



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

Anna Sophia Feix<sup>1</sup> , Teresa Cruz-Bustos<sup>1</sup>, Bärbel Ruttkowski<sup>1</sup>, Marlene Mötzt<sup>2</sup>, Till Rümenapf<sup>2</sup> and Anja Joachim<sup>1</sup> 

<sup>1</sup>Department of Pathobiology, Institute of Parasitology, University of Veterinary Medicine, Vienna, Austria and

<sup>2</sup>Department of Pathobiology, Institute of Virology, University of Veterinary Medicine, Vienna, Austria

## Research Article

**Cite this article:** Feix AS, Cruz-Bustos T, Ruttkowski B, Mötzt 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; *Isoospora suis*; qPCR

### Author for correspondence:

Anna Sophia Feix,

E-mail: [Anna.Feix@vetmeduni.ac.at](mailto: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 Coccidomorpha, 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. *Isoospora 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 (Scholtyssek *et al.*,

© The Author(s), 2021. Published by Cambridge University Press. This is an Open Access article, distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike licence (<http://creativecommons.org/licenses/by-nc-sa/4.0/>), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the same Creative Commons licence is included and the original work is properly cited. The written permission of Cambridge University Press must be obtained for commercial re-use.

**CAMBRIDGE**  
UNIVERSITY PRESS

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 (Hijawi 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 (Hijawi 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*.

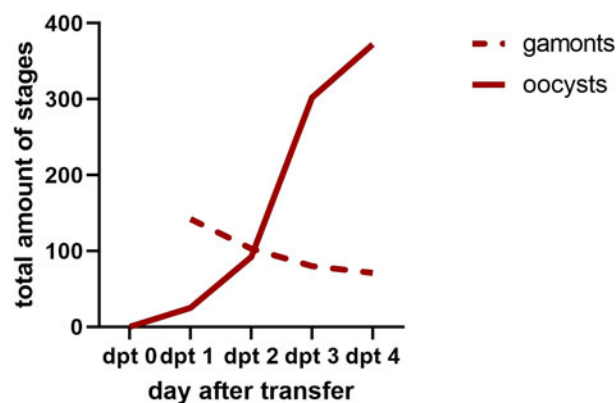
## Materials and methods

### 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,  $5 \times 10^3$  sporozoites were released into fresh Advanced<sup>®</sup> 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<sup>®</sup>  $\mu$ -slide (ibidi, Gräfelting, Germany) and were incubated at 40°C under 5% CO<sub>2</sub>. 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<sup>®</sup> (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<sup>®</sup> 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



**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. \*\*\*\* $P < 0.0001$ .

ibiTreat<sup>®</sup>  $\mu$ -slide (ibidi, Gräfelting, Germany) at a concentration of  $1.2 \times 10^5$  merozoites per mL medium and were incubated at 40°C under 5% CO<sub>2</sub>. The development of parasite stages was monitored daily, and stages were harvested at 3 and 4 dpt (days post transfer), pelleted and stored at  $-20^\circ\text{C}$ .

### 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  $\mu\text{L}$  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.

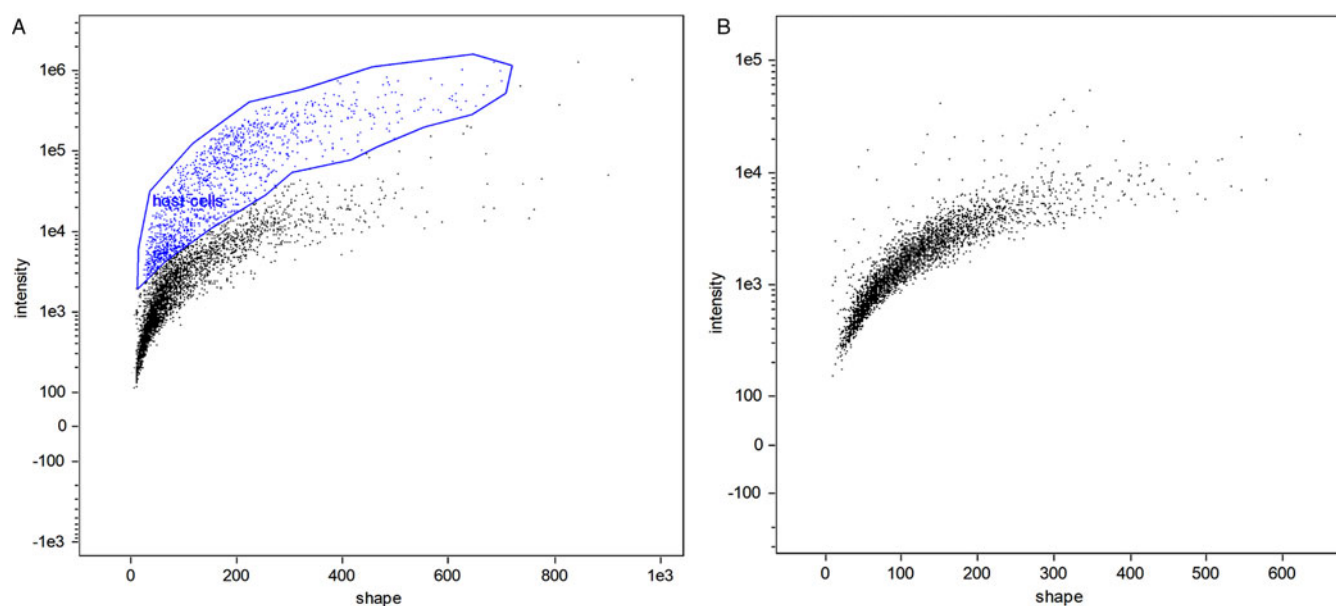
Digital microphotographs of all *C. suis* stages in culture were taken with an Olympus IX71 inverse microscope (Olympus, Shinjuku, Japan) at 400 $\times$  and 600 $\times$  magnification. Autofluorescence of oocysts was detected at UV 385 nm.

### Flow cytometry

Purified parasite stages harvested 4 dpt were stained with 5  $\mu\text{g}/\text{mL}$  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<sup>®</sup> FlowSight<sup>®</sup> 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<sup>®</sup> 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<sup>®</sup> 2000 (Thermo Fischer Science, Waltham, USA). For reverse transcription, an iScript<sup>®</sup> 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 dpi.

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  $\mu$ L of sample DNA (100 ng/ $\mu$ L), 5  $\mu$ L of SsoAdvanced™ Universal Probes Supermix (Bio-Rad) and 1.3  $\mu$ L 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

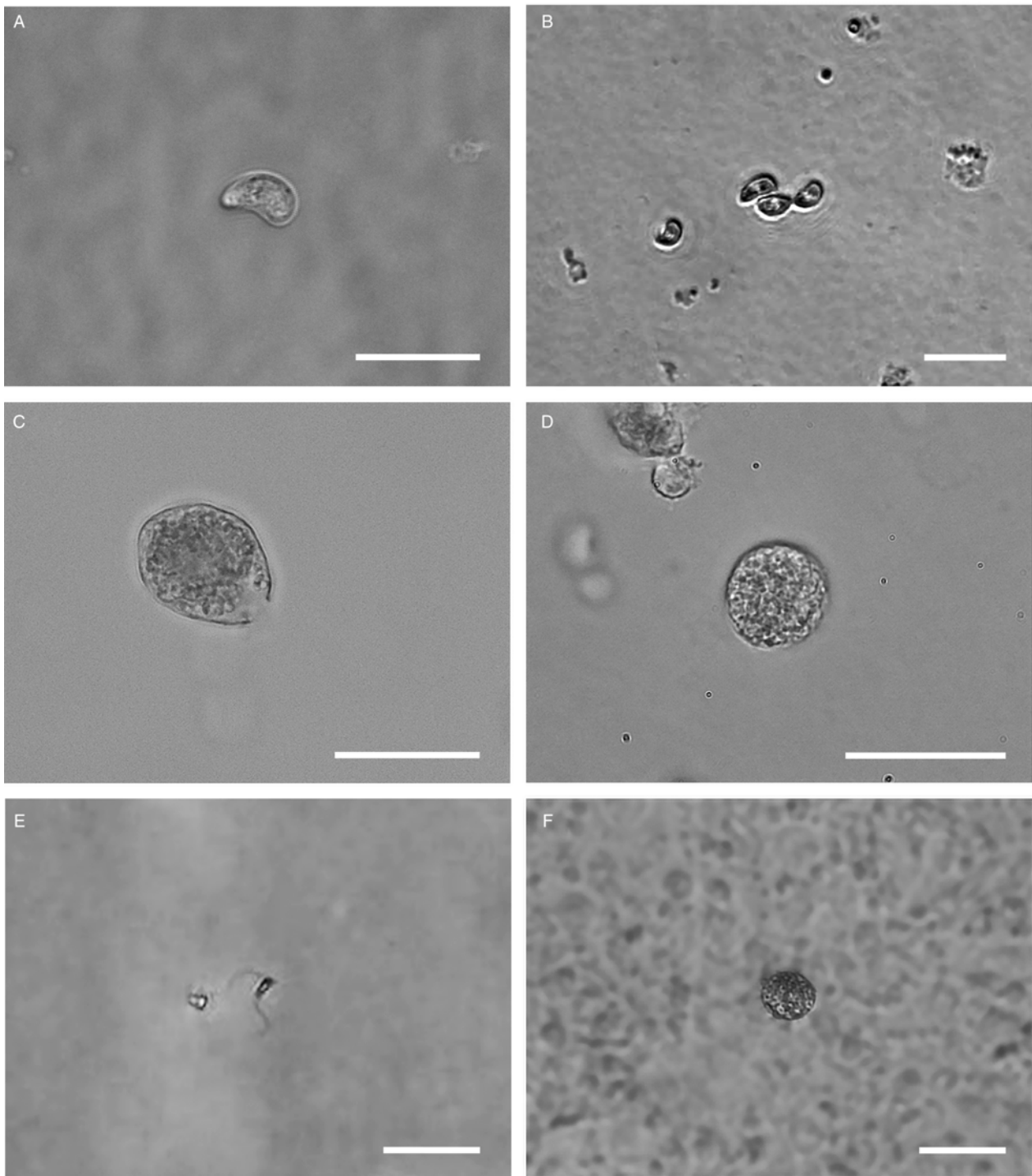
### Morphology

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  $1.2 \times 10^5$  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.

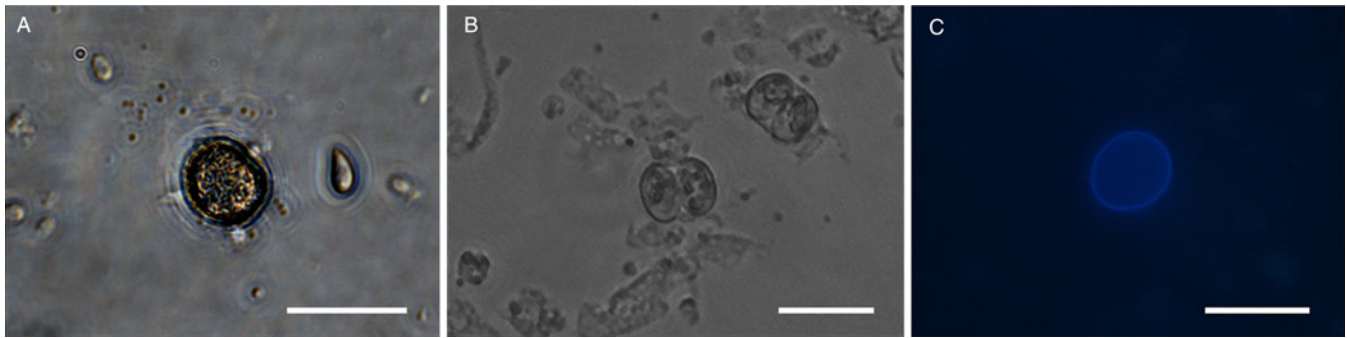
All life cycle stages were morphologically comparable to *in vitro* cell cultures (Fig. 3). Second-generation merozoites (Fig. 3A and B) could be detected 4 h after the start of the host cell-free culture. Previous studies showed male and female sexual stages and first oocysts from 120 h post infection *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  $\mu\text{m}$  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  $\mu\text{m}$ , 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  $\mu$ m.

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.*, 2012a; 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 microgamonts 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.*, 2012a, 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.

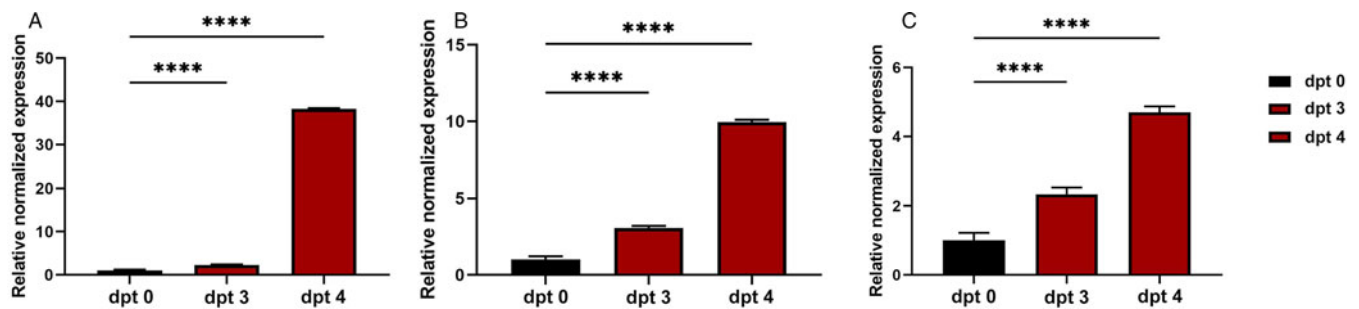
The sexual commitment has long been considered a crucial step in the development of Apicomplexan parasites (Kaushal *et al.*, 1980; Cornelissen, 1988; Bruce *et al.*, 1990); however, the underlying mechanisms are still mainly unclear (Smith *et al.*, 2002; Bechti and Waters, 2017). Sexually committed 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

	<i>In vivo</i> (Harleman and Meyer, 1984)	<i>In vitro</i> (Feix et al., 2020)	<i>In vitro</i> cell-free
Dose <sup>a</sup>	2.4 × 10 <sup>5</sup> sporulated oocysts/piglet	5 × 10 <sup>3</sup> sporozoites/ 4 × 10 <sup>5</sup> host cells	1.2 × 10 <sup>5</sup> merozoites per mL host cells <sup>b</sup>
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)

<sup>a</sup>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).

<sup>b</sup>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 (Bechtesi 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 *Isoospora* (Schneider 1881) species infecting birds and resurrection of *Cystoisospora* (Frenkel 1977) as the correct genus for *Isoospora* species infecting mammals. *Journal of Parasitology* **91**, 726–727.
- Bechtesi 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) monozyotic 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.
- Scholtyssek 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.