The transcriptome from asexual to sexual in vitro development of *Cystoisospora suis* (Apicomplexa: Coccidia)

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The apicomplexan parasite *Cystoisospora suis* is an enteropathogen of suckling piglets with worldwide distribution. As with all coccidian parasites, its lifecycle is characterized by asexual multiplication followed by sexual development with two morphologically distinct cell types that presumably fuse to form a zygote from which the oocyst arises. However, knowledge of the sexual development of *C. suis* is still limited. To complement previous in vitro studies, we analysed transcriptional profiles at three different time points of development (corresponding to asexual, immature and mature sexual stages) in vitro via RNASeq. Overall, transcription of genes encoding proteins with important roles in gametes biology, oocyst wall biosynthesis, DNA replication and axonema formation as well as proteins with important roles in merozoite biology was identified. A homologue of an oocyst wall tyrosine rich protein of *Toxoplasma gondii* was expressed in macrogametes and oocysts of *C. suis*. We evaluated inhibition of sexual development in a host-free culture for *C. suis* by antiserum specific to this protein to evaluate whether it could be exploited as a candidate for control strategies against *C. suis*. Based on these data, targets can be defined for future strategies to interrupt parasite transmission during sexual development.

*Cystoisospora suis* (syn. *Isospora suis*) is a protozoan parasite of the phylum Apicomplexa (Class Conoidasida, subclass Coccidiasiina, order Eucoecidiorida, family Sarcocystidae). This phylum contains almost exclusively obligate endoparasites of animals, including species of great medical and veterinary relevance such as *Plasmodium* spp., *Eimeria* spp., *Cryptosporidium* spp., and *Toxoplasma gondii*. *Cystoisospora suis* is the causative agent of neonatal porcine cystoisosporosis (coccidiosis). The disease is characterized by generally self-limiting 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. It has a worldwide distribution, and infections are very common, particularly in young animals.

The life-cycle of *C. suis* consists of asexual multiplication (sporogony, merogony) coupled with sexual development (gamogony) with the production of gamonts (syn. gametocytes). Merogony and gamogony take place entirely in one host and the sporogony in the environment. After ingestion of sporulated oocysts, invasive stages are released and infect the gut epithelial cells to reproduce asexually within an intracellular vacuole. The final generation of merozoites is considered to be sexually committed as the first step towards sexual development. The early gamonts, already differentiated into spherical micro- and macrogamonts (syn. micro- and macrogametocytes), are both immobile and morphologically very similar in shape and size. Gamonts develop further into clearly differentiated macro- and microgametes. The life cycle eventually proceeds with the fertilization by fusion of a motile flagellated microgamete with a large and immobile macrogamete, leading to the formation of a diploid zygote. After fertilization, the immature oocysts are excreted with the feces and undergo sporogony in the environment. Several divisions of the zygote by meiosis and mitosis result in infectious haploid sporozoites contained in the mature oocyst. Previous studies have shown that the development of *C. suis* in vitro through the entire lifecycle is comparable with the lifecycle in vivo. This makes it possible to observe, harvest and examine sexual stages during the short time frame in which they occur. After in vitro merogony in epithelial host
cells. C. suis can also continue gamogony in a host cell-free environment, suggesting that gamete production and fusion occurs extracellularly. The complete life cycle of C. suis in a cell line representing the natural host cell type and species provides a unique model among coccidial parasites and can be used to address a wide range of topics (RNAseq, proteomics), especially with regard to the sexual development of coccidia.

Comparative RNA-seq analysis can be exploited to uncover molecules and pathways critical to parasite biology. In recent years, transcriptomic and proteomic analyses carried out in Apicomplexa revealed different genes coding for proteins related to the sexual development and unrecognised key components common to it in different species. Based on profiling quantitative changes in gene transcription, stage-specific genes have been identified in oocysts, sporozoites, second and third-generation merozoites and gametocytes of E. tenella, E. maxima and E. acervulina, in tachyzoites, bradyzoites, sporozoites and oocysts of T. gondii, in tachyzoites of Neospora caninum, in tachyzoites of Besnoitia besnoiti, oocysts, sporozoites and intracellular stages of Cryptosporidium parvum, and in human and mosquito stages and gametocytes of Plasmodium falciparum and P. vivax. According to recent reevaluations of the coccidial phylogeny, the position of C. suis in the family Sarcocystidae constitutes an outgroup of the cluster containing the genera Neospora, Hammondia and Toxoplasma. The transcriptional profile of multiplying asexual stages (tachyzoites) of T. gondii, the closest relative to C. suis, revealed an upregulation in genes encoding proteins involved in host cell adhesion and invasion, intracellular development and multiplication, resistance to host stress, gliding motion and ribosomal proteins in comparison to resting stages (bradyzoites). The AP2 (ApiAP2) family was identified as a major class of transcriptional regulators that are found across all Apicomplexa and modulate key regulatory decisions of parasite development. Only a small number of asexual stages differentiates into gametes, and therefore this step is considered a bottleneck of development, and it can be assumed that this is relevant for all Apicomplexa that produce gametocytes. Proteins with important roles in sexual development have been described, including members involved in macrogamete development, oocyst wall formation, glycosylation and proteolytic cleavage of the oocyst wall proteins, axoneme and flagella assembly and construction, DNA replication, microgamete budding from microgamonts and gamete fusion.

In the present study we performed RNA-seq of C. suis harvested at different points during development – specifically, asexual stages (merozoites) as a baseline, and immature and mature sexual stages (microgametes, macrogametes and early oocysts) to provide a better understanding of the developmental process and regulation of sex differentiation of C. suis in vitro.

Results and discussion
Overview of RNA sequencing of C. suis merozoites and sexual stages. Transcriptome sequencing was carried out at three time points characterizing three different steps in the development of C. suis to determine the transcript levels. Epithelial cells were infected with freshly excysted sporozoites and culture supernatants containing developed parasite stages were harvested at different time points: time points T1 (days 6–8 after infection) for the merozoites, T2 (days 9–11), containing merozoites and immature sexual stages, and T3 (days 12–14), containing mainly mature sexual stages and oocysts. Total RNA was extracted from seven biological replicates for each time point, DNase-treated and quality assessed by automated gel electrophoresis. Parasite-specific large ribosomal RNA bands (26S and 18S) were detected in all samples. Although contamination with host RNA (28S) was also observed, this was not a strong concern since we performed read mapping to the C. suis genome. Approximately 21 million reads were generated for each sample. Data were mapped to the combined genomes of C. suis (strain Wien I) and the pig host, Sus scrofa (Sscrofa11.1). After filtering out S. scrofa, at least 21% of the mapped reads of each replicate were assigned to the C. suis genome, this provided a robust data for quantitative analysis of gene transcript levels (for details of the total number of reads per replicate, see Table S1).

Identification of differentially expressed genes. In order to identify upregulated or downregulated transcripts, quantified RNAseq mapping was used to generate quantitative profiles for individual differentially expressed genes (DEG) between developmental stages of C. suis harvested at different time points. Lowly expressed C. suis genes (4,418 out of 11,543) were excluded from subsequent analysis (filtering criteria: cpm > 4 and count > 30 for at least four replicates at each time point). The remaining 7,125 genes were tested for differential gene expression with a mixed model accounting for the repeated measures structure of our data. At a False Discovery Rate (FDR) cut-off of 5% and a minimum absolute log2 fold change of 1, we found 891 and 1,860 differentially expressed genes at T2 and T3 compared to T1, and 823 genes differentially expressed at T3 compared to T2, respectively (Fig. 1a).

The primary goal of this study was the identification of genes with elevated expression levels at sexual stages to identify genes and proteins that may be related to this last step of development of the Coccidia, including C. suis. In total, 937 upregulated and 1188 downregulated genes were identified in sexual stages compared to T1–T2 (Fig. 1b and c), representing 8.11% respectively 12.53% of all predicted C. suis genes. The gene identification, description and transcript abundance levels for each of these genes, at each developmental stage, are provided in Table S2.

qRT-PCR validation. The gene expression profile identified by RNAseq was validated by selecting six different genes for qRT-PCR analysis with specific primer sequences. The transcripts levels were calculated according to the 2-ΔΔCt values (see amplification efficacies for primers in Supplemental file S1). Using glyceraldehyde-3-phosphate (GAPDH) and actin as a reference genes, expression levels determined by qRT-PCR were consistent with those obtained by RNA-seq (Fig. 2), confirming the accuracy and reliability of the RNA-seq results. Thus, the data generated here can be used to investigate stage-specific expression of genes that show different expression levels among different developmental stages.
Figure 1. Identification of upregulated and downregulated genes in sexual stages using differential expression analysis. (a) Summary of the differential expression analysis of early sexual stages compared to merozoites (T1-T2), late sexual stages (T1-T3) and late sexual stages compared to early sexual stages (T2-T3) showing the number of genes up or down-regulated of all predicted *C. suis*. (b) Venn diagrams showing the overlap between the genes that were up- and down-regulated in early and late sexual stages compared with asexual stages. A total of 443 upregulated and 689 downregulated genes were identified in this overlapping region. (c) Summary of total number of genes (937 upregulated and 1188 downregulated) in early and late sexual stages.

Figure 2. Verification of the gene expression profiles by qRT-PCR. Six genes were selected randomly for validation of the RNA-seq data. According to the RNA-seq results, the expression levels of CSUI_008252, CSUI_003709, CSUI_000190 were upregulated at T2 and T3, and the expression levels of CSUI_005927, CSUI_003422 and CSUI_006265 were downregulated at T2 and T3. Glyceraldehyde-3-phosphate and actin were used for normalization. Values represent the mean ± standard deviation (SD). Asterisks represent significant difference (*P < 0.05, **P < 0.01***, P < 0.001, ****P < 0.0001).
Gene ontology classification. Gene ontology enrichment analysis was conducted by Fisher's exact test taking into account the GO hierarchy. At a significance cut-off of 5%, 27 biological processes, 17 molecular functions and eight cellular components were significantly enriched in upregulated genes. The top five GO terms enriched in upregulated genes were associated with localization, proteolysis, oxidation–reduction process, microtubule-based movement and cellular catabolic processes which together support subsequent gamogony, fertilization and oocyst wall formation. For the downregulated genes, 11 biological processes, 16 molecular functions and 4 cellular components were significantly enriched. The top five GO terms enriched in downregulated genes were protein phosphorylation, proteolysis, regulation of transcription, signal transduction and transmembrane transport which are implicated in host invasion, merozoite reproduction and gamogony as well as and parasite-host immunological interactions (Fig. 3).

Transcripts down- or upregulated in sexual stages. Within the subset of down- and upregulated transcripts identified in C. suis, a large proportion coded for hypothetical proteins (Fig. 4a and b)—an expected observation, given the still limited understanding of coccidial sexual biology. As expected, genes coding for previously characterised merozoite proteins with putative roles in host-cell attachment and invasion, motility, signaling, virulence and transport were most distinctly downregulated (Fig. 4a); detailed information on these is given in Sect. Transcripts down- or upregulated in sexual stages. Other putative functions (not discussed in detail) included proteolysis, redox activity and DNA/RNA related proteins. Genes coding for previously characterised gametocyte antigens and oocyst wall proteins are among the most highly transcribed gametocyte genes including proteins with putative roles in glycosylation, protease activity, redox activity and fatty acid metabolism, surface and oocyst wall formation, as well as components of microgamete flagella (Fig. 4b) and further details are given in “Macrogamete and oocyst-specific genes” and “Microgamete-specific genes” sections. Other putative functions (not discussed in detail) included: (1) metabolism, including the energy metabolism, aminoacid synthesis and carbon source, and (2) DNA/RNA binding, which may play a role in gene regulation that is not yet further specified for coccidia. About 20% of the proteins found in both sets of regulated transcripts have diverse functions with undefined roles in parasite biology, e.g., kinase activity, calcium and metal binding or membrane components.

Identification of genes downregulated in sexual stages. Asexual stages of Coccidia develop strictly intracellularly, and have developed various strategies to ensure cell invasion and intracellular persistence. The invasive stages have specialized cellular structures and organelles attached to their membranes. The apical polar complex is composed of secretory organelles (micronemes and rhoptries) and structural elements (conoid and polar rings). T. gondii secretes a broad spectrum of proteins to infiltrate its host cells and to regulate the expression of host proteins, including micronemal proteins (MICs) and PAN/Apple domains, rhoptry and rhoptry neck proteins (ROPs and RONs) and dense granules (GRAs). We detected 19 genes coding for nine different MICs and seven coding for PAN-Containing proteins, 32 ROPs and RONs and two dense granule proteins downregulated in sexual stages (Table S3). Internalization of asexual stages is achieved by active participation of the parasite. The process of gliding requires the coordinated secretion and translocation of proteins via the actin-based cytoskeleton. Parasites use the gliding motion to establish host cell adhesion to generate enough traction to drive themselves into the host cell. This initial contact is mediated by proteins released from the micronemes. Of these, the best characterized are the Apical Membrane Antigen 1 (AMA1) and yet anonymous thrombospondin-related proteins which bind directly to the motor complex of the adhesion site. We identified three genes related to these proteins that were downregulated in sexual compared to asexual stages.

Invasion, replication and egress require dynamic changes in the cellular architecture of the parasite. The inner membrane complex (IMC) is a structural element involved in these morphological changes. The IMC of T. gondii is a peripheral membrane system composed of flattened alveolar sacs (alveoli) underlying the plasma membrane, coupled to a supporting cytoskeletal network. The IMC plays major roles in parasite intracellular replication, motility and host cell invasion. The best studied group of IMC proteins are components of the motor complex—also referred to as the “glideosome” – in T. gondii. This actin-myosin motor complex powers the required cell motility, and the proteins identified include myosins, tubulins, actins and glideosome-associated proteins. Twenty-six genes coding for these proteins were identified in asexual stages of C. suis. Another interesting group of IMC proteins, such as the Inner Membrane Complex Protein 1 of T. gondii (TgIMC1) are the alveolins of which we identified 14 genes in C. suis. Beside the alveolins, other additional IMC-associated peripheral membrane proteins like the IMC subcompartment-proteins (ISPs) were identified. The specific signalling pathways which regulate the activity of the glideosome are still not known. Regulation of adhesin release from micronemes and glideosome activity are linked to environmental signals that ensure proper activation and suppression of gliding motility. Extracellular K+ and cytosolic Ca2+ concentrations have been implicated in the activation of gliding motility. A role for cyclic nucleotide signaling has been unveiled, and phosphorylation, and methylation events also regulate the gliding motility. A total of 43 genes encoding proteins that are involved in phosphorylation and cell signalling, signal transduction and calcium regulation were identified in C. suis (Table S3).

The surface of T. gondii tachyzoites and bradyzoites is covered with glycosylphosphatidylinositol (GPI)-anchored antigens, most of which are members of the large family of surface antigen (SAG)-related (SRS) proteins which includes the SAG1-like and the SAG2-like sequence branches, SAG and SUSA (SAG-unrelated surface antigens). These proteins have diverse functions. Presumably they facilitate adhesion to and invasion of host cells, and play a role in immune evasion and defining host specificity. In C. suis, 53 SAGs or SRSs were identified, and their downregulation indicates that sexual stages have less interactions with host cells and the
Figure 3. Gene ontology (GO) analysis of differently expressed genes. Differentially expressed genes (DEGs) are classified into three main categories: biological process (BP), molecular function (MF) and cellular component (CC). The identified functions for each corresponding GO category are shown in supplemental file 2.
host's immune system, maybe because the do not reinvade cells and are rather short-lived, progressing quickly from gamonts to oocysts11.

We could show that genes encoding proteins that play an active role in the invasion of host cells are down-regulated in the early and late sexual stages of C. suis, supporting the assumption that these stages do not invade host cells. These molecular clues consequently suggest that the fertilization process (and consequently oocyst formation) occurs extracellularly which facilitates and accelerates the discharge of oocysts into the environment – but at the same time makes these stages accessible to specific antibodies for immunological control.

**Genes involved in cell cycle regulation.** During the progression from asexual to sexual stages in in vitro culture, we identified orthologues of 12 genes coding for proteins found specifically in bradyzoites of T. gondii, involved in tissue cyst wall formation67,68, an orthologue of the bradyzoite antigen 121, a Myb-like transcription factor69, two heat shock proteins and one serpin found in this stage (Table S4). The existence of bradyzoites as persisting intracellular stages has never been demonstrated in C. suis, neither in vivo70 nor in vitro. However, other species of the genus Cystoisospora can develop resting monozoic tissue cyst stages71,72. The role of these putative proteins in merozoite biology of C. suis remains to be investigated. The commitment of type II merozoites to sexual differentiation during the final phase of asexual development is a key process during the life cycle of Apicomplexan parasites73. DNA binding proteins (ApiAP2 factors) are related to the APETALA family of transcription factors which play key roles in the development and environmental stress response pathways of plants74. The ApiAP2 family was discovered in the genomes of various Apicomplexan species75. In Plasmodium, they play a role in stage conversion76 and are related to sexual commitment of blood-stages77–79. Currently, 67 ApiAP2 domain-containing proteins are annotated in the Toxoplasma genome, with 24 being expressed cyclically during the tachyzoite division cycle18,80 and six in bradyzoite development81. The genome of C. suis encodes 64 AP2 factors of which we found 23 AP2 factors downregulated and 7 AP2 factors upregulated in the sexual stages, possibly linked to the observed stage conversion from type II merozoites to gamonts and onwards to gametes.

**Macrogamete and oocyst-specific genes.** The wall composition of coccidian oocysts has previously been characterized in great detail79. Oocyst wall proteins (OWP) and gametocyte-specific proteins (GAM)-proteins were previously characterized in Eimeria, Toxoplasma and Cryptosporidium as the main protein constituents of the oocyst wall82,83. In C. suis, 12 transcripts, originally described in oocysts of T. gondii and E. maxima, were confirmed to be upregulated at T2 and T3 (Tables 1 and S5). The putative oocyst wall proteins encoded by CSUI_008806 and CSUI_006207 showed homology to TgOWP6 and TgOWP1, respectively, cysteine-rich
oocyst wall proteins of the wall-forming bodies with a vital role in oocyst wall formation. Seven genes that
encode proteins of novel OWP candidates were not highly homologous with established OWPs although all of
them share characteristic cysteine repeats. The oocyst and sporocyst walls of T. gondii encode proteins of novel OWP candidates were not highly homologous with established OWPs although all of

| Gene ID     | logFC | FDR adj. pval | Annotation                  | Comparison                               | Function     |
|-------------|-------|---------------|-----------------------------|------------------------------------------|--------------|
| CSUI_008806 | 4.62  | 1.47E+04      | oocyst wall protein         | UT12_UT23_UT13                           | Oocyst wall  |
| CSUI_006207 | 1.75  | 1.04E+08      | oocyst wall protein         | UT23_UT13                                | Oocyst Wall  |
| CSUI_002027 | 5.47  | 1.88E+04      | toxoplasma gondii family a protein | UT12_UT23_UT13                           | Oocyst Wall  |
| CSUI_006555 | 1.78  | 9.10E-06      | toxoplasma gondii family a protein | UT12_UT13                                | Oocyst Wall  |
| CSUI_010157 | 4.32  | 4.37E+05      | toxoplasma gondii family a protein | UT12_UT23_UT13                           | Oocyst Wall  |
| CSUI_003908 | 3.39  | 2.68E-06      | toxoplasma gondii family d protein | UT12_UT13                                | Oocyst Wall  |
| CSUI_004489 | 3.29  | 2.89E-05      | toxoplasma gondii family d protein | UT12_UT13                                | Oocyst Wall  |
| CSUI_004212 | 2.21  | 1.92E+07      | toxoplasma gondii family d protein | UT23_UT13                                | Oocyst Wall  |
| CSUI_009196 | 1.67  | 2.45E-09      | toxoplasma gondii family d protein | UT13                                    | Oocyst Wall  |
| CSUI_000190 | 5.84  | 3.56E+03      | hypothetical protein-TyRP    | UT12_UT23_UT13                           | Oocyst Wall  |
| CSUI_001473 | 2.77  | 9.93E+05      | hypothetical protein-TyRP    | UT12_UT13                                | Oocyst Wall  |
| CSUI_001475 | 3.46  | 1.07E+05      | hypothetical protein-TyRP    | UT12_UT13                                | Oocyst Wall  |
| CSUI_007070 | 4.06  | 2.69E+03      | fasciclin domain protein    | UT12_UT23_UT13                           | Surface      |
| CSUI_006459 | 4.41  | 3.07E+04      | fasciclin-domain-containing protein | UT12_UT23_UT13                         | Surface      |
| CSUI_002476 | 1.08  | 4.52E+09      | outer omp85 family protein  | UT13                                    | Surface      |
| CSUI_006179 | 3.96  | 3.97E+01      | SAG domain-containing protein | UT12_UT23_UT13                           | Surface      |
| CSUI_004248 | 3.06  | 1.29E+06      | sag-related sequence sn26i   | UT12_UT23_UT13                           | Surface      |
| CSUI_005667 | 1.87  | 3.74E+08      | sag-related sequence sn28     | UT23_UT13                                | Surface      |
| CSUI_009850 | 3.09  | 6.33E+08      | proteophosphoglycan related  | UpT23_UpT13                              | Surface      |
| CSUI_006635 | 1.68  | 7.28E+05      | proteophosphoglycan related  | PstP23_UpT13                             | Surface      |
| CSUI_001278 | 2.85  | 3.81E+08      | proteophosphoglycan related  | UpT23_UpT13                              | Surface      |
| CSUI_001693 | 1.11  | 1.10E+08      | longevity-assurance protein-domain-containing protein | UT23            | Resistance   |
| CSUI_001900 | 5.63  | 2.06E+03      | late embryogenesis abundant domain protein | UT12_UT23_UT13                          | Resistance   |
| CSUI_001899 | 5.31  | 1.95E+04      | late embryogenesis abundant domain protein | UT12_UT23_UT13                      | Resistance   |
| CSUI_005059 | 1.46  | 4.91E+09      | late embryogenesis abundant domain protein | UT13                                    | Resistance   |

Table 1. Upregulated transcripts coding for proteins with known or putative roles in oocyst wall composition and surface. They are listed along with their transcript abundance (LogFC), annotation, comparison (upregulated transcripts (UT) in early sexual stages(2) compared to merozoites(1), UT12, late sexual stages (3) compared to merozoites (1), UT13, and late sexual stages (3) compared to early sexual stages(2), UT23) and biological function.
Most of the genes identified are related to macrogamete development and oocyst formation, predominating during the transition from asexual to sexual stages in cell culture. GAM are well characterized tyrosine-rich proteins of the oocyst wall of *Eimeria* [32,92,93,96]; they were previously developed as antigens for transmission-blocking vaccines targeting the gametocyte-specific proteins GAM56, GAM82 and GAM2295,97–100. These proteins are potent immunogens for the use as vaccines against chicken coccidiosis as they induce a diverse and robust immunity101. Oral applications of sera containing *E. tenella* gamont-specific monoclonal antibodies significantly reduced oocyst output and cecal lesions in chicken109. Studies in *Plasmodium* proposed the HAP2 fusion protein as a candidate for a transmission-blocking vaccine candidate for a transmission-blocking vaccine92,93,97–100. The transmission blocking potential of proteins specific to sexual stage as candidates for vaccination or drug targets has been suggested in related Coccidia and other Apicomplexa3. Oral application of sera containing *E. tenella* gamont-specific monoclonal antibodies significantly reduced oocyst output and cecal lesions in chicken109. Studies in *Plasmodium* proposed the HAP2 fusion protein as a candidate for a transmission-blocking vaccine [102–104]. All these findings indicate that inhibiting the fertilization of macrogametes by microgametes and the oocyst wall formation can effectively interfere with the parasite's developmental cycle.

### Microgamete-specific genes.

Scanning electron microscopy observations of *C. suis* showed that microgametes consisted of a small, spherical body with two opposing flagella8. The molecular characterisation of microgametes in the Coccidia is still limited. Microgametes use flagella to move quickly in search of macrogametes such as Pfs25 and Pfs230 are investigated in ongoing trials102–104. All these findings indicate that inhibiting the fertilization of macrogametes by microgametes and the oocyst wall formation can effectively interfere with the parasite's developmental cycle.

#### Table 2. Upregulated transcripts coding for proteins with known or putative roles in microgamete biology.

They are listed along with their transcript abundance (LogFC), annotation, comparison (upregulated transcripts (UT) in early sexual stages(2) compared to merozoites(1), UT12, late sexual stages (3) compared to merozoites (1), UT13, and late sexual stages (3) compared to early sexual stages(2), UT23) and biological function.

| Gene ID    | logFC | FDR_adj. pval | Annotation                                      | Comparison | Function                      |
|------------|-------|---------------|-------------------------------------------------|------------|-------------------------------|
| CSUI_007002| 1.23  | 5.46E+09      | centrin 2                                       | UT23_UT13  | Flagella                      |
| CSUI_006055| 4.27  | 3.82E+03      | flagellar associated protein                     | UT12_UT23  | Flagella                      |
| CSUI_006910| 2.80  | 3.12E+05      | flagellar associated protein                     | UT12_UT23  | Flagella                      |
| CSUI_003885| 1.23  | 8.85E+08      | kinesin motor-domain-protein                    | UT23_UT13  | Microtubule                   |
| CSUI_005407| 1.39  | 4.18E+09      | myosin a                                        | UT13       | Microtubule                   |
| CSUI_009311| 1.96  | 2.06E+08      | myosin heavy chain                              | UT13       | Microtubule                   |
| CSUI_000425| 1.38  | 5.57E+03      | myosin k                                        | UT13       | Microtubule                   |
| CSUI_009385| 1.18  | 2.96E+03      | myosin k                                        | UT13       | Microtubule                   |
| CSUI_005346| 1.10  | 2.12E+09      | myosin light chain                              | UT13       | Microtubule                   |
| CSUI_011403| 1.20  | 5.16E+00      | myosin regulatory light chain                    | UT13       | Microtubule                   |
| CSUI_000854| 1.58  | 1.43E+07      | non-muscle myosin heavy                         | UT13       | Microtubule                   |
| CSUI_007953| 3.02  | 1.69E+05      | chromosom-associated kinesin klp1               | UT12_UT23  | Microtubule                   |
| CSUI_007586| 3.41  | 8.12E+06      | dynin gamma flagellar outer                      | UT12_UT23  | Axoneme                       |
| CSUI_004717| 1.62  | 7.86E+07      | dynin light chain dlc                           | UT13       | Axonema                       |
| CSUI_001333| 1.08  | 1.22E+04      | dynin light chain roadblock-type 2               | UT13       | Axonema                       |
| CSUI_002604| 2.73  | 7.88E+05      | growth arrest–specific protein 8                 | UT12_UT23  | Axonema                       |
| CSUI_000245| 2.21  | 4.06E+07      | heavy chain 2 family protein                    | UT12_UT13  | Axonema                       |
| CSUI_000472| 3.19  | 2.40E+08      | male gamete fusion factor                       | UT12_UT23  | Gamete fusion                 |
| CSUI_002998| 3.20  | 1.95E+04      | morn repeat-containing protein                   | UT23_UT13  | Cell budding                  |
| CSUI_000048| 3.39  | 2.20E+08      | morn repeat-containing protein                   | UT13       | Cell budding                  |
| CSUI_004816| 1.70  | 2.78E+06      | morn repeat-containing protein                   | UT23_UT13  | Cell budding                  |

Based on the motile nature of the male sexual stages and the lack of invasion machinery genes in sexual stages, it is obvious that the fertilization process takes place extracellularly, rather than intracellularly as previously assumed80,100,106,108. The transmission blocking potential of proteins specific to sexual stage as candidates for vaccination or drug targets has been suggested in related Coccidia and other Apicomplexa3. Oral application of sera containing *E. tenella* gamont-specific monoclonal antibodies significantly reduced oocyst output and cecal lesions in chicken109. Studies in *Plasmodium* proposed the HAP2 fusion protein as a candidate for a transmission-blocking vaccine110–112. Recently, a HAP2-deficient *T. gondii* strain was created using the CRISPR/Cas9 approach and used as transmission blocking control strategy by immunising cats against a challenge with a *T. gondii* wildtype strain23. This in turn supports the assumption that intestinal sexual stages are accessible for specific antibodies which could be induced by vaccination or transferred by colostrum (as maternal antibodies).
For *C. suis* it was previously shown that high levels of colostral and possibly milk antibodies from superinfected sows exert significant protection of suckling piglets against experimental *C. suis* infection\(^9\). Although these antibodies were not characterised regarding the targeted proteins or parasite stages, it is conceivable that sexual-stage specific proteins could be implemented as a vaccine targets in this context.

**Immunolocalization of CSUI\_001473 antigens in macrogametes and oocysts.** The oocyst wall is a distinctive characteristic of coccidian development and the key stage of transmission\(^9\). It is described that vaccines incorporating antigens from magrogamete surface or oocyst wall significantly reduced the oocyst formation. We hypothesized that targeting these stages may be an effective approach in *C. suis* parasite control in the future. In our previous study applying qRT-PCR on stages derived from in vitro cultures of *C. suis*, transcript levels of CSUI\_001473 (CsTyRP) were highly upregulated with a peak on day 13 of in vitro culture or on day 4 of transfer to host-cell free medium and declined after that\(^7,11\), correlating with the distinct upregulation of transcript level in the current analysis. We selected CSUI\_001473 to test the proof of principle that targeting a sexual stage specific antigen could be used as a candidate for a transmission-blocking vaccine.

A single I463 bp CSUI\_001473 open reading frame encoded a protein of 353 amino acids with the predicted molecular mass of 39 kDa. The deduced amino acid sequence had a predicted N-terminal 19-amino acids signal peptide for entrance into the secretory pathway. No predicted transmembrane domains were identified. The recombinant CSUI\_001473 protein (rCSUI\_001473) revealed a major protein band of \(\sim 55\) kDa, higher than the predicted 46 kDa (Figure S1a), after induction with 1 mM IPTG for 4 h at 37 °C. Purification was performed under denaturing conditions. These antibodies recognized a single strong band of approximately 55 kDa, corresponding to rCSUI\_001473, and a lower molecular weight protein band which might be degraded products or truncations of rCSUI\_001473 (Figure S1b). Furthermore, to confirm that the chicken anti-rCSUI\_001473 serum recognized the native form of CSUI\_001473 protein, a crude extract of sexual stage proteins was probed with anti-rCSUI\_001473 serum in which a band of approximatively 48 kDa was recognized. As expected, negative chicken serum failed to detect any bands of the expected size in Western blot (Figure S1c and d).

To test the hypothesis that CSUI\_001473 is a component of the oocyst wall we performed immunolocalisation studies, again using chicken anti-rCSUI\_001473 serum. The protein localized to *C. suis* macrogametes (Fig. 5), specifically to the periphery of the parasite cell, and to the outer wall of the unsporulated and sporulated oocyst, but not to the the sporocyst wall. We did not detect antibody binding in merozoites or microgamonts. This confirms that CSUI\_001473 is homologous to the proteins identified in the oocyst proteome of *T. gondii* and is an oocyst wall protein member.

**Serum inhibition assay.** No genetic manipulation technique is currently available for *C. suis* to confirm the direct involvement of CSUI\_001473 in oocyst formation and/or development. In order to test whether CSUI\_001473 expression is essential for oocyst wall formation we tested whether chicken anti-rCSUI\_001473 serum can inhibit late sexual stage development. The novel host cell-free in vitro culture system for *C. suis* made it possible to evaluate the effects of culture conditions on the development of merozoites to sexual stages and oocysts\(^11\). The addition of antiserum did not significantly decrease the number of asexual stages compared to the it possible to evaluate the effects of culture conditions on the development of merozoites to sexual stages and oocysts\(^11\). The addition of antiserum did not significantly decrease the number of asexual stages compared to the

**Conclusions**

A comparative RNAseq transcriptomics approach led to the identification of genes specifically expressed in *C. suis* early and late sexual stages (gamonts and gametes) in comparison to asexual stages (merozoites) in vitro. We could describe global changes in gene expression during sexual differentiation and gamete maturation from merozoites to gametes and oocysts in vitro. This set of results represents a detailed overview of the biology of sexual development in this model coccidian in comparison to asexual intracellular replication. In addition, a previously uncharacterized protein of the oocyst wall of *C. suis* was investigated that may represent a candidate for a transmission-blocking vaccine against piglet cystoisosporosis. These new findings create a dataset that incorporates an initial comprehensive view of the mechanisms associated with sexual reproduction and oocyst formation in a range of taxa as a common denominator in the understanding of parasite biology and definition of intervention targets.

**Materials and methods**

**Cystoisospora suis oocyst collection.** *Cystoisospora suis* oocysts (strain Wien I) were obtained from experimentally infected suckling piglets as described previously\(^7,10\). Piglets were raised with the sow in the animal facilities of the Institute of Parasitology, University of Veterinary Medicine Vienna, Austria.

**In vitro culture.** Intestinal porcine epithelial cells (IPEC-1, ACC 705, Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Leibniz, Germany) were used as host cells in vitro and
Figure 5. Localization of CSUI_001473 antigens in different C. suis stages. (a) Merozoite from day 6 of in vitro culture. (b) Microgamont from day 9 of culture. (c) Macrogamont from day 9 of culture. (d) Unsporulated oocyst from day 14. (e) Sporulated oocyst ex vivo (isolated from the feces of experimentally infected piglets). (f) Sporozoite released from in vitro excysted oocysts. DIC, differential interference contrast microscopy; DAPI staining appears in blue; green indicates binding of anti-rCSUI-001473 antibodies, and turquoise indicates merged results. Scale bar = 10 μm.

seeded in a density of $4 \times 10^5$ cells per well in a 6-well plate (PAA, Pasching, Austria). A total of 21 plates were used with material from three pooled plates constituting a biological replicate. Cells were grown in DMEM/Ham’s F-12 medium (Gibco—Fisher Scientific GmbH, Schwerte, Germany) with 5% fetal calf serum (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 cells were infected with 5 × 10³ sporozoites/well released from excysted oocysts and incubated further at 40 °C under 5% CO₂.5,10.

**Experimental design, sampling and RNA-seq library preparation.** For the sampling of sexual stages released from host cells we collected cell culture supernatant every day, from day of cultivation (doc) 6 to day 14. The material was washed twice with phosphate-buffered saline (PBS; Gibco) and pelleted by centrifugation at 600 × g for 10 min. The numbers of merozoites, sexual stages and oocysts were counted in a Neubauer-counting chamber for each given time point. For each day, seven biological replicates were harvested and the mean numbers of each stage per biological replicate were calculated.

Pellets from the same wells were pooled to increase the number of parasites per sample and the analysis was performed for three time points:

1. pool of days 6, 7 and 8 (merozoites, type I and II) = Time point 1.
2. pool of days 9, 10 and 11 (merozoites type II and early sexual stages, i.e. gamonts) = Time point 2.
3. pool of days 12, 13 and 14 (mainly sexual stages, gametes, and unsporulated oocysts) = Time point 3.

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), and samples were sent for library preparation using a reverse stranded protocol with poly-A enrichment. Sequencing libraries were prepared at the Core Facility Genomics, Medical University of Vienna, using the NEBNext Poly(A) mRNA Magnetic Isolation Module™ and the NEBNext Ultra™ II Directional RNA Library Prep

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**Figure 6.** Serum Inhibition assay. (a) Inhibition rates for asexual, early (mostly gamonts) and late (mostly gametes) sexual stages of *C. suis* in a host-cell free culture 0–4 days after transfer of merozoites. (b to d) Total numbers of counted stages by day of cultivation in host-cell free culture. n.s.: not significant, *: P ≤ 0.05, **: P ≤ 0.01. Values represent the mean ± standard deviation (SD) from three independent experiments. n.s.: not significant, *: P ≤ 0.05, **: P ≤ 0.01.
Kit for Illumina according to manufacturer’s protocols (New England Biolabs, Ipswich, Massachusetts, USA). Libraries were QC-checked on a Bioanalyzer 2100® (Agilent Technologies, Santa Clara, CA, USA) using a High Sensitivity® DNA kit for correct insert size and quantified using Qubit dsDNA HS® assay (Invitrogen, Waltham, Massachusetts, USA). Pooled libraries were sequenced on a NextSeq500® instrument (Illumina, San Diego, California, USA) in 1×75 bp single-end sequencing mode. Approximately 21.5 million reads were generated per sample.

**RNA-Seq data analysis.** Sequencing reads were mapped against the concatenated fasta sequences of *C. suis* (version 48 from ToxoDB) and *S. scrofa* (version 1.11 from Ensembl, GCA_000003025.6) using STAR (version 2.7.3a with option –outSAMmultNmax 1) and the combined annotations of each genome (version 48 for *C. suis* and 11.1.98 for *S. scrofa*). Only the reads mapping to the *C. suis* genome were subsequently used for quantification and further analysis.

Quality control was performed with FastQC and QualiMap. RNA degradation was taken into account via the TIN (Transcript Integrity Number) values, which were measured for each gene and library with the RSeQC. It was used to assess gene body coverage (module geneBody_coverage.py with with option -l 500) and to calculate transcript integrity numbers (TIN scores, module tin.py with option –c 20). TIN is considered an accurate and reliable measurement of RNA integrity at the sample level. Gene expression was quantified with featureCounts (version 1.5.0a) with options -s 2 -Q 20 –primary.

**Identification and analysis of differentially expressed genes.** All statistical analysis were performed in R (version 4.1).

Given the repeated measures design of our experiment (briefly, gene expression was measured for seven samples at each of three timepoints) we employed a linear mixed model framework to account for the covariance structure in the data. Differential gene expression analysis between the three time points was performed via linear mixed models with the function `lmer` (R package variancePartition, version 1.18.3), which is a wrapper for the function `lmer` in package lme4. Replicate ID was fitted as random intercept, and hypothesis testing was carried out for a fixed categorical effect of time with the three time points as factor levels. We further included the median TIN scores, calculated across all genes in each library, as a continuously distributed (nuisance) covariate in our model. We filtered for genes with a minimum count of 30 and four counts per million reads in at least four out of seven replicates of each time point. The remaining counts were quantile normalized before differential gene expression analysis with the function `voomWithDreamWeights` (R package variancePartition). The p-values were adjusted for multiple testing according to Benjamini and Hochberg's false discovery rate (FDR) correction. Genes with FDR > 0.05 and absolute log2FC > 1 were considered significantly differentially expressed.

**Gene ontology enrichment analysis.** To explore the broader biological context of the identified genes, gene ontology (GO) enrichment analysis was performed via topGO with the Fisher’s Exact Test and the GO annotations from ToxoDB (version 50). The “Weighted01” algorithm which accounts for the GO hierarchy was applied.

**qRT-PCR validation of DEGs.** The cDNA samples were synthesised from DNase-treated total RNA used in RNASeq. Synthesis of cDNA was accomplished using the iScript® cDNA synthesis kit (Bio-Rad, Hercules, California, USA). 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 S7. Reaction mixtures contained 2.5 μl of sample cDNA (50 ng/μl), 5 μl of SsoAdvanced™ Universal Probes Supermix (Bio-Rad, Hercules, California, USA) and 1.3 μl of nuclease-free water with primers and probes at a final concentration of 100 and 200 nM, respectively. Activation of polymerase was performed at 95 °C for 2 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 30 s. Each sample was run in triplicate. The qPCR results were normalized against the mean of two reference genes, GAPDH and actin (see primers efficiencies in Supplemental file S1). Average gene expression relative to the endogenous control for each sample was calculated using the 2−ΔΔCq method. The relative fold change of gene expression was expressed as the mean and standard deviation. Statistical analysis were performed using the ANOVA one way test with the software GraphPad Prism 9.2 (GraphPad Software, San Diego, CA). Differences were considered statistically significant at P ≤ 0.05.

**Recombinant protein expression.** A Champion pET151 Directional TOPO* Expression Kit was used for the expression of recombinant proteins with N-terminal V5-6xHis tags. Coding sequences of the hypothetical gene CsTyRP (CSUI_001473) were amplified by PCR from cDNA using a Q5 high Fidelity® DNA Polymerase (New England Biolabs, Ipswich, Massachusetts, USA) according to manufacturer’s instructions. The gene-specific primers used for amplification and subsequent cloning into Champion pET151 Directional TOPO* are listed in Table S7 (primers no. 25–26). After verification of the correct cloning in BL21 Star® (DE3) and confirmation of the reading frames, plasmids with the correct inserts were used to transform One Shot® chemically competent *E. coli* (Thermo Fischer). Briefly, bacteria containing the recombinant plasmid were grown overnight in non-inducing LB medium at 37 °C on a culture shaker at 180 rpm. One milliliter of pre-cultured LB medium was then inoculated in 50 ml of fresh LB medium and incubated for 1 h at 37 °C, 220 rpm, until OD600 = 0.6, and the expression of the recombinant proteins was induced by adding 1 mM of IPTG (Sigma-Aldrich, St. Louis, Missouri, USA), followed by incubation for 4 h. The culture was then centrifuged at 4,000 × g for 30 min. The pellet was re-suspended in lysis buffer (20 mM NaHPO4; 8 M urea; 0.5 M NaCl, 5 mM imidazole, pH 8) under constant stirring for 1 h for solubilization and then centrifuged at 10,000 × g for 20 min. The lysates were analysed by SDS-PAGE (12.5%) followed by staining with Coomassie blue (BioRad, Hercules, California, USA).
Recombinant proteins were purified using a Ni-sepharose column (His GraviTrap®, GE Healthcare, Chicago, Illinois USA) following the manufacturer’s instructions.

**Antibody production.** The recombinant protein was used for immunizing two chicken according to a standard 87-day programme immunization procedure (Eurogentec, Seraing, Liège, Belgium). Before injection (day 0), preimmune egg yolk was collected (pre-immune serum), and subsequent immunizations (100 µg of antigen per injection) were made on days 14, 28, 56 and (as an additional booster) on day 99. Egg yolks were collected during three time periods (days 38–52, days 66–81 and days 109–121). The collected egg yolk sera were evaluated in conventional ELISA for checking their respective titers. The isolation of IgY from the egg yolk and their subsequent affinity purification were performed by Eurogentec.

**Western blots.** To test the quality and specificity of the sera produced, we loaded 2 and 10 µg of the recombinant protein and total protein from cell culture samples, respectively, mixed with 2 × Laemmli sample buffer, on two 12.5% SDS-PAGE gels, one was stained with Coomassie blue after electrophoresis, the other one was used to transfer protein bands onto a PVDF membrane (Mini ProBlott Membranes, Applied Biosystems, Foster City, CA, USA) using a Transblot device (Bio-Rad). Membrane strips were subsequently blocked for 30 min at room temperature in a TBS solution containing 1% casein and 0.05% Tween 20. After blocking, the membranes were incubated with chicken anti-r-CSU_001473 polyclonal sera, or negative chicken sera dilutions 1:500 in TTBS buffer (100 mM Tris, 0.9% NaCl, 0.1% Tween 20) at room temperature for 1 h. After rinsing with TTBS for 30 min, blots were exposed to biotinylated goat anti-chicken IgY (Vector Laboratories, Burlingame, CA, USA) as secondary antibody at 1:5000 dilution in TTBS buffer for 1 h at room temperature, incubated with avidin–biotin complex solution (Vector Laboratories) and finally detected by addition of 3,3′-5,5′-tetramethylbenzidine according to the manufacturer’s instructions (Vector Laboratories).

**Immunofluorescence microscopy.** Merozoites and gamonts from cell culture supernatants were washed once with PBS at room temperature and transferred to poly-L-lysine treated glass slides (Polysciences Inc., Hirschberg an der Bergstrasse, Germany) and air dried before fixation. Parasites were either fixed with 4% paraformaldehyde in PBS for 10 min followed by permeabilization with 0.25% TritonX-100 in PBS for 10 min or fixed in ice-cold 100% methanol for 10 min and then blocked with 4% bovine serum albumin (Sigma-Aldrich, St. Louis, Missouri, USA) in PBS for 2 h at room temperature. A 1:500 dilution of anti-r-CSU_001473 polyclonal sera was added and incubated for 2 h at room temperature followed by 1 h incubation with a 1:300 dilution of Alexa Fluor® (A488) goat anti-chicken IgY (Invitrogen, Eugene, OR, USA). The slides were washed five times with PBS for 25 min after each step described above. 4′,6-diamidino-2-phenylindole (DAPI) (5 µg/ml) was included in the Fluoromount-G® mounting medium (Thermo Fischer Scientific) for nuclear staining. Imaging was carried out with a Zeiss LSM 510 Meta-confocal laser scanning microscope (×63 oil immersion objective). Images were analyzed with Light Editions of Zen 2012 and 2009 (Carl Zeiss Microimaging GmbH, Jena, Germany).

**Inhibition of macrogametes and oocyst development by specific antibodies.** To determine inhibition of sexual stage development and oocyst formation by specific antibodies in vitro, we adapted a previously developed host cell-free culture for treatment of merozoites with egg yolk-derived chicken antibodies. Free merozoites were obtained from monolayer culture supernatant of intestinal porcine epithelial cells 6 days after infection with sporozoites. Purified merozoites were counted and treated with 2 µg/ml of chicken anti-r-CSU_001473 polyclonal sera or 2 µg/ml pre-immune chicken serum as a negative control. The treated merozoites were transferred to fresh Advanced DMEM/F-12 culture medium (Gibco) supplemented with 5% fetal calf serum (Gibco) and penicillin/streptomycin plus l-glutamine (Gibco) onto a new uncoated ibidi 8-well ibiTreat® μ-slide (ibidi, Gräfelfing, Germany) at a concentration of 1.2 × 10^5 merozoites per mL medium and were incubated at 40 °C under 5% CO2. The development of parasite stages was monitored daily. The numbers of asexual and early and late sexual stages and oocysts were monitored from the first day post treatment onwards. The numbers of stages were estimated in the host cell-free culture chambers and 10 µL of each well was counted in a Neubauer chamber at each given time point for calculation of the average numbers of sexual stages. Statistical analysis were performed using a multiple unpaired t-test with the software GraphPad® Prism 9.2 (GraphPad Software). Differences were considered statistically significant at P < 0.05 (*).

To show significance between the average number of stages on different culture days a multiple t-test was performed. In vitro inhibition percentage for each stage was calculated as follows:

\[
\% \text{ inhibition} = 100 \times \left(1 - \frac{\text{average no. of parasite stages in treated cultures}}{\text{average no. of parasite stages in untreated control cultures}}\right)
\]

**Gene annotation analyses.** Gene annotations available on www.toxodb.org were used for C. suis genes described in this study. The identification of potential homologues of C. suis hypothetical proteins was also carried out using a BLAST analyses on www.toxodb.org and www.plasmodb.org.

**Ethics approval.** All procedures in this study involving experimental animals were approved by the institutional ethics and animal welfare committee and the national authority according to §26ff. of the Animal Experiments Act, Tierversuchsgesetz 2021—TVG 2012 und der number 2021–0.030.760.. All efforts were made to minimize the number of animals used for C. suis oocyst generation. All methods were performed in accord-
ance with the guidelines and regulations approved by University of Veterinary Medicine Vienna and the national authority (Austrian Federal Ministry of Science, Health and Economy). The study is reported in accordance with ARRIVE guidelines.

Data availability
All data are contained in the publication.

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**Author contributions**

T.C.B. participated in the overall design of the study, carried out a majority of the experiments and data analysis, interpreted the genes identified and drafted the manuscript. M.L. and M.D. participated in the coordination of the RNA Sequencing and performed the transcriptomic data analysis. A.F. performed the immunolocalisation and the development inhibition assay. B.R. prepared the cell culture. A.J. provided the financial resources, conceived the study and helped draft the manuscript. All authors read and approved of the submitted version of the manuscript.

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**Competing interests**

The authors declare no competing interests.

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