.4	15.1	3.0	10.8	11.5
maximum	12.5	16.5	18.4	18.4	5.0	12.3	13.0

Table 2. Primers used in this work

	Primer	Sequences (5'@ 3')	Amplicon length (bp)
1	Fw-ACTIN	CTTGCTGGCCGTGATTTGAC	
2	Rv-ACTIN	ATATTGCCGTCCGGAAGCTC	203
3	Probe-ACTIN	CCTCCGCCGAGAAGGAAATT	
4	Fw-GAPDH	TTCAACGAGAAGGAGCCAAG	
5	Rv-GAPDH	CTTCGGAGGTGCAGACATG	150
6	Probe-GAPDH	CAAGGAAAAGGCTGAGGCGCAT	
7	Fw-HAP2	GGAACCCAGGAAATTTTGT	
8	Rv-HAP2	CATGTTGTTGATGTGCGTGA	155
9	Probe-HAP2	GCTGCTGGTGCAGTGAGGTC	
10	Fw-DLC1	TGCTATGGCCTGTTGATATGC	
11	Rv-DLC1	CTTCTGGTCGAGCTCCTTTT	137
12	Probe-DLC1	TGCTGCGCGTGA CTGTATAATCCA	
13	Fw-OWP1	CCAGAAGGATGTTTATTTGCCG	
14	Rv-OWP1	TGGGCAGATGTATTCAGGTTT	123
15	Probe-OWP1	AATCCGAAGGGCAGCGTTGTAGAA	
16	Fw-TyRP	GAAGTGGACGGTGATCGTGA	
17	Rv-TyRP	GCTCTCAATAAGTCCCTCAGAG	177
18	Probe-TyRP	CTCATGCGCTCGCTACCTGA	
19	Fw-Rad51	GCTTCGCTTTGCTTATTGTC	
20	Rv-Rad51	CAACAACAGCCACACCATAC	150
21	Probe-Rad51	TGCCACGGCCCTATACAGGT	
22	Fw-NIMA1	GCTGGAAACTGGTGTTTTAC	
23	Rv-NIMA1	GCATCGCAGTACTCCATAAC	169
24	Probe-NIMA1	GTGAACTTCGGCACCCCAAC	
25	Fw-NIMA2	AGGACAACACTACATCCGTGTC	
26	Rv-NIMA2	CGTGACATATATTTTCGCTGA	240
27	Probe-NIMA2	TCCAGCAAGCAAGAACGCAG	
28	Fw-NIMA4	AAAGAGTCGCAGATTCTCAG	
29	Rv-NIMA4	CCTGCGTATGATCAAGAAGT	172
30	Probe-NIMA4	AGAATTCGCTGGCGGATTT	
31	Fw- CSUI_005805	CCTGAAAGTCGCCTGTCCAT	
32	Rv- CSUI_005805	GACGCGTCAGCCGTTATAGT	224
33	Probe-CSUI_005805	CTCTCAGTTTCGCGGCACCT	

Table 3. Classification of genes used in this study from *C. suis* and their comparison with other coccidian parasites. Percentage values represent identities of *Toxoplasma*, *Eimeria* and *Neospora* genes with their corresponding *Cystoisospora* orthologs. All sequences are found in the genomic Resource database ToxoDB (<https://toxodb.org/toxo/>). Alignment analyses were performed using the Clustal omega (<https://www.ebi.ac.uk/Tools/msa/clustalo>).

	<i>Cystoisospora suis</i>	<i>Toxoplasma gondii</i>		<i>Eimeria necatrix</i>		<i>Neospora caninum</i>	
	Accession no.	Accession no.	Identity (%)	Accession no.	Identity (%)	Accession no.	Identity (%)
<i>Dynein light chain type 1 (DLC1)</i>	CSUI_000751	TGARI_244900	71.70	ENH_00055140	36.49	NCLIV_019360	74.53
<i>Male gamete fusion factor (HAP2)</i>	CSUI_000472	TGARI_285940	63.80	ENH_00067440	37.5	NCLIV_014480	61.28
<i>Oocyst Wall protein (OWP1)</i>	CSUI_006207	TGARI_204420	65.87	ENH_00062180	23.45	NCLIV_020820	66.87
<i>Tyrosine rich, "Eimeria gam-like"</i>	CSUI_001473	TGARI_237080	54.97	ENH_00047090	22.52	NCLIV_050960	48.83
<i>DNA repair proteinRad51/dmc1-like</i>	CSUI_004539	TGARI_272900	93.18	ENH_00059490	81.98	NCLIV_059840	46.23
<i>Nima-related protein kinase 1 Nima1</i>	CSUI_004317	TGARI_292140A TGARI_292140B	43.97	ENH_00060280	46.41	NCLIV_043340	42.44
<i>Nima-related protein kinase 2 Nima2</i>	CSUI_003099	TGARI_307640	86.62	ENH_00076430	65.94		
<i>Nima-related protein kinase 4 Nima4</i>	CSUI_000744	TGARI_244620	77.14	ENH_00003390	31.27	NCLIV_019170	83.46

Additional File Legends

Additional file 1: Video of free microgametes, a macrogamont containing motile microgametes and a macrogamete in close proximity to each other, in real time.

Additional file 2: Table S1. Reporting of significant results from statistical analyses from Figs. 4–7.

Figures

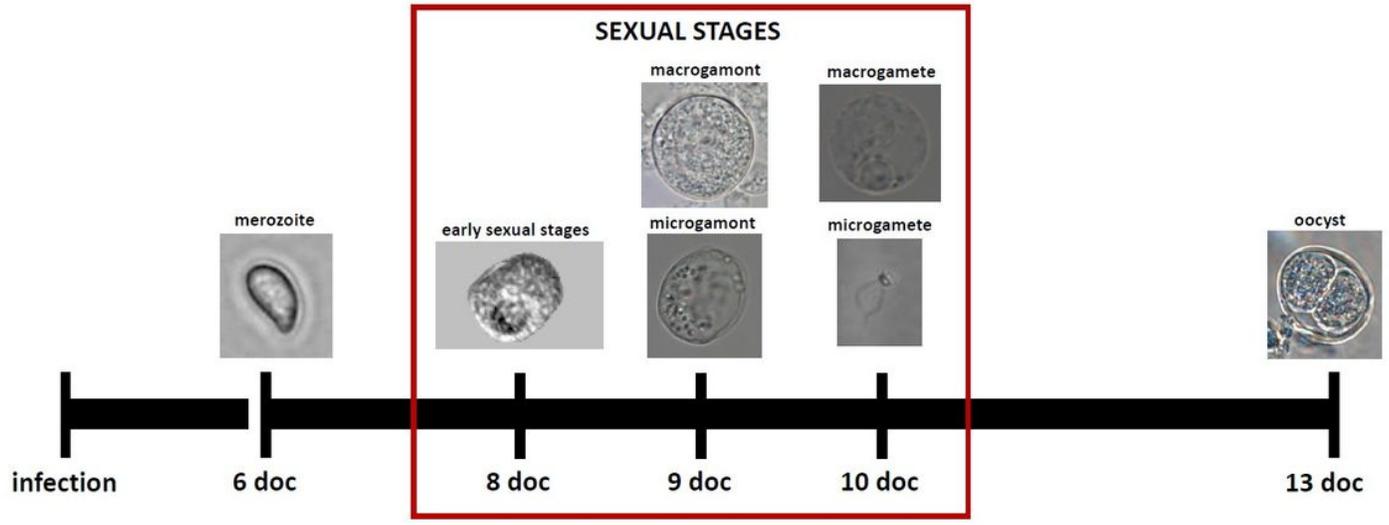


Figure 1

Time line of *C. suis* development in vitro. Early sexual stages could be found at 8 days of cultivation (doc) and gamonts from 9 doc onwards. Micro- and macrogametes could be detected from 10 doc onwards in vitro. Sporulated oocysts were found at 13 doc.

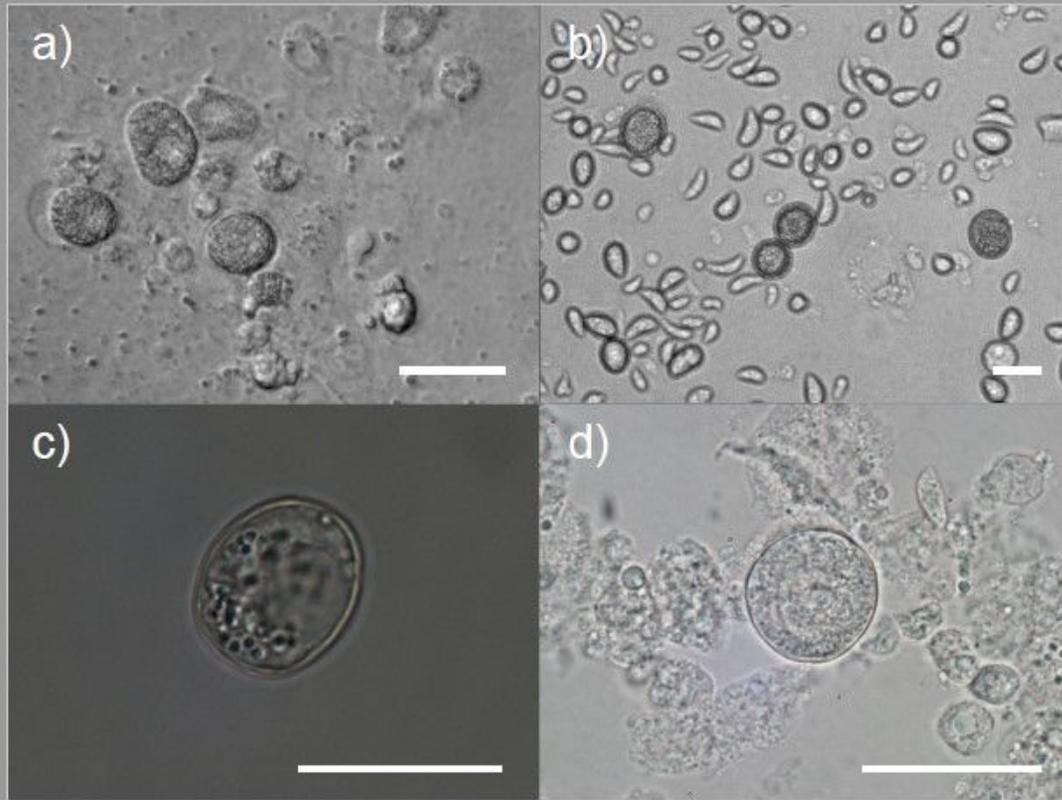


Figure 2

Light microscopy of different sexual stages of *C. suis* in vitro culture. (a) early sexual stages, 7 doc, differential interference contrast. (b) micro- and macrogamont in close proximity to each other, 8 doc, differential interference contrast. (c) microgamont, 8 doc. (d) macrogamont, 8 doc. Scale bar=20 μm.

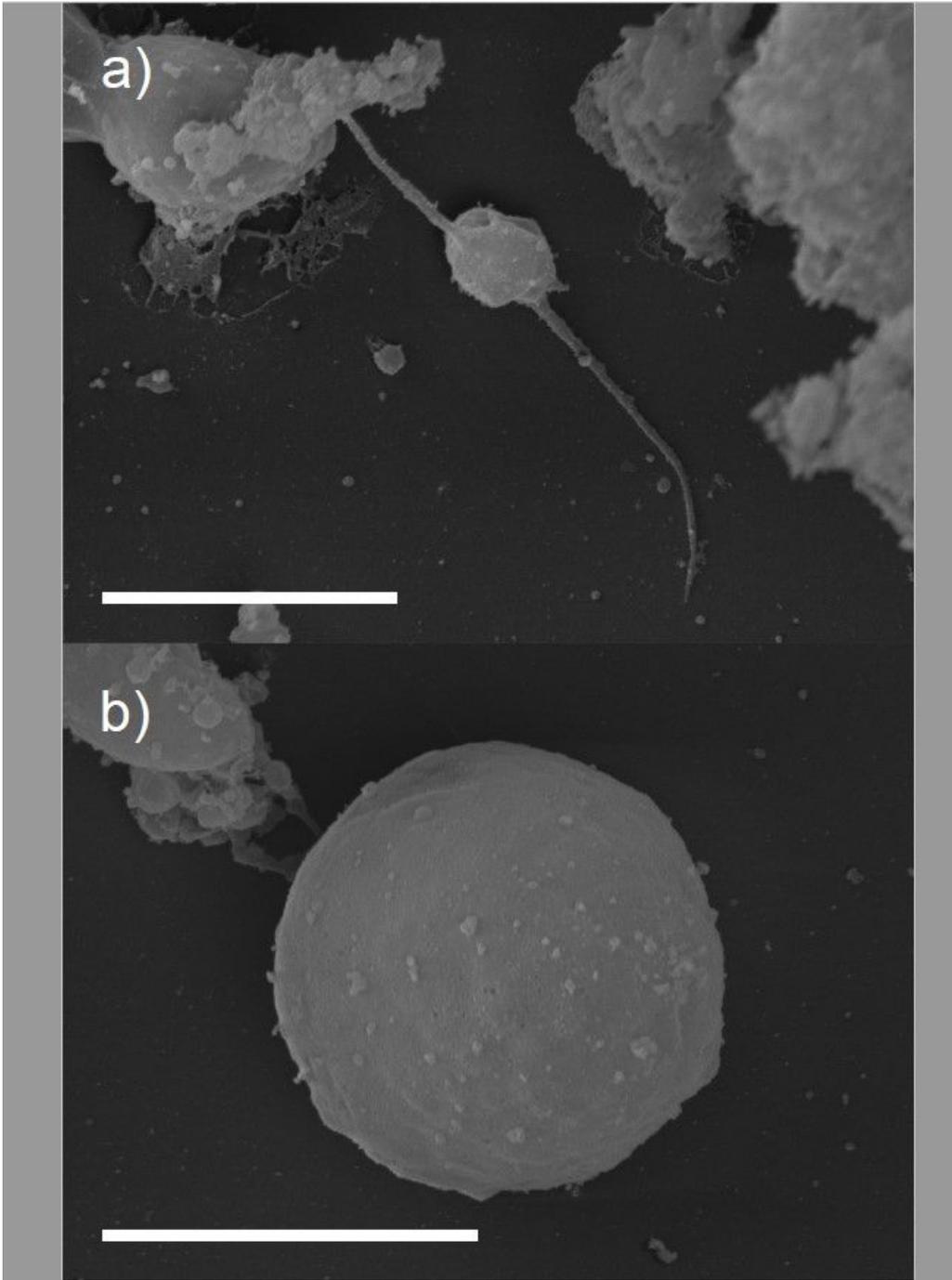


Figure 3

Scanning electron microscopy of adult sexual stages of *C. suis* in vitro culture. (a) microgamete 10 doc. (b) macrogamete from 10 doc. Scale bar=5 μ m.

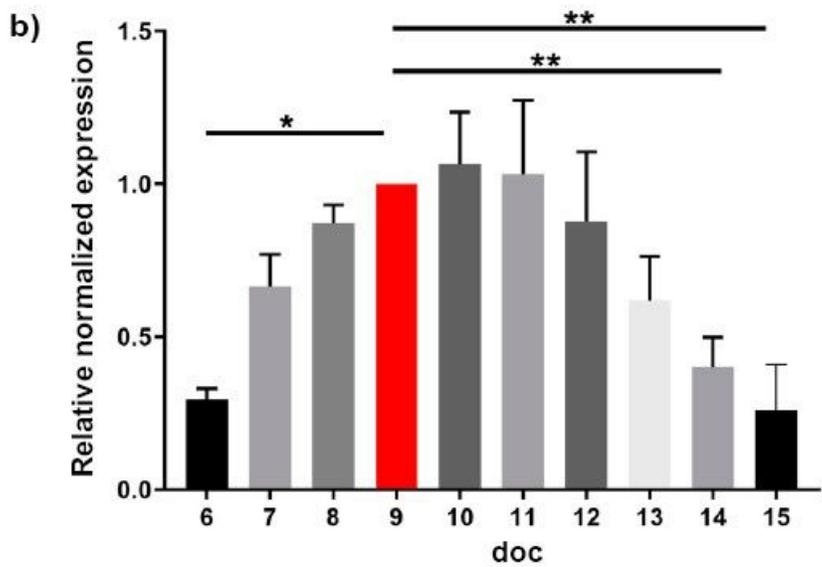
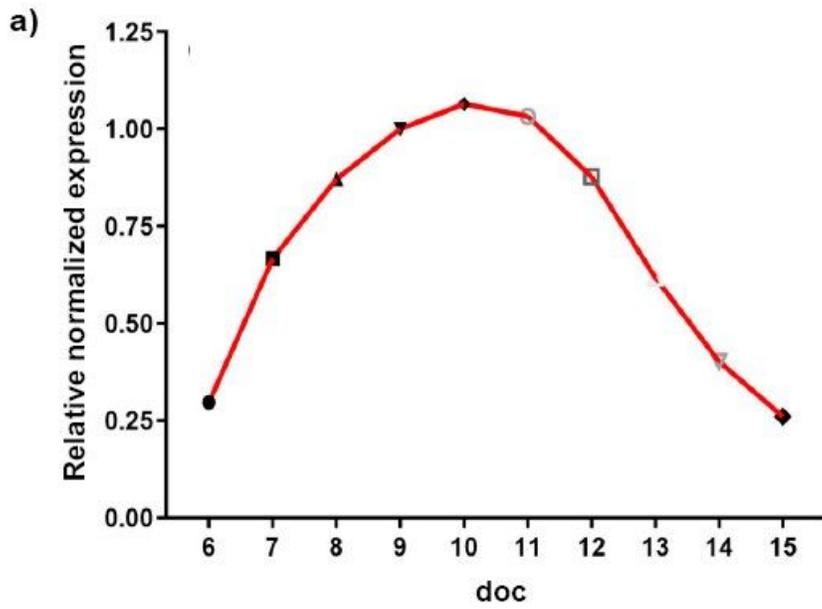


Figure 4

Relative mRNA expression levels of CSUI_005805 in intracellular parasites. Qualitative RT-PCR of intracellular parasites from 6–15 doc. Values are means \pm SEM (n=4). One-way ANOVA with multiple comparisons. **P<0.01. For details on statistical data see additional file 2.

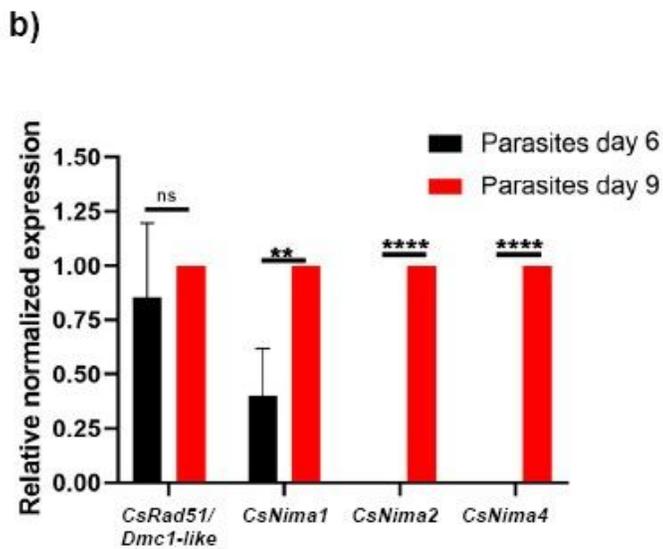
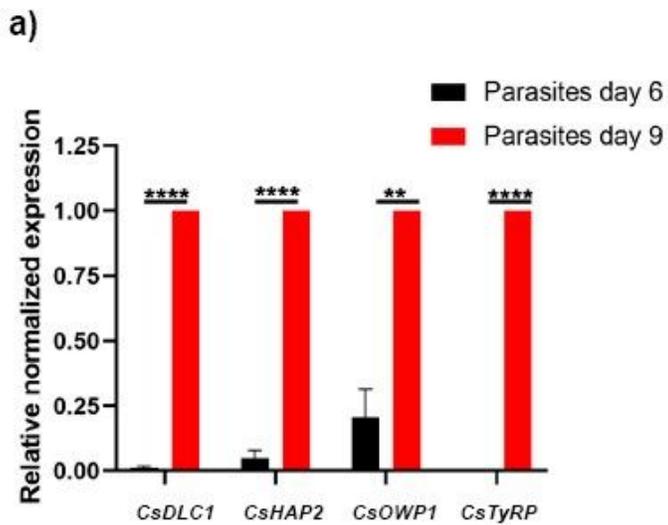


Figure 5

. Relative mRNA expression levels of sexual related genes, cellular division and meiosis genes in extracellular parasites. Qualitative RT-PCR of intracellular parasites 6 to and 9 days of cultivation. Values are means \pm SEM (n=4). Unpaired Student's t-test. ns: not significant ($P > 0.05$), ** $P < 0.01$, **** $P < 0.0001$. For details on statistical data see additional file 2.

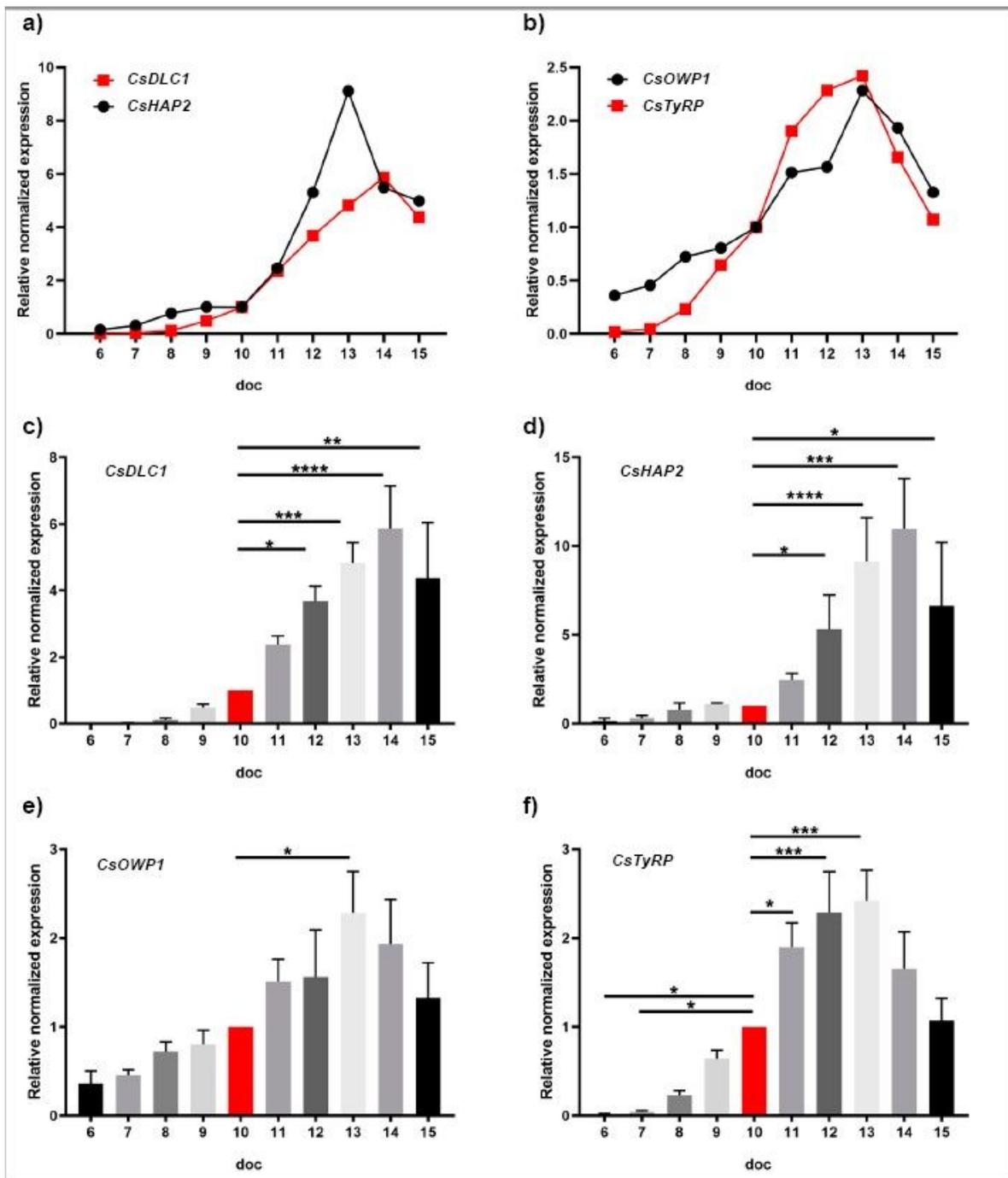


Figure 6

Relative mRNA expression levels of sexual related genes in intracellular parasites. qRT-PCR of intracellular parasites from day 6 to day 15. (a) microgametes related genes. (b) macrogametes related genes. (c) *CsDLC1*, (d) *CsHAP2*, (e) *CsOWP1* and (f) *CsTyRP* (CSUI_001473). Values are means \pm SEM from four independent experiments and are expressed as arbitrary absorbance units. Values are means \pm SEM (n=4). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; by one-way with multiple comparisons. For details on statistical data see additional file 2.

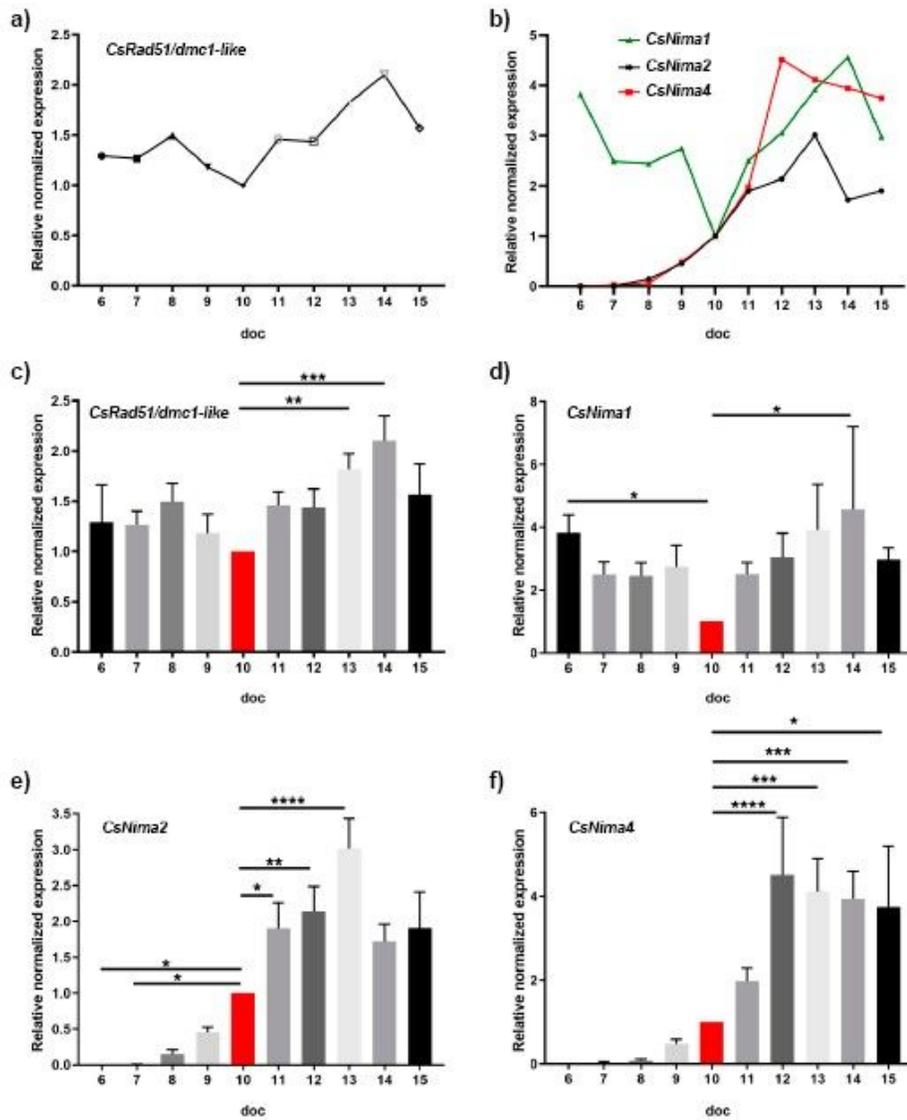


Figure 7

Relative mRNA expression levels of related to cellular division and meiosis genes in intracellular parasites. qRT-PCR of intracellular parasites from 6 to 15 days of cultivation. (a) CsRad51 related gene. (b) Nima-Nek related kinase genes. (c) CsRad51, (d) CsNima1, (e) CsNima2 and (f) CsNima4. Values are means \pm SEM from four independent experiments and are expressed as arbitrary absorbance units. Values are means \pm SEM (n=4). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; by one-way with multiple comparison. For details on statistical data see additional file 2.

Supplementary Files

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