From the Department of Pathobiology

University of Veterinary Medicine Vienna

Institute of Parasitology

(Head: Univ. Prof. Dr. med. vet. Anja Joachim)

CHARACTERIZATION OF *CYSTOISOSPORA SUIS* SEXUAL STAGES

Academic dissertation submitted to fulfill the requirements of the

Academic degree of

Doctor of Philosophy (PhD)

University of Veterinary Medicine Vienna, Austria

Submitted by

Anna Sophia Feix

Vienna, July 2022

PHD COMMITTEE

1st supervisor

Univ. Prof[in]. Dr[in]. med. vet. Anja Joachim

Department of Pathobiology

Institute of Parasitology

University of Veterinary Medicine Vienna

Veterinärplatz 1

1210 Vienna, Austria

2nd supervisor

Univ.-Prof[in] Dipl.-Ing[in] Dr[in] rer.nat. Monika Ehling-Schulz

Department of Pathobiology

Institute of Microbiology

University of Veterinary Medicine Vienna

Veterinärplatz 1

1210 Vienna, Austria

REVIEWER

Prof. Dr. rer. nat. Markus Meissner

Experimentelle Parasitologie

Tierärztliche Fakultät: Veterinärwissenschaftliches Department

Ludwig-Maximilians-Universität München

Lena-Christ-Str. 48

82152 Planegg-Martinsried, Germany

ACKNOWLEDGMENTS

This dissertation would not have been possible without the help and guidance of several individuals, who contributed directly or indirectly to it.

First, I would like to express my gratitude to my primary supervisor Anja Joachim, Institute of Parasitology, University of Veterinary Medicine Vienna for her excellent supervision during the whole project, her encouragement to follow up new ideas and her tireless patience. I also would like to thank her for leading me through the academic career path.

My special thanks go to Bärbel Ruttkowski for introducing me to *Cystoisospora suis* and the beautiful world of *in vitro* culture systems. Without her enthusiasm to constantly improve methods and develop new systems, many great ideas would have never flourished. I would also like to thank Roman Peschke, for sharing his experience and knowledge about different laboratory techniques with me. Thank you to Martin Glösmann and Ursula Reichart for introducing me to new microscopy techniques and Daniela Gruber, from the Core Facility Cell Imaging and Ultrastructure Research at the University of Vienna for an excellent collaboration.

My heartfelt thanks go to Teresa Cruz-Bustos, without whom the completion of this project would not have been possible. Her scientific expertise, but also moral support often helped me through tough times.

I also would like to thank my parents being ok with the fact that, their daughter frequently talks about parasites at the dinner table. Their support, encouragement and faith in me at the times of pain, hurdles and happiness have always motivated me to excel in my career. I also would like to thank my brother for his formatting and artistic support whenever needed.

Lastly, I would like to acknowledge the University of Veterinary Medicine Vienna for providing funds within the Pig and Poultry (PaP) Graduate School for successful completion of this PhD project.

TABLE OF CONTENTS

1. PUBLICATIONS INCLUDED IN THE THESIS AND AUTHORS CONTRIBUTIONS

Publication 1

Feix A, Cruz-Bustos T, Ruttkowski B, Joachim A. (2020) Characterization of *Cystoisospora suis* sexual stages *in vitro*. Parasites & Vectors 13:143. https://doi.org/10.1186/s13071-020-04014-4

5-year Impact Factor: 3.959

Authors' contributions:

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

Publication 2

Feix A, Cruz-Bustos T, Ruttkowski B, Mötz M, Rümenapf T, Joachim A. (2021) Progression of asexual to sexual stages of *Cystoisospora suis* in a host cell-free environment as a model for Coccidia. Parasitology 148 (12):1475-1481. https://doi.org/10.1017/S0031182021001074

5-year Impact Factor: 3.234

Authors' contributions:

AF drafted the manuscript, was responsible for the host cell-free culture system and processed and analysed all samples. AJ designed the study. BR provided oocysts, sporozoites and merozoites and was responsible for the maintenance of the cell culture. TCB provided the necessary expertise for the molecular analysis. TR and MM were involved in the establishment

and conduction of the flow cytometry. All authors read and approved of the final version of the manuscript

Publication 3

Cruz-Bustos T, **Feix A**, Lyrakis M, Dolezal M, Ruttkowski B, Joachim A. (2022) The transcriptome from asexual to sexual in vitro development of *Cystoisospora suis* (Apicomplexa : Coccidia). Sci. Rep. 1–17, https://doi.org/10.1038/s41598-022-09714-8

5-year impact Factor: 5.134

Authors' contributions:

AF took care the *in vitro* host cell free cultures and performed the immunolocalisation and the development inhibition assay. TCB participated in the overall design of the study, carried out the majority of the experiments and data analysis, interpreted the identified genes and drafted the manuscript. ML and MD participated in the coordination of the RNA sequencing and performed the transcriptomic data analysis. AJ provided the financial resources, conceived the study and helped to draft the manuscript. All authors read and approved of the submitted version of the manuscript.

All publication are published in full open access.

2. DECLARATIONS

I hereby declare that the work included in this thesis with the title "Characterization of *Cystoisospora suis* sexual stages" was performed during my PhD study at University of Veterinary Medicine Vienna, Austria, following the rules of Good Scientific Practice in all the aspects. In addition, I assure that no parts of this work have been submitted before to another academic institution for the fulfilment of any sort of degree or awards.

Vienna, July 2022,

Anna Sophia Feix

3. ABBREVIATIONS

CC Cell culture

C. *Cystoisospora*

Cr. *Cryptosporidium*

dpi days post infection

dat days after transfer

doc day of cultivation

E. *Eimeria*

FCS Foetal calf serum

HAP2 Hapless 2 protein

IPEC Intestinal porcine epithelial cells

OWP1 Oocyst wall forming protein

P. *Plasmodium*

SEM Scanning electron microscopy

TEM Transmission electron microscopy

T. *Toxoplasma*

TyRP Tyrosin rich protein

4. SUMMARY

Cystoisospora suis, an apicomplexan enteral parasite of pigs, causes severe economic losses in the livestock industry worldwide. It is characterized by a complex life cycle, during which asexual multiplication, with sporogony and merogony, is followed by sexual development with two morphologically distinct cell types, the micro- and macrogametes. Previous life cycle studies give an overview of the development *in vivo*; however, the detailed development of the sexual stages, i.e. fusion of macro- and microgametes and proteins involved in this is not yet known. However, this crucial step in the life cycle of *C. suis* may constitute a promising intervention target to interrupt parasite development and to prevent further formation of transmissible stages. This thesis aimed to provide a detailed morphological characterisation of all stages of the sexual development of *C. suis*, including fertilization, and to identify and characterise stage-specific proteins linked to sexual development by various cell cultivation, molecular and imaging techniques.

First and foremost, we could demonstrate that the detailed life cycle of *C. suis* can be produced *in vitro* and all life stages can be found at distinct timeslots. Early sexual stages of *C. suis* are first observed on day 8 of cultivation (doc) and look like sunny-side-up eggs, are immobile and have a size of 11.6×15.6 µm. Late sexual stages comprise of two types of gamonts, micro- and macrogamonts, which are similar in size and morphology. Mature micro- and macrogametes are mainly found at 9-11 doc and are often observed in close proximity to each other to support fusion. Mature microgametes consist of an uninucleate body (3-5 µm), with two flagella of 10.8-12.3 µm length on opposite sides, which makes them actively motile. Macrogametes are immobile, spherical with a smooth surface and have a diameter of 11.5-13.0 µm. After fusion a zygote is formed which is the stage preceding the infectious, transmissible sporozoites. From the proteins of the macrogamete an oocyst wall forms, and the environmentally resistant oocysts can be found *in vitro* between 10-13 doc.

The present work also identified genes linked to the developmental cycle of *C. suis* by RNASeq analysis and showed the expression changes of selected genes by quantitative real time PCR during the course of development. Genes related to the sexual stages (micro- or macrogametes) showed an upregulation from 6 doc onwards with a peak on the days when gamonts were also present *in vitro* (10-13 doc). For microgamonts and macrogametes an increased expression of DLC1 and HAP2, proteins related to fertilization, was shown. The occurrence of macrogamonts and macrogametes was correlated with elevated OWP1 and TyRP expression, proteins necessary for oocyst wall formation, both of which increased steadily during cultivation.

Moreover, a new host cell free culture system was developed during this project. For this, merozoites collected from *in vitro* IPEC monolayer cultures infected with *C. suis* were transferred to a host cell-free environment on the 6th doc developed further into sexual stages and thus continued their life cycle in the same time slots as in the cell culture system. Also, the gene expression levels of sexual stage specific genes were comparable in the host cell-free culture system to the already established cell *in vitro* system. This novel system provides a new tool for detailed research on the development of *C. suis* and possibly other Coccidia and will also be useful for evaluation of novel drug or vaccine targets in these parasites.

Furthermore, the gene transcribing for the TyRP in *C. suis* could be identified, and the native protein was bound by a recombinant anti-TyRP-antibody in host cell-free culture. Inhibition of TyRP stunned the formation of the oocyst wall, rendering the parasite non-infectious. Hence, during the course of this project, a possible candidate for the interruption of the *C. suis* life cycle could be described.

Overall, this work sheds light on the sexual development of an important intestinal parasite of pigs, and paves the ways to more applied research for the development of tools and methods to effectively interrupt the parasite's development as the basis of novel control options.

5. GENERAL INTRODUCTION

5.1 **The life cycle of *Cystoisospora suis***

The apicomplexan *Cystoisospora suis* (syn. *Isospora suis*) is an obligate intracellular protozoan parasite of the order Coccidia (1) and closely related to other parasites of veterinary and medical importance, such as *Toxoplasma gondii* or *Eimeria* spp. (2,3). Its lifecycle is completed within a single host, the pig, and it can cause severe diarrhoea and reduced weight gain in suckling piglets, considerably impairing animal health in the first three weeks of life (4).

Piglets take up infectious oocysts from contaminated surroundings. The oocysts of *C. suis* are round with a size of 22.5 × 19.2 µm and an autofluorescent oocysts wall (5). They contain a sporont with a peripherally located nucleus, which after nuclear division forms two sporocysts. Sporozoites are the actual infectious cellular entities of *C. suis* and are released during the gastrointestinal passage once the oocysts are ingested by the host. Free sporozoites are highly motile and will penetrate the porcine intestine epithelial cells by invagination of the host cell plasma membrane (6,7). In the host cell sporozoites undergo cellular division by merogony, which results in the formation of motile crescent-shaped merozoites (8,9). Merozoites released from host cells will re-invade other host cells in a defined number of cycles and finally convert to sexual stages - some will become microgamonts and others become macrogamonts. Microgamonts undergo multiple nuclear divisions, which results in the formation of numerous microgametes. The nucleus of macrogamonts, in contrast, does not divide (5,10,11). It is assumed that a macrogamete is fertilized by a single microgamete to form a zygote (3,5,10,12), which then forms a new oocyst, however the actual process of fertilization could not be observed *in vivo*.

The complete life cycle of *C. suis*, from the infection with oocysts to the shedding of a new generation of oocysts with the host's faeces, is completed within five days in the animal

host (10). It is restricted to the epithelial cells of the porcine intestine, with the highest parasite density in the mid-jejunum (4,13). However, research about sexual stages and oocyst development of *C. suis*, but also of other Apicomplexa is underrepresented in current research, as *in vitro* culture systems supporting all life cycle stages are still lacking.

5.2 *In vitro* systems in *Cystoisospora suis*

In case of *C. suis*, an *in vitro* cultivation system supporting the entire lifecycle in porcine intestinal epithelial cells (IPEC) is established and continuously improved (14,15). This system permits detailed studies of the parasite's biology and cellular host-parasite dynamics, and also allows for research on stage-specific gene transcription and translation, protein composition (and its changes during development) and developmental bottlenecks when the numbers of vital stages are momentarily low, which can be considered as targets for novel control options. *In vitro* culture systems provide the continuous access to the different parasite stages at defined time points in sufficient quantities for different studies (16,17).

In the present culture system, cells are initially infected with free sporozoites of *C. suis*, isolated from sporulated oocysts after excystation with taurocholate, and maintained with in a DMEM/Ham's F-12 medium with 5% foetal calf serum (FCS) at 40 °C under 5% CO_2. Free merozoites can be harvested on doc 6 to 8 and are isolated and cleaned by centrifugation. The number of parasite stages found on each cultivation day *in vitro* depends not only on the harvesting day, but also on the FCS concentration. A higher FCS concentration leads to a higher density of free and intracellular merozoites, but also returns an increased output of gamonts and oocysts. Furthermore, the infection dose also influences the output of gametes, revealing that a high initial infection dose does not necessarily improve the number of sexual stages on doc 9 (15).

Methods for isolation and purification of the sexual stages of *C. suis* were not established at the start of this project, so one of the goals was a viable isolation and purification of micro- and macrogametes for further analysis.

5.3 **Sexual stages in Coccidia**

All Coccidia undergo sexual reproduction within their species-restricted host range (11,18,19). However, the two families of the Coccidia differ greatly in their life cycles (2). Eimeriorina have a fixed number of merogonies, after which the merozoite infects a host cell and undergoes gametogenesis and differentiation of micro- and macrogametes (11,20–22). Completion of the whole life cycle, including gametogenesis resulting in syzygy, is necessary to complete sexual development and form a zygote (11,18).

However, the sexual development of Coccidia is still poorly investigated and only addressed in selected species. *Eimeria* spp. complete their endogenous life cycle in the same host, but have distinct species-specific host tissue preferences, mostly in the intestinal tract, to pursue their sexual development (18). After a fixed number of merogonies, two discernible sexual stages arise. Macrogametes are immobile and remain intracellular, whereas microgametes can move freely in between cells, as they often have one or several flagella, to find a compatible macrogamete and fuse (20). Upon fertilization a zygote is formed, followed by formation of a protective wall, until finally an oocyst arises (22–24). *Eimeria tenella* macrogametes show an upregulation of distinct wall forming protein transcripts and transcripts of oocyst surface proteins, both thought to be necessary for oocyst wall biosynthesis (23,25,26). Especially the tyrosin-rich glycoproteins EmGAM56 and EmGAM82 of *Eimeria maxima* (27) appear to link the oocyst wall structures, making it more robust. A subset of oocyst wall forming proteins (OWP) could be found in *E. tenella* (24).

In *Toxoplasma* the sexual cycle is restricted to feline hosts and begins after an unrestricted number of replication cycles of the asexual stages (28,29). Some of those stages

will penetrate the intestinal epithelia cells of the feline host after ingestion and develop into micro- and macrogamonts, which mature into micro- and macrogametes (28,30,31). In the macrogametes of *T. gondii* seven cysteine-rich OWPs could be detected (32,33) and are also necessary for the oocyst wall formation in this species. Both sexual stages subsequently have to fuse to form a zygote and continue the life cycle of this parasite. *Toxoplasma* not only shows an upregulation in cysteine- and tyrosine-rich proteins in the macrogamete, but also an upregulation of the fusion protein Hap2 in microgametes, which seems to be necessary for zygote formation and life cycle progression, as a knockout of this gene results in the interruption of the life cycle and suppression of oocyst development (34). These stage-specific proteins can also be found in other Eimeriorina (35–37).

During the development of *Sarcocystis* spp., muscle tissue with resting asexual parasite stages (bradyzoites) is consumed by a vertebrate host (18,38) and these bradyzoites then infect cells of the intestinal epithelium and transform into macro- and microgamonts. The microgamonts develop into microgametes with up to four flagella, which penetrate the macrogametes and form a zygote (39–41). Not a lot is known about the sexual genes of *Sarcocystis* species, however wall morphology of gametocytes and oocysts of different *Sarcocystis* species varies. But different wall forming proteins could not defined yet (42).

Cystoisospora suis also expresses distinct sexual genes and genes involved in the regulation of cellular events found in other coccidian species. These genes often code for proteins that play critical roles during the sexual development, including the oocyst wall formation, microgamete motility and fertilization process and zygote formation.

5.4 **Expected characteristics of proteins involved in *Cystoisospora suis* sexual stages**

Prior research in Coccidia has shown that the macrogamete develops from the macrogamont and contains numerous mitochondria and wall-forming bodies (43,44); the latter provide the basis for oocyst wall formation. After the formation of the oocyst wall from the

wall-forming bodies, the endogenous development of Coccidia is complete and the oocysts can be discharged into the environment, where they develop further, finally containing the infectious sporozoites enveloped in sporocysts (45). In the macrogametocyte stage of Coccidia two wall-forming bodies (Type 1 and Type 2) can be found in its bilayered wall, which are believed to be responsible for the development of the oocyst wall (43,46,47). The oocyst wall is composed of 90% protein (48,49), however only few of these proteins have been studied in Coccidia (33,50,51). One protein group is rich in cysteine and is presumed to crosslink via disulphide bridges (51,52). Another protein-group is rich in tyrosine (47). These proteins may derive from precursors which are found in the wall-forming bodies. Proteases catalyse the processing of the precursors into tyrosine-rich peptides, which are then oxidatively crosslinked in a reaction catalysed by peroxidases. Therefore, the oocyst wall has high levels of dityrosine bonds (32,33,47). These dityrosine-crosslinked proteins provide a structural matrix for assembly of the oocyst wall, making it extremely resilient to environmental stress (53). However, such wall-forming bodies have not yet been shown in *Cystoisospora suis*. Previous studies have detected the EtMIF wall-forming bodies of macrogametes in *E. tenella* merozoites (24). Furthermore, gene transcripts coding for EtGAM56 and other oocyst wall proteins (EtGAM22, EtGAM59, and EtGAM230) are confirmed for other Apicomplexan protozoa (51,54), and the family of EtOWP6 proteins is expressed in wall forming bodies of macrogametes and oocyst walls of *T. gondii* and *Cryptosporidium parvum* (33).

The microgamete flagella of Coccidia are believed to contain specific proteins for structure, transport and fusion, mainly dynein and HAP-proteins (36,55). Some of these are considered to be rather conserved between species so that comparison with previously characterized protozoan proteins should yield a high success rate for an annotation, while others are absent from, e.g., *Plasmodium* (56,57), the best-studied member of the Apicomplexa and causative agent of malaria. Conserved stage-specific proteins associated with the flagellum and

flagellar function in *Plasmodium* include dynein components and an armadillo-like repeat protein that is considered as essential for flagellum structure and function (58–60).

Conserved cell cycle proteins crucial for microgamete development can be found in *Plasmodium* and can be expected to be present and functional in other apicomplexan parasites (22). A highly conserved transmembrane protein GCS1 (Generative Cell Specific 1) has been described as an interaction factor between gametes of *Plasmodium berghei* involved in the membrane fusion process of the two cells. GCS1 is an ancient fusogen of eukaryotes expressed exclusively in male gametes (37,61) and it is necessary for membrane merging of gametes and thus fertilization in *Plasmodium* (62).

Prior studies on other Coccidia species imply that an intact microgamete attaches to the macrogamete surface and penetrates its cell membrane. Then the microgamete enters the macrogamete cytoplasm (46,63). During this fertilization process, the plasma membranes of the micro- and macrogametes fuse together and transfer their nuclear material. At the point of fusion, the gamete membranes are thickened and an aggregate of dense material lines the cytoplasmic side of the fusion area. Afterwards nucleoplasm from the microgamete passes into the cytoplasm of the macrogametes (64). Microtubules and mitochondria remain within the microgametes. However, assigning specific proteins to either gamete is rather new. Functions assigned to microgamete-specific or macrogamete-specific transcripts and proteins in the Coccidia is difficult due to the limited biological information on these stages (24). However, it is assumed that the process of fusion is similar in all Coccidia and works due to the action of a number of different proteins in their cell membranes. Proteins present during the fertilization process are not yet known; however, dynein and HAP-proteins might be responsible for the merging of micro- and macrogametes. Comparative analysis of *E. tenella* transcriptome subsets of asexual and sexual stages revealed macro- and microgamete-specific transcripts (24)

including HAPLESS protein 2 (65). In microgametes of *E. tenella* the EtHAP2 gamete fusion protein will probably be further investigated (24).

6. AIMS AND HYPOTHESES

6.1 General Aims

The main aim of this project was to characterize the development of sexual stages in *C. suis* and the role of micro- and macrogametes for parasite development by studying the morphology and composition of these stages.

Functional studies have so far been hampered by the lack of suitable *in vitro* models for the production and characterization of defined sexual stages. Detailed knowledge on the characteristics of the sexual stages, including their maturation and the process of fertilization on the cellular and molecular level, could provide new directions for the intervention of parasite development by interrupting the life cycle, which is considered one of the most promising ways of controlling coccidian parasites. *Cystoisospora suis* has the potential to clear the methodological barriers stated above and is therefore highly suitable to become a model for coccidian pathogens in general. Previous life cycle studies have shown details of the development of *C. suis in vivo*. However, the accurate development of the sexual stages, i.e. fusion of macro- and microgametes has not been described in much detail, although it is an important step in the development of control strategies against infection with this parasite, as *C. suis* to date is the most common parasite in pigs, inducing high economic losses in the piglet-producing industry(38,66,67). Prior research at the Institute of Parasitology of the University of Veterinary Medicine Vienna has produced a promising *in vitro* protocol to produce sexual stages, allowing a continuous observation of the development of the sexual stages of *C. suis*, as well as the study of fertilization in this organism. To characterize the process of fertilization in *C. suis* as well as the role of micro- and macrogametes for parasite development, we intended to study the morphology and composition of these stages in parasite development and, further on, their possible roles in host-parasite interactions.

6.2 **Hypotheses and aims**

From previous works three hypotheses were formulated and addressed in the work described here:

1) Micro- and macrogametes of. *C. suis* developing *in vitro* are similar to those described histologically from infected animals.

2) Their development and maturation *in vitro* can be described from the sexual differentiation of merozoites to the fusion of the cells and the development of a zygote in morphological detail.

3) Conserved proteins described for other Apicomplexa are present in the genome of *C. suis* and expressed specifically during sexual development *in vitro*.

Consequently, the goals of this PhD-project were a detailed morphological and molecular characterisation of all stages of the sexual development of *C. suis* with different imaging techniques (scanning electron microscopy, transmission electron microscopy and time-lapse live imaging) and molecular methods (quantitative real-time PCR, RNASeq). For this a series of experiments was conducted showing the morphology, protein composition and the function of selected stage-specific proteins. This work was published in three papers in international, peer-reviewed journals, which are shown in the next chapters.

7. PUBLICATIONS

7.1 Publication 1

Characterization of *Cystoisospora suis* sexual stages *in vitro*

Feix A, Cruz-Bustos T, Ruttkowski B, Joachim A. (2020) Parasites & Vectors 13:143.

https://doi.org/10.1186/s13071-020-04014-4

Own contributions:

- isolation and preparation of stages from the *in vitro* culture
- accomplishment of all morphological laboratory experiments
- data analysis
- drafting of the original manuscript and correction of the revised version

Feix *et al. Parasites Vectors* (2020) 13:143
https://doi.org/10.1186/s13071-020-04014-4

Parasites & Vectors

RESEARCH Open Access

Characterization of *Cystoisospora suis* sexual stages *in vitro*

Anna Sophia Feix[†], Teresa Cruz-Bustos[*†], Bärbel Ruttkowski and Anja Joachim

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. *Dynein light chain type 1* and male gamete fusion factor *HAP2* are restricted to microgametes. Tyrosine-rich proteins and oocyst wall proteins are a part of the oocyst wall. The Rad51/Dmc1-like protein and Nima-related protein kinases are associated with the cell cycle and fertilization process. 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* 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. Transcription levels were compared for parasites in culture supernatants at 6–9 days of cultivation (doc) and intracellular parasites at 6–15 doc.

Results: Sexual stage of *C. suis* was detected during 8–11 doc *in vitro*. Microgamonts (16.8 ± 0.9 µm) and macrogamonts (16.6 ± 1.1 µm) are very similar in shape and size. Microgametes had a round body (3.5 ± 0.5 µm) and two flagella (11.2 ± 0.5 µm). Macrogametes were spherical with a diameter of 12.1 ± 0.5 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 study linked gene expression changes to the detailed morphological description of *C. suis* sexual development *in vitro*, including fertilization, meiosis and oocyst formation in this unique model for coccidian parasites. Following this process at the cellular and molecular level will elucidate details on potential bottlenecks of *C. suis* development (applicable for coccidian parasites in general) which could be exploited as a novel target for control.

Keywords: *Isospora suis*, Gametes, *DLC-1*, *HAP2*, *OWP*, *RAD51/Dmc*, *Nima*-related protein kinases

Background

Cystoisospora suis (syn. *Isospora suis* Biester & 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

*Correspondence: Teresa.Cruz-Bustos@vetmeduni.ac.at
[†]Anna Sophia Feix and Teresa Cruz-Bustos contributed equally to this work
Institute for Parasitology, Department of Pathobiology, University of Veterinary Medicine Vienna, Veterinaerplatz 1, Vienna 1210, Austria

delayed [7]. This makes it possible to observe, harvest and examine sexual stages in the short time frame in which they occur. Macrogametes of coccidians 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 certain 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 (i) 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 (ii) 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 s with Precellys® glass beads (Peqlab, 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 6-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% CO_2. After 24 h of cell growth IPEC-1 were infected with 5×10^3 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 KH_2PO_4, 9000.0 mg/l NaCl, 795.0 mg/l Na_2HPO_4-$7H_2O$) 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 coverslips 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% ethanol 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 s 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 calculation of percentage identity 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

Table 1 Primers used in this study

	Primer	Sequence (5′–3′)
1	Fw-ACTIN	CTTGCTGGCCGTGATTTGAC
2	Rv-ACTIN	ATATTGCCGTCCGGAAGCTC
3	Probe-ACTIN	CCTCCGCCGAGAAGGAAATT
4	Fw-GAPDH	TTCAACGAGAAGGAGCCAAG
5	Rv-GAPDH	CTTCGGAGGTGCAGACATG
6	Probe-GAPDH	CAAGGAAAAGGCTGAGGCGCAT
7	Fw-HAP2	GGAACCCAGGGAAATTTTGT
8	Rv-HAP2	CATGTTGTTGATGTGCGTGA
9	Probe-HAP2	GCTGCTGGTGCAGTGAGGTC
10	Fw-DLC1	TGCTATGGCCTGTTGATATGC
11	Rv-DLC1	CTTCTGGTCGAGCTCCTTTT
12	Probe-DLC1	TGCTGCGCGTGACTGTATAATCCA
13	Fw-OWP1	CCAGAAGGATGTTTATTTGCCG
14	Rv-OWP1	TGGGCAGATGTATTCAGGTTC
15	Probe-OWP1	AATCCGAAGGGCAGCGTTGTAGAA
16	Fw-TyRP	GAACTGGACGGTGATCGTGA
17	Rv-TyRP	GCTCTCAATAAGTCCCTCAGAG
18	Probe-TyRP	CTCATGCGCTCGCTACCTGA
19	Fw-Rad51	GCTTCGCTTTGCTTATTGTC
20	Rv-Rad51	CAACAACAGCCACACCATAC
21	Probe-Rad51	TGCCACGGCCCTATACAGGT
22	Fw-NIMA1	GCTGGAAACTGGTGTTTTAC
23	Rv-NIMA1	GCATCGCAGTACTCCATAAC
24	Probe-NIMA1	GTGAACTTCGGCACCCCAAC
25	Fw-NIMA2	AGGACAACTACATCCGTGTC
26	Rv-NIMA2	CGTGACATATATTTCGCTGA
27	Probe-NIMA2	TCCAGCAAGCAAGAACGCAG
28	Fw-NIMA4	AAAGAGTCGCAGATTCTCAG
29	Rv-NIMA4	CCTGCGTATGATCAAGAAGT
30	Probe-NIMA4	AGAATTCGCCTGGCGGATTT
31	Fw- CSUI_005805	CCTGAAAGTCGCCTGTCCAT
32	Rv- CSUI_005805	GACGCGTCAGCCGTTATAGT
33	Probe-CSUI_005805	CTCTCAGTTTCGCGGCACCT

out on a Mx3000P thermal cycler (Agilent Technologies, Santa Clara, CA, USA). The primers for gene amplification are listed in Table 1. 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 s and 60 °C for 30 s. 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 $2^{-\Delta\Delta Cq}$ 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 were detected in appreciable numbers from day 6 doc. Sexual stages occured in appreciable numbers between 8–11 doc and were 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 coccidians, 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 on average 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 on average 16.8 ± 0.9 µm ($n = 50$), whereas macrogamonts were 16.6 ± 1.1 ($n = 50$) in average diameter (Table 2). In light microscopy, unstained microgamonts were recognized by their large vacuole and motile microgametes inside (Fig. 2b, c). Both micro- and macrogamonts were often found in close proximity to each other (Fig. 2b, d, Additional file 1), and the egress of microgametes from microgamonts could be observed during a 4-hour time frame. Each microgamont contained between 30–40 microgametes.

Scanning electron microscopy observations showed that microgametes consisted of a small, spherical (3.5 ± 0.5 µm, $n = 50$) body with two opposing flagella, 11.2 ± 0.5 µm in length ($n = 50$, Fig. 3a), which enabled the quick movement of the microgamete in search for

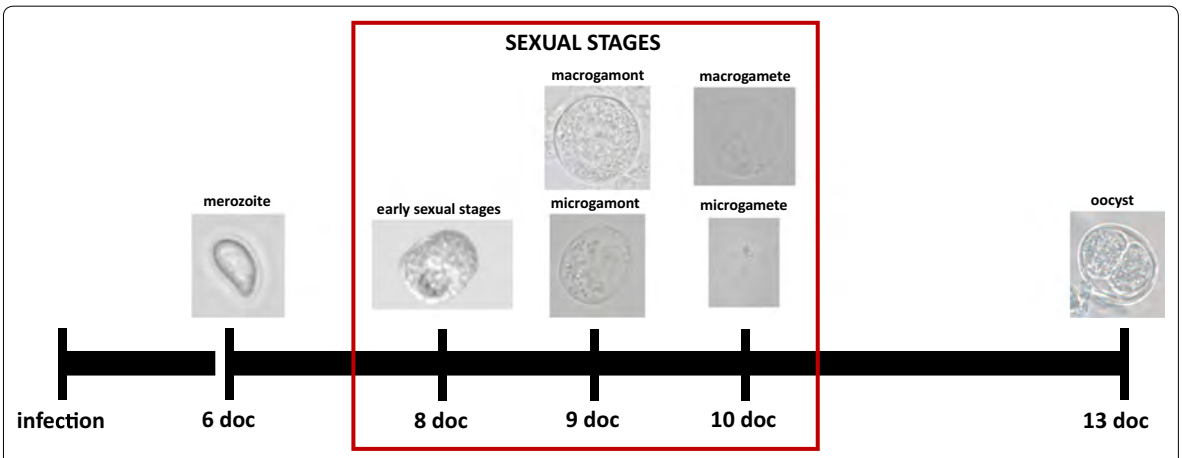


Fig. 1 Time line of *C. suis* development *in vitro*. Early sexual stages could be found at 8 days of cultivation (doc) and gamonts from 9 doc onwards. Micro- and macrogametes could be detected from 10 doc onwards *in vitro*. Sporulated oocysts were found at 13 doc

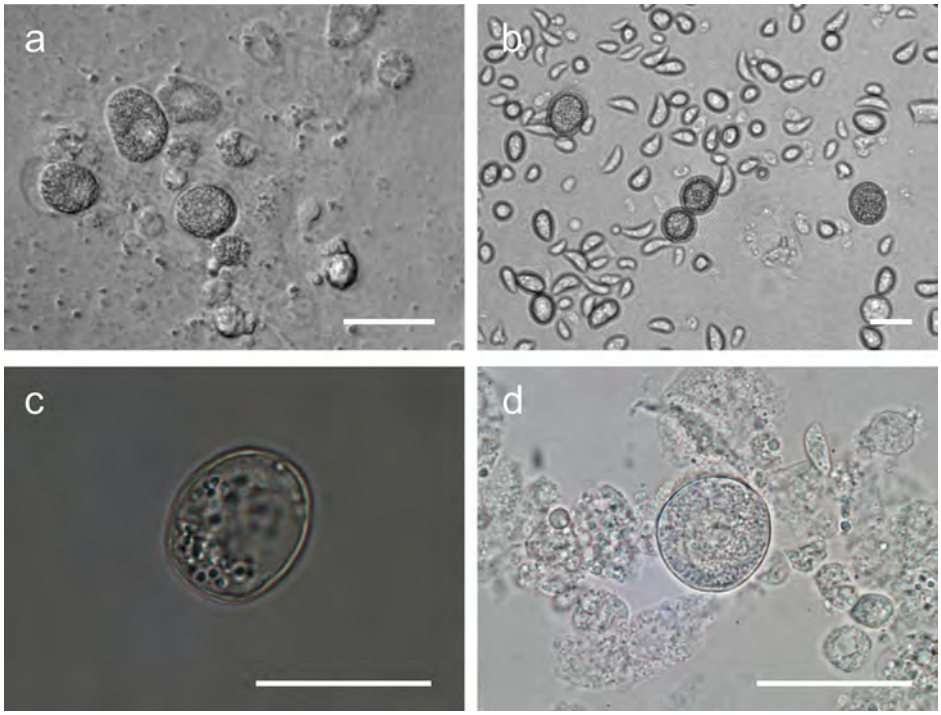


Fig. 2 Light microscopy of different sexual stages of *C. suis in vitro* culture. **a** Early sexual stages, 7 days of cultivation (doc), differential interference contrast. **b** Micro- and macrogamont in close proximity to each other, 8 doc, differential interference contrast. **c** Microgamont, 8 doc. **d** Macrogamont, 8 doc. *Scale-bars*: 20 µm

Table 2 Overview of mean, standard deviation, variance and range of *C. suis* sexual stages *in vitro* ($n = 50$)

	Early sexual stages		Microgamonts	Macrogamonts	Microgametes		Macrogamets
	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

Note: All measurements are in micrometers

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 2).

To our knowledge, this 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: 11.8×8.4 µm; immature macrogamonts: 9.4×6.5 µm) while the shape and size of mature micro- and macrogamonts are comparable to those in the early sexual stages [6]. Other closely related coccidian species show similar morphology of the sexual stages [37, 38]; however, microgametes of *T. gondii* and *Eimeria* [17] have flagella on the posterior end, whereas in *C. suis* they are clearly positioned on opposite sides, which might also affect microgamete movement on the search for a macrogamete. With an *in vitro* system that

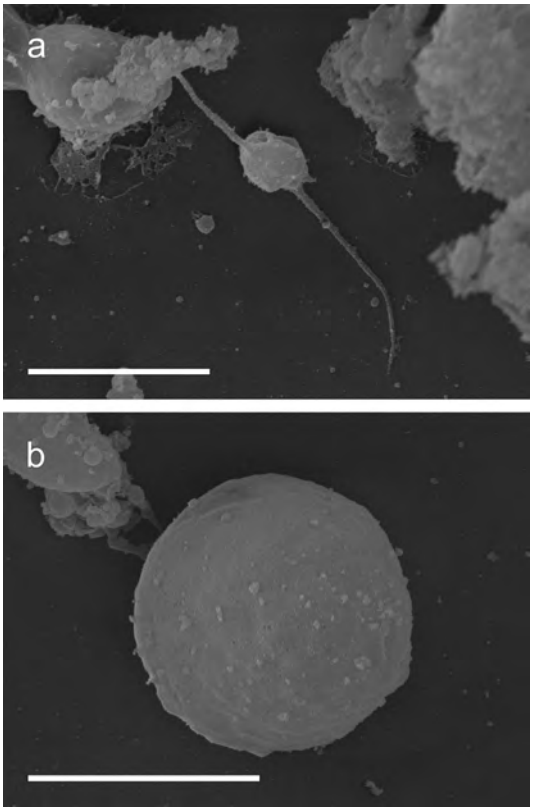


Fig. 3 Scanning electron microscopy of adult sexual stages of *C. suis in vitro* culture. **a** Microgamete, 10 days of cultivation (doc). **b** Macrogamete from 10 doc. *Scale-bars*: 5 µm

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 protozoans. 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 1).

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 1).

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 1). 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].

Table 3 Classification of genes used in this study from *C. suis* and their comparison with other coccidian parasites

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

Notes: 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)

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 signalling, 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 8–9-fold 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 microgamete-related genes transcripts reach peaks on 13–14 doc and the increase was 10–90-fold higher compared to merozoites at 6 doc (Fig. 6) which is in agreement with results for RNA seq comparing merozoites from third-generation

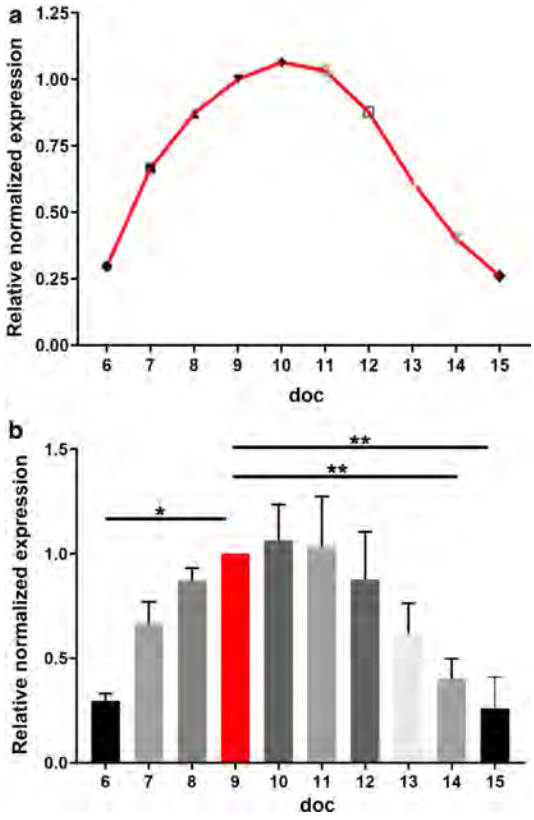


Fig. 4 Relative mRNA expression levels of CSUI_005805 in intracellular parasites. Qualitative RT-PCR of intracellular parasites from 6–15 days of cultivation (doc). Values represent the mean ± standard error (SE) ($n = 4$). One-way ANOVA with multiple comparisons. $^{**}P < 0.01$. For details on statistical tests see Additional file 2: Table S1

merozoites and gamonts of *Eimeria* [11]. The levels for *DLC1* and *HAP2* (Fig. 6a, c, d) transcripts on 13 and 14 doc were 30-fold 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 9–15 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

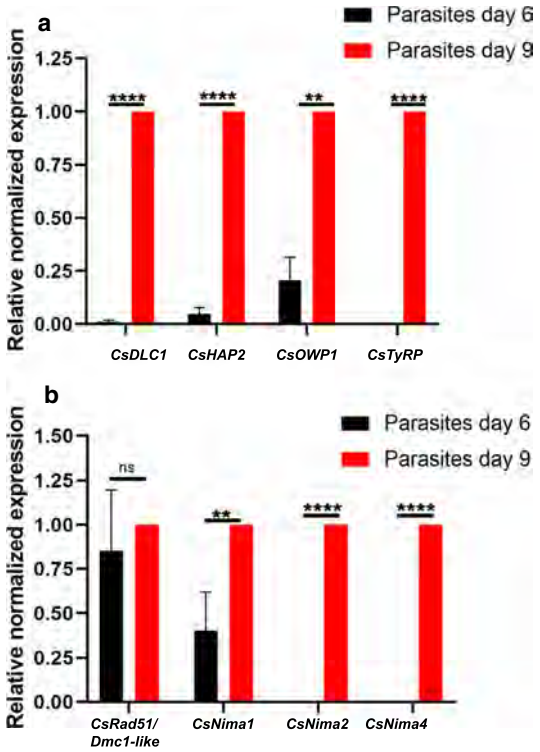


Fig. 5 Relative mRNA expression levels of sexual related genes, cellular division and meiosis genes in extracellular parasites. Qualitative RT-PCR of intracellular parasites 6 to and 9 days of cultivation (doc). Values represent the mean ± standard error (SE) ($n = 4$). Unpaired Student's t-test: ns: not significant ($P > 0.05$), $^{**}P < 0.01$, $^{****}P < 0.0001$. For details on statistical tests see Additional file 2: Table S1

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

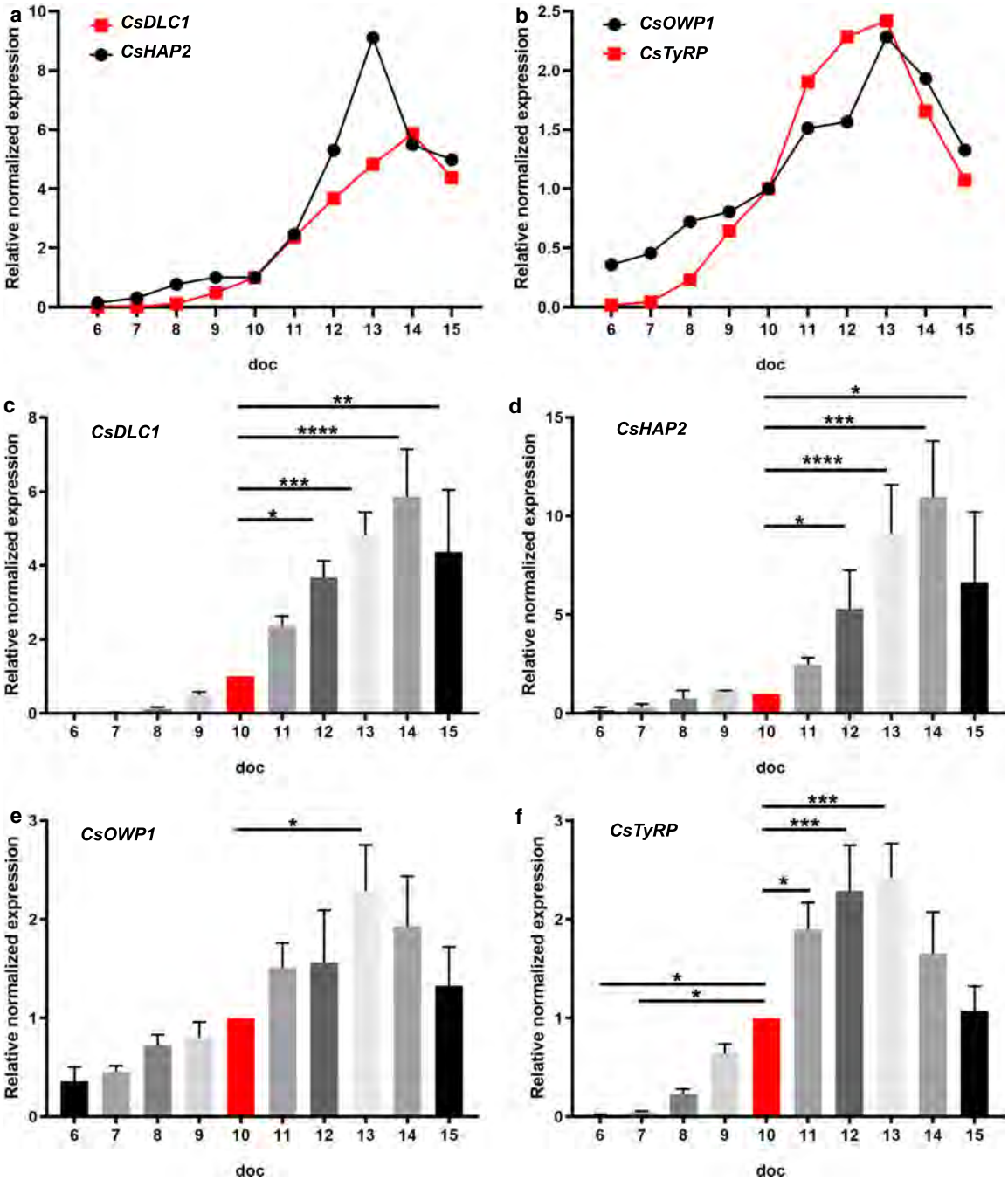


Fig. 6 Relative mRNA expression levels of sexual related genes in intracellular parasites. qRT-PCR of intracellular parasites from day 6 to day 15 of cultivation. **a** Microgamete-related genes. **b** Macrogamete-related genes. **c** *CsDLC1*. **d** *CsHAP2*. **e** *CsOWP1*. **f** *CsTyRP* (CSUI_001473). Values represent the mean ± standard error (SE) from four independent experiments and are expressed as arbitrary absorbance units ($n = 4$). *$P < 0.05$, **$P < 0.01$***, $P < 0.001$, ****$P < 0.0001$. For details on statistical tests (one-way ANOVA with multiple comparisons) see Additional file 2: Table S1

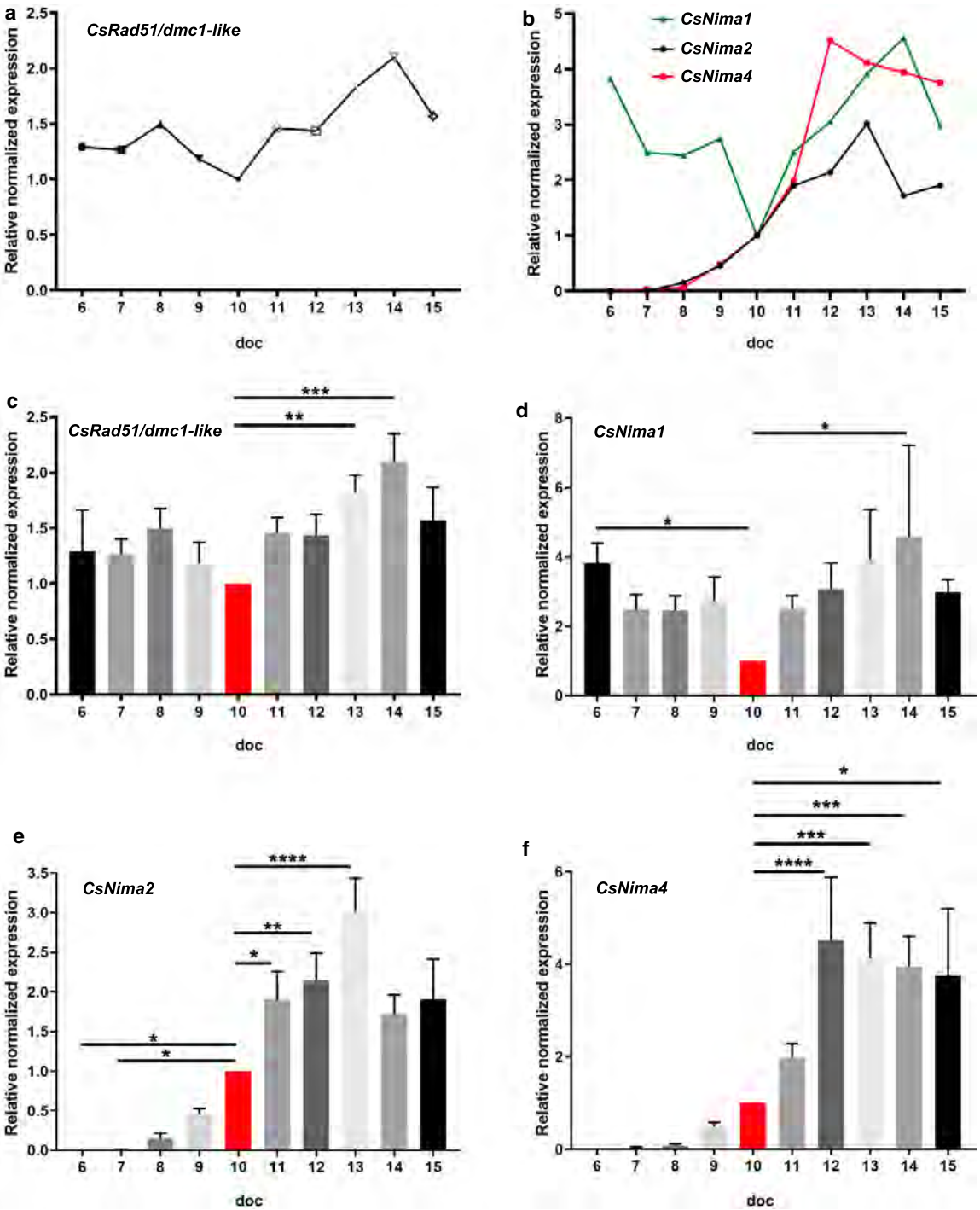


Fig. 7 Relative mRNA expression levels of related to cellular division and meiosis genes in intracellular parasites. qRT-PCR of intracellular parasites from 6 to 15 days of cultivation (doc). **a** *CsRad51*-related gene. **b** *Nima-Nek*-related kinase genes. **c** *CsRad51*. **d** *CsNima1*. **e** *CsNima2*. **f** *CsNima4*. Values represent the mean ± standard error (SE) from four independent experiments and are expressed as arbitrary absorbance units ($n = 4$). *$P < 0.05$, **$P < 0.01$***, $P < 0.001$, ****$P < 0.0001$. For details on statistical tests (one-way ANOVA with multiple comparisons) see Additional file 2: Table S1

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 and 14 doc, respectively (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*.

Conclusions

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 coccidians, *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.*

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13071-020-04014-4.

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, 5, 6, 7.

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.

Acknowledgements

We would like to thank the animal technicians Sonja Rohrer and Martina Lastufka for taking care of the piglets used for oocyst collection for this study, as well as the Core Facility Cell Imaging and Ultrastructure Research, University of Vienna-member of the Vienna Life-Science Instruments (VLSI) and especially Mag. Daniela Gruber for her expertise in all performed scanning electron microscopy work.

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 the final manuscript.

Funding

ASF is funded through the Graduate School "Pig and Poultry Medicine" of the University of Veterinary Medicine Vienna.

Availability of data and materials

All data and materials of the experiments described here are included in this published article and its additional files.

Ethics approval and consent to participate

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 number of animals used for ***C. suis*** oocyst generation.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Received: 19 December 2019 Accepted: 10 March 2020
Published online: 18 March 2020

References

1. Martineau GP, Del Castillo J. Epidemiological, clinical and control investigations on field porcine coccidiosis: clinical, epidemiological and parasitological paradigms? Parasitol Res. 2000;86:834–7.
2. Stuart BP, Lindsay DS. Coccidiosis in swine. Vet Hum Toxicol. 1987;29:65–7.
3. Stuart BP, Lindsay DS, Ernst JV, Gosser HS. *Isospora suis* enteritis in piglets. Vet Pathol. 1980;17:84–93.

4. Joachim A, Shrestha A. Coccidiosis of pigs. In: Dubey JP, editor. Coccidiosis in livestock, companion animals and humans. Boca Raton: CRC Press; 2020. p. 125–45.
5. Lindsay DS, Stuart BP, Wheat BE, Ernst JV. Endogenous development of the swine coccidium, *Isospora suis* Biester 1934. J Parasitol. 1980;66:771–9.
6. Lindsay DS, Quick DP, Steger AM, Toivio-Kinnucan MA, Blagburn BL. Complete development of the porcine coccidium *Isospora suis* Biester, 1934 in cell cultures. J Parasitol. 1998;84:635.
7. Worliczek HL, Ruttkowski B, Schwarz L, Witter K, Tschulenk W, Joachim A. *Isospora suis* in an epithelial cell culture system—an *in vitro* model for sexual development in coccidia. PLoS One. 2013;8:e69797.
8. Smith TG, Walliker D, Ranford-Cartwright LC. Sexual differentiation and sex determination in the Apicomplexa. Trends Parasitol. 2002;18:315–23.
9. Mai K, Sharman PA, Walker RA, Katrib M, Souza DDE, McConville MJ, et al. Oocyst wall formation and composition in coccidian parasites. Mem Inst Oswaldo Cruz. 2009;104:281–9.
10. Mouafo A, Weck-Heimann A, Dubremetz JF, Entzeroth R. Monoclonal antibodies specific for the two types of wall-forming bodies of *Eimeria tenella* macrogametes (Coccidia, Apicomplexa). Parasitol Res. 2002;88:217–24.
11. Su S, Hou Z, Liu D, Jia C, Wang L, Xu J, et al. Comparative transcriptome analysis of *Eimeria necatrix* third-generation merozoites and gametocytes reveals genes involved in sexual differentiation and gametocyte development. Vet Parasitol. 2018;252:35–46.
12. Possenti A, Fratini F, Fantozzi L, Pozio E, Dubey JP, Ponzi M, et al. Global proteomic analysis of the oocyst/sporozoite of *Toxoplasma gondii* reveals commitment to a host-independent lifestyle. BMC Genomics. 2013;14:183.
13. Wallach MG, Mencher D, Yarus S, Pillemer G, Halabi A, Pugatsch T. *Eimeria maxima*: identification of gametocyte protein antigens. Exp Parasitol. 1989;68:49–56.
14. Spano F, Puri C, Ranucci L, Putignani L, Crisanti A. Cloning of the entire COWP gene of *Cryptosporidium parvum* and ultrastructural localization of the protein during sexual parasite development. Parasitology. 1997;114:427–37.
15. Lippuner C, Ramakrishnan C, Basso WU, Schmid MW, Okoniewski M, Smith NC, et al. RNA-Seq analysis during the life cycle of *Cryptosporidium parvum* reveals significant differential gene expression between proliferating stages in the intestine and infectious sporozoites. Int J Parasitol. 2018;48:413–22.
16. Belli SI, Wallach MG, Luxford C, Davies MJ, Smith NC. Roles of tyrosine-rich precursor glycoproteins and dityrosine- and 3,4-dihydroxyphenylalanine-mediated protein cross-linking in development of the oocyst wall in the coccidian parasite *Eimeria maxima*. Eukaryot Cell. 2003;2:456–64.
17. Walker RA, Ferguson DJP, Miller CMD, Smith NC. Sex and *Eimeria*: a molecular perspective. Parasitology. 2013;140:1701–17.
18. Xu J, Zhang Y, Tao J. Efficacy of a DNA vaccine carrying *Eimeria maxima* Gam56 antigen gene against coccidiosis in chickens. Korean J Parasitol. 2013;51:147–54.
19. Jang SI, Lillehoj HS, Lee SH, Lee KW, Park MS, Cha SR, et al. *Eimeria maxima* recombinant Gam82 gametocyte antigen vaccine protects against coccidiosis and augments humoral and cell-mediated immunity. Vaccine. 2010;28:2980–5.
20. Rafiqi SI, Garg R, Ram H, Reena KK, Asari M, Kumari P, et al. Immunoprophylactic evaluation of recombinant gametocyte 22 antigen of *Eimeria tenella* in broiler chickens. Parasitol Res. 2019;118:945–53.
21. Possenti A, Cherchi S, Bertuccini L, Pozio E, Dubey JP, Spano F. Molecular characterisation of a novel family of cysteine-rich proteins of *Toxoplasma gondii* and ultrastructural evidence of oocyst wall localisation. Int J Parasitol. 2010;40:1639–49.
22. Templeton TJ, Lancto CA, Vigdorovich V, Liu C, London NR, Hadsall KZ, et al. The *Cryptosporidium* oocyst wall protein is a member of a multigene family and has a homolog in *Toxoplasma*. Infect Immun. 2004;72:980–7.
23. Briggs LJ, Davidge JA, Wickstead B, Ginger ML, Gull K. More than one way to build a flagellum: comparative genomics of parasitic protozoa. Curr Biol. 2004;14:611–2.
24. Khan SM, Franke-Fayard B, Mair GR, Lasonder E, Janse CJ, Mann M, et al. Proteome analysis of separated male and female gametocytes reveals novel sex-specific *Plasmodium* biology. Cell. 2005;121:675–87.
25. Ferguson DJP, Sahoo N, Pinches RA, Bumstead JM, Tomley FM, Gubbels M-J. MORN1 has a conserved role in asexual and sexual development across the apicomplexa. Eukaryot Cell. 2008;7:698–711.
26. Fédry J, Liu Y, Péhau-Arnaudet G, Pei J, Li W, Tortorici MA, et al. The ancient gamete fusogen HAP2 is a eukaryotic class II fusion protein. Cell. 2017;168:904–15.
27. Walker RA, Sharman PA, Miller CM, Lippuner C, Okoniewski M, Eichenberger RM, et al. RNA Seq analysis of the *Eimeria tenella* gametocyte transcriptome reveals clues about the molecular basis for sexual reproduction and oocyst biogenesis. BMC Genomics. 2015;16:94.
28. Angrisano F, Sala KA, Da DF, Liu Y, Pei J, Grishin NV, et al. Targeting the conserved fusion loop of HAP2 inhibits the transmission of *Plasmodium berghei* and *falciparum*. Cell Rep. 2017;21:2868–78.
29. Ramakrishnan C, Maier S, Walker RA, Rehrauer H, Joekel DE, Winiger RR, et al. An experimental genetically attenuated live vaccine to prevent transmission of *Toxoplasma gondii* by cats. Sci Rep. 2019;9:1474.
30. Weedall GD, Hall N. Sexual reproduction and genetic exchange in parasitic protists. Parasitology. 2015;142(Suppl. 1):120–7.
31. Fritz HM, Bowyer PW, Bogyo M, Conrad PA, Boothroyd JC. Proteomic analysis of fractionated *Toxoplasma* oocysts reveals clues to their environmental resistance. PLoS One. 2012;7:e29955.
32. Carvalho TG, Doerig C, Reininger L. Nima- and Aurora-related kinases of malaria parasites. Biochim Biophys Acta. 2013;1834:1336–45.
33. Gonzalez-Vallina R, Wang H, Zhan R, Berschneider HM, Lee RM, Davidson NO, et al. Lipoprotein and apolipoprotein secretion by a newborn piglet intestinal cell line (IPEC-1). Am J Physiol. 1996;271:249–59.
34. Diesing AK, Nossol C, Panther P, Walk N, Post A, Kluess J, et al. Mycotoxin deoxynivalenol (DON) mediates biphasic cellular response in intestinal porcine epithelial cell lines IPEC-1 and IPEC-J2. Toxicol Lett. 2011;200:8–18.
35. Schierack P, Nordhoff M, Pollmann M, Weyrauch KD, Amasheh S, Lodemann U, et al. Characterization of a porcine intestinal epithelial cell line for *in vitro* studies of microbial pathogenesis in swine. Histochem Cell Biol. 2006;125:293–305.
36. Matuschka F, Heydorn AO. Die Entwicklung von *Isospora suis* Biester und Murray 1934 (Sporozoa:Coccidia:Eimeriidae) im Schwein. 1980;26:405–76.
37. Madden PA, Vetterling JM. Scanning electron microscopy of *Eimeria tenella* microgametogenesis and fertilization. J Parasitol. 1977;63:607–10.
38. Scholtyseck E, Mehlhorn H, Hammond DM. Electron microscope studies of microgametogenesis in coccidia and related groups. Zeitschrift für Parasitenkd. 1972;38:95–131.
39. Matos LVS, McEvoy J, Tzipori S, Bresciani KDS, Widmer G. The transcriptome of *Cryptosporidium* oocysts and intracellular stages. Sci Rep. 2019;9:7856.
40. Fritz HM, Buchholz KR, Chen X, Durbin-Johnson B, Rocke DM, Conrad PA, et al. Transcriptomic analysis of *Toxoplasma* development reveals many novel functions and structures specific to sporozoites and oocysts. PLoS One. 2012;7:e29998.
41. Su S, Hou Z, Liu D, Jia C, Wang L, Xu J, et al. Comparative transcriptome analysis of second- and third-generation merozoites of *Eimeria necatrix*. Parasit Vectors. 2017;10:388.
42. Wass MN, Stanway R, Blagborough AM, Lal K, Prieto JH, Raine D, et al. Proteomic analysis of *Plasmodium* in the mosquito: progress and pitfalls. Parasitology. 2012;139:1131–45.
43. Morahan B, Garcia-Bustos J. Kinase signalling in *Plasmodium* sexual stages and interventions to stop malaria transmission. Mol Biochem Parasitol. 2014;193:23–32.
44. Shrestha A, Palmieri N, Abd-Elfattah A, Ruttkowski B, Pagès M, Joachim A. Cloning, expression and molecular characterization of a *Cystoisospora suis* specific uncharacterized merozoite protein. Parasit Vectors. 2017;10:68.
45. Morrissette N. Targeting *Toxoplasma* tubules: tubulin, microtubules, and associated proteins in a human pathogen. Eukaryot Cell. 2015;14:2–12.
46. Qureshi BM, Hofmann NE, Arroyo-Olarte RD, Nickl B, Hoehne W, Jungblut PR, et al. Dynein light chain 8a of *Toxoplasma gondii*, a unique conoid-localized β-strand-swapped homodimer, is required for an efficient parasite growth. FASEB J. 2012;27:1034–47.
47. Wong JL, Johnson MA. Is HAP2-GCS1 an ancestral gamete fusogen? Trends Cell Biol. 2010;20:134–41.
48. Hussein HE, Bastos RG, Schneider DA, Johnson WC, Adham FK, Davis WC, et al. The *Babesia bovis hap2* gene is not required for blood stage replication, but expressed upon *in vitro* sexual stage induction. PLoS Negl Trop Dis. 2017;11:e0005965.
49. Jonscher E, Erdbeer A, Günther M, Kurth M. Two COWP-like cysteine rich proteins from *Eimeria nieschulzi* (coccidia, apicomplexa) are expressed

during sporulation and involved in the sporocyst wall formation. Parasit Vectors. 2015;8:395.
50. Belli SI, Smith NC, Ferguson DJP. The coccidian oocyst: a tough nut to crack! Trends Parasitol. 2006;22:416–23.
51. Wallach M. Role of antibody in immunity and control of chicken coccidiosis. Trends Parasitol. 2010;26:382–7.
52. Joachim A, Ruttkowski B, Sperling D. Detection of *Cystoisospora suis* in faeces of suckling piglets—when and how? A comparison of methods. Porc Heal Manag. 2018;4:20.
53. Brown MS, Grubb J, Zhang A, Rust MJ, Bishop DK. Small Rad51 and Dmc1 complexes often co-occupy both ends of a meiotic DNA double strand break. PLoS Genet. 2015;11:e1005653.
54. Reininger L, Garcia M, Tomlins A, Müller S, Doerig C. The *Plasmodium falciparum*, Nima-related kinase Pfnek-4: a marker for asexual parasites committed to sexual differentiation. Malar J. 2012;11:250.
55. Reininger L, Tewari R, Fennell C, Holland Z, Goldring D, Ranford-Cartwright L, et al. An essential role for the *Plasmodium* Nek-2 Nima-related protein kinase in the sexual development of malaria parasites. J Biol Chem. 2009;284:20858–68.

Publisher's Note

7.2 Publication 2

Progression of asexual to sexual stages of *Cystoisospora suis* in a host cell-free environment as a model for Coccidia

Feix A, Cruz-Bustos T, Ruttkowski B, Mötz M, Rümenapf T, Joachim A. (2021) Parasitology 148 (12):1475-1481. https://doi.org/10.1017/S0031182021001074

Own contributions:

- development of the host cell-free culture system
- processing of all samples
- data analysis
- drafting of the original manuscript and correction of the revised version

Parasitology

cambridge.org/par

Progression of asexual to sexual stages of *Cystoisospora suis* in a host cell-free environment as a model for Coccidia

Anna Sophia Feix[1], Teresa Cruz-Bustos[1], Bärbel Ruttkowski[1], Marlene Mötz[2], Till Rümenapf[2] and Anja Joachim[1]

[1]Department of Pathobiology, Institute of Parasitology, University of Veterinary Medicine, Vienna, Austria and [2]Department of Pathobiology, Institute of Virology, University of Veterinary Medicine, Vienna, Austria

Research Article

Cite this article: Feix AS, Cruz-Bustos T, Ruttkowski B, Mötz M, Rümenapf T, Joachim A (2021). Progression of asexual to sexual stages of *Cystoisospora suis* in a host cell-free environment as a model for Coccidia. *Parasitology* **148**, 1475–1481. https://doi.org/10.1017/S0031182021001074

Received: 22 January 2021
Revised: 11 June 2021
Accepted: 15 June 2021
First published online: 1 July 2021

Keywords:
Cell-free culture; gametes; gametogenesis; gamonts; *Isospora suis*; qPCR

Author for correspondence:
Anna Sophia Feix,
E-mail: Anna.Feix@vetmeduni.ac.at

Abstract

Coccidia display a characteristic life cycle, where the parasites switch between asexual and sexual development, resulting in an environmental stage, the oocyst. The entero-pathogenic *Cystoisospora suis*, a coccidian parasite of swine and close relative to *Toxoplasma gondii*, undergoes development in one host-cycle. Despite the well-described intracellular development of Coccidia, the *C. suis* life cycle can progress in an *in vitro*, host cell-free system after initial intracellular development of merozoites. A novel host cell-free cultivation method was developed by transferring purified merozoites from cell culture supernatant (dpi 6) to culture medium and incubating them for 5 days to induce their progression to sexually differentiated stages. The development of sexual stages in the absence of host cells was verified by morphological studies, flow cytometry and the transcription analysis of three genes linked to sexual stages (HAP2, OWP and TyRP). The host cell-free culture permits the sexual development (and with this, the complete life cycle progression from sporozoites to oocysts) of *C. suis in vitro* and provides a new tool for detailed research on the development of *C. suis* and possibly other Coccidia. This will also be useful for the evaluation of novel drug or vaccine targets in these parasites.

Introduction

Protozoa of the order Coccidia [infraphylum Apicomplexa, class Coccidomorphea, subclass Coccidia (Ruggiero *et al.*, 2015)] are a large group of parasites that contains some of the most important parasitic pathogens of humans and animals, including members of the genera *Cryptosporidium*, *Toxoplasma*, *Neospora*, *Eimeria* and *Cystoisospora* (Current *et al.*, 2019). Their life cycle is complex, as it includes a change from asexual to the sexual stage, and the latter displays a characteristic sexual dimorphism (Smith *et al.*, 2002). Generally, sporozoites infect a host and undergo asexual proliferation inside host cells to form merozoites, which infect further host cells. Subsequently, merozoites undergo several rounds of cellular division and develop into sexual stages, which then fuse to form a zygote and eventually oocysts. In most *Eimeria* and in *Toxoplasma gondii* (Barta *et al.*, 2005; Jonscher *et al.*, 2015), sporozoites penetrate cells of the gut, form an intermediate stage and start merogony. Newly formed merozoites egress from the host cell and spread the infection to neighbouring epithelial cells. Once merogony is finished (after a genus-specific number of cycles or generations) merozoites develop into micro- and macrogametes which mature, fuse and form a zygote that subsequently develops into an infectious oocyst (Smith *et al.*, 2002; Feix *et al.*, 2020).

The coccidian parasite *Cystoisospora suis* [syn. *Isospora suis* (Biester and Murray, 1934)] is an important entero-pathogen of swine in their first weeks of live (Joachim and Shrestha, 2020) considerably impairing animal health. It is a member of the family Sarcocystidae (Barta *et al.*, 2005) and thus closely related to *T. gondii* (Smith *et al.*, 2002; Palmieri *et al.*, 2017). As with all Coccidia, the life cycle of *C. suis* is characterized by a switch from asexual to sexual development with micro- and macrogametes. The microgametes form as a consequence of the division of microgamonts and develop into small uninucleate bodies with flagella which they use for their quick movement. Macrogametes do not divide but constitute a large, usually immobile cell. Presumably, micro- and macrogametes fuse to a zygote and complete their life cycle with the formation of an oocyst (Lindsay *et al.*, 1998; Smith *et al.*, 2002).

The development of these parasites is presumably regulated by genes linked to cell cycle progression which are, amongst others, involved in meiosis and gamete fusion (Walker *et al.*, 2013; Shibuya and Watanabe, 2018; Tandel *et al.*, 2019). The class II gamete fusogen HAP2 is important for the function of male sexual stages in many eukaryotic kingdoms (Fédry *et al.*, 2017). In apicomplexan parasites (including the Coccidia), it appears to be essential for fusion of the gamete membranes and consequently for fertilization (Wong and Johnson, 2010). The oocyst is the characteristic stage of the Coccidia and maintains parasite transmission between individual hosts which is supported by its environmental resistance and longevity (Belli *et al.*, 2006). For the formation of the oocyst wall, oocyst-wall forming bodies (WFBs) are assembled in the macrogamete and later translocated (Scholtyseck *et al.*,

1972). As described for the genus *Eimeria*, WFB 1 gives rise to the outer layer of the oocyst wall, WFB 2 forms the inner layer (Scholtyseck *et al.*, 1972; Belli *et al.*, 2006; Mai *et al.*, 2009). WFBs are present in female sexual stages, macrogamonts and macrogametes, as well as in early unsporulated oocysts (Templeton *et al.*, 2004; Possenti *et al.*, 2010). In addition, oocyst wall formation requires tyrosine-rich proteins (TRPs). Studies on *Eimeria* suggest that tyrosine-rich precursor glycoproteins, i.e. gam 56 and gam 82, are processed and incorporated into the oocyst wall by TRPs crosslinking *via* tyrosine residues through peroxidase activity followed by translocation (Belli *et al.*, 2003).

Of the coccidia, only *Cryptosporidium* species have been propagated in a host cell-free environment (Boxell *et al.*, 2008). In *Cryptosporidium hominis*, all asexual stages as well as gamonts were detected over a 9-day incubation period (Hijjawi *et al.*, 2010) and the production of newly formed oocysts was possible. The complete development of *Cryptosporidium parvum* in a host cell-free culture could also be demonstrated, with both unstained and stained life cycle stages (Hijjawi *et al.*, 2004; Boxell *et al.*, 2008). During its development in culture, the parasite underwent changes in its DNA levels, indicating cell division and multiplication (Zhang *et al.*, 2009).

The current study describes a novel host cell-free culture system for *C. suis* as a representative of the intestinal Coccidia and compares the parasite's morphological development with the transcription profile of selected sexual genes previously identified. A *C. suis in vitro* model employing intestinal host cell monolayer cultures is already established and is suitable for several applications (Worliczek *et al.*, 2013; Feix *et al.*, 2020; Joachim and Ruttkowski, 2021). We hypothesized that (i) after the intracellular propagation of merozoites, *C. suis* can complete its life cycle in a host cell-free environment which can be demonstrated by the development of gamonts, gametes, unsporulated and sporulated oocysts; and that (ii) the expression of genes related to the sexual development of *C. suis* is comparable to the *in vitro* expression in cell cultures infected with *C. suis*.

Materials and methods

Seeding of a host cell-free in vitro *culture with sporozoites*

To check whether the *C. suis* life cycle can exclusively occur outside of a host cell, 5×10^3 sporozoites were released into fresh Advanced® DMEM/F-12 culture medium (Gibco) supplemented with 5% fetal calf serum (Gibco) and penicillin/streptomycin plus L-glutamine 100x (Gibco) onto a new uncoated ibidi 8-well ibiTreat® μ-slide (ibidi, Gräfelfing, Germany) and were incubated at 40°C under 5% CO_2. The development of parasite stages was monitored daily.

Infection of a host cell-free in vitro *culture with merozoites*

To start the host cell-free culture, free merozoites were obtained from monolayer culture supernatant of intestinal porcine epithelial cells 6 days after infection with sporozoites (Feix *et al.*, 2020). Merozoites were washed with phosphate-buffered saline (PBS; Gibco, Thermo Fisher Science, Waltham, USA) and purified by density gradient centrifugation on a Percoll® (GE Healthcare, Uppsala, Sweden) gradient (60, 40 and 20%, topped up with the merozoite suspension in PBS) at $600 \times g$ for 10 min at 20°C in a MegaStar 3.0R swing bucket centrifuge (VWR International, Leuven, Belgium). Both acceleration and deceleration were at the lowest possible setting. Purified 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 100x (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% CO_2. The development of parasite stages was monitored daily, and stages were harvested at 3 and 4 dpt (days post transfer), pelleted and stored at −20°C.

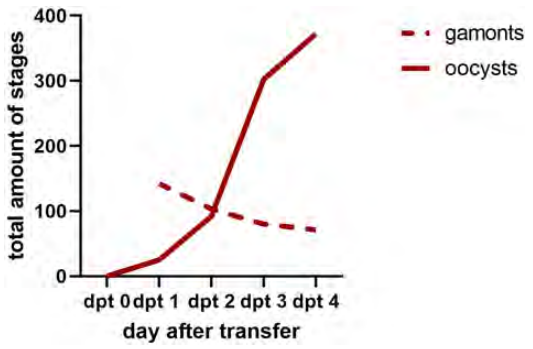


Fig. 1. Total amount of gamonts and oocysts of *Cystoisospora suis* in a host cell-free culture. Values represent the mean from five independent experiments. ****$P < 0.0001$.

Parasite stage evaluation, harvest and microphotography

The numbers of merozoites, sexual stages and oocysts were monitored from the first dpt onwards. The numbers of gamonts and oocysts 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 and oocysts, and checked for regular morphology. To show significance between the average number of gamonts and oocysts on different culture days an unpaired Student's *t*-test was performed.

Digital microphotographs of all *C. suis* stages in culture were taken with an Olympus IX71 inverse microscope (Olympus, Shinjuku, Japan) at 400× and 600× magnification. Autofluorescence of oocysts was detected at UV 385 nm.

Flow cytometry

Purified parasite stages harvested 4 dpt were stained with 5 μg/mL DAPI (4′,6-diamidino-2-phenylindol) working solution, washed with PBS and transferred to 1 mL of PBS solution to be immediately processed. The different parasite stages were subjected to fluorescence analysis in an Amnis® FlowSight® imaging flow cytometer (Luminex, Austin, USA) and were evaluated by size, shape and fluorescence intensity. A total of 10 000 recordings were measured for every replicate ($n = 5$) and gated with software IDEAS® 6.2.64.0 (Luminex) based on size, which we used for previous morphological studies, and intensity, which made it possible to differentiate debris from different parasite cells.

Transcription levels of C. suis *sexual stages in a host cell-free culture*

To quantify the transcript levels of three genes related to sexual stages, quantitative real-time polymerase chain reaction (qPCR) was used. Total RNA was isolated from pelleted host cell-free cultures using an RNeasy mini kit (Qiagen, Hilden, Germany), and treated with RNase-free DNase (Qiagen) according to the manufacturer's instructions. Total RNA was quantified using a NanoDrop® 2000 (Thermo Fischer Science, Waltham, USA). For reverse transcription, an iScript® cDNA synthesis kit (Bio-Rad, Hercules, USA) was used.

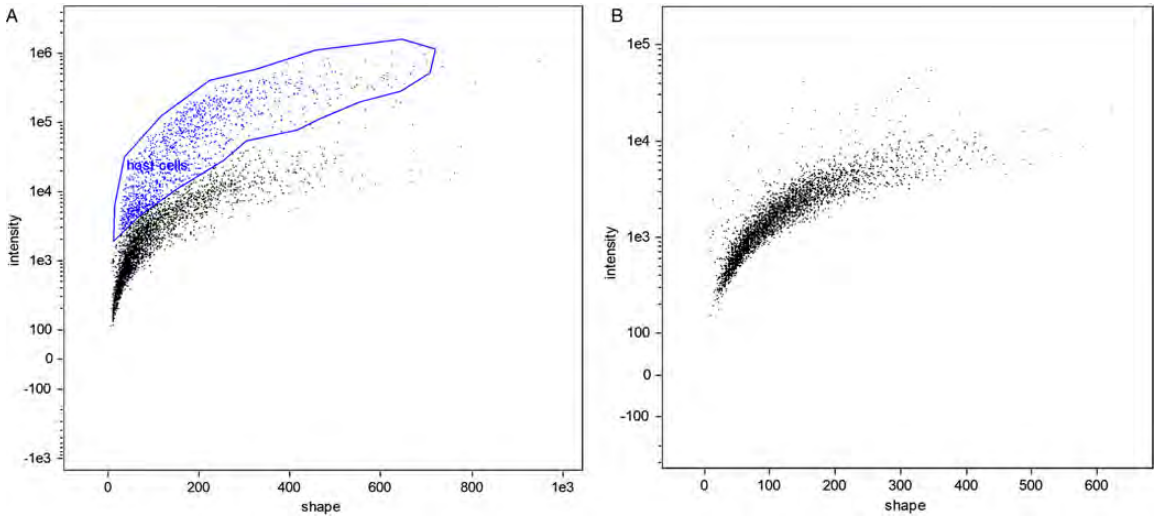


Fig. 2. Comparison of flow cytometry of an *in vitro* cell culture and the cell-free *in vitro* culture of parasite cells. (A) Microgamonts, macrogamonts and macrogametes (black) and host cells and few intact microgametes in the top right corner (blue), *in vitro* culture, 10 dpi. (B) Exclusively parasite stages (black) are found in the host cell-free culture 4 dpt.

Transcription levels were analysed 3 and 4 dpt for HAP2, OWP and TyRP, when sexual stages could be detected. The primers for gene amplification used in this study are described in previous studies (Feix *et al.*, 2020). Quantitative PCR amplification of cDNA was carried out on an Mx3000P thermal cycler. The reaction mixtures each 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. 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. The qPCR results were normalized against each of the two reference genes, glyceraldehyde-3-phosphate and actin, and the complete experiment was performed in five separate biological replicates. The samples were quantified according to the percent threshold cycle (CT) method, and all the assays were technically performed at least three times.

Results and discussion

Morphology

In order to confirm the presence of various developmental stages of *C. suis* in a host cell-free environment, microphotographs of sexual stages and oocysts were captured at different time points of cultivation. An increase of gamonts (2 dpt) and oocysts (4 dpt), followed by a decrease of gamonts, could be shown in the host cell-free environment (Fig. 1). Although the total number of parasite stages at each time point was lower than in *in vitro* cultures with host cell monolayers (Feix *et al.*, 2020), a significant difference between the days was noted ($P < 0.0001$). From an initial amount of 1.2×10^5 merozoites (a mixture of types 1 and 2) up to 200 gamonts developed. Approximately 400 oocysts (unsporulated and sporulated) were detected by 4 dpt. The number of developed gamonts might be related to the numbers of the different merozoite types seeded into the host cell-free culture, since we assume that only type II-merozoites develop to viable sexual stages and consequently to oocysts. This hints at a sexual commitment of *C. suis* merozoites, which has not yet been investigated. For *Plasmodium falciparum* (Poran *et al.*, 2017; Brancucci *et al.*, 2018; Neveu *et al.*, 2020) and *T. gondii* (Ramakrishnan *et al.*, 2019), sexual commitment of merozoites preceding sexual differentiation has been shown and represents an important developmental switch in the life cycle of apicomplexan parasites (Poran *et al.*, 2017; Ramakrishnan *et al.*, 2019; Neveu *et al.*, 2020).

In flow cytometry, a difference between unstained cells and DAPI-stained cells was evident; however, microgamonts, macrogamonts and macrogametes have a similar shape and fluorescence intensity, making differentiation by flow cytometry not possible (Fig. 2). Microgametes are distinguishable by this technique, due to their significantly smaller size, their flagella are highly breakable, decreasing the amount of complete microgametes found by flow cytometry. Similar to previous studies on *C. parvum* using COWP1-tdTomato staining (Tandel *et al.*, 2019), sexual stages were only visible at an increased intensity of UV-light, whereas uninfected cells were still visible at a lower intensity. Nevertheless, cell sorting clearly differentiated between debris, dead cells and living parasite stages, and will be an improvement for the isolation of different *C. suis* stages in the future. Further experiments with antibodies against stage-specific proteins should make it possible to sort out single parasite cells of a specific developmental stage and will be conducted in the future; as such antibodies do not exist for *C. suis* yet.

All life cycle stages were morphologically comparable to *in vitro* cell cultures (Fig. 3). Second-generation merozoites (Fig. 3A and B) could be detected 4 h after the start of the host cell-free culture. Previous studies showed male and female sexual stages and first oocysts from 120 h post infection *in vivo* (Matuschka and Heydorn, 1980). Previous studies *in vitro* first detected gamonts at 216 h post infection of cell cultures and first oocysts after 240 h, so that the *in vitro* life cycle of *C. suis* takes roughly twice as long as *in vivo* (Feix *et al.*, 2020). The sexual development of *C. suis* in a host cell-free environment takes as long as that in *in vitro* cell culture. Lindsay *et al.* (1998) demonstrated the complete development of *C. suis* in a swine testicle cell line, and detected oocysts at 12 dpi. Therefore, the developmental delay of both sexual stages and oocysts *in vitro* might be influenced by the culture conditions and the used cell lines. The numbers of newly developed micro- and macrogamonts increased until 3 dpt (Fig. 3C and D). At that time point, microgamonts

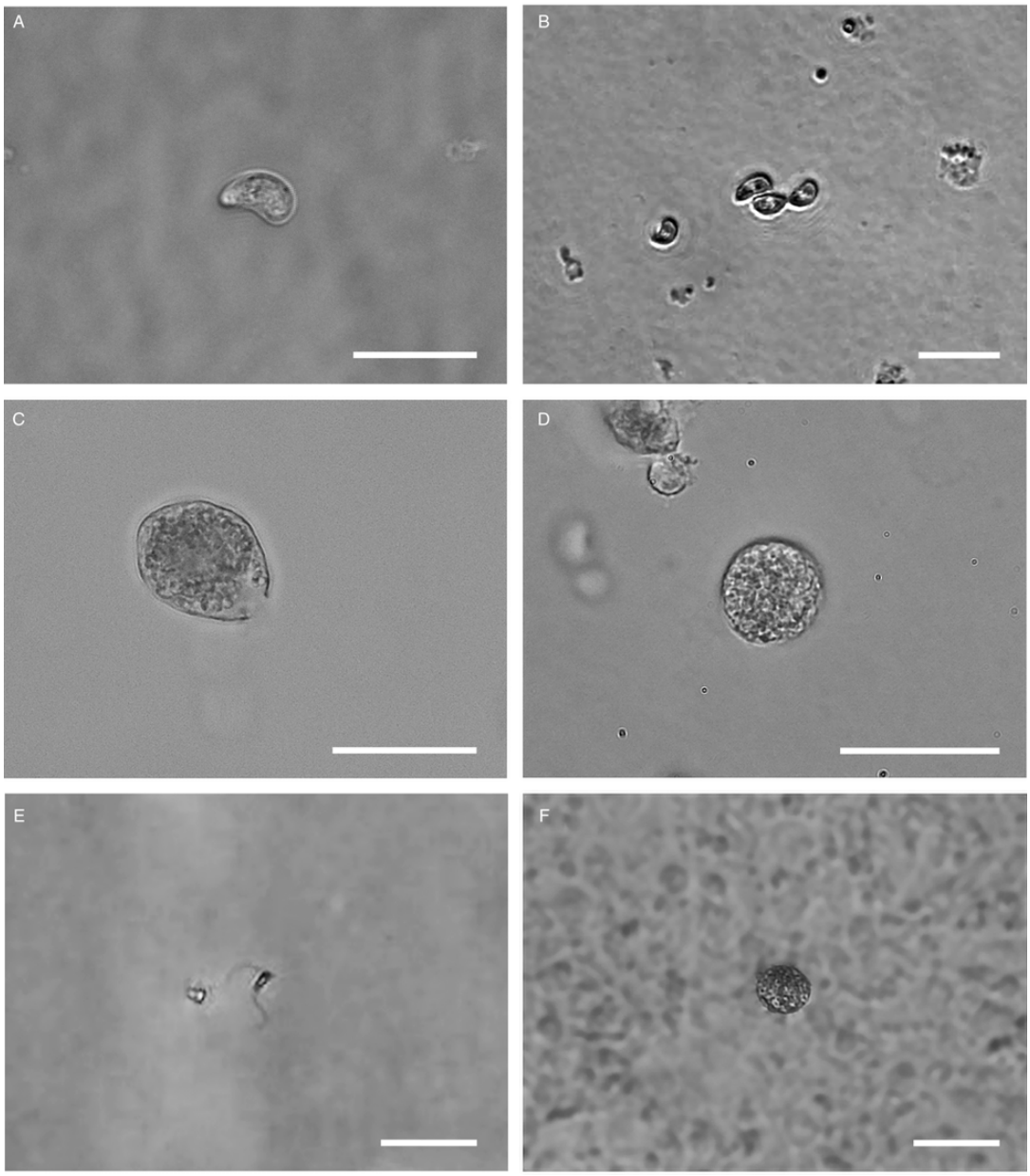


Fig. 3. Light microscopy of different stages of *C. suis* in host cell-free culture, captured with differential interference contrast (DIC) or brightfield microscopy. Scale bars: 20 μm unless indicated otherwise. (A) First-generation merozoite, 0 dpt, DIC. (B) Second-generation merozoite, 0 dpt, brightfield microscopy. (C) Microgamont with an opening on one end and moving microgametes in the centre, 3 dpt, DIC. (D) Macrogamont, 3 dpt, DIC. (E) Microgamete, 4 dpt, scale bar: 5 μm. DIC. (F) Macrogamete, 4 dpt, DIC.

contained motile microgametes (Supplementary material file V1). Free, motile microgametes (Fig. 3E) and macrogametes (Fig. 3F) could be detected from 3 dpt onwards. Microgametes from host cell-free culture had a central body and two opposing flagella and showed no obvious morphological differences compared to the cell culture, and they also displayed their typical extremely fast movement (Supplementary material file V2). Macrogametes were spherical and appeared to have a thinner outer layer in comparison with *in vivo* and previous *in vitro* studies (Fig. 3F). Micro- and macrogametes could frequently be found in close proximity to each other, as observed previously (Feix *et al.*, 2020). Microgametes appeared to adhere to the macrogamete before (presumably) fusing with it, and newly formed oocysts were found in the same area approximately 24 h later. Both unsporulated and sporulated oocysts were present by 4 dpt. Characteristic, clear oocyst walls were seen in unsporulated oocysts (Fig. 4A). Unsporulated oocysts later matured and developed two sporocysts (Fig. 4B). Sporulated oocysts appeared to have thinner oocyst walls, in comparison with oocysts sampled from *in vitro* cell cultures. Harleman and Meyer (1984) demonstrated that merozoites collected at different time points in their development still conclude their life cycle in their specific time

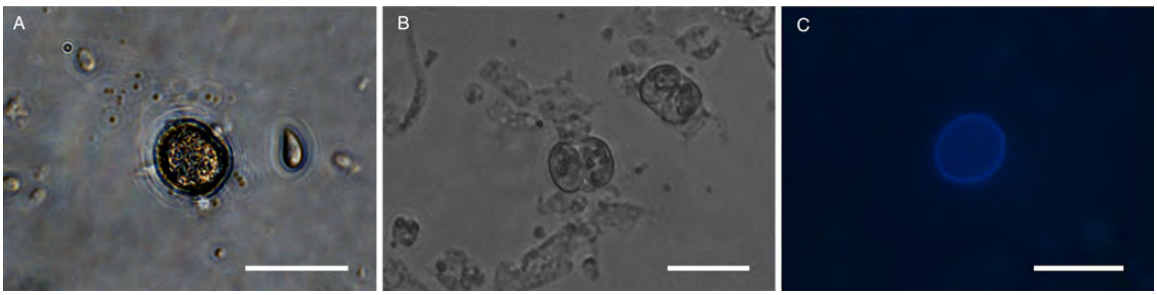


Fig. 4. Light microscopy of oocysts of *C. suis* in a host cell-free culture. (A) Unsporulated oocyst, 4 dpt. (B) Sporulated oocysts, 4 dpt. (C) Autofluorescent, unsporulated oocyst, 4 dpt. Scale bars: 20 μm.

frame. Second-generation merozoites were shown at 3–4 days *in vivo*, which then develop into third-generation meronts at 4–5 days and further into sexual stages at 5–6 dpi. In this study, we could show that *C. suis* still follows its time line, as merozoites become sexual stages despite the absence of host cells.

In vivo the life cycle of *C. suis* takes place in a slightly shorter time frame (Harleman and Meyer, 1983), as seen *in vitro*. The life cycle progression in the host cell-free *in vitro* culture, however, continues at the same pace as in other *in vitro* cultures (Table 1). However, the morphology of *C. suis* sexual stages and oocysts does not differ between *in vivo* (Joachim *et al.*, 2018; Lindsay *et al.*, 2014) or *in vitro* systems.

Genes linked to sexual stages

In addition to the morphological description of all sexual stages, we analysed three genes with upregulated transcripts in microgametes, macrogametes and oocysts, based on previous studies on *C. suis* (Feix *et al.*, 2020).

To evaluate the transcription level of genes related to sexual stages in *C. suis* (HAP2, OWP and TyRP) the time points 3 and 4 dpt were chosen as the total amount of sexual stages was highest on these days, and compared to 0 dpt (i.e. to merozoites from cell cultures 6 days after infection of cells, before transfer to cell-free culture). The transcription levels of all three examined genes increased during the cell-free cultivation, and significant differences between all days were shown. The transcription of HAP2 had its peak 4 dpt with a 40-fold increase (Fig. 5A). It is usually only found in microgamonts, microgametes and unsporulated oocysts (Fritz *et al.*, 2012*a*; Jonscher *et al.*, 2015; Fédry *et al.*, 2017), which correlates with the present and previous results for *C. suis in vitro* (Feix *et al.*, 2020). Enhanced transcription of HAP2 in a host cell-free culture system supports the hypothesis that sexual stages can develop outside the host cell and that fertilization and oocyst production can occur extracellularly.

As newly formed oocysts could be detected at 4 dpt, the oocyst wall formation must have taken place at that time point already. We could show a continuous transcriptional increase of OWP until 4 dpt when it was 9-fold increased (Fig. 5B). *Cryptosporidium parvum* also showed increased levels of OWP after 72 h of cultivation (Tandel *et al.*, 2019). As the expression of OWP in the current study increased during the days in which macrogametes and oocysts were present, the slight morphological deviations of *C. suis* oocyst morphology in the host cell-free culture did not seem to be correlated with changes in the expression of OWPs.

Upregulation could also be detected for TyRP (Fig. 5C) which is characteristic for macrogamonts and –gametes as well as oocysts. Autofluorescence of *C. suis* oocysts occurs due to dityrosine bonds between TRPs in the oocyst wall (Fritz *et al.*, 2012*a*, *b*). Oocysts derived from the host cell-free culture system could be detected by autofluorescence microscopy (Fig. 4C) and TyRP was distinctly upregulated on dpt 3 and 4 similar to the development in *in vitro* cell cultures (Feix *et al.*, 2020), oocyst wall formation can be assumed to be possible also in a cell-free environment. TyRP already seems to be present in macrogametes, but the cross-linking of TRPs only occurs in oocysts (Silvestrini *et al.*, 2005). This specific oocyst wall structure seems to be responsible for the resistance to environmental stress oocysts have to deal with (Silvestrini *et al.*, 2005; Jonscher *et al.*, 2015). The oocyst-related gene transcript of TyRP reached its peaks on dpt 4, with a 3-fold increase compared to the reference time point (Fig. 5C), which is comparable with our previous results for cell culture, where we could show the same increase in transcription. This upregulation can also be found in *T. gondii in vitro* and *in vivo*, demonstrating a key function of TyRP in oocyst development (Silvestrini *et al.*, 2005; Jonscher *et al.*, 2015).

Both male and female sexual stages (and their respective specific genes) could be detected simultaneously, hinting at a mandatory combination (and fusion) of both gametes to continue the parasite life cycle, as described in previous studies (Tandel *et al.*, 2019; Feix *et al.*, 2020). However, the relative quantitative expression of the analysed male sexual stage specific protein was almost 45-fold higher than those for female sexual stage specific proteins.

In the current study, we could demonstrate that merozoites derived from cell cultures infected with sporozoites 6 days earlier can complete their development to sporulated oocysts in a cell-free environment. It appears that the early events of the endogenous development of *C. suis* require an intracellular niche because initial experiments with cell-free cultivation of sporozoites were unsuccessful. Sporozoites from freshly excysted oocysts could only survive for a few hours and did not develop to merozoites (data not shown).

From these findings we draw three conclusions: (1) host cells are necessary for the asexual development but not for sexual development; (2) fertilization takes place outside the host cells (*in vitro*, but also *in vivo*, in the lumen of the gut) and (3) merozoites seeded into cell-free cultures are already sexually committed.

The sexual commitment has long been considered a crucial step in the development of Apicomplexan parasites (Kaushal *et al.*, 1980; Cornelissen, 1988; Bruce *et al.*, 1990); however, the underlying mechanisms are still mainly unclear (Smith *et al.*, 2002; Bechtsi and Waters, 2017). Sexually committed merozoites undergo a developmental determination to develop either into male or female gametocytes prior to their actual sexual development (Jeninga *et al.*, 2019). During schizont segregation in *Plasmodium* sp. sexual commitment (based on the increase in their transcript abundance) could be shown, similar to what we

Table 1. Comparison of the life cycle progression and morphology of some stages in all three systems used in *Cystoisospora suis*, i.e. *in vivo*, *in vitro* (cell culture) and the novel *in vitro* cell-free culture

	In vivo (Harleman and Meyer, 1984)	*In vitro* (Feix et al., 2020)	*In vitro* cell-free
Dose[a]	2.4×10^5 sporulated oocysts/ piglet	5×10^3 sporozoites/ 4×10^5 host cells	1.2×10^5 merozoites per mL host cells[b]
Harvested mixture of unsporulated and sporulated oocysts	50– 201 200 per g of feces	900 per mL culture medium	400 per mL culture medium
First occurrence of (days after infection/ transfer)			
– merozoites	2	6	0
– sexual stages	5–6	8	2
– sporulated oocysts	11–14	13	4
Size of gamonts (μM)	–	16.8 ± 0.9 (n = 50)	16.6 ± 0.8 (n = 20)
Size of unsporulated oocysts (μM)	22.5 × 19.2 (n = not specified)	22.3 × 19.2 (n = 50)	21.8 × 19.1 (n = 20)

[a]Sporulated oocysts were used to infect piglets *in vivo* (Harleman and Meyer, 1984), sporozoites (derived from oocysts after excystation *in vitro*) were used to infect IPEC cell cultures (Feix *et al.*, 2020).
[b]In the current study, mixed type 1 and 2 merozoites were used to seed the *in vitro* cell-free culture.

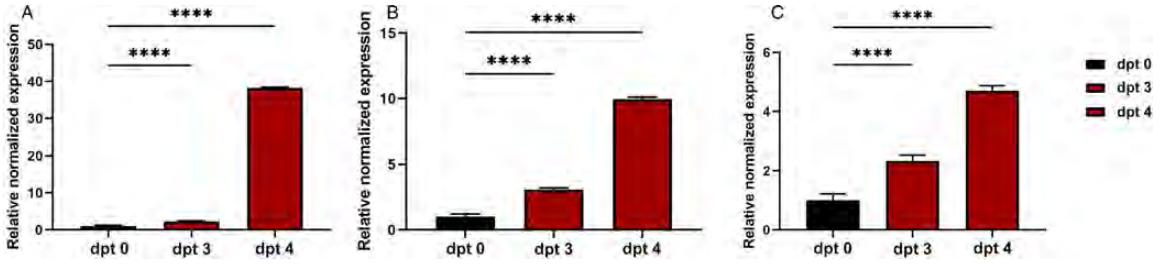


Fig. 5. Relative normalized expression levels of genes related to sexual development in a host cell-free culture evaluated by qRT-PCR 0, 3 and 4 dpt. (A) Microgamete-related gene HAP2. (B) Macrogamete related gene OWP1. (C) Macrogamete related gene TyRP. Values represent the mean ± standard error (S.E.) from five independent experiments. ****$P < 0.0001$.

observed in the current study. Sexual commitment could be key in the development of transmission-blocking agents (Bechtsi and Waters, 2017). *Cystoisospora suis* life cycle progression in a host cell-free environment hints at sexual commitment also in this protozoan species. We hope to be able to analyse this phenomenon in future studies, since we assume that *C. suis* exhibits sexual commitment in second-generation merozoites which determines the life cycle progression of the parasite independent of its environment and could also be a target for intervention.

Previous studies have shown that blocking the fusion of micro- and macrogametes could also serve as a novel tool for intervention in the control of coccidial infections. In *T. gondii* HAP2 knockout parasites failed to produce sporulated oocysts *in vivo* (Ramakrishnan *et al.*, 2019), which would support the hypothesis that fertilization (and, more broadly, sexual development) is a potential bottleneck in the life cycle progression of coccidian parasites, and interfering with these crucial events to block further development will preclude transmission of the parasite *via* infectious oocysts.

Taken together, with the newly developed host cell-free culture system it would be possible to evaluate the effects of targeting sexual stages of *C. suis in vitro*, opening new avenues for the control of this and possibly related parasites by vaccination or selective drugs.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182021001074.

Data. All data are accessible in the manuscript.

Author contributions. AJ designed the study. BR provided oocysts, sporozoites and merozoites and was responsible for the maintenance of the cell culture. ASF drafted the manuscript, was responsible for the host cell-free culture system and processed and analysed all samples. TCB provided the necessary expertise for the molecular analysis. TR and MM were involved in the establishment and conduction of the flow cytometry. All authors read and approved of the final version of the manuscript.

Financial support. ASF is funded through the Graduate School 'Pig and Poultry Medicine' of the University of Veterinary Medicine Vienna. TCB is funded by the Austrian Science fund (FWF; project no.: P 33123).

Conflict of interest. The authors declare that they have no competing interests.

References

Barta JR, Schrenzel MD, Carreno R and Rideout BA (2005) The genus *Atoxoplasma* (Garnham 1950) as a junior objective synonym of the genus *Isospora* (Schneider 1881) species infecting birds and resurrection of *Cystoisospora* (Frenkel 1977) as the correct genus for *Isospora* species infecting mammals. *Journal of Parasitology* **91**, 726–727.

Bechtsi DP and Waters AP (2017) Genomics and epigenetics of sexual commitment in *Plasmodium*. *International Journal for Parasitology* **47**, 425–434.

Belli SI, Wallach MG, Luxford C, Davies MJ and Smith NC (2003) Roles of tyrosine-rich precursor glycoproteins and dityrosine- and 3,4-dihydroxyphenylalanine-mediated protein cross-linking in development of the oocyst wall in the coccidian parasite *Eimeria maxima*. *Eukaryotic Cell* **2**, 456–464.

Belli SI, Smith NC and Ferguson DJP (2006) The coccidian oocyst: a tough nut to crack!. *Trends in Parasitology* **22**, 416–423.

Biester HE and Murray C (1934) Studies in infectious enteritis of Swine. In *12th International Veterinary Congress*, pp. 207–219.

Boxell A, Hijjawi N, Monis P and Ryan U (2008) Comparison of various staining methods for the detection of *Cryptosporidium* in cell-free culture. *Experimental Parasitology* **120**, 67–72.

Brancucci NMB, De Niz M, Straub TJ, Ravel D, Sollelis L, Birren BW, Voss TS, Neafsey DE and Marti M (2018) Probing *Plasmodium falciparum* sexual commitment at the single-cell level. *Wellcome Open Research* **3**, 70.

Bruce MC, Alano P, Duthie S and Carter R (1990) Commitment of the malaria parasite *Plasmodium falciparum* to sexual and asexual development. *Parasitology* **100**, 191–200.

Cornelissen A (1988) Sex determination and sex differentiation in malaria parasites. *Biological Reviews* **63**, 379–394.

Current WL, Upton SJ and Long PL (2019) Taxonomy and life cycles. In Long PL (ed.), *Coccidiosis of Man and Domestic Animals*. Boca Raton, USA: CRC Press, pp. 1–16.

Fédry J, Liu Y, Péhau-Arnaudet G, Pei J, Li W, Tortorici MA, Traincard F, Meola A, Bricogne G, Grishin NV, Snell WJ, Rey FA and Krey SJ (2017) The ancient gamete fusogen HAP2 is a eukaryotic class II fusion protein. *Cell* **168**, 904–915.e10.

Feix AS, Cruz-Bustos T, Ruttkowski B and Joachim A (2020) Characterization of *Cystoisospora suis* sexual stages *in vitro*. *Parasites & Vectors* **13**, 143.

Fritz HM, Bowyer PW, Bogyo M, Conrad PA and Boothroyd JC (2012a) Proteomic analysis of fractionated *Toxoplasma* oocysts reveals clues to their environmental resistance. *PLoS One* **7**, e29955–e29955.

Fritz HM, Buchholz KR, Chen X, Durbin-Johnson B, Rocke DM, Conrad PA and Boothroyd JC (2012b) Transcriptomic analysis of *Toxoplasma* development reveals many novel functions and structures specific to sporozoites and oocysts. *PLoS One* **7**, e29998.

Harleman J and Meyer R (1983) *Isospora* infection in piglets – a review. *The Veterinary Quarterly* **5**, 178–185.

Harleman J and Meyer R (1984) Life cycle of *Isospora suis* in gnotobiotic and conventionalized piglets. *Veterinary Parasitology* **17**, 27–39.

Hijjawi NS, Meloni BP, Ng'anzo M, Ryan UM, Olson ME, Cox PT, Monis PT and Thompson RCA (2004) Complete development of *Cryptosporidium parvum* in host cell-free culture. *International Journal for Parasitology* **34**, 769–777.

Hijjawi N, Estcourt A, Yang R, Monis P and Ryan U (2010) Complete development and multiplication of *Cryptosporidium hominis* in cell-free culture. *Veterinary Parasitology* **169**, 29–36.

Jeninga MD, Quinn JE and Petter M (2019) Apiap2 transcription factors in apicomplexan parasites. *Pathogens (Basel, Switzerland)* **8**, 1–24.

Joachim A and Ruttkowski B (2021) *Cystoisospora suis* merozoite development assay for screening of drug efficacy *in vitro*. *Experimental Parasitology* **220**, 108035.

Joachim A and Shrestha A (2020) Coccidiosis of pigs. In Dubey JP (ed.), *Coccidiosis in Livestock, Poultry, Companion Animals and Humans*. Boca Raton, USA: CRC Press, pp. 125–145.

Joachim A, Ruttkowski B and Sperling D (2018) Detection of *Cystoisospora suis* in faeces of suckling piglets – when and how? A comparison of methods. *Porcine Health Management* **4**, 20.

Jonscher E, Erdbeer A, Günther M and Kurth M (2015) Two COWP-like cysteine rich proteins from *Eimeria nieschulzi* (Coccidia, Apicomplexa) are expressed during sporulation and involved in the sporocyst wall formation. *Parasites & Vectors* **8**, 395.

Kaushal DC, Carter R, Miller LH and Krishna G (1980) Gametocytogenesis by malaria parasites in continuous culture. *Nature* **286**, 490–492.

Lindsay DS, Quick DP, Steger AM, Toivio-Kinnucan MA and Blagburn BL (1998) Complete development of the porcine coccidium *Isospora suis* Biester, 1934 in cell cultures. *Journal of Parasitology* **84**, 635–637.

Lindsay DS, Houk AE, Mitchell SM and Dubey JP (2014) Developmental biology of *Cystoisospora* (Apicomplexa: Sarcocystidae) monozoic tissue cysts. *Journal of Parasitology* **100**, 392–398.

Mai K, Sharman PA, Walker RA, Katrib M, De Souza D, McConville MJ, Wallach MG, Belli SI, Ferguson DJP and Smith NC (2009) Oocyst wall formation and composition in coccidian parasites. *Memorias do Instituto Oswaldo Cruz* **104**, 281–289.

Matuschka F and Heydorn AO (1980) Die entwicklung von *Isospora suis* Biester und Murray 1934 (sporozoa: coccidia: eimeriidae) im Schwein. *Zoologische Beiträge* **26**, 405–476.

Neveu G, Beri D and Kafsack BF (2020) Metabolic regulation of sexual commitment in *Plasmodium falciparum*. *Current Opinion in Microbiology* **58**, 93–98.

Palmieri N, Shrestha A, Ruttkowski B, Beck T, Vogl C, Tomley F, Blake DP and Joachim A (2017) The genome of the protozoan parasite *Cystoisospora suis* and a reverse vaccinology approach to identify vaccine candidates. *International Journal for Parasitology* **47**, 189–202.

Poran A, Nötzel C, Aly O, Mencia-Trinchant N, Harris CT, Guzman ML, Hassane DC, Elemento O and Kafsack BFC (2017) Single-cell RNA sequencing reveals a signature of sexual commitment in malaria parasites. *Nature* **551**, 95–99.

Possenti A, Cherchi S, Bertuccini L, Pozio E, Dubey JP and Spano F (2010) Molecular characterisation of a novel family of cysteine-rich proteins of *Toxoplasma gondii* and ultrastructural evidence of oocyst wall localisation. *International Journal for Parasitology* **40**, 1639–1649.

Ramakrishnan C, Maier S, Walker RA, Rehrauer H, Joekel DE, Winiger RR, Basso WU, Grigg ME, Hehl AD, Deplazes P and Smith NC (2019) An experimental genetically attenuated live vaccine to prevent transmission of *Toxoplasma gondii* by cats. *Scientific Reports* **9**, 1474.

Ruggiero MA, Gordon DP, Orrell TM, Bailly N, Bourgoin T, Brusca RC… and Kirk PM (2015) A higher level classification of all living organisms. *PLoS One* **10**, e0119248.

Scholtyseck E, Mehlhorn H and Hammond DM (1972) Electron microscope studies of microgametogenesis in coccidia and related groups. *Zeitschrift für Parasitenkunde* **38**, 95–131.

Shibuya H and Watanabe Y (2018) Live-cell microscopy of meiosis in spermatocytes. *Methods in Cell Biology* **145**, 269–277.

Silvestrini F, Bozdech Z, Lanfrancotti A, Di Giulio E, Bultrini E, Picci L, deRisi JL, Pizzi E and Alano P (2005) Genome-wide identification of genes upregulated at the onset of gametocytogenesis in *Plasmodium falciparum*. *Molecular and Biochemical Parasitology* **143**, 100–110.

Smith TG, Walliker D and Ranford-Cartwright LC (2002) Sexual differentiation and sex determination in the Apicomplexa. *Trends in Parasitology* **18**, 315–323.

Tandel J, English ED, Sateriale A, Gullicksrud JA, Beiting DP, Sullivan MC, Pinkston B and Striepen B (2019) Life cycle progression and sexual development of the apicomplexan parasite *Cryptosporidium parvum*. *Nature Microbiology* **4**, 2226–2236.

Templeton TJ, Lancto CA, Vigdorovich V, Liu C, London NR, Hadsall KZ and Abrahamsen MS (2004) The *Cryptosporidium* oocyst wall protein is a member of a multigene family and has a homolog in *Toxoplasma*. *Infection and Immunity* **72**, 980–987.

Walker RA, Ferguson DJP, Miller CMD and Smith NC (2013) Sex and *Eimeria*: a molecular perspective. *Parasitology* **140**, 1701–1717.

Wong JL and Johnson MA (2010) Is HAP2-GCS1 an ancestral gamete fusogen? *Trends in Cell Biology* **20**, 134–141.

Worliczek HL, Ruttkowski B, Schwarz L, Witter K, Tschulenk W and Joachim A (2013) *Isospora suis* in an epithelial cell culture system – an *in vitro* model for sexual development in coccidia. *PLoS One* **8**, e69797–e69797.

Zhang L, Sheoran AS and Widmer G (2009) *Cryptosporidium parvum* DNA replication in cell-free culture. *Journal of Parasitology* **95**, 1239–1242.

7.3 Publication 3

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

Cruz-Bustos T, **Feix A**, Lyrakis M, Dolezal M, Ruttkowski B, Joachim A. (2022) Sci. Rep. 1–17, https://doi.org/10.1038/s41598-022-09714-8

Own contributions:

- isolation and preparation of stages from the *in vitro* host cell-free culture
- immunolocalization study
- development of the inhibition assay
- contribution in compilation of the manuscript

scientific reports

Check for updates

OPEN

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

Teresa Cruz-Bustos[1]✉, Anna Sophia Feix[1], Manolis Lyrakis[2], Marlies Dolezal[2], Bärbel Ruttkowski[1] & Anja Joachim[1]

The apicomplexan parasite *Cystoisospora suis* is an enteropathogen of suckling piglets with woldwide 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*[1]) is a protozoan parasite of the phylum Apicomplexa (Class Conoidasida, subclass Coccidiasina, order Eucoccidiorida, 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*[2]. *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[3,4]. It has a worldwide distribution, and infections are very common, particularly in young animals[5].

The life-cycle of *C. suis* consists of asexual multiplication (sporogony, merogony) followed by 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[6]. 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[7]. 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[8,9]. Previous studies have shown that the development of *C. suis* in vitro through the entire lifecycle is comparable with the lifecycle *in vivo*[10]. 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

[1]Institute of Parasitology, Department of Pathobiology, University of Veterinary Medicine Vienna, Veterinärplatz 1, 1210 Vienna, Austria. [2]Platform for Bioinformatics and Biostatistics, Department of Biomedical Sciences, University of Veterinary Medicine Vienna, Veterinärplatz 1, 1210 Vienna, Austria. ✉email: Teresa.Cruz-Bustos@vetmeduni.ac.at

cells *C. suis* can also continue gamogony in a host cell-free environment, sugesting that gamete production and fusion occurs extracellularly[11]. The complete life cycle of *C. suis* in a cell line representing the natural host cell type and species provides a unique model among coccidian 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 unravelled 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*[12–17], in tachyzoites, bradyzoites, sporozoites and oocysts of *T. gondii*[18–24], in tachyzoites of *Neospora caninum*[25], in tachyzoites of *Besnoitia besnoiti*[26], oocysts, sporozoites and intracellular stages of *Cryptosporidium parvum*[27], and in human and mosquito stages[28–30] and gametocytes of *Plasmodium falciparum* and *P. vivax*[31–33].

According to recent reevaluations of the coccidian phylogeny, the position of *C. suis* in the family Sarcocystidae constitutes an outgroup of the cluster containing the genera *Neospora*, *Hammondia* and *Toxoplasma*[34,35]. 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[23]. Only a small number of asexual stages differentiates into gametes, and therefore this step is considered a bottleneck of development[36], and it can be assumed that this is relevant for all Apicomplexa that produce gametocytes[37]. Proteins with important roles in sexual development have been described, including members involved in macrogamete development, oocyst wall formation, glycosilation and proteolytic cleavage of the oocyst wall proteins[38,39], axoneme and flagella assembly and construction, DNA replication, microgamete budding from microgamonts and gamete fusion[40,41].

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).

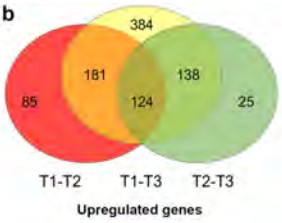
The primary goal of this study was the identification of genes with elevated expression levels at sexual stages to identify genes and proteins that may be related to this last step of development of the Coccidia, including *C. suis*. In total, 937 upregulated and 1188 downregulated genes were identified 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[112] (see amplication 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.

a

Genes	T1-T2	T2-T3	T1-T3
Non-significant changes	6234	6302	5265
Upregulated	390	287	827
Downregulated	501	536	1033
Total regulated	891	823	1860

b



c

No. of genes significantly (FRD cut-off 5%) Up- and Downregulated		
Log_2 FC	up	down
1	704	802
2	126	275
3	48	100
4	36	11
5	21	0
6	2	0

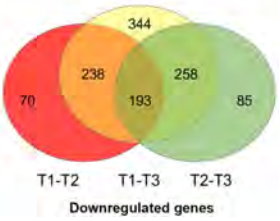


Figure 1. Identification of upregulated and downregulated genes in sexual stages using differential expression analysis. (**a**) Summary of the differential expression analysis of early sexual stages compared to merozoites (T1-T2), late sexual stages (T1-T3) and late sexual stages compared to early sexual 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.

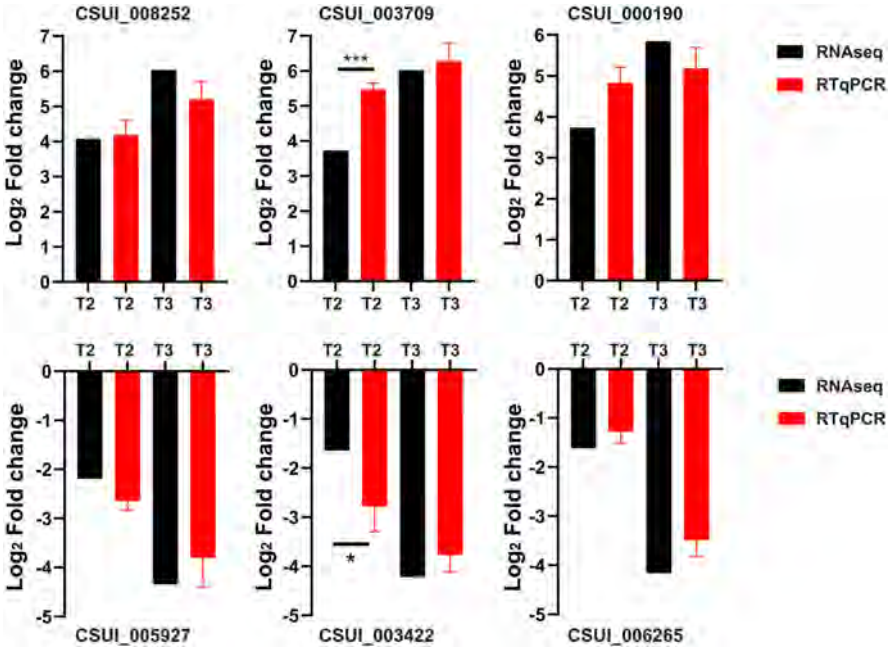


Figure 2. Verification of the gene expression profiles by qRT-PCR. Six genes were selected randomly for validation of the RNA-seq data. According to the RNA-seq results, the expression levels of CSUI_008252, CSUI_003709, CSUI_000190 were upregulated at T2 and T3, and the expression levels of CSUI_005927, 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 coccidian sexual biology[14,41,42]. 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[43]. 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)[44–47]. We detected 19 genes coding for nine different MICs and seven coding for PAN-domain 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[48–50]. 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 themself into the host cell[51,52]. This initial contact is mediated by proteins released from the micronemes[44]. 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[53–55]. 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[56–58]. The best studied group of IMC proteins are components of the motor complex—also referred to as the "glideosome" – in *T. gondii*[59,60]. 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)[56] are the alveolins of which we identifed 14 genes in *C. suis*. Beside the alveolins, other additional IMC-associated peripheral membrane proteins like the IMC subcompartment-proteins (ISPs) were identified[61]. 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[49]. Extracellular K^+ and cytosolic Ca_2^+ concentrations have been implicated in the activation of gliding motility[62]. A role for cyclic nucleotide signaling has been unveiled, and phosphorylation, and methylation events also regulate the gliding motility[63]. 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[64–66]. In *C. suis*, 53 SAGs or SRSs were identified, and their downregulation indicates that sexual stages have less interactions with host cells and the

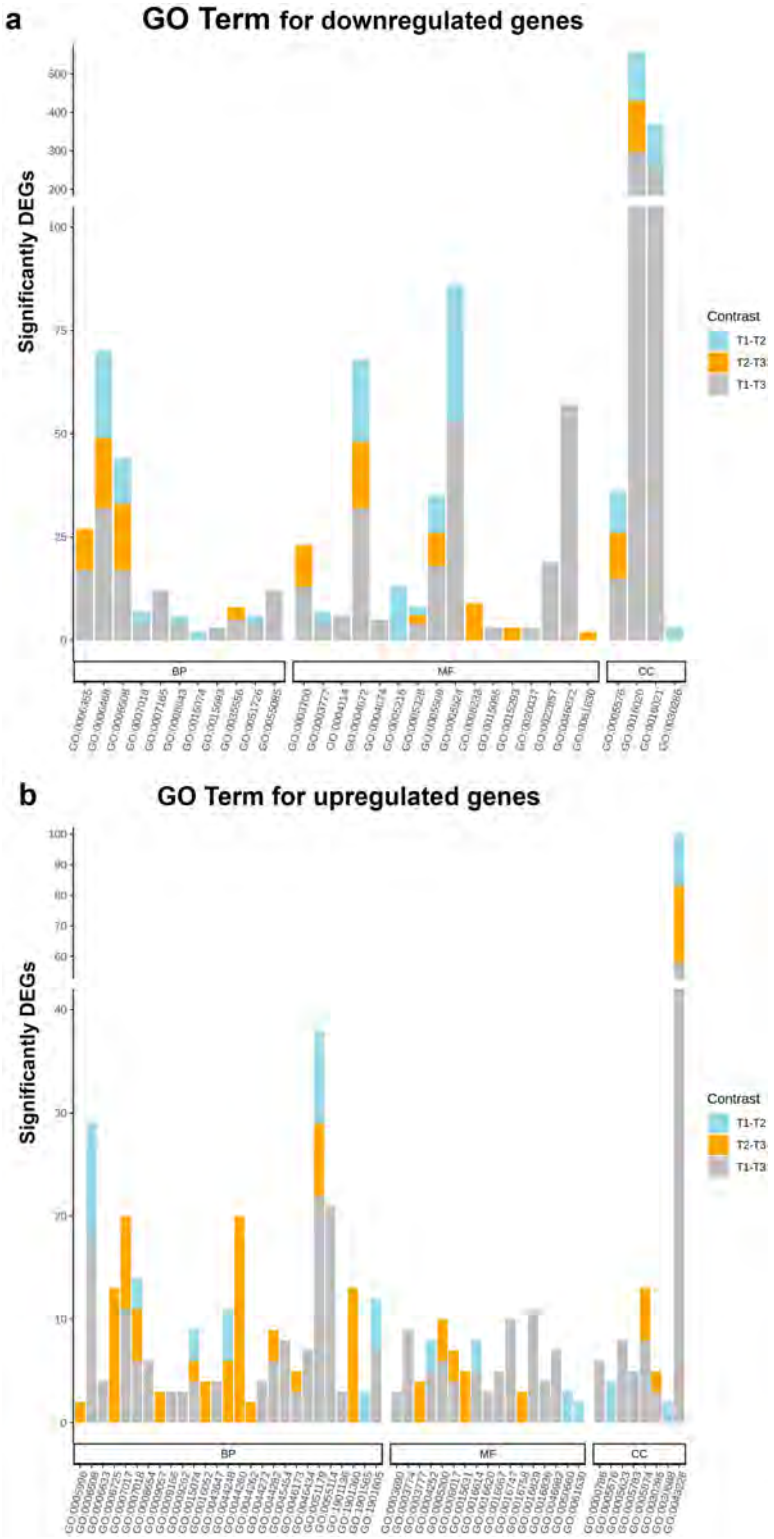


Figure 3. Gene ontology (GO) analysis of differently expressed genes. Differentially expressed genes (DEGs) are classified into three main categories: biological process (BP), molecular function (MF) and cellular component (CC). The identified functions for each corresponding numbers GO category are shown in supplemental file 2.

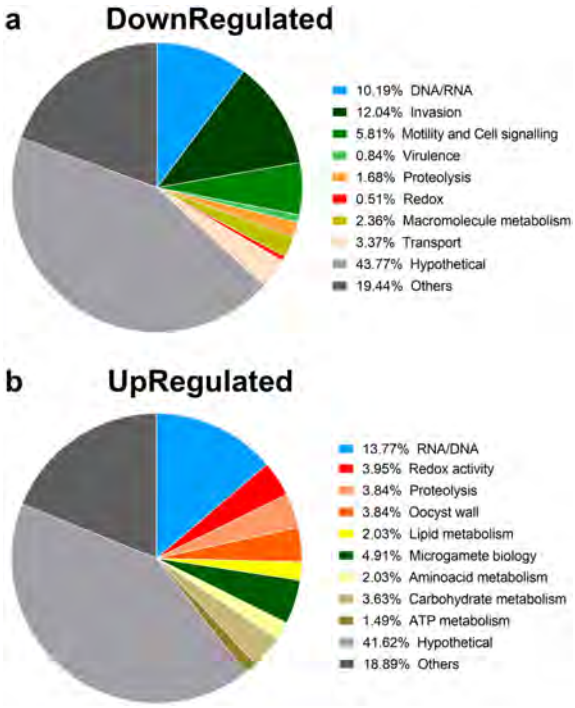


Figure 4. Biological functions of proteins coded by downregulated **(a)** and upregulated **(b)** genes. The biological functions of proteins coded by each of the 1188 down- and 937 upregulated transcripts was assigned manually based on ToxoDB®, Blast2Go® or previously published annotations. The pie charts represent the relative proportion of these different biological functions within up- and down regulated genes.

host's immune system, maybe because the do not reinvade cells and are rather short-lived, progressing quickly from gamonts to oocysts[11].

We could show that genes encoding proteins that play an active role in the invasion of host cells are downregulated 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 enviroment – but at the same time makes these stages accesible 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 formation[67,68], an orthologue of the bradyzote antigen 1[21], a Myb-like transcription factor[69], 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 vivo*[70] nor in vitro. However, other species of the genus *Cystoisospora* can develop resting monozoic tissue cyst stages[71,72]. 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 parasites[73]. 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 plants[74]. The ApiAP2 family was discovered in the genomes of various Apicomplexan species[75]. In *Plasmodium*, they play a role in stage conversion[76] and are related to sexual commitment of blood-stages[77–79]. Currently, 67 ApiAP2 domain-containing proteins are annotated in the *Toxoplasma* genome, with 24 being expressed cyclically during the tachyzoite division cycle[18,80] and six in bradyzoite development[81]. 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 detail[39]. 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 wall[38,82–86]. 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

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_006655	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 srs26i	UT12_UT23_UT13	Surface
CSUI_005667	1.57	3,74E+08	sag-related sequence srs28	UT23_UT13	Surface
CSUI_009850	3.09	6,33E+08	proteophosphoglycan related	UpT12_UpT13	Surface
CSUI_006635	1.68	7,28E+05	proteophosphoglycan related	UpT23_UpT13	Surface
CSUI_001278	2.85	3,81E+08	proteophosphoglycan related protein	UpT12_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.

oocyst wall proteins of the wall-forming bodies with a vital role in oocyst wall formation[87]. Seven genes that encode proteins of novel OWP candidates were not highly homologous with established OWPs although all of them share characteristic cysteine repeats[86]. The oocyst and sporocyst walls of *C. suis* display autofluorescence under 405 nm laser light, presumably due to the dityrosine bonds formed between tyrosine-rich proteins in the oocyst wall[88]. Three hypothetical proteins which are tyrosine-rich (> 14% and 7% of tyrosine) are homologous to genes identified in the oocyst proteome of *T. gondii* and the sporocyst wall of *E. tenella*[20,89,90]. Recent studies on glycosylation in *Toxoplasma* demonstrated its importance for cyst wall rigidity and parasite persistence in the environment[91]. In *Eimeria*, glycoproteins expressed specifically in the sexual stages are important components of the oocyst wall[92]. In our study we identified eleven genes involved in protein glycosylation. The tyrosine rich proteins undergo proteolysis into smaller tyrosine-rich peptides before oocyst wall assembly[88]. In the present study, 36 genes coding for enzymes proposed to be involved in the proteolysis of dityrosine bond formation in the oocyst wall were identified, including, among others, five subtilisins, four peptidases, seven proteasome units, four microneme and two pan domain-proteins. The subtilisins are particularly interesting with regard to dityrosine bond formation[8]. Cross-linking of the smaller tyrosine-rich proteins to dityrosine imparts further stability to the oocyst wall, and the role of peroxidases in catalysing this reaction is implicated[93]. A total of 37 proteins involved in oxidoreductase activities were identified. The oocyst wall consists mainly of proteins and lipids[39]. Polysaccharide granules and lipid droplets are also found in the cytoplasm of mature macrogamonts in *T. gondii* and *E. maxima*[39,94,95]. Recent studies indicate that the coccidian oocyst wall architecture is comprised not only of glycoproteins but also of an outer layer of acid-fast lipids[39]. Nineteen genes coding for proteins with predicted roles in synthesis, metabolim or remodeling of acid-fast lipids were found in *C. suis*. Consistent with the presence of triglycerides in oocyst walls, mRNAs of diacylglycerol acyltransferases and three putative acyl coenzyme A (acyl-CoA) cholesterol acyltransferases were also found to be upregulated. The roles of these putative proteins in sexual stage biology need to be further investigated. Additional proteins considered to be of interest were surface proteins and proteins involved in oocyst wall resistance. Among these are nine surface proteins, including three SAGs, two fasciclins and three proteophosphoglycans, one outer omp85 family protein, one longevity-assurance domain-containing protein and three late embryogenesis abundant domain proteins (LEA). All these proteins were previously identifed in *T. gondii* oocysts and in *Eimeria* gametocytes and oocysts[14,20,38,89] (Tables 1 and S5).

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_UT13	Flagella
CSUI_006910	2.80	3,12E+05	flagellar associated protein	UT12_UT23_UT13	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	UT23_UT13	Microtubule
CSUI_000425	1.38	5,37E+03	myosin k	UT13	Microtubule
CSUI_009385	1.18	2,96E+03	myosin k	UT13	Microtubule
CSUI_005546	1.10	2,12E+09	myosin light chain	UT13	Microtubule
CSUI_011403	1.20	5,16E+0	myosin regulatory light chain	UT13	Microtubule
CSUI_000854	1.58	1,43E+07	non-muscle myosin heavy	UT23_UT13	Microtubule
CSUI_007953	3.02	1,69E+05	chromosome-associated kinesin klp1	UT12_UT23_UT13	Microtubule
CSUI_007586	3.41	8,12E+06	dynein gamma flagellar outer	UT12_UT23_UT13	Axonema
CSUI_004717	1.62	7,86E+07	dynein light chain dlc	UT23_UT13	Axonema
CSUI_001333	1.08	1,22E+04	dynein light chain roadblock-type 2	UT13	Axonema
CSUI_002604	2.73	7,88E+05	growth arrest-specific protein 8	UT12_UT23_UT13	Axonema
CSUI_000245	2.21	4,06E+07	heavy chain 2 family protein	UT23_UT13	Axonema
CSUI_000472	3.19	2,40E+08	male gamete fusion factor	UT12_UT23_UT13	Gamete fusion
CSUI_002998	3.20	1,95E+04	morn repeat-containing protein	UT12_UT23_UT13	Cell budding
CSUI_000048	1.30	2,22E+07	morn repeat-containing protein	UT13	Cell budding
CSUI_004816	1.70	2,78E+06	morn repeat-containing protein	UT23_UT13	Cell budding

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.

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*[82,95,96]; they were previously developed as antigens for transmission-blocking vaccines targeting the gametocyte-specific proteins GAM56, GAM82 and GAM22[95,97–100]. These proteins are potent immunogens for the use as vaccines against chicken coccidiosis as they induce a diverse and robust immunity[101]. In *Plasmodium*, vaccines incorporating magrogamete surface antigens significantly reduced oocyst formation, and several surface proteins of *Plasmodium* macrogametes such as Pfs25 and Pfs230 are investigated in ongoing trials[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 flagella[7]. The molecular characterisation of microgametes in the Coccidia is still limited. Microgametes use flagella to move quickly in search of macrogametes and fertilize them, leading to the formation of the zygote. Although there are few data on the molecular mechanisms underlying this development, some proteins and genes were predicted to be involved in DNA replication, microgamete budding from microgamonts, axoneme/flagellar formation and gamete fusion[14,17,20,105,106]. We detected 46 upregulated transcripts coding for proteins with a putative role in microgamete biology. Of these, CSUI_004019 and CSUI_006055 were the two most abundant transcripts, coding for a tubulin beta-chain and a flagella-associated protein. In sexual stages, tubulins are the building block proteins of the microtubules that form the flagellar axoneme, basal bodies, and centrioles. They are components of the flagella that are essential for microgamete motility and fertilisation. We also found six proteins involved in motor activity and microtubule movement, 25 axonema and microtubule-associated proteins, four involved in cell budding and one in gamete fusion (Table 2 and S6).

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 assumed[40,107,108]. 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 Apicomplexa[2]. Oral application of sera containing *E. tenella* gamont-specific monoclonal antibodies significantly reduced oocyst output and cecal lesions in chicken[109]. Studies in *Plasmodium* proposed the HAP2 fusion protein as a candidate for a transmission-blocking vaccine[110–112]. 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 strain[22]. This in turn supports the assumption that intestinal sexual stages are accesible 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[113]. Although these antibodies were not characterised regarding the targeted proteins or parasite stages, it is conceivable that sexual-stage specific proteins could be implemented a vaccine targets in this context.

Inmunolocalization of CSUI_001473 antigens in macrogametes and oocysts. The oocyst wall is a distinctive characteristic of coccidian development and the key stage of transmission[39]. 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*, transcrip levels of CSUI_001473 (*Cs*TyRP) 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 1463 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 identifed. The recombinant CSUI_001473 protein (rCSUI_001473) revealed a major protein band of ~ 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 aproximatively 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 pre-immune serum, merozoite growth was inhibited by only 20% (Fig. 6a and b). The numbers of newly developed early sexual stages increased until three days after merozoite transfer, the late sexual stages (macrogametes and free motile microgametes) could be detected from three days after transfer, and both unsporulated and sporulated oocysts were present by day four post transfer. Treatment with positive serum significantly inhibited the development of early and late sexual stages (Fig. 6c and d). Development of early sexual stages was inhibited by 50%, while the late sexual stages were reduced by 75% (Fig. 6a).

Taken together our previous studies with the current results on the protein localization on the surface of macrogametes and oocyst wall and the development inhibition of early and late sexual stages confirm that CSUI_001473 transcripts encode a protein that plays a decisive functional role in the development and/or formation of the oocyst wall.

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

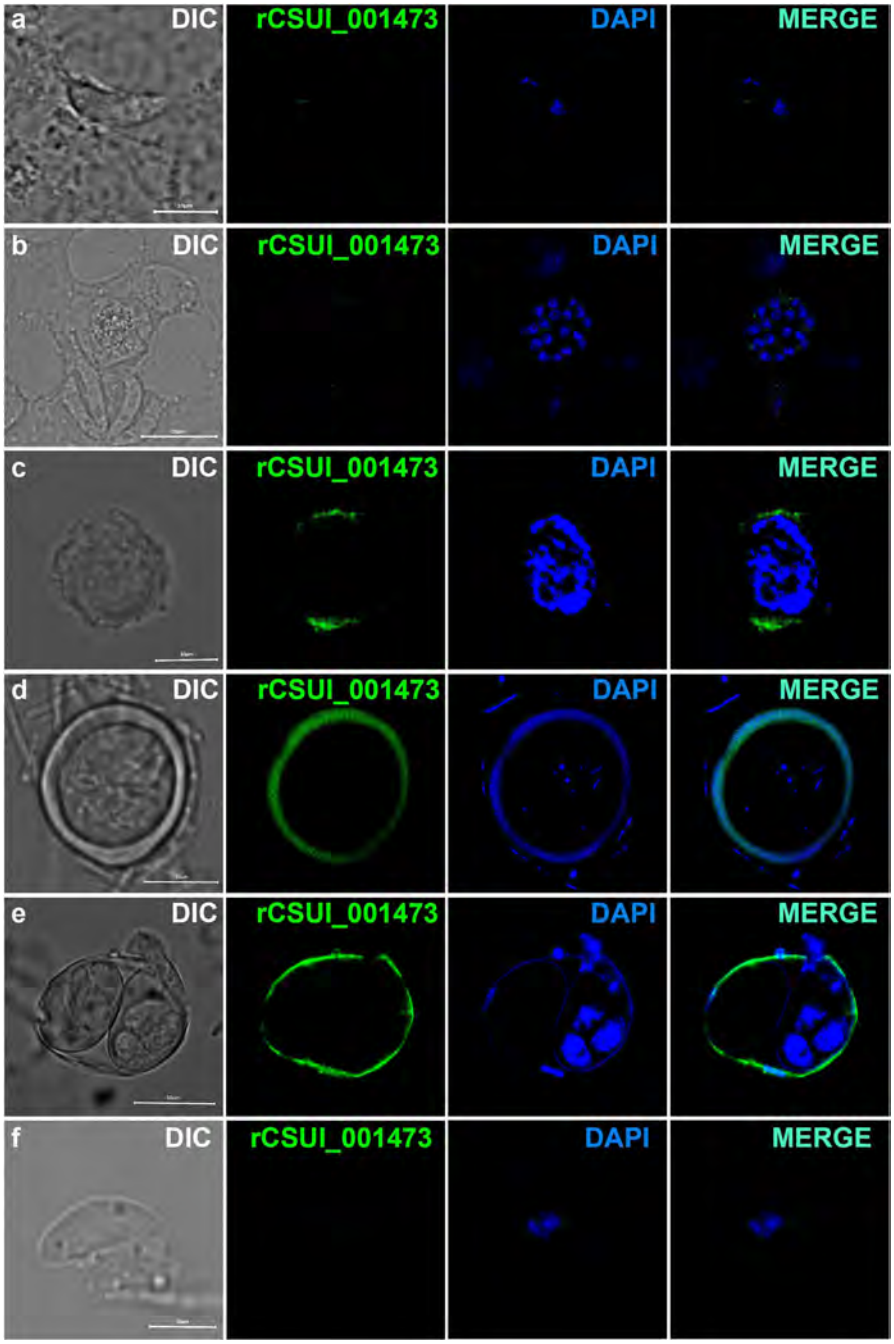


Figure 5. Localization of CSUI_001473 antigens in differents *C. suis* stages. (**a**) Merozoite from day 6 of in vitro culture. (**b**) Microgamont from day 9 of culture. (**c**) Macrogamont from day 9 of culture. (**d**) Unsporulated oocyst from day 14. (**e**) Sporulated oocyst ex vivo (isolated from 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 4×10^5 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 in DMEM/Ham's F-12 medium (Gibco—Fisher Scientific GmbH, Schwerte, Germany) with 5% fetal calf serum (Gibco)

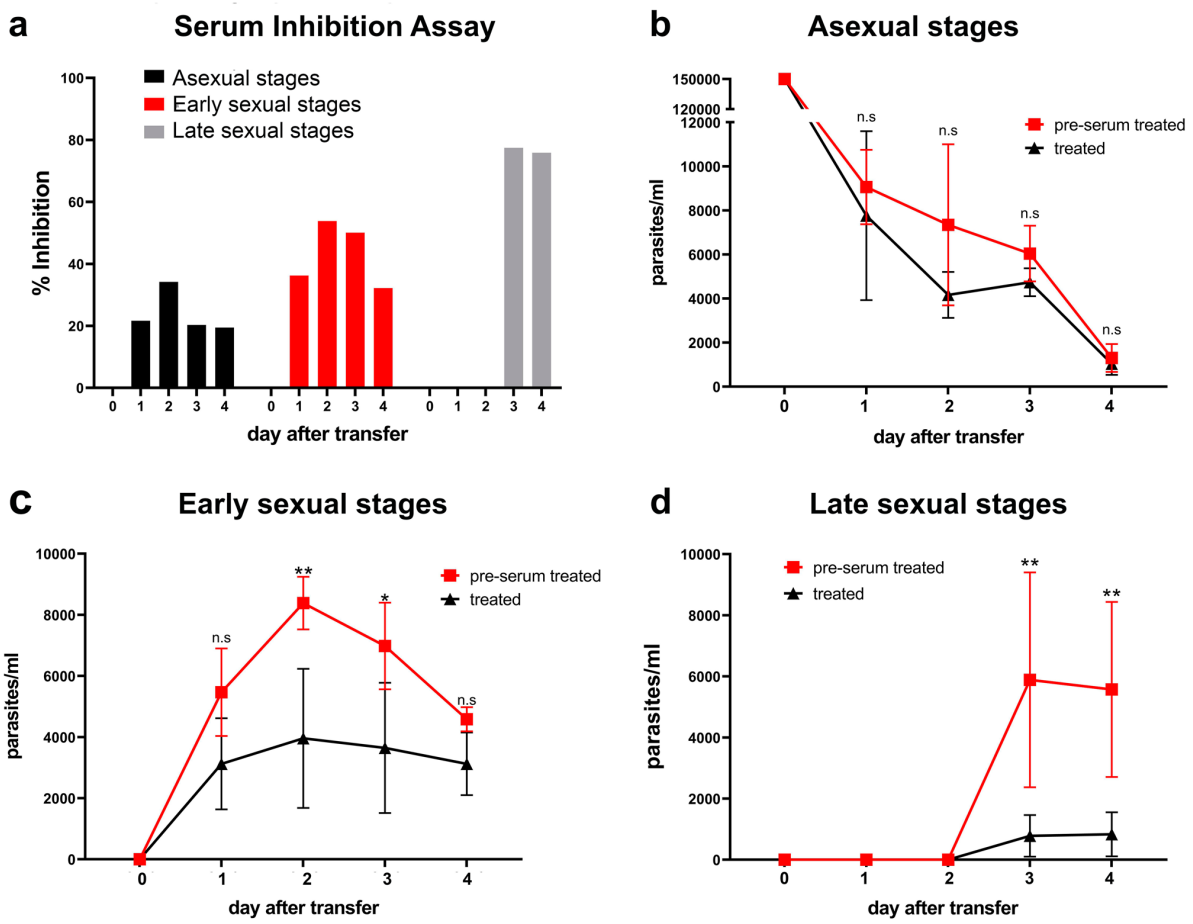


Figure 6. Serum Inhibition assay. (**a**) Inhibition rates for asexual, early (mostly gamonts) and late (mostly gametes) sexual stages of *C. suis* in a host-cell free culture 0–4 days after transfer of merozoites. (**b** to **d**) Total numbers of counted stages by day of cultivation in host-cell free culture. n.s.: not singificant, *: $P \leq 0.05$, **: $P \leq 0.01$. Values represent the mean ± standard deviation (SD) from three independent experiments. n.s.: not singificant, *: $P \leq 0.05$, **: $P \leq 0.01$.

and 100 U/ml penicillin and 0.1 mg/ml streptomycin (PAA, Pasching, Austria) at 37 °C in 5% CO_2. After 24 h of cell growth IPEC-1 cells were infected with 5×10^3 sporozoites/well released from excysted oocysts and incubated further at 40 °C under 5% CO_2[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

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)[114] and *S. scrofa* (version 1.11 from Ensembl, GCA_000003025.6)[115,116] using STAR (version 2.7.3a with option –outSAMmultNmax 1)[117] 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[118] and QualiMap[119]. RNA degradation was taken into account via the TIN (Transcript Integrity Number) values, which were measured for each gene and library with the RSeQC[120]. 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[120,121]. Gene expression was quantified with featureCounts (version 1.5.0a)[122] with options -s 2 -Q 20 –primary.

Identification and analysis of differentially expresed genes. All statistical analysis were performed in R (version 4.1)[123].

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[124] 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 *dream* (R package variancePartition, version 1.18.3)[124,125], 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[126]. 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[127] 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 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 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 $2-\Delta\Delta Cq$ 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 $P \leq 0.05$.

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 $OD_{600} = 0.6$, 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 Na_2HPO_4; 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 imunizations (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 blotting. 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 them with Coomassie blue after electrophoresis, the other one was used to transferprotein bands onto a PVDF membrane (Mini ProBlott Membranes, Applied Biosystems, Foster City, CA, USA) using a Transblot device (Bio-Rad). Membrane strips was 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-rCSUI_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).

Inmunofluorescence 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-rCSUI_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[10] 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-rCSUI_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% CO_2. 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 transfer 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 $P \leq 0.05$ (*).

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 committeee and the national authority according to §§26ff. of the Animal Expriments 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.

Received: 16 December 2021; Accepted: 15 March 2022
Published online: 08 April 2022

References

1. Barta, J., Schrenzel, M., Carreno, R. & Rideout, B. The Genus *Atoxoplasma* (Garnham 1950) as a Junior Objective Synonym of the Genus *Isospora* (Schneider 1881) Species Infecting Birds and Resurrection of *Cystoisospora* (Frenkel 1977) as the Correct Genus for *Isospora* Species Infecting Mammals. *J. Parasitol.* **91**, 726–727 (2005).
2. Cruz-Bustos, T., Feix, A. S., Ruttkowski, B. & Joachim, A. Sexual development in non-human parasitic apicomplexa: just biology or targets for control?. *Anim. Open Access J. MDPI* **11**, 2891 (2021).
3. Lindsay, D. S., Dubey, J. P. & Blagburn, B. L. Biology of *Isospora* spp. from humans, nonhuman primates, and domestic animals. *Clin. Microbiol. Rev.* **10**, 19–34 (1997).
4. Joachim, A. & Shrestha, A. Coccidiosis of pigs. In *Coccidiosis in livestock, poultry, companion animals, and humans* (ed. Dubey, J. P.) 125–145 (Milton Park, Taylor & Francis Group, 2019).
5. Shrestha, A. *et al. Cystoisospora suis*—A model of mammalian cystoisosporosis. *Front. Vet. Sci.* **2**, 1–9 (2015).
6. Shrestha, A. *et al.* Shifts in the fecal microbial community of *Cystoisospora suis* infected piglets in response to Toltrazuril. *Front. Microbiol.* **11**, 983 (2020).
7. Feix, A. S., Cruz-Bustos, T., Ruttkowski, B. & Joachim, A. Characterization of *Cystoisospora suis* sexual stages in vitro. *Parasit. Vectors* **13**, 143 (2020).
8. Mai, K. *et al.* Oocyst wall formation and composition in coccidian parasites. *Mem. Inst. Oswaldo Cruz.* **104**, 281–289 (2009).
9. Weedall, G. D. & Hall, N. Sexual reproduction and genetic exchange in parasitic protists. *Parasitology* **142**(Suppl), S120–S127 (2015).
10. Worliczek, H. L. *et al. Isospora suis* in an epithelial cell culture system - an in vitro model for sexual development in coccidia. *PLoS ONE* **8**, e69797–e69797 (2013).
11. Feix, A. S. *et al.* Progression of asexual to sexual stages of *Cystoisospora suis* in a host cell-free environment as a model for Coccidia. *Parasitology* **148**(12), 1–25 (2021).
12. Novaes, J. *et al.* A comparative transcriptome analysis reveals expression profiles conserved across three *Eimeria* spp. of domestic fowl and associated with multiple developmental stages. *Int. J. Parasitol.* **42**, 39–48 (2012).
13. Wang, X. *et al.* RNA Sequencing analysis of chicken cecum tissues following *Eimeria tenella* infection in vivo. *Genes (Basel)* **10**, 420 (2019).
14. Walker, R. A. *et al.* RNA Seq analysis of the *Eimeria tenella* gametocyte transcriptome reveals clues about the molecular basis for sexual reproduction and oocyst biogenesis. *BMC Genomics* **16**, 94 (2015).
15. Amiruddin, N. *et al.* Characterisation of full-length cDNA sequences provides insights into the *Eimeria tenella* transcriptome. *BMC Genomics* **13**, 21 (2012).
16. Su, S. *et al.* Comparative transcriptome analysis of second- and third-generation merozoites of *Eimeria necatrix*. *Parasit. Vectors* **10**, 388 (2017).
17. Su, S. *et al.* Comparative transcriptome analysis of *Eimeria necatrix* third-generation merozoites and gametocytes reveals genes involved in sexual differentiation and gametocyte development. *Vet. Parasitol.* **252**, 35–46 (2018).
18. Behnke, M. S. *et al.* Coordinated progression through two subtranscriptomes underlies the Tachyzoite cycle of *Toxoplasma gondii*. *PLoS ONE* **5**, e12354 (2010).
19. Radke, J. R. *et al.* The transcriptome of *Toxoplasma gondii*. *BMC Biol.* **3**, 26 (2005).
20. Fritz, H. M. *et al.* Transcriptomic analysis of *Toxoplasma* development reveals many novel functions and structures specific to sporozoites and oocysts. *PLoS ONE* **7**, e29998–e29998 (2012).
21. Chen, L.-F. *et al.* Comparative studies of *Toxoplasma gondii* transcriptomes: insights into stage conversion based on gene expression profiling and alternative splicing. *Parasit. Vectors* **11**, 402 (2018).
22. Ramakrishnan, C. *et al.* An experimental genetically attenuated live vaccine to prevent transmission of *Toxoplasma gondii* by cats. *Sci. Rep.* **9**, 1474 (2019).
23. Xue, Y. *et al.* A single-parasite transcriptional atlas of *Toxoplasma Gondii* reveals novel control of antigen expression. *Elife* **9**, e54129 (2020).
24. Garfoot, A. L., Wilson, G. M., Coon, J. J. & Knoll, L. J. Proteomic and transcriptomic analyses of early and late-chronic *Toxoplasma gondii* infection shows novel and stage specific transcripts. *BMC Genomics* **20**, 859 (2019).
25. Horcajo, P. *et al.* Integrative transcriptome and proteome analyses define marked differences between *Neospora caninum* isolates throughout the tachyzoite lytic cycle. *J. Proteomics* **180**, 108–119 (2018).
26. Jiménez-Meléndez, A., Ramakrishnan, C., Hehl, A. B., Russo, G. & Álvarez-García, G. RNA-Seq Analyses Reveal That Endothelial Activation and Fibrosis Are Induced Early and Progressively by *Besnoitia besnoiti* Host Cell Invasion and Proliferation . *Frontiers in Cellular and Infection Microbiology* **10**, (2020).
27. Matos, L. V. S., McEvoy, J., Tzipori, S., Bresciani, K. D. S. & Widmer, G. The transcriptome of *Cryptosporidium* oocysts and intracellular stages. *Sci. Rep.* **9**, 7856 (2019).
28. Le Roch, K. G. *et al.* Discovery of gene function by expression profiling of the malaria parasite life cycle. *Science (80-)* **301**, 1503–1508 (2003).
29. Chappell, L. *et al.* Refining the transcriptome of the human malaria parasite *Plasmodium falciparum* using amplification-free RNA-seq. *BMC Genomics* **21**, 395 (2020).
30. Otto, T. D. *et al.* New insights into the blood-stage transcriptome of *Plasmodium falciparum* using RNA-Seq. *Mol. Microbiol.* **76**, 12–24 (2010).
31. Prajapati, S. K. *et al.* The transcriptome of circulating sexually committed *Plasmodium falciparum* ring stage parasites forecasts malaria transmission potential. *Nat. Commun.* **11**, 6159 (2020).
32. Silvestrini, F. *et al.* Genome-wide identification of genes upregulated at the onset of gametocytogenesis in *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **143**, 100–110 (2005).
33. van Biljon, R. *et al.* Hierarchical transcriptional control regulates *Plasmodium falciparum* sexual differentiation. *BMC Genomics* **20**, 920 (2019).
34. Ogedengbe, M. *et al.* Molecular phylogenetic analyses of tissue coccidia (sarcocystidae; apicomplexa) based on nuclear 18s RDNA and mitochondrial COI sequences confirms the paraphyly of the genus Hammondia. *Parasitol. Open* **2**, (2016).

35. Samarasinghe, B., Johnson, J. & Ryan, U. Phylogenetic analysis of *Cystoisospora* species at the rRNA ITS1 locus and development of a PCR-RFLP assay. *Exp. Parasitol.* **118**, 592–595 (2008).
36. Schneider, P. & Reece, S. E. The private life of malaria parasites: Strategies for sexual reproduction. *Mol. Biochem. Parasitol.* **244**, 111375 (2021).
37. Smith, R. C., Vega-Rodríguez, J. & Jacobs-Lorena, M. The *Plasmodium* bottleneck: malaria parasite losses in the mosquito vector. *Mem. Inst. Oswaldo Cruz.* **109**, 644–661 (2014).
38. Possenti, A. *et al.* Global proteomic analysis of the oocyst/sporozoite of *Toxoplasma gondii* reveals commitment to a host-independent lifestyle. *BMC Genomics* **14**, 183 (2013).
39. Belli, S. I., Smith, N. C. & Ferguson, D. J. P. The coccidian oocyst: a tough nut to crack!. *Trends Parasitol.* **22**, 416–423 (2006).
40. Walker, R. A., Ferguson, D. J. P., Miller, C. M. D. & Smith, N. C. Sex and *Eimeria*: a molecular perspective. *Parasitology* **140**, 1701–1717 (2013).
41. Martorelli Di Genova, B. & Knoll, L. J. Comparisons of the sexual cycles for the coccidian parasites *Eimeria* and *Toxoplasma*. *Front. Cell. Infect. Microbiol.* **10**, 776 (2020).
42. Ramakrishnan, C. & Smith, N. C. Recent achievements and doors opened for coccidian parasite research and development through transcriptomics of enteric sexual stages. *Mol. Biochem. Parasitol.* **243**, 111373 (2021).
43. Attias, M. *et al.* The life-cycle of *Toxoplasma gondii* reviewed using animations. *Parasit. Vectors* **13**, 588 (2020).
44. Dubois, D. J. & Soldati-Favre, D. Biogenesis and secretion of micronemes in *Toxoplasma gondii*. *Cell. Microbiol.* **21**, e13018 (2019).
45. Wang, J.-L. *et al.* Functional Characterization of Rhoptry Kinome in the Virulent *Toxoplasma gondii* RH Strain. *Front. Microbiol.* **8**, 84 (2017).
46. Dubremetz, J. F. Rhoptries are major players in *Toxoplasma gondii* invasion and host cell interaction. *Cell. Microbiol.* **9**, 841–848 (2007).
47. Mercier, C., Cesbron-Delauw, M. & Ferguson, D. Dense Granules of the Infectious Stages of *Toxoplasma gondii*: Their Central Role in the Host-Parasite Relationship, In Toxoplasma. (2007).
48. Shen, B. & Sibley, L. D. The moving junction, a key portal to host cell invasion by apicomplexan parasites. *Curr. Opin. Microbiol.* **15**, 449–455 (2012).
49. Frénal, K., Dubremetz, J.-F., Lebrun, M. & Soldati-Favre, D. Gliding motility powers invasion and egress in Apicomplexa. *Nat. Rev. Microbiol.* **15**, 645–660 (2017).
50. Sibley, L. D. Intracellular parasite invasion strategies. *Science (80-).* **304**, 248–253 (2004).
51. Håkansson, S., Morisaki, H., Heuser, J. & Sibley, L. D. Time-lapse video microscopy of gliding motility in *Toxoplasma gondii* reveals a novel, biphasic mechanism of cell locomotion. *Mol. Biol. Cell* **10**, 3539–3547 (1999).
52. Heintzelman, M. B. Gliding motility in apicomplexan parasites. *Semin. Cell Dev. Biol.* **46**, 135–142 (2015).
53. Kato, K. How does *Toxoplama gondii* invade host cells?. *J. Vet. Med. Sci.* **80**, 1702–1706 (2018).
54. Bargieri, D. Y. *et al.* Apical membrane antigen 1 mediates apicomplexan parasite attachment but is dispensable for host cell invasion. *Nat. Commun.* **4**, 2552 (2013).
55. Mital, J., Meissner, M., Soldati, D. & Ward, G. E. Conditional expression of *Toxoplasma gondii* apical membrane antigen-1 (TgAMA1) demonstrates that TgAMA1 plays a critical role in host cell invasion. *Mol. Biol. Cell* **16**, 4341–4349 (2005).
56. Harding, C. R. & Meissner, M. The inner membrane complex through development of *Toxoplasma gondii* and *Plasmodium*. *Cell. Microbiol.* **16**, 632–641 (2014).
57. Ferreira, J. L. *et al.* The dynamic roles of the inner membrane complex in the multiple stages of the malaria parasite. *Front. Cell. Infect. Microbiol.* **10**, 841 (2021).
58. Harding, C. R. & Frischknecht, F. The riveting cellular structures of apicomplexan parasites. *Trends Parasitol.* **36**, 979–991 (2020).
59. Frénal, K. & Soldati-Favre, D. Un complexe moléculaire unique à l'origine de la motilité et de l'invasion des Apicomplexes. *Med. Sci.* **29**, 515–522 (2013).
60. Boucher, L. E. & Bosch, J. The apicomplexan glideosome and adhesins - Structures and function. *J. Struct. Biol.* **190**, 93–114 (2015).
61. Tosetti, N. *et al.* Essential function of the alveolin network in the subpellicular microtubules and conoid assembly in *Toxoplasma gondii*. *Elife* **9**, e56635 (2020).
62. Lourido, S. & Moreno, S. N. J. The calcium signaling toolkit of the Apicomplexan parasites *Toxoplasma gondii* and *Plasmodium* spp. *Cell Calcium* **57**, 186–193 (2015).
63. Uboldi, A. D., Wilde, M.-L., Bader, S. M. & Tonkin, C. J. Environmental sensing and regulation of motility in *Toxoplasma*. *Mol. Microbiol.* **115**, 916–929 (2021).
64. Hehl, A. B. *et al.* Asexual expansion of *Toxoplasma gondii* merozoites is distinct from tachyzoites and entails expression of non-overlapping gene families to attach, invade, and replicate within feline enterocytes. *BMC Genomics* **16**, 66 (2015).
65. Blader, I. J. & Saeij, J. P. Communication between *Toxoplasma gondii* and its host: impact on parasite growth, development, immune evasion, and virulence. *APMIS* **117**, 458–476 (2009).
66. Lekutis, C., Ferguson, D. J. P., Grigg, M. E., Camps, M. & Boothroyd, J. C. Surface antigens of *Toxoplasma gondii*: variations on a theme. *Int. J. Parasitol.* **31**, 1285–1292 (2001).
67. Craver, M. P. J., Rooney, P. J. & Knoll, L. J. Isolation of *Toxoplasma gondii* development mutants identifies a potential proteophosphogylcan that enhances cyst wall formation. *Mol. Biochem. Parasitol.* **169**, 120–123 (2010).
68. Tomita, T. *et al.* The *Toxoplasma gondii* Cyst wall protein CST1 is critical for cyst wall integrity and promotes bradyzoite persistence. *PLOS Pathog.* **9**, e1003823 (2013).
69. Sharma, J., Rodriguez, P., Roy, P. & Guiton, P. S. Transcriptional ups and downs: patterns of gene expression in the life cycle of *Toxoplasma gondii*. *Microbes Infect.* **22**, 525–533 (2020).
70. Pinckney, R. D., Lindsay, D. S., Toivio-Kinnucan, M. A. & Blagburn, B. L. Ultrastructure of *Isospora suis* during excystation and attempts to demonstrate extraintestinal stages in mice. *Vet. Parasitol.* **47**, 225–233 (1993).
71. Lindsay, D. S., Houk, A. E., Mitchell, S. M. & Dubey, J. P. Developmental Biology of *Cystoisospora* (Apicomplexa: Sarcocystidae) Monozoic Tissue Cysts. *J. Parasitol.* **100**, 392–398 (2014).
72. Ferreira, R., Borges-Silva, W., de Jesus, R. F. & Gondim, L. F. P. Development of *Cystoisospora felis* in cell culture and in vitro formation of monozoic tissue cysts. *Front. Vet. Sci.* **6**, 361 (2019).
73. Dubey, J. P. Re-evaluation of merogony of a *Cystoisospora ohioensis*-like coccidian and its distinction from gametogony in the intestine of a naturally infected dog. *Parasitology* **146**, 740–745 (2019).
74. Riechmann, J. L. & Meyerowitz, E. M. The AP2/EREBP family of plant transcription factors. *Biol. Chem.* **379**, 633–654 (1998).
75. Balaji, S., Babu, M. M., Iyer, L. M. & Aravind, L. Discovery of the principal specific transcription factors of Apicomplexa and their implication for the evolution of the AP2-integrase DNA binding domains. *Nucleic Acids Res.* **33**, 3994–4006 (2005).
76. Painter, H. J., Campbell, T. L. & Llinás, M. The Apicomplexan AP2 family: integral factors regulating *Plasmodium* development. *Mol. Biochem. Parasitol.* **176**, 1–7 (2011).
77. Ralph, S. A. & Cortés, A. *Plasmodium* sexual differentiation: how to make a female. *Mol. Microbiol.* **112**, 1627–1631 (2019).
78. Josling, G. A. *et al.* Dissecting the role of PfAP2-G in malaria gametocytogenesis. *Nat. Commun.* **11**, 1503 (2020).
79. Neveu, G., Beri, D. & Kafsack, B. F. C. Metabolic regulation of sexual commitment in *Plasmodium falciparum*. *Curr. Opin. Microbiol.* **58**, 93–98 (2020).

80. Radke, J. B. *et al.* Transcriptional repression by ApiAP2 factors is central to chronic toxoplasmosis. *PLoS Pathog.* **14**, e1007035–e1007035 (2018).
81. Sokol-Borrelli, S. L., Coombs, R. S. & Boyle, J. P. A Comparison of Stage Conversion in the Coccidian Apicomplexans *Toxoplasma gondii, Hammondia hammondi*, and *Neospora caninum*. *Front. Cell. Infect. Microbiol.* **10**, 608283 (2020).
82. Wallach, M. G. *et al. Eimeria maxima*: Identification of gametocyte protein antigens. *Exp. Parasitol.* **68**, 49–56 (1989).
83. Spano, F., Puri, C., Ranucci, L., Putignani, L. & Crisanti, A. Cloning of the entire COWP gene of *Cryptosporidium parvum* and ultrastructural localization of the protein during sexual parasite development. *Parasitology* **114**, 427–437 (1997).
84. Lippuner, C. *et al.* RNA-Seq analysis during the life cycle of *Cryptosporidium parvum* reveals significant differential gene expression between proliferating stages in the intestine and infectious sporozoites. *Int. J. Parasitol.* **48**, 413–422 (2018).
85. Jonscher, E., Erdbeer, A., Günther, M. & Kurth, M. Two COWP-like cysteine rich proteins from *Eimeria nieschulzi* (coccidia, apicomplexa) are expressed during sporulation and involved in the sporocyst wall formation. *Parasit. Vectors* **8**, 395 (2015).
86. Salman, D. *et al.* Evaluation of novel oocyst wall protein candidates of *Toxoplasma gondii*. *Parasitol. Int.* **66**, 643–651 (2017).
87. Possenti, A. *et al.* Molecular characterisation of a novel family of cysteine-rich proteins of *Toxoplasma gondii* and ultrastructural evidence of oocyst wall localisation. *Int. J. Parasitol.* **40**, 1639–1649 (2010).
88. Belli, S. I., Wallach, M. G., Luxford, C., Davies, M. J. & Smith, N. C. Roles of tyrosine-rich precursor glycoproteins and dityrosine- and 3,4-dihydroxyphenylalanine-mediated protein cross-linking in development of the oocyst wall in the coccidian parasite *Eimeria maxima*. *Eukaryot. Cell* **2**, 456–464 (2003).
89. Fritz, H. M., Bowyer, P. W., Bogyo, M., Conrad, P. A. & Boothroyd, J. C. Proteomic analysis of fractionated *Toxoplasma* oocysts reveals clues to their environmental resistance. *PLoS ONE* **7**, e29955–e29955 (2012).
90. Walker, R. A. *et al.* Discovery of a tyrosine-rich sporocyst wall protein in *Eimeria tenella*. *Parasit. Vectors* **9**, 124 (2016).
91. Bandini, G., Albuquerque-Wendt, A., Hegermann, J., Samuelson, J. & Routier, F. H. Protein O- and C-Glycosylation pathways in *Toxoplasma gondii* and *Plasmodium falciparum*. *Parasitology* **146**, 1755–1766 (2019).
92. Walker, R. A., Slapetova, I., Slapeta, J., Miller, C. M. & Smith, N. C. The glycosylation pathway of *Eimeria tenella* is upregulated during gametocyte development and may play a role in oocyst wall formation. *Eukaryot. Cell* **9**, 127–135 (2010).
93. Mai, K. *et al.* Peroxidase catalysed cross-linking of an intrinsically unstructured protein via dityrosine bonds in the oocyst wall of the apicomplexan parasite, *Eimeria maxima*. *Int. J. Parasitol.* **41**, 1157–1164 (2011).
94. Wiedmer, S. *et al.* Correlative light and electron microscopy of wall formation in *Eimeria nieschulzi*. *Parasitol. Res.* **119**, 2667–2678 (2020).
95. Ferguson, D. J. P., Belli, S. I., Smith, N. C. & Wallach, M. G. The development of the macrogamete and oocyst wall in *Eimeria maxima*: immuno-light and electron microscopy. *Int. J. Parasitol.* **33**, 1329–1340 (2003).
96. Wallach, M. *et al.* Maternal immunization with gametocyte antigens as a means of providing protective immunity against *Eimeria maxima* in chickens. *Infect. Immun.* **60**, 2036–2039 (1992).
97. Ding, J., Liu, Q.Q.-R., Han, J.-P., Qian, W.-F. & Liu, Q.Q.-R. Anti-recombinant gametocyte 56 protein IgY protected chickens from homologous coccidian infection. *J. Integr. Agric.* **11**, 1721–1728 (2012).
98. Wiedmer, S. *et al.* Passive immunization with *Eimeria tenella* gametocyte antigen 56 (EtGAM56) specific antibodies and active immunization trial with the epitope containing peptide. *Vet. Parasitol.* **247**, 100–107 (2017).
99. Wallach, M. G., Ashash, U., Michael, A. & Smith, N. C. Field application of a subunit vaccine against an enteric protozoan disease. *PLoS ONE* **3**, e3948–e3948 (2008).
100. Jang, S. I. *et al. Eimeria maxima* recombinant Gam82 gametocyte antigen vaccine protects against coccidiosis and augments humoral and cell-mediated immunity. *Vaccine* **28**, 2980–2985 (2010).
101. Venkatas, J. & Adeleke, M. A. A review of *Eimeria* antigen identification for the development of novel anticoccidial vaccines. *Parasitol. Res.* **118**, 1701–1710 (2019).
102. Chichester, J. A. *et al.* Safety and immunogenicity of a plant-produced Pfs25 virus-like particle as a transmission blocking vaccine against malaria: a Phase 1 dose-escalation study in healthy adults. *Vaccine* **36**, 5865–5871 (2018).
103. Coelho, C. H. *et al.* A human monoclonal antibody blocks malaria transmission and defines a highly conserved neutralizing epitope on gametes. *Nat. Commun.* **12**, 1750 (2021).
104. Gwadz, R. W., Carter, R. & Green, I. Gamete vaccines and transmission-blocking immunity in malaria. *Bull. World Health Organ.* **57**(Suppl 1), 175–180 (1979).
105. Lasonder, E. *et al.* Integrated transcriptomic and proteomic analyses of *P. falciparum* gametocytes: molecular insight into sex-specific processes and translational repression. *Nucleic Acids Res.* **44**, 6087–6101 (2016).
106. Talman, A. M. *et al.* Proteomic analysis of the *Plasmodium* male gamete reveals the key role for glycolysis in flagellar motility. *Malar. J.* **13**, 315 (2014).
107. Ferguson, D. J. P. *Toxoplasma gondii* and sex: essential or optional extra?. *Trends Parasitol.* **18**, 351–355 (2002).
108. Tomasina, R. & Francia, M. E. The Structural and Molecular Underpinnings of Gametogenesis in *Toxoplasma gondii*. *Front. Cell. Infect. Microbiol.* **10**, 608291 (2020).
109. Larsen, N. C., Rasmussen, K. R. & Healey, M. C. Production and partial characterization of monoclonal antibodies specific for the gamonts of *Eimeria tenella*. *J. Parasitol.* **77**, 1012–1015 (1991).
110. Blagborough, A. & Sinden, R. *Plasmodium berghei* HAP2 induces strong malaria transmission-blocking immunity in vivo and in vitro. *Vaccine* **27**, 5187–5194 (2009).
111. Qiu, Y. *et al.* Evaluation of *Plasmodium vivax* HAP2 as a transmission-blocking vaccine candidate. *Vaccine* **38**, 2841–2848 (2020).
112. Angrisano, F. *et al.* Targeting the conserved fusion loop of HAP2 inhibits the transmission of *Plasmodium berghei* and *falciparum*. *Cell Rep.* **21**, 2868–2878 (2017).
113. Schwarz, L., Worliczek, H. L., Winkler, M. & Joachim, A. Superinfection of sows with *Cystoisospora suis* ante partum leads to a milder course of cystoisosporosis in suckling piglets. *Vet. Parasitol.* **204**, 158–168 (2014).
114. Palmieri, N. *et al.* The genome of the protozoan parasite *Cystoisospora suis* and a reverse vaccinology approach to identify vaccine candidates. *Int. J. Parasitol.* **47**, 189–202 (2017).
115. Warr, A. *et al.* An improved pig reference genome sequence to enable pig genetics and genomics research. *bioRxiv* 668921 (2019). https://doi.org/10.1101/668921
116. Groenen, M. A. M. *et al.* Analyses of pig genomes provide insight into porcine demography and evolution. *Nature* **491**, 393–398 (2012).
117. Dobin, A. *et al.* STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics* **29**, 15–21 (2013).
118. Andrews, S. No Title. *FastQC: a quality control tool for high throughput sequence data* (2010). Available at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc.
119. Okonechnikov, K., Conesa, A. & García-Alcalde, F. Qualimap 2: advanced multi-sample quality control for high-throughput sequencing data. *Bioinformatics* **32**, 292–294 (2016).
120. Wang, L., Wang, S. & Li, W. RSeQC: quality control of RNA-seq experiments. *Bioinformatics* **28**, 2184–2185 (2012).
121. Wang, L. *et al.* Measure transcript integrity using RNA-seq data. *BMC Bioinformatics* **17**, 58 (2016).
122. Liao, Y., Smyth, G. K. & Shi, W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* **30**, 923–930 (2014).
123. R Core Team (2020). — European Environment Agency. Available at: https://www.eea.europa.eu/data-and-maps/indicators/oxygen-consuming-substances-in-rivers/r-development-core-team-2006. (Accessed: 26th November 2021)

124. Hoffman, G. E. & Schadt, E. E. variancePartition: Interpreting drivers of variation in complex gene expression studies. *BMC Bioinformatics* **17**, 483 (2016).
125. Hoffman, G. E. & Roussos, P. Dream: powerful differential expression analysis for repeated measures designs. *Bioinformatics* **37**, 192–201 (2021).
126. Benjamini, Y. & Hochberg, Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B* **57**, 289–300 (1995).
127. Alexa, A. & Rahnenfuhrer, J. topGO: Enrichment Analysis for Gene Ontology. *R package version 2.40.0* (2020).

Acknowledgements
This work is dedicated to the memory of Prof. David Lindsay (1955-2021) for his impact in the field of veterinary parasitology and his groundbreaking work on *Isospora suis*. We are grateful to the staff at the "Core Facilities of the Medical University of Vienna, a member of VLSI, for carrying out RNA Seq experiments and for providing ongoing technical support.

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 identificated 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.

Funding
This research was funded by the Austrian Science Fund (FWF), Grant Number: P 33123-B.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-022-09714-8.

Correspondence and requests for materials should be addressed to T.C.-B.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

8. UNPUBLISHED DATA

Coccidia are characterized by the environmentally resistant oocyst, as the result of the fusion of gametes. During this developmental step the motile microgamete tries to find a macrogamete to fertilize, hence both must appear at the same time and be compatible with each other. Although at the transcriptomic and proteomic level gamete fusion is already well described for many Coccidia, a morphological description of the fusion could not be obtained yet. Since the gametes of *C. suis* can be observed *in vitro* their morphology (ref. **Publication 1**), their behavior and their spatial proximity was part of this project.

For this, IPEC *in vitro* cultures were infected with *C. suis* sporozoites, and as soon as free moving microgametes could be detected, a 24 hour-observation was started. For this, the parasite was observed with an inverse microscope and a 3 sec video was taken every 30 min for exactly 24 h. These data sets were further processed with the data processing tool Fiji (GNU General Public License). To calculate the velocity, measure the proximity of sexual stages to each other and determine the different movement behavior, the plugin Track Mate was used.

During the 24h observation time, most host cells died due to the constant light and heat emitted by the microscope. However, the amount of gametes and their proximity to each other could be shown during this process. Micro- and macrogametes and gamonts could be found in close proximity (15-20 µm) to each other. This might be so that the smaller, motile microgamete finds a macrogamete to readily fuse with (Figure 1).

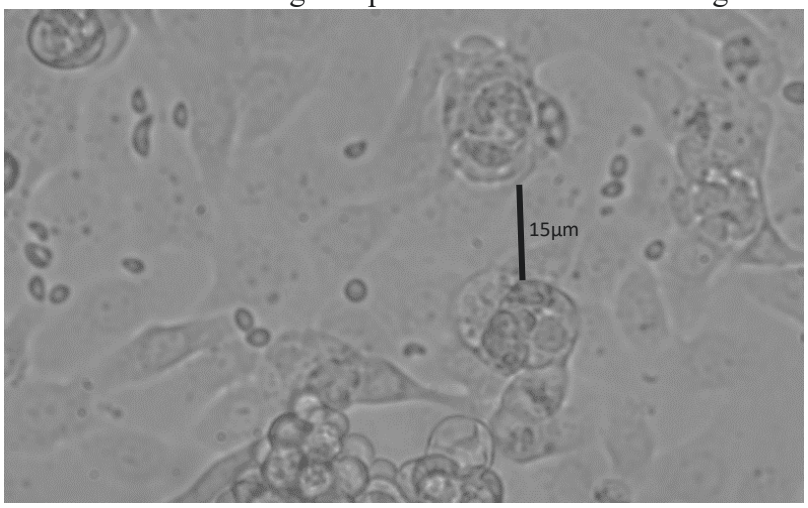


Figure 1: Proximity of micro- and macrogamonts to each other (Feix 2020, unpublished).

Microgamonts include a large vacuole from which 30-40 microgametes arise and freely move forward through the culture.

During this time microgametes in search of macrogametes show two different forms of movement behavior. First, microgametes search for a macrogametes by randomly twitching outside of and between host cells until a macrogamete is found (Figure 2a). The mean velocity of *C. suis* microgametes was 22 µm/s. This searching behavior is displayed until a macrogamete is found. As soon as a viable macrogamete is discovered, the movement pattern changes into a circular movement (Figure 2b). Here the microgamete circles the macrogamete for up to several minutes until it has found a location where it can fuse.

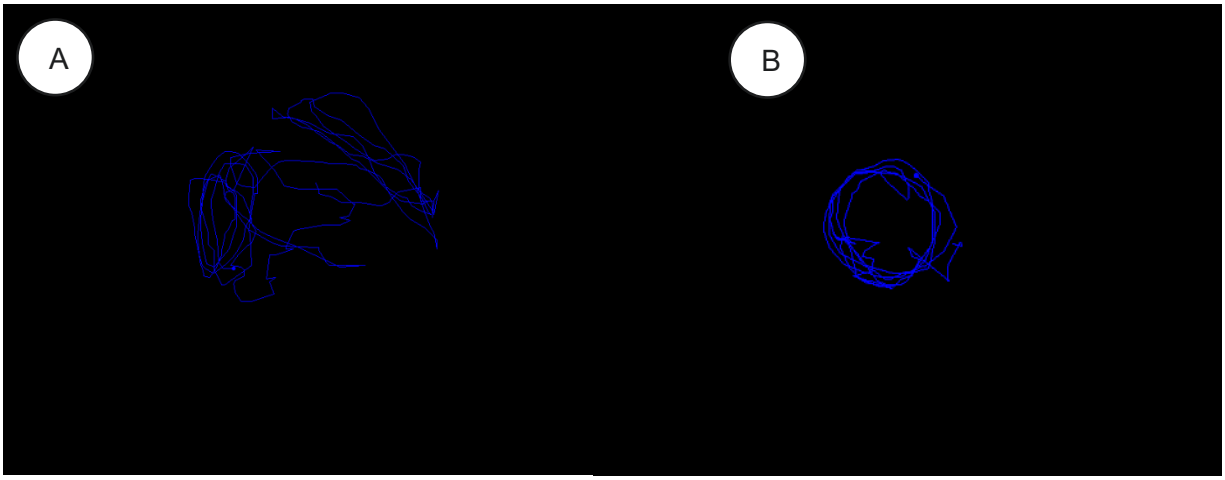


Figure 2: (a) searching behaviour of microgametes, (b) circular movement (Feix 2020, unpublished).

9. DISCUSSION

9.1 Improvement and development of *in vitro* culture systems for *C. suis*

In vitro cultivation is an important tool in biological research, however the intimate association of apicomplexan parasites with their host does not readily permit *in vitro* propagation throughout their life cycle. *C. suis* has been shown to be an exception, as it can be propagated *in vitro* through its whole life cycle including the sexual development as shown in **Publication 1**. During this project the previously established *in vitro* culture (15,68) was optimised, so that a higher output of sexual stages and oocysts could be achieved.

Under these culture conditions, the whole life cycle of *C. suis* takes 13 days and is comparable with previous *in vivo* studies (5,10). However, *in vitro* culture systems for the generation of sexual stages are still lacking and only a few systems are currently known. Morphological studies on *Cr. parvum* showed a production of sexual stages in a HCT8 cell-line (69), however transcriptomic data is not available to further support these observations. The viability of HCT8 cells, however, supported the improvement and development of *in vitro* systems for other *Cryptosporidium* species (70–73). The sexual development of *Eimeria* can be studied in chicken cell lines (74,75). For other more prominent Apicomplexa, such as *T. gondii,* cellular systems supporting the production of sexual stages and oocysts are still lacking, although the sexual development is precisely described with the help of transcriptomic and proteomic methods.

What distinguished the improved *in vitro* culture system used in **Publication 1** and **Publication 3** from the previous one is that not only a full morphological description of all sexual stages was possible, but that the sexual stages also appeared during very specific time slots, characteristic for each sexual stage, i.e merozoites at 6 and 7 doc, micro- and macrogametes on 9-10 doc and oocysts at 10-13 doc. This enables the harvest of clean parasite

stages, so that they can be used for further research on the morphological, transcriptomic and proteomic level.

The viability of the *C. suis in vitro* culture system, was shown by the fact that all described stages are morphologically comparable with stages already described in *in vivo* studies in tissue sections of the small intestine (13,76) and morphological descriptions in other related species (12,77). 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.

Furthermore, a new host cell free culture system for *C. suis* was developed as a result of this PhD-project (**Publication 2**). To initiate the host cell-free culture, free merozoites were obtained from the before mentioned *in vitro* culture of *C. suis* in IPEC cells and transferred to fresh, supplemented Advanced DMEM/F-12 culture medium on uncoated 8-well slides for further incubation. Of the coccidia, only *Cryptosporidium* species have so far been propagated in a host cell-free environment (71,72). In *Cr. hominis*, all asexual stages as well as gamonts were detected over a 9-day incubation period (71)and the production of newly formed oocysts was possible. The complete development of *Cr. parvum* in a host cell-free culture could also be demonstrated, with both unstained and stained lifecycle stages (72,78). *In vitro* cultivation of multiplying stages increases the amount of available parasitic stages, however for host cell-free culture systems the amount of parasitic stages that can be harvested is limited since the parasite can transform to the next stage but not multiply in the absence of host cells, which often precludes their usefulness for molecular techniques (71).

The development of all sexual stages of *C. suis*, however, follows the same time frame as in *in vitro* cultures with IPEC cells and their morphology does not change, although the parasite has no possibility for interactions with the host. Only sporulated oocysts seem to have a thinner oocyst wall than those from cell culture, as they appear lopsided.

Publication 2 also showed that the parasite undergoes changes in its DNA levels, indicating cell division and multiplication in the host cell-free culture system. This supports two main theories; first, the assumption that the fertilization of macrogametes and microgametes is located outside of the host cell, and second the hypothesis that merozoites already express genes transcribing for sexual stage specific proteins. As it was shown in this thesis that the microgametes of *C. suis* freely move extracellularly and also macrogametes are found outside host cells, it can be assumed that extracellular location of mature gametes is a prerequisite for successful fertilization. The fact that the life cycle of *C. suis* continues in a host cell-free environment supports this assumption.

Furthermore, **Publication 2** showed that the expression patterns of genes encoding sexual stage-specific proteins are comparable with those of other Apicomplexa. For *Plasmodium falciparum* and *T. gondii* sexual commitment of merozoites preceding sexual differentiation has been demonstrated by expression of the respective genes and represents an important developmental switch in the life cycle of apicomplexan parasites (79–81). We also showed that a progression of the *C. suis* life cycle to the formation of sexual stages in cell-free culture is only possible after the development of merozoites, but not directly from sporozoites, which hints at a sexual commitment of *C. suis* merozoites. This phenomenon has not been investigated in *C. suis* in detail; however, we could show that the parasite undergoes changes in its DNA levels, indicating preparation for cell division and multiplication.

The newly developed host cell-free culture system is the first viable host cell free culture system for an intracellular parasite. It is possible to produce parasite stages in clearly defined timeframes and to harvest all stages separately, unlike in *in vitro* cell culture, opening new avenues for research on the control of this and possibly related parasites targeting specific stages during the life cycle by vaccination or selective drugs. For instance, the novel host cell-free culture system allows the testing of specific recombinant antibodies in culture conditions that

exclude influences of the host cells, as shown in **Publication 3**. We therefore can be sure that the recombinant antibody and not external influences due to cell apoptosis or changing culture conditions are the reason for inhibited growth of parasite numbers.

9.2 Genes of male gametocyte-specific proteins

A transcriptomic analysis with RNA sequencing of *C. suis* sexual stages identified genes coding for proteins that play critical roles in the sexual biology of coccidian parasites, including functions in motility, fertilization or oocyst wall formation (**Publication 3**).Consequently, genes coding for proteins with those functions are highly upregulated during the appearance of gamonts, gametes or oocysts.

During this project upregulated transcripts coding for proteins with putative function in microgametes biology could be detected, including involvement in DNA replication, microgamete budding from microgamonts, flagellum formation and gamete fusion (24,58,82–85). The gene CSUI_000472 could be identified in transcriptomic analysis (**Publication 3**) and was also highly upregulated on all days of cultivation, whenever microgamont or microgametes were present, both in *in vitro* cell cultures (**Publication 1**) and the novel host cell-free culture system (**Publication 2**). This gene codes for the protein HAP2, a class II gamete fusogen, which is important for male sexual stages in many eukaryotic taxa (37,61). In apicomplexan parasites, it appears to be essential for fusion of the gamete membranes and consequently for fertilization. Furthermore, a similar upregulation of genes coding for tubulins, dyneins, HAP2 and intraflagellar transport proteins was observed by RNA Seq analysis of *E. tenella* sexual stages (86).

The enhanced transcription of HAP2 in the host cell-free culture system, shown in **Publication 2**, also further strengthens the hypothesis that sexual stages can develop outside the host cell and that fertilization and oocyst production occur extracellularly. However, the full fertilization

process could not be demonstrated, as microgametes were short-lived and could not be followed during gamete fusion. We see that microgamonts occur in close proximity to macrogamonts, and microgametes show a searching behaviour after egress. After a macrogamete was found the microgametes showed circling behaviour around the macrogametes until the flagella adhere to the surface of the macrogametes. After that the microgamete's body collapses. The behavioural observation approach is unique to the host cell-free culture of *C. suis*, and precludes comparison with other Apicomplexa impossible, unlike comparative molecular approaches that are more tangible.

It is already known that HAP2 is essential for the fusion of gametes surface membrane and subsequent fertilization and hence can be used as a potential target for transmission-blocking control strategies (87,88). In *Babesia* HAP2 is expressed only in tick-infecting stages, and specific antibodies block zygote formation during gametogenesis *in vitro* (36,89). Furthermore, Hap2-deficient *T. gondii* parasites fail to complete their development and do not undergo oocyst formation in cats (34). Antibodies targeting HAP2 inhibited *P. berghei* transmission *in vivo* up to 58.9 % (35,87). Anti-rHAP2 serum could also inhibit the development of *C. suis in vitro* (Feix et al., unpublished), indicating that this protein constitutes a potential intervention target in this species.

9.3 Genes expressing for female gametocyte specific proteins

Most of the genes identified with RNA sequencing of sexual stages are related to macrogamete development and oocyst formation. Previous studies in *Eimeria* (27,90) and *Cryptosporidium* (91) already identified two families of proteins participating in oocyst wall formation. The first family includes tyrosin-rich proteins (GAMs) the second family includes cysteine-rich proteins (OWPs). Proteins of the first family are found in the wall forming bodies type 1. Proteases break down GAM proteins into tyrosine-rich peptides, which are then oxidatively crosslinked in a reaction by peroxidases and incorporated into the oocyst wall as dityrosine bonded peptids (92).

Interestingly, these di-tyrosine bonds are also responsible for the autofluorescence typical of coccidian oocysts under UV-light (27,93), a phenomenon that is utilized for detection of *C. suis* oocysts in piglet feces (94,95). The second group are cysteine-rich proteins called oocyst wall proteins, OWP. These proteins are found in the wall forming bodies type 2. The cysteine residues form disulfide bridges and are responsible for the stabilization and formation of the oocyst wall and sporocyst wall, conferring additional strength and rigidity to the wall.

Transcriptomic analysis of *C. suis* sexual stages (**Publication 3**), could identify two genes (CSUI_008806 and CSUI_006207) that show homology to the cysteine-rich TgOWP6 and TgOWP1 in *Toxoplasma* (33) and three hypothetical proteins which are tyrosine-rich and homologous to genes identified in the oocyst proteome of *T. gondii* and the sporocyst wall of *E. tenell*a (24,49,96).

Furthermore, genes expected to code for the protein OWP1 in *C. suis* showed higher transcription levels on doc 10 and 13 in both *in vitro* culture systems used in this project (**Publication 1**), which was expected, as the amount of macrogametes and oocysts is highest during this period. Even in the host cell free *in vitro* system the transcription level of OWP1 was 9-fold higher than on cultivation days when only asexual stages occurred. The oocyst wall, however, appeared morphologically altered in the host cell-free culture, as the oocysts seem lopsided, so the expression on OWPs alone does not necessarily correlate with the morphology of the oocysts (**Publication 2**). However *Cr. parvum* also showed increased levels of OWP after 72 h of cultivation in a host cell free culture system (72), which supports our findings.

Most interestingly, similar transcription levels were shown for the gene CSUI_001473 in **Publication 1**, which encodes for the tyrosin rich protein (TyRP) found in female gametocytes and oocysts and is an orthologue with a 54% of homology with the gene coding a tyrosine rich protein in Toxoplasma (TGME49_037080) (96). Oocysts of *C. suis* display characteristic autofluorescence due to their dityrosine bonds between tyrosine-rich proteins in the oocyst wall

(95,97). Moreover, oocysts derived from the host cell-free culture system also showed autofluorescence, and TyRP was distinctly upregulated on doc 9 and doc 10, as also shown in the development in *in vitro* cell cultures (**Publication 2**). This also demonstrates that oocyst wall formation is possible in a cell-free environment.

To test if CSUI_001473 is a component of the oocyst wall we performed immunolocalisation studies, against TyRP using chicken anti-rCSUI_001473 serum (**Publication 3**). TyRP localizes in *C. suis* macrogametes, specifically to the periphery of the parasite cell, and to the outer wall of the unsporulated and sporulated oocyst. This also confirms that *C. suis* is a member of the oocyst wall protein family and that the crosslinking of tyrosine-rich proteins occurs in oocysts. This specific oocyst wall structure seems to be responsible for the resistance to extreme environmental stress oocysts have to deal with (49,96).

Currently no genetic manipulation technique is available for *C. suis* to confirm the direct involvement of CSUI_001473 in oocyst formation. To test whether TyRP 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, as seen in Publication 2. Furthermore we showed in **Publication 3** that the treatment of *C. suis* with the recombinant antibody significantly decreased the numbers of early and late sexual stages. Especially the formation of late sexual stages and oocysts was reduced by 75% in comparison with a non-treated control group. This indicates that the TyRP would be a sufficient candidate for a transmission-blocking vaccine.

10. CONCLUSION AND OUTLOOK

In conclusion, this study provides an extensive characterisation of the stages involved in the fertilization process, with a focus on the role of micro- and macrogametes for parasite development, of *C. suis*, by studying the morphology and composition of these stages, as well as the transcription levels of selected genes that transcribe for stage-specific proteins. Furthermore, the *in vitro* cell culture system was improved to achieve the highest output of sexual stages and a novel host cell free *in vitro* culture system was developed. This new knowledge and methodology opens up new possibilities to evaluate the intervention effects targeting sexual stages of *C. suis*, and also opens up new avenues for the control of this, but also possibly related parasites by vaccination or selective drugs.

To date no commercial vaccines for controlling *C. suis* are available, and toltrazuril is the only licensed drug against *C. suis*, which arises the risks of generating toltrazuril-resistant parasites, which was already detected *in vitro* (98). Hence vaccination strategies against cystoisosporosis are a promising alterative for the future. Previous studies, in other Apicomplexan parasites, showed that blocking the fusion of micro- and macrogametes can be a novel tool for intervention in the control of coccidial infections. In *T. gondii* HAP2 knockout parasites fail to produce sporulated oocysts *in vivo* (88) and in *E. maxima* two glycosylated tyrosine-rich proteins of the wall-forming bodies were shown to induce a strong antibody response to other *Eimeria* species as well (33,47). A commercial vaccine, CoxAbic® with the native gametocyte antigens of *E. maxima* oocyst wall forming proteins as antigens that stimulate an immune response that blocked the construction of the oocyst wall, was developed and marketed for the vaccination of chicken in 2002 (89,90,91).

In this project we could also show that the Tyrosin-rich protein can be significantly inhibited by specific antibodies. The inhibition of TyRP stuns the formation of the oocyst wall, rendering the parasite non-infectious. The biological function of TyRP is presumably the same as in other

coccidian parasites, making it also a candidate for transmission-blocking strategies in *C. suis* and related species with a sexual development during its life cycle. Hence, anti-rTyRP could also block the construction of the oocyst wall in other coccidian parasites closely related to *C. suis*, similarly to the already commercialized vaccination strategy for four *Eimeria* species.

However, we have found a variety of possible candidates for vaccination strategies against cystoisosporiosis during our transcriptomic analysis that should be evaluated and characterized further in the future. Prohibiting a potential fusion of the gametes and further formation of the oocyst wall might be the best strategy to interrupt the parasites life cycle and prevent the formation of further infectious stages.

11. REFERENCES

1. Biester BW, Murray C. Studies in infections enteritis of swine. J Am Vet Med Assoc 1934;85:207-19.

2. Morrison DA. Evolution of the Apicomplexa: where are we now? Trends Parasitol. 2009;25(8):375–82.

3. Ruggiero MA, Gordon DP, Orrell TM, Bailly N, Bourgoin T, Brusca RC, et al. Correction: A higher level classification of all living organisms. PLoS One. 2015;10(6):1–54.

4. Stuart BP, Lindsay DS, Ernst J V., Gosser HS. *Isospora suis* Enteritis in Piglets. Vet Pathol. 1980;17(1):84–93.

5. Harleman JH, Meyer RC. Life cycle of *Isospora suis* in gnotobiotic and conventionalized piglets. Vet Parasitol. 1984;17(1):27–39.

6. Pinckney RD, Lindsay DS, Toivio-Kinnucan MA, Blagburn BL. Ultrastructure of *Isospora suis* during excystation and attempts to demonstrate extraintestinal stages in mice. Vet Parasitol. 1993;47(3–4):225–33.

7. Lindsay DS, Current WL, Ernst J V. Excystation of *Isospora suis* Biester, 1934 of swine. Zeitschrift für Parasitenkd Parasitol Res. 1983;69(1):27–34.

8. Lindsay DS, Houk AE, Mitchell SM, Dubey JP. Developmental Biology of Cystoisospora (Apicomplexa: Sarcocystidae) Monozoic Tissue Cysts . J Parasitol. 2014;100(4):392–8.

9. Worliczek HL, Buggelsheim M, Saalmüller A, Joachim A. Porcine isosporosis: Infection dynamics, pathophysiology and immunology of experimental infections. Wien Klin Wochenschr. 2007;119(3):33–9.

10. Harleman JH, Meyer RC. Isospora suis infection in piglets. A review. Vet Q. 1983;5(4):178–85.

11. Smith TG, Walliker D, Ranford-Cartwright LC. Sexual differentiation and sex determination in the Apicomplexa. Trends Parasitol. 2002;18(7):315–23.

12. Walker RA, Ferguson DJP, Miller CMD, Smith NC. Sex and Eimeria: A molecular perspective. Parasitology. 2013;140(14):1701–17.

13. Lindsay DS, Stuart BP, Wheat BE, Ernst J V. Endogenous development of the swine coccidium, *Isospora suis* Biester 1934. J Parasitol. 1980;66(5):771—779.

14. Mundt HC, Joachim A, Becka M, Daugschies A. *Isospora suis*: An experimental model for mammalian intestinal coccidiosis. Parasitol Res. 2006;98(2):167–75.

15. Worliczek HL, Ruttkowski B, Schwarz L, Witter K, Tschulenk W, Joachim A. *Isospora suis* in an epithelial cell culture system - an *in vitro* model for sexual development in coccidia. PLoS One. 2013;8(7):e69797–e69797.

16. Müller J, Hemphill A. In vitro culture systems for the study of apicomplexan parasites in farm animals. Int J Parasitol. 2013;43(2):115–24.

17. Ahmed N. Cultivation of parasites. Trop Parasitol. 2014;4(2):80.

18. Cruz-Bustos T, Feix AS, Ruttkowski B, Joachim A. Sexual development in non-human parasitic apicomplexa: Just biology or targets for control? Animals. 2021;11(10):1–18.

19. Gubbels MJ, Keroack CD, Dangoudoubiyam S, Worliczek HL, Paul AS, Bauwens C, et al. Fussing About Fission: Defining Variety Among Mainstream and Exotic Apicomplexan Cell Division Modes. Front Cell Infect Microbiol. 2020;10(June).

20. Scholtyseck E, Mehlhorn H, Hammond DM. Electron microscope studies of microgametogenesis in coccidia and related groups. Zeitschrift fur Parasitenkd. 1972;38(2):95–131.

21. Burrell A, Tomley FM, Vaughan S, Marugan-Hernandez V. Life cycle stages, specific organelles and invasion mechanisms of Eimeria species. Parasitology. 2020;147(3):263–78.

22. Walker RA, Ferguson DJP, Miller CMD, Smith NC. Sex and Eimeria: A molecular perspective. Parasitology. 2013;140(14):1701–17.

23. Klimes B, Bootes DG, Tanielian Z. Sexual differentiation of merozoites of *Eimeria tenella*. Parasitology. 1972;65(1):131–6.

24. Walker RA, Sharman PA, Miller CM, Lippuner C, Okoniewski M, Eichenberger RM, et al. RNA Seq analysis of the *Eimeria tenella* gametocyte transcriptome reveals clues about the molecular basis for sexual reproduction and oocyst biogenesis. BMC Genomics. 2015;16(1):94.

25. Madden PA, Vetterling JM. Scanning electron microscopy of *Eimeria tenella* microgametogenesis and fertilization. J Parasitol. 1977;63(4):607–10.

26. Press A. Scanning Electron Microscopy of *Eimeria tenella* Microgametogenesis and Fertilization Author (s): Philip A . Madden and John M . Vetterling Published by : Allen Press on behalf of The American Society of Parasitologists Stable URL : https://www.jstor.org. 2018;63(4):607–10.

27. Belli SI, Wallach MG, Luxford C, Davies MJ, Smith NC. Roles of tyrosine-rich precursor glycoproteins and dityrosine- and 3,4-dihydroxyphenylalanine-mediated protein cross-linking in development of the oocyst wall in the coccidian parasite *Eimeria maxima*. Eukaryot Cell. 2003;2(3):456–64.

28. Ferguson DJP, Dubremetz JF. The Ultrastructure of *Toxoplasma gondii* [Internet]. Second Edi. *Toxoplasma gondii*: The Model Apicomplexan - Perspectives and Methods: Second Edition. Elsevier; 2013: 19–59

29. Dubey JP. History of the discovery of the life cycle of *Toxoplasma gondii*. Int J Parasitol. 2009;39(8):877–82.

30. Dubey JP. Life cycle of *Cystoisospora felis* (Coccidia: Apicomplexa) in cats and mice. J Eukaryot Microbiol. 2014;61(6):637–43.

31. Attias M, Teixeira DE, Benchimol M, Vommaro RC, Crepaldi PH, De Souza W. The life-cycle of *Toxoplasma gondii* reviewed using animations. Parasites and Vectors . 2020;13(1):1–13.

32. Possenti A, Fratini F, Fantozzi L, Pozio E, Dubey JP, Ponzi M, et al. Global proteomic analysis of the oocyst/sporozoite of *Toxoplasma gondii* reveals commitment to a host-independent lifestyle. BMC Genomics. 2013;14:183.

33. Possenti A, Cherchi S, Bertuccini L, Pozio E, Dubey JP, Spano F. Molecular characterisation of a novel family of cysteine-rich proteins of *Toxoplasma gondii* and ultrastructural evidence of oocyst wall localisation. Int J Parasitol. 2010;40(14):1639–49.

34. Ramakrishnan C, Maier S, Walker RA, Rehrauer H, Joekel DE, Winiger RR, et al. An experimental genetically attenuated live vaccine to prevent transmission of *Toxoplasma gondii* by cats. Sci Rep. 2019 ;9(1):1474.

35. Qiu Y, Zhao Y, Liu F, Ye B, Zhao Z, Thongpoon S, et al. Evaluation of *Plasmodium vivax* HAP2 as a transmission-blocking vaccine candidate. Vaccine. 2020;38(13):2841–8.

36. Camacho-Nuez M, Hernández-Silva DJ, Castañeda-Ortiz EJ, Paredes-Martínez ME, Rocha-Martínez MK, Alvarez-Sánchez ME, et al. Hap2, a novel gene in *Babesia bigemina* is expressed in tick stages, and specific antibodies block zygote formation. Parasites and Vectors. 2017;10(1):1–9.

37. Fédry J, Liu Y, Péhau-Arnaudet G, Pei J, Li W, Tortorici MA, et al. The Ancient Gamete Fusogen HAP2 Is a Eukaryotic Class II Fusion Protein. Cell. 2017 Feb;168(5):904-915.e10.

38. Long PL. Coccidiosis of Man and Domestic Animals. Coccidiosis of Man and Domestic Animals. 2019.

39. Lindsay DS, Dubey JP. Neosporosis, Toxoplasmosis, and Sarcocystosis in Ruminants: An Update. Vet Clin North Am - Food Anim Pract. 2020;36(1):205–22.

40. Prakas P, Butkauskas D. Protozoan parasites from genus Sarcocystis and their investigations in Lithuania. Ekologija. 2012;58(1):45–58.

41. Verma SK, Lindsay DS, Grigg ME, Dubey JO. Isolation, Culture and Cryptopreservation of *Sarcocystis* species. Curr. Protoc. Microbiol. 2018; 45: 20D.1.1–20D.1.27.

42. Fayer R, Esposito DH, Dubey JP. Human infections with Sarcocystis species. Clin Microbiol Rev. 2015;28(2):295–311.

43. Scholtyseck E, Hammond DM. Electron microscope studies of macrogametes and fertilization in *Eimeria bovis*. Zeitschrift für Parasitenkd. 1970;34(4):310–8.

44. Ferguson DJP. *Toxoplasma gondii* and sex: Essential or optional extra? Trends Parasitol. 2002;18(8):351–5.

45. Levine N. Progress in Taxonomy of the Apicomplexan Protozoa. Rev Inst Med Trop Sa'o Paulo. 1988;35(4):518–20.

46. COLLEY FC. Fine Structure of Microgametocytes and Macrogametes of *Eimeria nieschulzi*. J Protozool. 1967;14(4):663–74.

47. Mai K, Sharman PA, Walker RA, Katrib M, de Souza D, McConville MJ, et al. Oocyst wall formation and composition in coccidian parasites. Mem Inst Oswaldo Cruz. 2009;104(2):281–9.

48. Su S, Hou Z, Liu D, Jia C, Wang L, Xu J, et al. Comparative transcriptome analysis of *Eimeria necatrix* third-generation merozoites and gametocytes reveals genes involved in sexual differentiation and gametocyte development. Vet Parasitol. 2018;252:35–46.

49. Fritz HM, Bowyer PW, Bogyo M, Conrad PA, Boothroyd JC. Proteomic analysis of fractionated Toxoplasma oocysts reveals clues to their environmental resistance. PLoS One. 2012/01/18. 2012;7(1):e29955–e29955.

50. Sharman PA, Smith NC, Wallach MG, Katrib M. Chasing the golden egg: Vaccination against poultry coccidiosis. Parasite Immunol. 2010;32(8):590–8.

51. Katrib M, Ikin RJ, Brossier F, Robinson M, Slapetova I, Sharman PA, et al. Stage-specific expression of protease genes in the apicomplexan parasite, *Eimeria tenella*. BMC Genomics. 2012;13:685.

52. Templeton TJ, Lancto CA, Vigdorovich V, Liu C, London NR, Hadsall KZ, et al. The Cryptosporidium oocyst wall protein is a member of a multigene family and has a homolog in Toxoplasma. Infect Immun. 2004;72(2):980–7.

53. Mai K, Sharman PA, Walker RA, Katrib M, Souza D De, McConville MJ, et al. Oocyst wall formation and composition in coccidian parasites . Vol. 104, Memórias do Instituto Oswaldo Cruz . scielo ; 2009. p. 281–9.

54. Krücken J, Hosse RJ, Mouafo AN, Entzeroth R, Bierbaum S, Marinovski P, et al. Excystation of *Eimeria tenella* sporozoites impaired by antibody recognizing gametocyte/oocyst antigens GAM22 and GAM56. Eukaryot Cell. 2008;7(2): 202–11.

55. Angrisano F, Sala KA, Da DF, Liu Y, Pei J, Grishin N V., et al. Targeting the Conserved Fusion Loop of HAP2 Inhibits the Transmission of *Plasmodium berghei* and falciparum. Cell Rep. 2017;21(10):2868–78.

56. Briggs LJ, Davidge JA, Wickstead B, Ginger ML, Gull K. More than one way to build a flagellum: comparative genomics of parasitic protozoa. Curr Biol. 2004 ;14(15):R611–2.

57. Yubuki N, Čepička I, Leander BS. Evolution of the microtubular cytoskeleton (flagellar apparatus) in parasitic protists. Mol Biochem Parasitol. 2016;209(1–2):26–34.

58. Wass MN, Stanway R, Blagborough AM, Lal K, Prieto JH, Raine D, et al. Proteomic analysis of Plasmodium in the mosquito: progress and pitfalls. Parasitology. 2012/02/16. 2012 ;139(9):1131–45.

59. Sinden RE. Malaria, sexual development and transmission: Retrospect and prospect. Parasitology. 2009;136(12):1427–34.

60. Miao J, Chen Z, Wang Z, Shrestha S, Li X, Li R, et al. Sex-Specific Biology of the

Human Malaria Parasite Revealed from the Proteomes of Mature Male and Female Gametocytes. Mol Cell Proteomics. . 2017;16(4):537–51.

61. Wong JL, Johnson MA. Is HAP2-GCS1 an ancestral gamete fusogen? Trends Cell Biol. 2010 ;20(3):134–41.

62. Liu Y, Tewari R, Ning J, Blagborough AM, Garbom S, Pei J, et al. The conserved plant sterility gene HAP2 functions after attachment of fusogenic membranes in Chlamydomonas and Plasmodium gametes. Genes Dev. 2008;22(8):1051–68.

63. Tohno M, Ueda W, Azuma Y, Shimazu T, Katoh S, Wang JM, et al. Molecular cloning and functional characterization of porcine nucleotide-binding oligomerization domain-2 (NOD2). Mol Immunol. 2008;45(1):194–203.

64. Paterson B, Desser S. An Ultrastructural Study of Microgametogenesis and the Microgamete in *Eimeria iroquoina* Molnar and Fernando , 1974 , in Experimentally Infected Fathead Minnows (*Pimephales promelas* , Cyprinidae): W . Brockley Paterson and Sherwin S . Desser. J Parasitol. 1981;67(3):314–24.

65. Hirai M, Mori T. Fertilization is a novel attacking site for the transmission blocking of malaria parasites. Acta Trop. 2010;114(3):157–61.

66. Stuart BF, Bedell DM, Lindsay DS. Coccidiosis in swine: effect of disinfectants on *in vitro* sporulation of *Isospora suis* oocysts. Vet Med Small Anim Clin. 1981;76(8):1185–6.

67. Joachim A, Shrestha A. Coccidiosis of Pigs. Coccidiosis Livestock, Poultry, Companion Anim Humans. 2019;125–45.

68. Shrestha A, Abd-Elfattah A, Freudenschuss B, Hinney B, Palmieri N, Ruttkowski B, et al. *Cystoisospora suis* - A model of mammalian cystoisosporosis. Front Vet Sci. 2015;2):1–9.

69. Maillot C, Favennec L, Francois A, Ducrotte P, Brasseur P. Sexual and Asexual Development of *Cryptosporidium parvum* in Five Oocyst- or Sporozoite-Infected Human Enterocytic Cell Lines. J Euk Microbiol. 1997;44(6):582–5.

70. Tandel J, English ED, Sateriale A, Gullicksrud JA, Beiting DP, Sullivan MC, et al. Life cycle progression and sexual development of the apicomplexan parasite *Cryptosporidium parvum*. Nat Microbiol. 2019;4(12):2226–36.

71. Hijjawi N, Estcourt A, Yang R, Monis P, Ryan U. Complete development and multiplication of *Cryptosporidium hominis* in cell-free culture. Vet Parasitol. 2010;169(1–2):29–36.

72. Hijjawi NS, Meloni BP, Ng'anzo M, Ryan UM, Olson ME, Cox PT, et al. Complete development of *Cryptosporidium parvum* in host cell-free culture. Int J Parasitol. 2004;34(7):769–77.

73. Hijjawi NS, Meloni BP, Ryan UM, Olson ME, Thompson RCA. Successful in vitro cultivation of *Cryptosporidium andersoni*: Evidence for the existence of novel extracellular stages in the life cycle and implications for the classification of Cryptosporidium. Int J Parasitol. 2002;32(14):1719–26.

74. Kurth M, Entzeroth R. Reporter gene expression in cell culture stages and oocysts of *Eimeria nieschulzi* (Coccidia, Apicomplexa). Parasitol Res. 2009;104(2):303–10.

75. Bussière FI, Niepceron A, Sausset A, Esnault E, Silvestre A, Walker RA, et al. Establishment of an in vitro chicken epithelial cell line model to investigate *Eimeria tenella* gamete development. Parasites and Vectors. 2018;11(1):1–8.

76. Matuschka F -r., Heydorn AO. Die Entwicklung von *Isospora suis* Biester und Murray 1934 (Sporozoa:Coccidia:Eimeriidae) im Schwein. 1980;26(3):405–76.

77. Wiedmer S, Erdbeer A, Volke B, Randel S, Kapplusch F, Hanig S, et al. Identification and analysis of *Eimeria nieschulzi* gametocyte genes reveal splicing events of gam genes and conserved motifs in the wall-forming proteins within the genus Eimeria (Coccidia, Apicomplexa). Parasite. 2017;24(2).

78. Girouard D, Gallant J, Akiyoshi DE, Nunnari J, Tzipori S. Failure to propagate *Cryptosporidium spp.* in cell-free culture. J Parasitol. 2006;92(2):399–400.

79. Poran A, Nötzel C, Aly O, Mencia-Trinchant N, Harris CT, Guzman ML, et al. Single-cell RNA sequencing reveals a signature of sexual commitment in malaria parasites. Nature. 2017;551(7678):95–9.

80. Brancucci NMB, De Niz M, Straub TJ, Ravel D, Sollelis L, Birren BW, et al. Probing *Plasmodium falciparum* sexual commitment at the single-cell level. Wellcome Open Res. 2018;3:70.

81. Neveu G, Beri D, Kafsack BF. Metabolic regulation of sexual commitment in *Plasmodium falciparum*. Curr Opin Microbiol. 2020;58:93–8.

82. Talman AM, Prieto JH, Marques S, Ubaida-Mohien C, Lawniczak M, Wass MN, et al. Proteomic analysis of the Plasmodium male gamete reveals the key role for glycolysis in flagellar motility. Malar J. 2014;13(1).

83. Lasonder E, Rijpma SR, Van Schaijk BCL, Hoeijmakers WAM, Kensche PR, Gresnigt MS, et al. Integrated transcriptomic and proteomic analyses of P. Falciparum gametocytes: Molecular insight into sex-specific processes and translational repression. Nucleic Acids Res. 2016;44(13):6087–101.

84. Khan SM, Franke-Fayard B, Mair GR, Lasonder E, Janse CJ, Mann M, et al. Proteome Analysis of Separated Male and Female Gametocytes Reveals Novel Sex-Specific PlasmodiumBiology. Cell. 2005;121(5):675–87.

85. Su S, Hou Z, Liu D, Jia C, Wang L, Xu J, et al. Comparative transcriptome analysis of *Eimeria necatrix* third-generation merozoites and gametocytes reveals genes involved in sexual differentiation and gametocyte development. Vet Parasitol. 2018;252:35–46.

86. Matos LVS, McEvoy J, Tzipori S, Bresciani KDS, Widmer G. The transcriptome of Cryptosporidium oocysts and intracellular stages. Sci Rep. 2019;9(1):7856.

87. Angrisano F, Sala KA, Da DF, Liu Y, Pei J, Grishin N V, et al. Targeting the Conserved Fusion Loop of HAP2 Inhibits the Transmission of *Plasmodium berghei* and falciparum. Cell Rep. 2017;21(10):2868–78.

88. Feng J, Dong X, Decosta A, Su Y, Angrisano F, Sala KA, et al. Structural basis of Malaria transmission blockade by a monoclonal antibody to gamete fusogen HAP2. Elife. 2021;10:1–23.

89. Hussein HE, Bastos RG, Schneider DA, Johnson WC, Adham FK, Davis WC, et al. The *Babesia bovis* hap2 gene is not required for blood stage replication, but expressed upon *in vitro* sexual stage induction. PLoS Negl Trop Dis. 2017;11(10):e0005965–e0005965.

90. Wallach MG, Mencher D, Yarus S, Pillemer G, Halabi A, Pugatsch T. *Eimeria maxima*: Identification of gametocyte protein antigens. Exp Parasitol. 1989 ;68(1):49–56.

91. Spano F, Puri C, Ranucci L, Putignani L, Crisanti A. Cloning of the entire COWP gene of *Cryptosporidium parvum* and ultrastructural localization of the protein during sexual parasite development. Parasitology. 1997;114(5):427–37.

92. Carvalho TG, Doerig C, Reininger L. Nima- and Aurora-related kinases of malaria parasites. Biochim Biophys Acta - Proteins Proteomics. 2013;1834(7):1336–45.

93. Varea M, Clavel A, Doiz O, Castillo FJ, Rubio MC, Gómez-Lus R. Fuchsin fluorescence and autofluorescence in Cryptosporidium, Isospora and Cyclospora oocysts. Int J Parasitol. 1998;28(12):1881–3.

94. Daugschies A, Bialek R, Joachim A, Mundt HC. Autofluorescence microscopy for the detection of nematode eggs and protozoa, in particular *Isospora suis*, in swine faeces. Parasitol Res. 2001;87(5):409–12.

95. Joachim A, Ruttkowski B, Sperling D. Detection of *Cystoisospora suis* in faeces of suckling piglets -- when and how? A comparison of methods. Porc Heal Manag. 2018 ;4(1):20.

96. Fritz HM, Buchholz KR, Chen X, Durbin-Johnson B, Rocke DM, Conrad PA, et al. Transcriptomic analysis of toxoplasma development reveals many novel functions and structures specific to sporozoites and oocysts. PLoS One. 2012;7(2):e29998–e29998.

97. Worliczek HL, Ruttkowski B, Joachim A, Saalmüller A, Gerner W. Faeces, FACS, and functional assays - Preparation of *Isospora suis* oocyst antigen and representative controls for immunoassays. Parasitology. 2010;137(11):1637–43.

98. Shrestha A, Freudenschuss B, Jansen R, Hinney B, Ruttkowski B, Joachim A. Experimentally confirmed toltrazuril resistance in a field isolate of *Cystoisospora suis*. Parasites and Vectors. 2017;10(1):1–9.

12. APPENDIX

12.1 Conference contributions with oral presentation

1. Feix Anna Sophia, Cruz-Bustos Teresa, Ruttkowski Bärbel, Joachim Anja. Untersuchungen an sexuellen Entwicklungsstadien von *Cystoisospora suis.* Tagung der DVG-Fachgruppe "Parasitologie und parasitäre Krankheiten". 17.-19.06.2019. Leipzig, Germany

2. Feix Anna Sophia, Cruz-Bustos Teresa, Ruttkowski Bärbel, Joachim Anja. *Cystoisospora suis* as an example for coccidian sexual stages. Parasitologische Fachgespräche, 08.11.2019, Wien

3. Feix Anna Sophia, Cruz-Bustos Teresa, Ruttkowski Bärbel, Joachim Anja. Die Befruchtung der sexuellen Stadien bei *Cystoisospora suis.* Tagung der DVG-Fachgruppe "Parasitologie und parasitäre Krankheiten". 15-17.10 2020. Online

4. Feix Anna Sophia, Cruz-Bustos Teresa, Ruttkowski Bärbel, Mötz Marlene, Rümenapf Till, Joachim Anja. Erste Einblicke in ein wirtszellenfreies *in vitro* System für *Cystoisospora suis* . Tagung der DVG-Fachgruppe "Parasitologie und parasitäre Krankheiten". 28-30.06.2021. Online

5. Feix Anna Sophia, Cruz-Bustos Teresa, Ruttkowski Bärbel, Mötz Marlene, Rümenapf Till, Joachim Anja. Life cycle progression in a novel host cell-free environment of *Cystoisospora suis* as a model for Coccidia. WAAVP. 19-22.07.2021. Dublin, Ireland

6. Feix Anna Sophia, Cruz-Bustos Teresa, Ruttkowski Bärbel, Joachim Anja. Wie man durch das gezielte Hemmen von für sexuelle Stadien exprimierende Proteine den Lebenszyklus von *C. suis* unterbrechen kann. Tagung der DVG-Fachgruppe "Parasitologie und parasitäre Krankheiten". 23-25.05.2022. Berlin, Germany

12.2 Conference contributions with poster presentation

1. Feix Anna Sophia, Cruz-Bustos Teresa, Ruttkowski Bärbel, Joachim Anja. Comparison of *Cystoisospora suis* developmental stages in cell culture and in a novel cell free culture system, APICOWPLEXA, 2 -4 October 2019, Berlin, Germany

2. Feix Anna Sophia, Cruz-Bustos Teresa, Ruttkowski Bärbel, Joachim Anja. Entwicklungsstadien von *Cystoisospora suis* in einem zellfreien Kultivierungssystem, Tagung der DVG-Fachgruppe "Parasitologie und parasitäre Krankheiten". 15-17.10. 2020. online

12.3 Awards

Thomas-Schnieder-Nachwuchspreis: first price for a first-time presentation, Jahrestagung der DVG-Fachgruppe „Parasitologie und parasitäre Krankheiten" in Leipzig, 17. -19.6. 2021, Presentation: „Untersuchungen zu sexuellen Entwicklungsstadien von *Cystoisospora suis*"

Early Career Research Award 2022: for papers published in the journal "Parasitology" (Cambridge University Press) in 2021 for Feix A, Cruz-Bustos T, Ruttkowski B, Mötz M, Rümenapf T, Joachim A. (2021) Progression of asexual to sexual stages of *Cystoisospora suis* in a host cell-free environment as a model for Coccidia. Parasitology 148 (12):1475-1481. https://doi.org/10.1017/S0031182021001074