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Lifecycle Progression And The Sexual Development Of The Apicomplexan Parasite Cryptosporidium Parvum

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Abstract

Cryptosporidium has emerged as one of the leading causes of diarrhea induced-mortality in children and immunocompromised HIV+ individuals. Other than the acute infection, chronic and asymptomatic cryptosporidiosis results in stunted physical and mental development in children. Drugs and vaccines are needed to combat cryptosporidiosis, and a better understanding of the biology of the parasite will help in developing therapeutics against the parasite. Cryptosporidium has a single host lifecycle. Ingested meiotic spores called oocysts release invasive sporozoites in gut. Sporozoites infect intestinal enterocytes where parasites multiply asexually followed by sexual differentiation. Parasites have sex and then undergo sporulation in the host to produce mature oocysts. Oocysts re-infect the host or are transmitted via feces. Cryptosporidium infection in cancerous cell lines (HCT-8 and Caco-2) lasts for only three days but mice stay infected for a month We engineered a strain that allows to discern different stages and used it to study the developmental kinetics in HCT-8 cells and mice. Parasites replicated asexually in culture followed by sexual differentiation of the 80% of the total population after 48 hours. However, parasites failed to fertilize in culture. Contrastingly, parasites undergo mating, post-fertilization development and sporulation in mice. These studies suggested that sex and renewed production of oocyst is necessary to maintain infection in a host. As a result, disruption of sexual development or mating should break the cycle of infection in mice. This requires an in-depth understanding of sexual stage processes. We identified sexual stage-specific markers and engineered male- and female-specific reporter strains to isolate sexual stages from infected mice and culture for RNA sequencing. Sexual stages were enriched for genes required for meiosis, oocyst development, gamete recognition and fusion. Transcriptional analyses further confirmed four sex specific ApiAP2 genes, and ApiAP2s in Plasmodium are involved in stage-specific development. We intend to disrupt sexual development by targeting one of these ApiAP2s, and cgd4_1110 was confirmed as an essential, female specific ApiAP2. We engineered rapamycin inducible DiCre gene KO system to conditionally disrupt AP2-F. Our next step is to conditionally disrupt AP2-F in infected mice to test essentiality of sex to maintain infection.

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LIFECYCLE PROGRESSION AND SEXUAL DEVELOPMENT OF THE APICOMPLEXAN PARASITE *CRYPTOSPORIDIUM PARVUM*

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LIFE CYCLE PROGRESSION AND SEXUAL DEVELOPMENT OF THE APICOMPLEXAN PARASITE *CRYPTOSPORIDIUM PARVUM*

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ABSTRACT

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Jayesh Tandel
Boris Striepen

Cryptosporidium has emerged as one of the leading causes of diarrhea induced-mortality in children and immunocompromised HIV^+ individuals. Other than the acute infection, chronic and asymptomatic cryptosporidiosis results in stunted physical and mental development in children. Drugs and vaccines are needed to combat cryptosporidiosis, and a better understanding of the biology of the parasite will help in developing therapeutics against the parasite. *Cryptosporidium* has a single host lifecycle. Ingested meiotic spores called oocysts release invasive sporozoites in gut. Sporozoites infect intestinal enterocytes where parasites multiply asexually followed by sexual differentiation. Parasites have sex and then undergo sporulation in the host to produce mature oocysts. Oocysts re-infect the host or are transmitted via feces. *Cryptosporidium* infection in cancerous cell lines (HCT-8 and Caco-2) lasts for only three days but mice stay infected for a month We engineered a strain that allows to discern different stages and used it to study the developmental kinetics in HCT-8 cells and mice. Parasites replicated asexually in culture followed by sexual differentiation of the 80% of the total population after 48 hours. However, parasites failed to fertilize in culture. Contrastingly, parasites undergo mating, post-fertilization development and sporulation in mice. These studies suggested that sex and renewed production of oocyst is necessary to maintain infection in a host. As a result, disruption of sexual development or mating should break the cycle of infection in mice. This requires an in-depth understanding of sexual stage processes. We identified sexual stage-specific markers

and engineered male- and female-specific reporter strains to isolate sexual stages from infected mice and culture for RNA sequencing. Sexual stages were enriched for genes required for meiosis, oocyst development, gamete recognition and fusion. Transcriptional analyses further confirmed four sex specific ApiAP2 genes, and ApiAP2s in *Plasmodium* are involved in stage-specific development. We intend to disrupt sexual development by targeting one of these ApiAP2s, and cgd4_1110 was confirmed as an essential, female specific ApiAP2. We engineered rapamycin inducible DiCre gene KO system to conditionally disrupt AP2-F. Our next step is to conditionally disrupt AP2-F in infected mice to test essentiality of sex to maintain infection.

Table of Contents

List of Tables

List of Illustrations

List of Abbreviations

AIDS, Acquired Immunodeficiency Syndrome

ANOVA, Analysis of Variance

ApiAP2, Apicomplexan Apetela-2

AR, Acrosome Reaction

bp, base pair

BSA, Bovine Serum Albumin

cAMP, 3',5'-cyclic adenosine monophosphate

Cas9, CRISPR-associated protein-9

Catsper, Cation channels of Sperm

CDC, Center for Disease Control

COWP, *Cryptosporidium* Oocyst Wall Protein

CRISPR, Clustered Regularly Interspaced Palindromic Repeats

DALYs, Disability-Adjusted Life Years

DAPI, 4′,6-diamidino-2-phenylindole

DiCre, Dimerizable cre recombinase

DMC1, Disruption of Meiotic Control-1

DMRT-1, Doublesex and Mab-3 Related Transcription factor 1

DNA, Deoxyribose Nucleic Acid

EDTA, ethylenediaminetetraacetic acid

EdU, 5-ethynyl-2’-deoxyuridine

ETEC, Enterotoxigenic Escherichia coli

FACS, Fluorescence-Activated Cell Sorting

FDR, False Discovery Rate

G1, Gap1

G2, Gap2

GCS-1, Generative Cell Specific-1

GE, General Electrics

GEMS, Global Enteric Multicenter Study

GO, Gene Ontology

gp60, glycoprotein 60

gp900, glycoprotein 900

GSEA, Gene Set Enrichment Analysis

H2B, Histone 2B

H3K9Ac, Histone 3-K9 Acetylated

HA, Hemeagglutinnin

HAP2, Hapless-2

HEPES, hydroxyethyl piperazineethanesulfonic acid

HIV, Human Immunodeficiency Virus

HOP2, Homologous-pairing protein-2

HORMA, HOP1, REV7 and MAD2

HSP70, Heat Shock Protein-70

ICZN, International Code of Zoological Nomenclature

IFN-γ, Interferon-γ

KO, Knockout

lysoPC,Lysophosphatidyl choline

M, Metaphase

MAL-ED, Malnutrition and Enteric Disease Study

mNG, monomeric Neon-Green

MPA, *Macula pomifera* Agglutinin

mRNA, messenger Ribonucleic Acid

NIH, National Institute of Health

NIMA, Never In Mitosis Gene-A

PBS, Phosphate Buffered Saline

PCA, Principal Component Analysis

PCR, Polymerase Chain Reaction

PFA, Paraformaldehyde

RAG-1, Recombination Activating-1

RBC, Red Blood Cells

RNA, Ribonucleic Acid

RPMI, Roswell Park Memorial Institute

rRNA, ribosomal Ribonucleic acid

SCID, Severe and Combined Immunodeficiency

SRY, Sex-determining Region Y

SSU, Small Subunit

TK, Thymidine Kinase

TRAP-C1, Thrombospondin-Related Adhesive Protein of *Cryptosporidium*-1

TrpB, Tryptophan Synthase B

U.K., United Kingdom

U.S., United States

U.V., Ultraviolet

VVL, *Vicia Villosa* Lectin

WHO, World Health Organization

WI, Wisconsin

YFP, Yellow Fluorescent Protein

Chapter 1: Introduction

Cryptosporidium, an apicomplexan parasite, recently emerged a leading cause of diarrheal illness and mortality in young children and immunocompromised individuals.(Khalil et al., 2018a; Kotloff et al., 2013; Liu et al., 2012) Ernest Edward Tyzzer was the first to observe *Cryptosporidium* in the gastric glands of laboratory mice(Tyzzer, 1907), and it was initially thought of as a benign commensal frequently observed in vertebrates.(Levine, 1980) However, multiple studies confirmed *Cryptosporidium* as the causative agent of acute and chronic enteric disease in children(Khalil et al., 2018b; Kotloff et al., 2013; Liu et al., 2012; Shoultz et al., 2016) and individuals suffering from HIV-AIDS(Ma, 1984; Ma and Soave, 1983), respectively. Many reports of *Cryptosporidium* outbreaks(Gharpure, 2019) and findings of longitudinal studies(Khalil et al., 2018b; Kotloff et al., 2013; Liu et al., 2012) have confirmed *Cryptosporidium* as one of the major water- and food-borne pathogens. Unfortunately, early efforts to develop anti-cryptosporidial drugs(Amadi et al., 2009) and vaccines(Lemieux et al., 2018; Mead, 2010, 2014) have been unsuccessful. *Cryptosporidium* has been a difficult pathogen to study in the laboratory, and the lack of a culture system and genetic tools have limited our understanding of the biology of the parasite and our ability to develop effective countermeasures.

In 1907, Tyzzer described the morphological appearance and the developmental sequence of asexual and sexual stages of *Cryptosporidium* in the gut of infected mice.(Tyzzer, 1907) Many subsequent studies have further characterized asexual and sexual stages of *Cryptosporidium* in a variety of infected hosts in ultrastructural detail.(Current and Reese, 1986; Ostrovska and Paperna, 1990) However, limited progress has been made in understanding the molecular mechanisms underlying the

complex developmental transitions that *Cryptosporidium* undergoes throughout its lifecycle. Recent breakthroughs like the development of genetic tools(Vinayak et al., 2015) and continuous culture systems(Heo et al., 2018; RePass et al., 2017; Wilke et al., 2019) have now made it possible to dissect the molecular biology of the *Cryptosporidium* lifecycle.

Apicomplexan parasites have a complex lifecycle that alternates between an asexual and a sexual phase.(Cowman et al., 2016) The associated transformations from one lifecycle stage to another require complex molecular changes modulated by developmental regulators that change patterns of gene expression.(Hehl et al., 2015; Painter et al., 2017) The asexual replication phase allows apicomplexan parasites to amplify in numbers and often compete with other pathogens or genotypes for resources. Contrastingly, sexual development allows parasites to diversify their gene pools. Apicomplexans such as *Toxoplasma*(Dubey et al., 2011), *Eimeria*(Graat et al., 1994) and *Cryptosporidium*(Ostrovska and Paperna, 1990) are transmitted through environmentally resilient cyst forms. Ingested *Eimeria*(Graat et al., 1994) oocysts infect the gut of a host and initiate asexual replication followed by obligatory sexual development. The sexual stages of *Eimeria* mate and the zygotic stage develops into an immature, unsporulated oocyst (not infectious). Unsporulated oocysts are shed through feces where they undergo a process of sporogony to produce sporulated oocysts (infectious form) with mature sporozoites.(Graat et al., 1994) *Toxoplasma* in felids undergoes a developmental process similar to *Eimeria*.(Dubey et al., 2011) *Cryptosporidium* undergoes a similar lifecycle development like *Eimeria*.(Ostrovska and Paperna, 1990) However, the zygotic stages of *Cryptosporidium* mature into infectious, sporulated oocysts within the host that reinfect the same host.(Current and Reese, 1986;

Tyzzer, 1907) This feature allows sex in *Cryptosporidium* to serve as the source of infection and transmission and raises the following questions about the lifecycle of the parasite: **1. Does the parasite undergo obligatory sexual development after limited rounds of asexual amplification? 2. If yes then will the disruption of sexual development or sex attenuate infection in a host?**

To address these questions in my thesis, I developed and used a variety of molecular approaches. I have engineered reporter strains to distinguish different stages of *Cryptosporidium* and to observe the progression of the asexual and sexual phases of the parasite. I have exploited sex-specific molecular markers to engineer sex-specific reporter strains. This has allowed me to isolate different stages of the parasites and to define the molecular processes unique to each of them using transcriptomic approaches. Finally, I have developed a conditional gene knockout system and used it to disrupt the lifecycle and test whether sex is an obligatory requirement of continued infection in a host. These findings have profound implications for how the infection may be best treated and prevented.

1.1. Cryptosporidiosis is a major public health concern.

Cryptosporidium infection has been observed in a wide range of vertebrate animals including rhesus monkeys(Levine, 1980), snakes(Levine, 1980) and cattle(Pohlenz et al., 1978). *Cryptosporidium* was first detected in humans in a 3-year old child suffering from acute enterocolitis.(Nime et al., 1976) The rectal biopsy confirmed severe tissue changes but was inconclusive as to whether *Cryptosporidium* was the causative agent of the entercolitis.(Nime et al., 1976) A report published in 1980 unequivocally documented

human infection and the entire lifecycle of *Cryptosporidium* by electron microscopy of biopsy samples from patients with chronic diarrhea.(Bird and Smith, 1980) 6 of the 7 *Cryptosporidium*-positive individuals were found to be immunocompromised.(Bird and Smith, 1980). Multiple reports published in the 1980s established *Cryptosporidium* as a causative agent of acute and chronic diarrhea in children and adults (mostly immunocompromised).(Tzipori and Widmer, 2008)

1.1.1 Cryptosporidiosis in immunocompromised individuals.

Cryptosporidiosis is characterized by watery diarrhea, dehydration, nausea and vomiting, and these symptoms in immunocompetent individuals typically last from 1-2 weeks (but maybe protracted in some).(Mac Kenzie et al., 1994) Consistent with initial findings of *Cryptosporidium* in immunocompromised individuals(Bird and Smith, 1980), HIV^+ individuals were found to be extensively affected by cryptosporidiosis(Hunter and Nichols, 2002). Hunter and Nichols have reviewed the presence of *Cryptosporidium* in individuals immunocompromised due to HIV infection, genetic disorders, cancer chemotherapy, malnutrition and organ transplantation.(Hunter and Nichols, 2002)

Cryptosporidiosis is often found to take a much more chronic course (>5 weeks) in HIV^+ individuals.(Blanshard et al., 1992; Manabe et al., 1998) In addition to chronic illness, transient and recurring infections have also been reported in HIV^+ individuals.(Blanshard et al., 1992; Manabe et al., 1998) The severity of the symptoms and prognosis was predicted by $CD4^+$ T cell counts. A London-based study of HIV^+ individuals established that patients with a $CD4^+$ T count of less than $50/mm^3$ exhibited severe dehydration (passage of more than 2L stool/day).(Manabe et al., 1998) Similarly, an American study found that HIV^+ patients with $CD4^+$ T count $>53/mm^3$ had a median survival of 1,119

days compared to 204 days in individuals with $CD4^+$ T count <53/mm^3 (relative hazard of death, 2.01; 95% CI, 1.38 to 2.93).(Blanshard et al., 1992) Consistently, amelioration of $CD4^+$ T count by antiretroviral therapy has resulted in the resolution of symptoms(Maggi et al., 2000) and/or complete elimination of the disease(Miao et al., 2000). These empirical observations have been replicated in mouse models lacking T-cells(Sateriale et al., 2019), and control of the infection has been attributed to interferon-γ (IFN-γ)-dependent effector function of $CD4^+$ T cells(Hayward et al., 2000; Mead, 2014; Theodos et al., 1997). Hence, it can be concluded that the susceptibility of AIDS patients to *Cryptosporidium* is due to the loss of CD4 cells.

Primary immunodeficiencies are genetic disorders that impair the normal functions of the immune system often leading to severely increased susceptibility to infectious agents.(McCusker et al., 2018) Since such immune disorders are rare, longitudinal studies of cryptosporidiosis prevalence in this population are lacking.(Hunter and Nichols, 2002) However, there are reports of *Cryptosporidium* in patients affected by severe and combined immunodeficiency (SCID)(Kocoshis et al., 1984), X-linked hyper-immunoglobulin M syndrome(Levy et al., 1997), CD4 lymphopenia(Wolska-Kusnierz et al., 2007) and immunoglobulin A(Jacyna et al., 1990) deficiencies.

There does not seem to be strong evidence for higher susceptibility for cryptosporidiosis in patients suffering from cancer, but multiple reports suggest that patients receiving bone marrow transplantation might be more susceptible to the disease (Hunter and Nichols, 2002). An Italian study described cryptosporidiosis in 20 patients suffering from hematological malignancies that have undergone chemotherapy and bone marrow transplantation.(Gentile et al., 1991) These patients were either asymptomatic or had mild to severe diarrhea.(Gentile et al., 1991) Similar to cancer patients, there is no clear

evidence of a higher risk of cryptosporidiosis in organ transplant recipients(Hunter and Nichols, 2002). However, a Turkish study of 69 renal transplant recipients revealed that the organ recipients had a higher incidence of infection.(Ok et al., 1997)

1.1.2 Cryptosporidiosis in children.

Infectious diseases accounted for 64% of the total deaths in children under 5 years in 2010.(Liu et al., 2012) Pneumonia and diarrhea were two of the leading causes of mortality in children due to infection.(Liu et al., 2012) The Global Enteric Multicenter Study (GEMS) published in 2013 was designed to identify the causative agents of diarrhea in children under 5 years.(Kotloff et al., 2013) This large-scale study included at least 9000 cases of moderate-to-severe diarrhea along with a case-control sample of equivalent size.(Kotloff et al., 2013) GEMS identified rotavirus, *Cryptosporidium*, enterotoxigenic *Escherichia coli* producing stable toxin (ST-ETEC) and *Shigella* as the leading causative agents of moderate-to-severe diarrhea.(Kotloff et al., 2013) Infection with *Cryptosporidium* was identified to carry a major risk of death in children who are 12-23 months old (Hazard Ratio 2·3; 95% CI 1·3–4·3).(Kotloff et al., 2013) The negative outcome of cryptosporidiosis on children health was also confirmed in a MAL-ED studies in multiple country.(Korpe et al., 2018)

Malnourished children are more susceptible to *Cryptosporidium* infection and the reason is not well understood. A West Indian study published in 1987 was the first to establish a link between malnutrition and susceptibility to cryptosporidiosis in children.(Macfarlane and Horner-Bryce, 1987) Out of 513 children tested, 77 were found to be malnourished.(Macfarlane and Horner-Bryce, 1987) 19.5% of these malnourished children were found to be positive for *Cryptosporidium*, and they exhibited typical

symptoms including diarrhea, vomiting, fever and dehydration that lasted for two weeks.(Macfarlane and Horner-Bryce, 1987) A similar observation was made in a case-controlled study in Peru that found more malnutrition among *Cryptosporidium*-positive children than among the non-infected.(Sarabia-Arce et al., 1990) Many other studies carried out in Mexico(Javier Enriquez et al., 1997), India(Jaggi et al., 1994), Gabon(Duong et al., 1995) and Tanzania(Cegielski et al., 1999) have found a higher prevalence of *Cryptosporidium* in malnourished children.

Even in the absence of overt disease, cryptosporidiosis can negatively affect the growth outcome in children.(Korpe et al., 2018) Asymptomatic cryptosporidiosis results in reduced nutritional status (as measured by height and weight) and delayed growth catch-up.(Agnew et al., 1998; Checkley et al., 1997, 1998; Korpe et al., 2018) Mechanism(s) of cryptosporidiosis induced malnourishment are currently unknown but diminished nutrient absorption due to changes in tissue structure and physiology, inflammation, and dysbiosis are among the underlying factors currently considered.(Korpe and Petri, 2012) *Cryptosporidium* infection is thus both cause and consequence of malnourishment and the cause-and-effect between cryptosporidiosis and malnourishment is complex and further studies are needed to unravel this relationship.

1.2. *Cryptosporidium* outbreaks

Cryptosporidium oocysts are highly resilient to environmental stresses and they are transmitted through contaminated water and food. *Cryptosporidium* oocysts have a thick wall composed of complex macromolecules, including proteinaceous components that

are crosslinked via disulfide bridges.(Jenkins et al., 2010; Spano et al., 1997) Chemical and ultrastructural analyses of oocysts suggest that the oocyst wall is a tripartite structure composed of a surface glycoconjugate layer followed by lipid and proteinaceous layers.(Jenkins et al., 2010) *Cryptosporidium* oocyst walls are enriched with mycolipids and fatty alcohols that might prevent the absorption of commonly used disinfectants.(Fayer, 1995; Jenkins et al., 2010) *Cryptosporidium* encodes for genes required for trehalose synthesis which might also explain the ability of oocysts to withstand desiccation and dehydration.(Elbein et al., 2003)

The resilience of *Cryptosporidium* oocysts to different environmental stresses have been reviewed systematically(Carey et al., 2004). Many of the standard waste-water treatment disinfectants like chlorine, chlorine dioxide and chloramine did not affect the infectivity of oocysts.(Korich et al., 1990) However, ozone and U.V. treatments have proven to be effective in neutralizing the parasite oocysts.(Keegan et al., 2003) The ability of the oocyst to withstand harsh environmental and chemical assaults and to persist for a prolonged period in feces(Robertson et al., 1992) allows for easy transmission of the parasite leading to outbreaks(Gharpure, 2019).

1.2.1 Waterborne Outbreaks

Of all the major cryptosporidiosis U.S outbreaks between 2009-2013, 67.2% were waterborne outbreaks.(Gharpure, 2019) Out of 183 waterborne outbreaks documented in this period, 156 of them were found to be due to exposure to treated recreational water.(Gharpure, 2019) This highlights the ineffectiveness of contemporary water treatment procedures to prevent *Cryptosporidium* transmission. One of the earliest reports of recreational waterborne outbreaks was documented in Los Angeles county in

1988 where 44 individuals developed the disease.(Porter et al., 1988) The source of the *Cryptosporidium* was found to be an individual who had defecated unintentionally in the swimming pool several days before the outbreak.(Porter et al., 1988) Since then many such outbreaks have been documented in several states in the United States.(Gharpure, 2019)

The 1993 outbreak of *Cryptosporidium* in Milwaukee, WI was the largest ever documented with 403,000 reported cases of diarrhea.(Mac Kenzie et al., 1994) The outbreaks was traced to two water-treatment plants that we're unable to filter *Cryptosporidium* oocysts adequately.(Mac Kenzie et al., 1994) The median age of the affected individuals was 4 years (ranging from 1-40 years). Around 90% of the patients reported stereotypical symptoms like diarrhea and stomach cramps.(Mac Kenzie et al., 1994) The symptoms lasted for a median of 14 days (1-30 days).(Mac Kenzie et al., 1994) Many of the patients affected during the outbreak were children(Mac Kenzie et al., 1994) but an elderly population of afflicted was also identified(Naumova et al.).

The total financial cost of the Milwaukee outbreak was estimated to be $96.2 million.(Corso et al.) Of that, $31.7 million were incurred as direct medical cost and $64.6 million were due to productivity losses.(Corso et al.) *Cryptosporidium* outbreaks due to exposure to recreational water continue to be the major issue in the United States(Gharpure, 2019) and Europe(Putignani and Menichella, 2010).

In certain regions of Africa and Asia, access to safe drinking water is limited, thus making it one of the largest sources of *Cryptosporidium* transmission.(Putignani and Menichella, 2010) In Africa, *Cryptosporidium* is highly prevalent in healthy, immunocompetent adults due to a lack of proper water disposal facilities.(Gait et al.,

2008) However the disease in immunocompetent individuals is rare.(Kotloff et al., 2013) A heavy burden of *Cryptosporidium* oocysts has been documented in effluent discharges due to direct contact with the fecal material.(Gait et al., 2008) *Cryptosporidium*-laden discharge is often diluted into open sources of water which are used as a source of drinking water.(Gait et al., 2008) Similarly, 72% of the water samples collected from potable water sources in Taiwan were found to be *Cryptosporidium*-positive.(Hsu et al., 1999) A study in India on the prevalence of *Cryptosporidium* in calves concluded that the infection often peaked in the monsoon and humid summer seasons.(Paul et al., 2009) This peak in infection might be due to mixing of the agricultural and livestock runoffs with the water sources.(Paul et al., 2009)

1.2.2 Foodborne Outbreaks

Foodborne transmission was the fourth largest source (22 out of 444 cases) of *Cryptosporidium* transmission in the U.S. in the period between 2009-2013.(Gharpure, 2019) Unpasteurized milk, apple cider,and fresh produce were found to be the common sources of foodborne transmission.(Gharpure, 2019) According to the 2015 WHO (World Health Organization) report, there were 8.6 million cases of foodborne *Cryptosporidium* outbreaks in 2010, with 3759 deaths and 296,156 DALYs (disability-adjusted life years).(WHO, 2015). Around 16 different species of *Cryptosporidium* have been identified for foodborne transmission, with *Cryptosporidium hominis* and *Cryptosporidium parvum* being the predominant species.(Xiao, 2010) A 2014 CDC survey revealed that 65% of the foodborne outbreaks occur in the restaurants(Dewey-Mattia et al. 2014), and elderly and immunosuppressed individuals being the most susceptible populations(Skovgaard, 2009).

Two of the major food-borne outbreaks in the U.K. were found to be due to salad contamination.(WHO, 2015) Improperly ozonized apple cider(WHO, 2015) and contaminated leafy vegetables(Quiroz et al., 2000) were implicated in two different foodborne outbreaks in the U.S. affecting 152 and 160 individuals, respectively. Due to the complex nature of food chain management, it is often difficult to trace the source of the contamination.(WHO, 2015) Use of contaminated water for irrigation and pesticides, handling of food by infected employees and use of contaminated currency notes have all been identified as sources of *Cryptosporidium* contamination.(WHO, 2015)

1.2.3 Zoonotic and Anthroponotic transmissions

Animals can get infected with multiple species of *Cryptosporidium* and they can transmit the infection to humans. (Xiao et al., 2004) The majority of the cryptosporidiosis cases in humans are attributable to *C. hominis* and *C. parvum* species that are responsible for anthroponotic and zoonotic cases, respectively.(Xiao, 2010) Anthroponotic cases are common in nursing homes, child-care settings, schools, colleges, etc.(Gharpure, 2019) Contrastingly, zoonotic cases are most prevalent in rural and agricultural settings where exposure to farm animals like cattle, goats, sheep, etc. is common.(Gharpure, 2019)

Many of the *Cryptosporidium* species have oocysts of similar size and shape, often making it difficult to diagnose the species by morphology.(Xiao et al., 2004) Broad host range of multiple *Cryptosporidium* species, the coexistence of different genotypes in the same host and abundant sexual recombination often confounds the species and strain definition in *Cryptosporidium*.(Xiao et al., 2004) The classical definition of species is a population that interbreeds and is reproductively isolated from other populations.(Hey et al., 2005) It has been technically challenging to conduct genetic crossing studies

between different *Cryptosporidium* isolates to define different genotypes as species in accordance to the classical definition.(Xiao et al., 2004) Oocyst morphology, multi-locus genotyping (using SSU rRNA, HSP-70, TRAP-C1, COWP, gp60, gp900, etc.), cross-transmission studies, infection site, developmental biology, oocyst shedding, etc. have been used in various combinations as criteria to define the species of the genus *Cryptosporidium*.(Xiao et al., 2004) The International Code of Zoological Nomenclature (ICZN) has defined the following four criteria to define a species of *Cryptosporidium*: compliance with ICZN guidelines, oocysts features, natural host specificity, and molecular genotyping.(ICZN, 1999) Based on this definition, a total of 13 different species have been recognized in four different branches of vertebrates. *C. parvum*, *C. hominis*, *C. wrairi*, *C. andersoni*, *C. muris*, *C. canis* and *C. felis* have been recognized as mammal-specific species.(Xiao et al., 2004) Non-mammalian species are *C. meleagridis*, *C. baileyi* and *C. galli* in birds; *C. saurophilum* and *C. serpentis* in reptiles; and *C. molnari* in fishes.(Xiao et al., 2004)

C. parvum is one of the most ubiquitous species of *Cryptosporidium* which has been identified in 150 mammalian species including humans.(Xiao, 2010) It was first identified by Tyzzer in 1907 and its infection was exclusively observed in the small intestines(Xiao et al., 2001) Morphological and transmission-based validations for *C. parvum* being distinct from *C. muris* were provided by multiple studies.(Current and Reese, 1986; Upton and Current, 1985) *C. parvum* prominently infected small intestines in calves and mice, but the infection was more modest in mice when compared to *C. muris*.(Current and Reese, 1986; Upton and Current, 1985)

C. hominis is the major *Cryptosporidium* species to infect humans.(McLauchlin et al., 1999) It was initially categorized as *C. parvum* genotype H, but many studies have

validated it as a species of its own. Cross-transmission(Morgan-Ryan et al., 2002) and molecular phenotyping studies(Awad-El-Kariem et al., 1998; Bonnin et al., 1996) have confirmed that *C. hominis* has a narrower host range infecting mostly humans (and gnotobiotic piglets as an experimental model). A U.K.-based study of 218 patient samples identified that 68% of the patients were affected by *C. hominis* while the rest were positive for *C. parvum*, suggesting a higher prevalence and/or susceptibility to *C. hominis*.(McLauchlin et al., 1999) Besides, patients affected by *C. hominis* shed more oocysts compared to *C. parvum*-positive individuals, potentially indicating adaptation of *C. hominis* towards humans.(McLauchlin et al., 1999) This has also been corroborated by a Peruvian study that concluded that patients affected by *C. hominis* shed oocysts for a longer period (Mean= 13.9 days) compared to *C. parvum* afflicted individuals (Mean= 6.4 days).(Xiao et al., 2001)

Other species of *Cryptosporidium* like *C. hominis, C. canis, C. felis* and *C. meleagridis* have been identified in humans(Morgan et al., 1998; Xiao et al., 1999a, 1999b) irrespective of the immunological status of the individual(Pedraza-Díaz et al., 2000, 2001; Xiao et al., 2001). It is hypothesized that the relative prevalence of different species of *Cryptosporidium* can vary depending upon the geographical location, socio-economic status, season and environmental changes.(Xiao, 2010)

1.3 The single-host lifecycle of *Cryptosporidium*

Cryptosporidium has a single-host lifecycle, and it undergoes asexual replication and sexual development in the same host (Fig.1.1). *Cryptosporidium* is transmitted through an extracellular, meiotic spore-like structure called oocyst that harbors four

sporozoites.(Current and Reese, 1986; Pohlenz et al., 1978) Sporozoites are released from the oocysts presumably due to certain cues signaling arrival in the gut(Feng et al., 2006; Koh et al., 2014), they then invade enterocytes to establish an intracellular. The intracellular vacuole is unique as it lies beneath the host cell membrane but is not cytoplasmic.(Bartošová-Sojková et al., 2015; Ostrovska and Paperna, 1990) This initial, single nucleus containing stage is known as the trophozoite. Trophozoite stages replicate by schizogony (the term merogony is used interchangeably) to produce type I meront that contains 6-8 merozoites.(Ostrovska and Paperna, 1990) Type I merozoites egress from the vacuole and infect another enterocyte to replicate by type I merogony or differentiate into type II meronts which are characterized by 4 nuclei.(Ostrovska and Paperna, 1990) Type II merozoites are programmed to differentiate into sexual stages, microgamont (males) and macrogamont (females) within the next infected cell.(Ostrovska and Paperna, 1990) The microgamont produces 16 miicrogametes which egress to find and fertilize intracellular macrogametes. The resulting zygote stage undergoes meiosis and sporogony inside the host cells.(Current and Reese, 1986; Ostrovska and Paperna, 1990) Sporogony is characterized by the development of four sporozoites, and oocyst wall(s) around it produce either a thick-walled (double membrane) or thin-walled (single-membrane) oocysts. Thick-walled oocysts are shed in the feces to be transmitted while thin-walled oocysts excyst within the gut to further auto-infect the same host.(Ostrovska and Paperna, 1990)

1.3.1 Sex as the source of infection and transmission: A phenomenon unique to *Cryptosporidium*

Toxoplasma is the causative agent of *Toxoplasmosis* that can result in organ damage and congenital birth defects.(Saadatnia and Golkar, 2012) *Toxoplasma* infects a broad

range of warm-blooded animals and is transmitted through the food chain by either ingesting infected meat or oocysts in the environment transmitted by defecation by cats (Fig. 1.2).(Dubey et al., 2011; Saadatnia and Golkar, 2012) The feline phase of the lifecycle of *Toxoplasma* encompasses limited merogony followed by gametogenesis and sex in the cat intestine.(Dubey et al., 2011; Saadatnia and Golkar, 2012) Sex results in unsporulated oocysts that undergo sporogony (2-3 days post defecation) once outside the host.(Dubey et al., 2011) *Toxoplasma* does thus not cause oocyst-mediated autoinfection in cats. The same phenomenon is observed in *Eimeria* a related apicomplexan that infects cattle and poultry. Meiosis and formation of infective sporozoites in *Eimeria* occur after oocysts are shed from the host.(del Cacho et al., 2010; Mesfin and Bellamy, 1978)

Lastly, the apicomplexan *Plasmodium*, causes malaria a disease driven by continuous asexual replication of parasites in red blood cells. A minor population of merozoites exits the asexual program and commits to becoming precursor sexual stages known as gametocytes (Fig. 1.3).(Cowman et al., 2016; Phillips et al., 2017) Gametocytes are taken up by a mosquito where gametocytes become mature gametes and eventually mate. Hence, *Plasmodium* sexual stages have no role in maintaining the infection. This has been validated in mutant strains that are deficient in producing gametocytes.(Kafsack et al., 2014; Sinha et al., 2014) Hence in the case of *Plasmodium, Eimeria* and *Toxoplasma*, sex serves solely as the source of transmission but not as a source of continued infection.

Sex in *Cryptosporidium* is followed by sporulation which occurs within the infected host cells.(Current and Reese, 1986; Ostrovska and Paperna, 1990) The sporulated oocyst is then released from the host cell and can either be shed with the feces or immediately

excyst in the gut lumen to release infectious sporozoites. (Current and Reese, 1986; Ostrovska and Paperna, 1990) This unique feature of producing sporulated oocysts within the host cells makes it possible for *Cryptosporidium* to maintain infection through successive rounds through the entire lifecycle including sex. For *Cryptosporidium*, asexual replication, and sexual development thus both contribute to parasite multiplication and maintenance in the host.

1.3.2 Two plausible models of *Cryptosporidium* lifecycle and their translational significance

Formally, *Cryptosporidium* infection might be sustained through asexual replication, and sex is only required for transmission, or sex and oocyst-mediated autoinfection may be a necessity for continued infection. This leads me to consider two models of the lifecycle, according to the model A (Fig. 1.4), parasites undergo asexual replication and sexual development simultaneously. Under this model, *Cryptosporidium* sustains the infection in a host through asexual amplification and oocyst biogenesis. If this is true, then a therapeutic strategy aimed at specifically targeting the sexual stages will only block transmission (as observed in *Plasmodium*). However, as I will detail in this thesis many of our experimental findings are inconsistent with this model. We have observed time dependent sexual differentiation of essentially the entire parasite population. This suggests that a second model (Model B; Fig. 1.4) where all asexual stages follow an obligatory developmental path towards sexual differentiation followed by renewed initiation of the lifecycle and the infection from newly formed oocysts. This would suggest that sex and oocyst biogenesis are crucial for the parasite to maintain infection in a host.

The main objective of my dissertation is to understand the role of parasite sex in cryptosporidiosis and discern between the two lifecycle models I just outline for *Cryptosporidium*. Through my dissertation work, I will develop genetic and cell biological approaches to put both hypotheses and their predictions to a rigorous experimental test.

1.4 Dissertation aims and questions

The goal of my dissertation is to understand the lifecycle of *Cryptosporidium* and in particular the role that the sexual phase plays in this infection. My research has approached this by addressing three specific questions:

1.4.1 Why is it not possible to maintain *Cryptosporidium* continuously in culture and may this be related to the developmental biology of the parasite?

Cryptosporidium cannot be continuously propagated in tissue culture. Transformed cell lines derived from intestinal epithelia cells can be infected and sustain parasite growth, but this growth is limited to a period of 3 days. We will observe *Cryptosporidium* lifecycle progression in culture and animals and test whether and how continued parasite growth is linked to its developmental programming.

To aide this goal we will engineer genetically encoded reporter strains to discern different stages of *Cryptosporidium*. We will use these reporters in mice and culture to observe and compare progression through different stages. In particular, we will be interested to study the transition between the asexual and sexual phase of the lifecycle, and we will develop a quantitative staging system based on rigorous molecular characters. These comparative experiments will provide us with empirical insights into

the programming of the lifecycle of *Cryptosporidium* help us to define whether the parasite experiences developmental arrest and if so at which point in the lifecycle this prevents the continuous propagation of the parasite.

1.4.2 Which biological processes are unique to the sexual stages of *Cryptosporidium*?

The biological processes that unfold over the parasite lifecycle are only poorly defined. We plan to use differential gene expression between stages, in particular between asexual and sexual stages to discover pathways associated with lifecycle progression. We will use reporter parasites developed in Aim1 to isolate specific stages using flow cytometry and we will subject them to RNA sequence analysis. We will then compare the patterns of relative gene expression to home in on genes that are vital for sexual development. We will pay particular attention to the differential expression of Apetala-2 type transcriptional regulators (ApiAP2) as these have been identified as a stage-specific developmental regulators in other apicomplexans. They thus may be candidates to perturb the development of specific stages to test our lifecycle hypotheses.

1.4.3 What is the effect of the perturbation of sexual development on infection in mice?

The central hypothesis that emerged from my research in tissue culture models is that the continued growth of the parasite depends on sex and an obligatory developmental reset. I will test whether this is also true *in vivo* in a rigorous genetic experiment. I will disrupt genes that encode transcriptional regulators that essential for sexual development and I will measure the impact of this disruption in a mouse model of infection. As sex is critical to the transmission, I will need to devise an experimental

system of conditional gene ablation. Our transcriptional analysis of female gametes has identified three female-specific ApiAP2 genes, and conventional perturbation of one of these genes (cgd4_1110; AP2-F) was lethal for the parasite. Hence, a conditional (preferably a chemically inducible) gene perturbation strategy is required to rigorously approach this specific aim. We aim to develop a rapamycin inducible DiCre recombinase system that has been used in other systems.(Knuepfer et al., 2017) We aim to specifically target AP2 factors restricted to female gametes to understand their role in the development of females, oocyst biogenesis and continued infection.

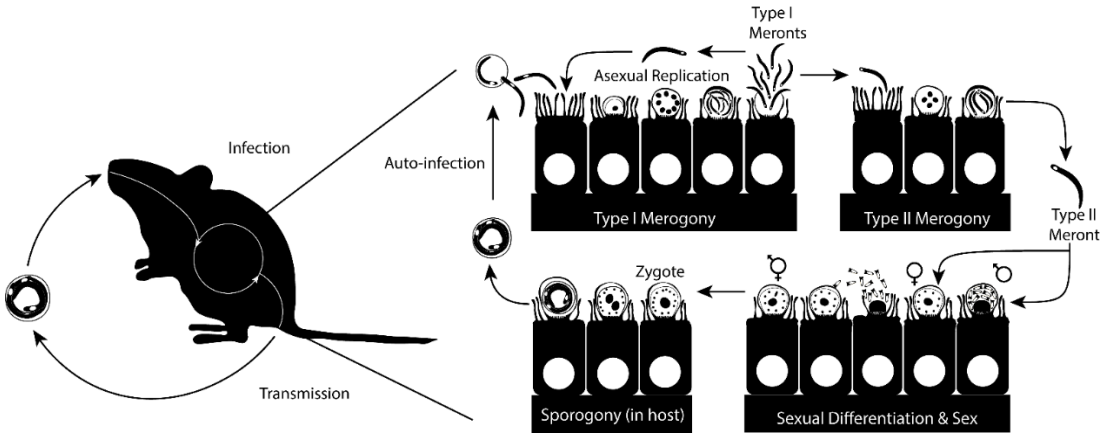

Figure 1.1: The single-host lifecycle of *Cryptosporidium*. Sporozoites excysted from ingested oocysts infect enterocytes and unfold following phases of the lifecycle sequentially in the same host: type I merogony, type II merogony, gamogony sex, meiosis and sporogony. The ability of the *Cryptosporidium* to undergo sporogony in the host result in mature oocysts that can autoinfect and reset the lifecycle in the same host. Hence in the case of *Cryptosporidium*, sex serves as the source of infection and transmission.

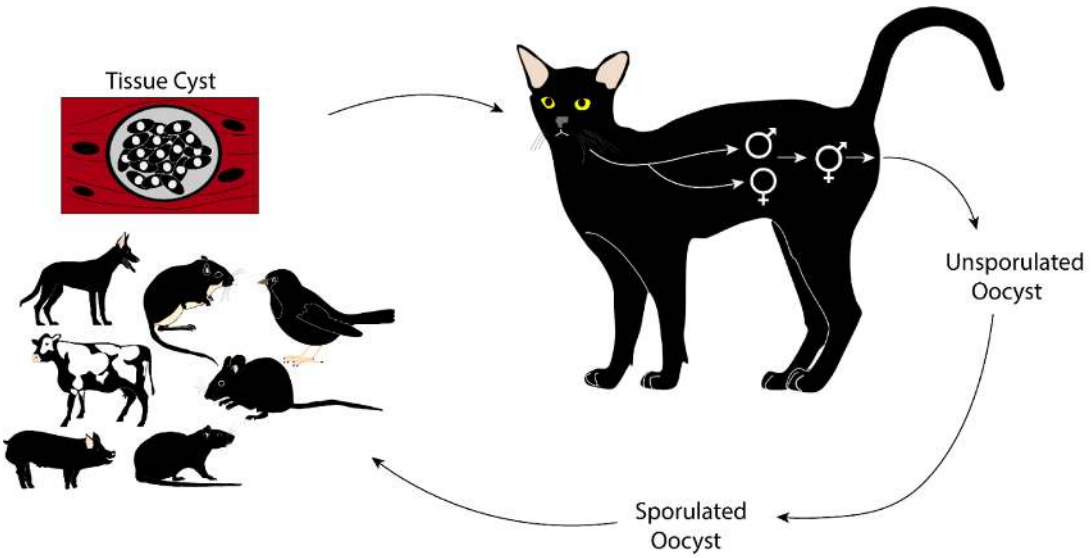

Figure 1.2: The feline phase of the lifecycle of *Toxoplasma*. *Toxoplasma*, though promiscuous in its host preference, only undergoes gametogenesis in cats. Cats become infected by ingesting tissue cyst (bradyzoites) infested prey. *Toxoplasma* then undergoes merogony, gametogenesis and sex to produce unsporulated oocysts. Unsporulated oocysts are then shed through feces where they undergo sporulation to produce mature, infectious oocysts that can be transmitted to other hosts.

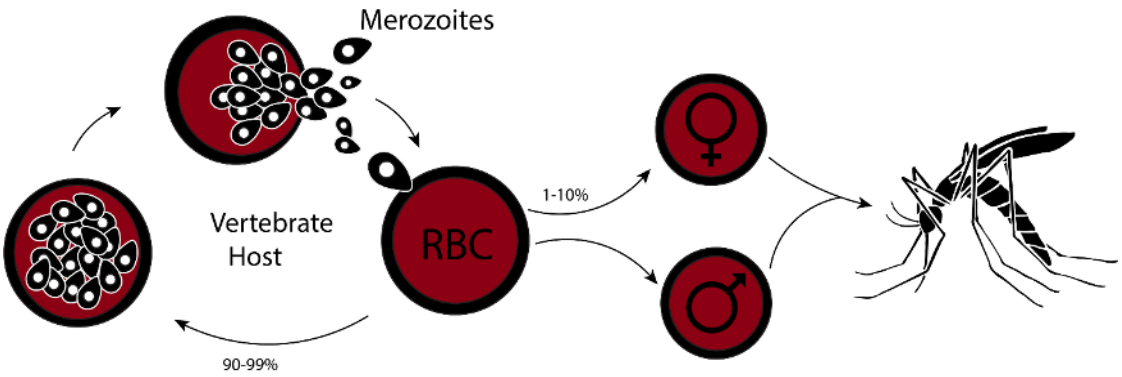

Figure 1.3: Merogony and gametogenesis in *Plasmodium*. *Plasmodium* merozoites replicate asexually in the RBC of the vertebrate host. After completing merogony, a small population of merozoites (1-10%) differentiates into gametocytes that are taken up by a mosquito for further transmission.

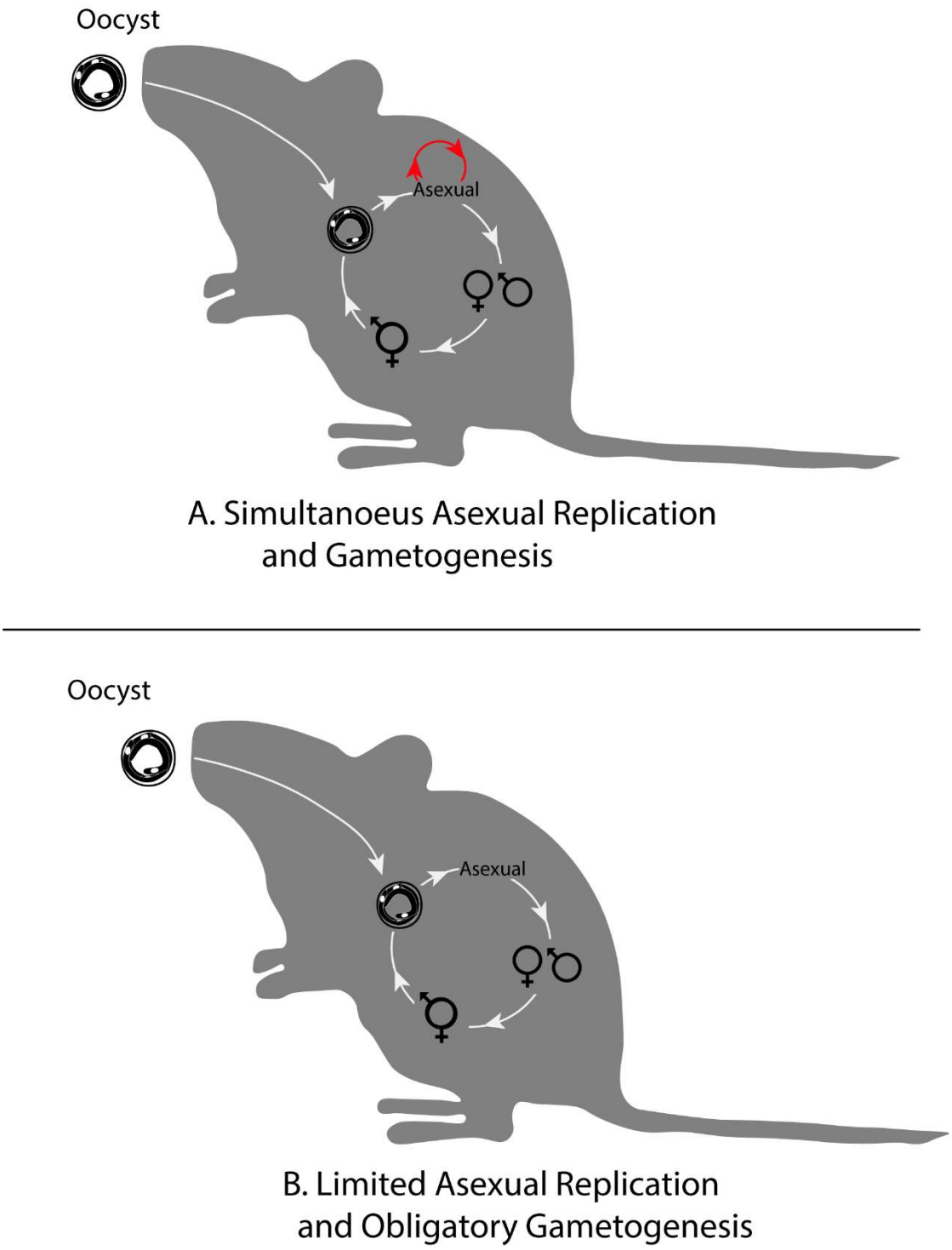

Figure 1.4: Two proposed models of the lifecycle of *Cryptosporidium*. The two proposed models of the lifecycle of *Cryptosporidium* differ in emphasizing the significance of sex (and sporogony) in maintaining the infection in a host.

Chapter 2: Materials and Methods

Plasmid construction. Guide oligonucleotides (Sigma-Aldrich) were introduced into the *C. parvum* Cas9/U6 plasmid16 by restriction cloning. See Pawlowic et al.(Pawlowic et al., 2017) for a detailed discussion of guide design for *C. parvum*. Transfection plasmids were constructed by Gibson assembly using NEB Gibson Assembly Master Mix (New England Biolabs).

Generation of transgenic parasites. To generate transgenic parasites, 5×10^7 C. parvum oocysts Iowa II strain (obtained from Bunchgrass Farms or the University of Arizona) were incubated at 37°C for 1 h in 0.8% sodium taurocholate to induce excystation. Excysted sporozoites were then transfected using an Amaxa 4D Electroporator (Lonza) with parasites suspended in SF buffer using program EH100 and 50 µg of each Cas9/U6 plasmid and PCR repair construct. The repair encodes the neomycin phosphotransferase drug-selection marker fused to a nanoluciferase reporter flanked by 50 bp homologous regions to guide insertion into the parasite genome. Ifng−/− mice were infected with transfected parasites by oral gavage. Stomach acid was neutralized with 100 µl of 8% $NaHCO_3$ solution by gavage before infection. Note that this modification replaces surgery-based infection and significantly streamlines the protocol. Stable transformants were selected with paromomycin, given to mice ad libitum in their drinking water (16 mg/ml) and parasite shedding was monitored by measuring nanoluciferase activity in the feces of infected mice. To purify transgenic parasites from collected feces, we used sucrose flotation followed by a CsCl gradient48. In brief, collected mouse feces were homogenized in tap water using a LabGen 125 homogenizer (Cole-Parmer) and filtered through a 250 µm mesh filter. This filtrate was diluted 1:1 with a saturated sucrose solution (specific gravity, 1.33) and centrifuged at

1,000g for 5 min. The supernatant was collected, resuspended in 0.85% saline solution, and overlaid onto CsCl solution (specific gravity, 1.15) and centrifuged at 16,000g for 3 min. Purified oocysts were collected from the saline–CsCl interface and resuspended in cold PBS.

Immunofluorescence assay. HCT-8 cells were infected with bleached and washed oocysts. Infected cells were maintained in RPMI-1640 medium (Sigma- Aldrich) containing 1% fetal bovine serum. Infected cells were fixed with 4% paraformaldehyde (Electron Microscopy Science) in PBS and then permeabilized with PBS containing 0.25% Triton X-100. Cells were blocked with 3% bovine serum albumin (BSA) solution, followed by incubation with primary antibodies. Cells were washed with PBS and then incubated with appropriate fluorophore-conjugated secondary antibodies and counterstained with DAPI. Coverslips were then mounted on glass slides with fluorogel (Electron Microscopy Science) mounting medium.

For *in vivo* staining, infected mice were killed, and the small intestine was resected and flushed with 10% neutral buffered formalin (Sigma-Aldrich), then 'swiss-rolled' and fixed overnight in 4% paraformaldehyde followed by overnight incubation in 30% sucrose solution. Samples were embedded in OCT medium (Tissue-Tek, Sakura Finetek) and cryosectioned. Tissue sections were blocked with 10% BSA and 0.1% Triton X-100 in PBS. Sections were stained with antibodies in PBS with 0.1% Triton X-100 as described above, counterstained with DAPI and mounted.

Super-resolution structured illumination microscopy was conducted using a Carl Zeiss Elyra (UGA Biomedical Microscopy Core) or a GE OMX (PennVet Imaging Core) microscope. Widefield microscopy was performed using a Leica LAS X microscope

(PennVet Imaging Core) and images were processed and analysed using Carl Zeiss ZEN v.2.3 SP1, GE Softworx, and NIH ImageJ software.

Cre–loxP-based fertilization assay. To measure sex between two different strains, Ifng−/− mice were infected with 50,000 oocysts of either the Cre or COWP1–HA flox tdNeon strain or coinfected with both the strains. Oocysts were purified from fecal samples that were collected at days 3–10 after infection. Oocysts were fixed with 4% PFA, stained with biotinylated *Macula pomifera* agglutinin49 (Vector Laboratories), washed and incubated with Streptavidin-594, and settled onto polyl- lysine (Sigma-Aldrich) coated coverslips before mounting with fluorogel. The same strains were used to infect HCT-8 cells. HCT-8 cells infected with oocysts obtained from Cre × COWP1–HA flox tdNeon coinfection were used as positive controls. Cells were fixed 48 h and 72 h after infection and parasites were stained using rabbit anti-TrpB antibodies.

EdU labelling to detect DNA synthesis. HCT-8 cells were infected with 100,000 oocysts of the COWP1–HA strain. EdU was added to cultures 36 h after infection to a final concentration of 10 μM and cells were fixed 12 h later. A Click-iT EdU Alexa-Fluor 594 kit (Thermo Fischer Scientific) was used to label incorporated EdU. Parasites were stained with anti-HA antibody or fluorescein conjugated *Vicia villosa* lectin (Vector Laboratories).

Flow sorting of intracellular stages and RNA extraction. HCT-8 cells grown in 6-well plates were infected with 300,000 oocysts of eno–tdNeon (constitutive reporter strain) or COWP1–tdTomato (female reporter strain) and AP2-14 tdNeon (male-reporter strain). Infected cultures were trypsinized with TrypLE Express Enzyme (Thermo Fischer Scientific), extensively washed with PBS, passed through a 40 μm filter (BD

Biosciences) and pelleted. Cells were resuspended in 400 µl of buffer and sorted using a BD FACSJazz sorter (BD Biosciences). Uninfected HCT-8 control cells were used to gate on the singlet host cell population. Then, 10,000 positive cells were directly sorted into the RLT lysis buffer of the micro RNA extraction kit (Qiagen).

Four Ifng−/− mice were infected with 200,000 oocysts of COWP1–tdTomato reporter strain. Mice were killed 2 d after infection, the small intestine was resected, cut into small pieces and incubated in RPMI-1640 medium containing 10% FBS, 25 mM HEPES, 5 mM EDTA, 50 µM β-mercaptoethanol and 0.145 mg ml−1 dithiothreitol for 20 min. The cell suspension was filtered through 70 µm and 40 µm kitchen-mesh filters (BD Biosciences). Cells were pelleted, resuspended in buffer, stained with anti-CD45.2 antibodies and sorted. Intestinal cells isolated from uninfected mice were used as controls. Then, 1,000 tdTomato-positive cells from each replicate was sorted directly into 350 µl of RLT lysis buffer. The Qiagen micro RNA extraction kit was used to extract RNA from sorted cells. RNA was finally eluted in RNase-free water and samples were then stored at −80 °C.

RNA sequencing of sorted cells. cDNA was generated using the SMART-Seq v4 Ultra Low Input RNA Kit (Takara Bio USA), and barcoded, sequence-ready libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina). Total RNA and libraries were quality checked and quantified on an Agilent Tapestation 4200 (Agilent Technologies) and Qubit 3 (Thermo Fischer Scientific), respectively. All the samples were pooled, and single-end reads were generated using an Illumina NextSeq 500 sequencer.

RNA sequencing of sporozoites and 24 h and 48 h infected bulk cultures.

Oocysts from the Sterling laboratory (University of Arizona) were first induced to excyst through resuspension in 0.8% sodium deoxytaurocholate and incubation at 37 °C for 2 h. RNA from the released sporozoites was then isolated using an RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. Each biological replicate was isolated from 40 million sporozoites. For the 24 h and 48 h in vitro time points, oocysts from the Sterling laboratory (University of Arizona) were first treated with dilute household bleach (1:4 in dH_2O) for 10 min on ice to sterilize them. The oocysts were then washed twice with cold PBS and resuspended in 0.8% sodium deoxytaurocholate and incubated for 10 min on ice. Oocysts were washed once more with cold PBS and then used to infect HCT-8 cell monolayers grown to 80% confluency in a 24-well plate. For the 24 h time points, 1 million oocysts were infected into each well with three biological replicates. For the 48 h timepoints, 100,000 oocysts were infected into each well with three biological replicates. During infection, standard RPMI growth medium was used supplemented with 1% fetal bovine serum. RNA from infected cells was isolated using an RNAeasy Mini Kit (Qiagen) according to the manufacturer's protocols. Sequencing libraries for sporozoites and in vitro time points were prepared using a Nextera XT DNA Library Preparation Kit (Illumina) and 150-bp paired-end reads were collected using an Illumina MiSeq (Illumina).

RNA-sequencing analysis. Raw reads were mapped to the *C. parvum* Iowa II reference (Ensembl, ASM16534v1) using Kallisto v.0.45.0(Bray et al., 2016). All subsequent analyses were carried out using the statistical computing environment R v.3.6 in RStudio v.1.1.463 and Bioconductor. In brief, transcript-level quantification data were summarized to genes using the tximport package and data were normalized using the TMM method (implemented in EdgeR). Only genes with more than 10 counts per

million in at least 3 or samples (depending on the analysis) were carried forward for analysis. Precision weights were applied to each gene on the basis of the mean–variance relationship using the VOOM function in Limma. Linear modelling and Bayesian statistics carried out in Limma were used to identify differentially expressed genes with a FDR-adjusted P of ≤0.01 and an absolute $\log_2$-transformed fold change of ≥1 after correcting for multiple testing using the Benjamini–Hochberg procedure. When necessary, batch correction was carried out using the empirical Bayes-moderated adjustment for unwanted covariates function (empiricalBayesLM) in the WGCNA package. All code used in these analyses is available in Supplementary File 7(Tandel et al., 2019) and on GitHub(Tandel et al., 2019). For *P. berghei*, files were downloaded from the NCBI Sequence Read Archive BioProject ID: PRJNA374918 (ref. 21) and forward reads were mapped as described above for the sorted C. parvum samples to the *P. berghei* reference transcriptome (Ensembl, PBANKA01). For *E. tenella*, differentially expressed gametocyte genes were obtained from Walker et al(Walker et al., 2015). Cross-species comparisons and orthologue identifications were performed using EuPathDB (https://eupathdb.org/). See Supplementary File 7 of Tandel et. al.(Tandel et al., 2019) for full details, including a link to all of the code used for the RNA analyses performed here. Functional enrichment analysis. GSEA was carried out using GSEA software(Bray et al., 2016). Four custom gene signatures for *C. parvum* were generated using Gene Ontology or community datasets available at CryptoDB (https://CryptoDB.org). A 28-gene signature for ‘carbohydrate metabolism’ was generated using the Gene Ontology term GO:0005975. A 63-gene signature for ‘DNA metabolic process’ was generated using GO:0006259. A 48-gene signature for ‘oxidation–reduction’ was generated using GO:0055114. An 85-gene oocyst signature was generated by using CryptoDB to mine a published oocyst wall proteome dataset

from Truong and Ferrari(Truong and Ferrari, 2006) to retrieve only genes that had ≥20 unique peptide sequences per sample. All four signatures were used for GSEA with 1,000 permutations of gene sets to generate P values, and multiple testing correction was applied to generate FDR-adjusted P values. GSEA results were used to create enrichment plots in DataGraph v.4.4 (Visual Data Tools).

Transient transfection assay for engineering DiCre conditional system. $1X10^7$ *C. parvum* bunchgrass oocysts (University of Arizona) were washed, pelleted, and resuspended in 1 ml of 0.8% sodium taurocholate and incubated at 37°C for an hour. Parasites were pelleted and transfected using an Amaxa 4D Electroporator (Lonza) with parasites suspended in SF buffer using program EH100 and 10 µg of the plasmid. Transfected parasites were resuspended in RPMI-1640 media containing 1% FBS and then split into three equal volumes to infect three 24 well plates containing HCT-8 cells. Nanoluciferase activity was measured after 48 hours of infection by resuspending cell monolayer in 200 µl of 1:50 NanoGlo substrate (Promega Corporation). Luciferase activity was measured by using the NanoGlo assay on Promega luciferase reader. For measuring the DiCre activity, 10 µg each of DiCre and floxed plasmids were transfected.

Rapamycin-induced activation of DiCre in stable transgenics. Parasites were washed, pelleted, and then used to infect HCT-8 cells. DiCre activity was induced by incubating cells with 100nM rapamycin in RPMI-1640 media containing 1% FBS. Control samples were only maintained in RPMI-1640 media containing 1% FBS. For PCR assay, cells were washed and used for DNA isolation by using Quick-Start Protocol DNeasy® Blood and Tissue Kit (Qiagen). 50,000 oocysts were used for infection for PCR analysis. For IFA assay, 20,000 oocysts were used to infect HCT-8 cells grown on coverslips in 96 well plates. Cells were fixed with 4% paraformaldehyde solution and then stained.

Statistical methods. GraphPad PRISM was used for all statistical analyses. When measuring the difference between the two populations, we used a standard Student's t-test. No statistical tests were used to predetermine sample size and no animals were excluded from results. ANOVA was used to compare the means of multiple groups followed by Tukey's post hoc test for pair-wise comparisons.

Animal ethics statement. All the protocols for animal experimentation were approved by the Institutional Animal Care and Use Committee of the University of Georgia (protocol A2016 01-028-Y1-A4) and/or the Institutional Animal Care and Use Committee of the University of Pennsylvania (protocol number 806292). Four-week-old Ifng−/− and Rag1 knockout female mice strains of *Mus musculus* were used for all the experiments. No statistical tests were used to predetermine the sample size of mice used for experiments. Mice were not randomized, and investigators were not blinded before any of the experiments.

Data availability RNA-sequencing data generated in this study are available from GEO database repository under accession number GSE129267.(Yeoh et al., 2017)

Code availability All code used in these analyses is available in Supplementary File 7 of Tandel et. al.(Tandel et al., 2019) and on GitHub**.**

Table 1: Primers used in this study

Primer name	5'-3' Sequence
tkintgbbsf	gttggaagtaaatacttattagca
tkintgbbsr	aaactgctaataagtatttacttc
tkguide#4f	gttggaagaatacaatttctaagg
tkguide#4r	aaacccttagaaattgtattcttc
cgd6_2090g4858f	gttggttgtttcatatgacacaat
cgd6_2090g4858r	aaacattgtgtcatatgaaacaac
cgd8_2220g2bbsf	gttggagccaagaaagtttaagtca
cgd8_2220g2bbsr	aaactgacttaaactttcttggctc
enotkintf	tccagtactatgctatggtttgagaacagactttaagggaaatttatttgatggggaaactaaatatactgaaattcggt
tkinteno3r	tagctttttttgccacagcgacaaatagttttgatttcagtaagtttatcaccatagctgcgccaaattttgc
c13utrtkf	tccagtactatgctatggtttgagaacagactttaagggaaatttatttgaaagtagtttggccttttctagataatttt
eno3utrtkr	tagctttttttgccacagcgacaaatagttttgatttcagtaagtttatcaaattaagataaaaagaaaaacttaatcgatactatcctacac
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creatubr	actccaagaacaatattgatctagacagatctaaggccgcta
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Chapter 3: Comparison of lifecycle progression and sexual development of *Cryptosporidium parvum* in culture and mice.

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3.1 Abstract

Cryptosporidium is one of the leading causes of diarrhea-induced mortality in children. Therapeutic intervention against cryptosporidiosis is lacking. *Cryptosporidium* undergoes asexual replication and sexual development in the same host. However, the molecular programming of the single-host lifecycle of *Cryptosporidium* is poorly understood. *Cryptosporidium parvum* cannot be cultured continuously by infecting intestinal epithelial, cancerous cell lines like HCT-8 and Caco-2. Previous studies suggest the presence of asexual and sexual stages in the culture but rigorous studies to identify the lifecycle block *in vitro* are lacking. Identifying aspects of the *Cryptosporidium* lifecycle that are blocked *in vitro* can help us gain insights on the programming of the lifecycle of the parasite. Hence to rigorously study the kinetics of lifecycle progression, we developed a reporter strain that allows us to discern district stages of *Cryptosporidium*. Kinetic studies in culture show robust sexualization (>80%) of the parasite population. However, fertilization, sporogony and oocyst development are not observed in culture. Contrastingly, parasites undergo successful mating, post-fertilization development and oocyst biogenesis in infected mice. Transcriptional analyses of females sorted from mice show upregulation of glideosome-associated genes (required for sporogony) when compared to females sorted from HCT-8 infected cells. Hence, the transcriptional analyses corroborate our finding that sex and post-fertilization development are lacking in the culture. To rigorously test for fertilization, we devised a two-component genetic-crossing assay using a reporter that is activated by Cre recombinase. Our findings suggest obligate developmental progression towards sex in *Cryptosporidium*, which has important implications for the treatment and prevention of the infection.

3.2 Introduction

Diarrheal diseases account for 9% of global child mortality(Liu et al., 2012) and infection with *Cryptosporidium* is a leading cause of severe pediatric diarrhoea(Kotloff et al., 2013). Malnourished children are particularly susceptible to cryptosporidiosis, which results in recurrent or persistent infection and death.(Checkley et al., 2015; Kotloff et al., 2013; Platts-Mills et al., 2015) *Cryptosporidium* is also an important cause of malnutrition(Korpe and Petri, 2012), and infection can result in lasting growth defects(Scallan et al., 2011). Even in high-income countries, outbreaks are frequent and more than 50% of waterborne infections in the United States are due to *Cryptosporidium*(Painter et al., 2015; Scallan et al., 2011). The current treatment of cryptosporidiosis is of limited efficacy for those patients who have the most urgent need of treatment(Amadi et al., 2009).

Cryptosporidium is a member of the eukaryotic phylum Apicomplexa and has a life cycle that alternates between asexual and sexual reproduction. However, in contrast to most other apicomplexans, the entire cycle occurs in a single host. Sex results in the production of oocysts, which are environmentally hardy meiotic spores. Sex and production of oocysts are therefore essential to transmission but may also play a role in the continued infection of the host.(Current and Reese, 1986) The chronic infection could be sustained by asexual replication with facultative sex, driving the host-to-host transmission. Alternatively, progression to sexual stages might be obligatory. *Cryptosporidium* oocysts are unique in that they mature within the host tissue and are autoinfective. Thus, they could reset the developmental cycle and maintain infection. Which of these two models applies is a fundamental, yet unanswered, question that has important implications for the disease and the development of drugs and vaccines. Here

we develop molecular markers to observe and analyze the progression of the *Cryptosporidium* life cycle and use these markers to demonstrate that a block in fertilization limits parasite growth in culture, supporting a model of obligate sexual developmental progression to maintain infection.

3.3 Results

Using a reporter parasite to track *Cryptosporidium* life cycle progression.

In the absence of adaptive immunity, humans and mice develop long-lasting *Cryptosporidium* infections and the parasite replicates continuously (Fig. 3.1a). Immortalized epithelial cell lines such as Caco2, HT-29, and HCT-8 are readily infected, but growth ceases after 3 d and the infection cannot be maintained by serial passage(Upton et al., 1994) (Fig. 3.1b). During this period, morphological stages that are consistent with asexual and sexual development have been observed, and different sets of genes appear to be expressed in succession(Current and Reese, 1986; Mauzy et al., 2012; Wilke et al., 2018). However, rigorous stage-specific markers are lacking. We, therefore, sought to engineer transgenic parasites that delineate life cycle progression and took advantage of well-documented changes in the nuclear morphology of the parasites(Current and Reese, 1986; Ostrovska and Paperna, 1990). We introduced a fusion of *Cryptosporidium parvum* histone H2B (cgd5_3170) with the fluorescent reporter mNeon16 (Supplementary Fig. 3.1). HCT-8 cells infected with these parasites were fixed after 24 h and 48 h and then imaged by super-resolution structured illumination microscopy. All of the parasites showed nuclear fluorescence. We recorded morphometric data for each parasite and its nucleus (Fig. 3.1d) and were able to distinguish multiple stages. At 24 h, we observed trophozoites, which are small rounded intracellular stages with a single nucleus, and stages of increasing size with an unsegmented cytoplasm and two or four nuclei that we interpret as intermediate stages. We also observed mature meronts with eight nuclei, before and during egress, as well as free merozoites (Fig. 3.1c).

At 48 h, we observed sexual stages (we use the terms male and female gametes according to the convention of the extensive literature on sex in the malaria parasite *Plasmodium*(Josling and Llinás, 2015)). Female or macrogametes had a single nucleus that was significantly larger (0.89 μm^2) than the nuclei of asexual stages (0.43 μm^2; $P <$ 0.0001, unpaired Student's *t*-test) and male or microgametes, which had dense, bullet-shaped nuclei (0.15 μm^2; $P < 0.0001$, unpaired Student's *t*-test). We found up to 16 of these nuclei in males or microgamonts (the precursor stage of the male gamete). We next conducted time-course experiments and assigned a stage to all of the parasites observed using the morphometric characteristics that are defined above. Initially, all parasites in culture were asexual meronts and trophozoites. After 36 h, the culture rapidly sexualizes, with gamonts and gametes representing >80% of all stages after 72 h (Fig. 3.1e).

Post-fertlization stages and oocyst development are only observed in vivo but not in vitro.

We infected HCT-8 cells with a strain that has its *Cryptosporidium* Oocyst Wall Protein-1 (COWP1, cgd6_2090; detailed in chapter 4) tagged with 3XHA to look for oocyst development *in* vitro. Oocysts made only 0.1% of the total population at 48h post-infection. To determine whether these oocysts were produced *de novo*, we labelled newly synthesized DNA using the thymidine kinase analogue, EdU. As reported previously, *C. parvum* readily incorporates thymidine-kinase-activated tracers(Striepen et al., 2004). In vitro, none of the oocysts observed at 48 h after infection were labelled (Fig. 3.2a-b), indicating that these were from the inoculum and not formed in culture. We

next studied whether male gametes mature and go on to fertilize females in culture. At 48 h after infection, we found that 10.6 ± 0.8% of all male gametes are released from gamonts and we frequently observed them to be attached to female gametes (Fig. 3.2c). At this time point, 16.1 ± 2.2% of all females identified by H2B–mNeon featured an attached male. Note that attachment was polar with the HAP2 (cgd8_2220; male-specific fusogen detailed in chapter 4)-marked end oriented towards the female. However, we did not observe female gametes with an internalized male gamete. To investigate how this compares with in vivo infection, in which we know that fertilization occurs, segments of the small intestine were recovered from infected mice, cryosectioned and processed for immunofluorescence. When using the H2B–mNeon line we rarely observed males attached to females, yet we frequently observed parasites that contained both an identifiable female and male nucleus (~5% of all stages; Fig. 3.2d). *In vivo*, zygotes and various intermediates of meiosis with one, two and four nuclei were readily observable and these post-fertilization stages accounted for 35% of all parasites (Fig. 3.2e). As these stages mature, they grow, and their size significantly exceeds that of the female gametes observed in culture ($P = 0.0023$; Fig. 3.2f). We made very similar observations when studying the COWP1–HA (female-specific marker; detailed in chapter 4) strain *in vivo*. Meiotic divisions precede and partially overlap with the deposition of the oocyst wall (Fig. 3.2g). We also observed strong labelling of these stages with RAD51 (cgd5_410), a DNA repair protein that has an important role in homologous cross-over during meiosis (Fig. 3.2h).

Genes required for post-fertilization development are upregulated in *in vivo* females but not in *in vitro* females

Fertilization of females *in vivo* and lack thereof in culture should result in transcriptional differences between *in vitro* and *in vivo* females. Hence, we specifically isolated female stages (tdTomato-positive) by flow cytometry from infected mice and HCT-8 culture (Fig. 3.3a) for transcriptional analyses (detailed in chapter 4). Both *in vitro* and *in vivo* females were found to have similar levels of expression of genes required for amylopectin synthesis (carbohydrate, GO:0005975), meiosis (DNA; GO:0006259), redox processes (redox; GO:0055114) and oocyst wall biogenesis(Truong and Ferrari, 2006) as confirmed by Gene Set Enrichment Analysis (GSEA, Fig. 3.3c) and PCA analysis (Fig. 4.2c). This suggests that developmental competence of in vitro females is normal. We also transcriptionally profiled sporozoites released from oocysts and infected bulk culture after 24 h and 48 h for comparison, and we found that sporozoites are moderately more similar to females *in vivo* than females *in vitro* (Fig. 4.2c and Supplementary Fig. 4.8). Overall, we conclude that a fraction of in vivo sorted cells moved beyond fertilization to the production of sporozoites. This is consistent with the expression of the protein components of the gliding machinery required for the motility of invasive stages that is only observed *in vivo* females (Fig 3.3e).

A genetic two-component assay demonstrates gamete fusion *in vivo* but not *in vitro*.

Our experiments suggested a lack of fertilization *in vitro*. To test this rigorously, we devised a genetic assay for *Cryptosporidium* gamete fusion. We engineered a two-

component system that produces a reporter signal only after cytoplasmic fusion of two strains. The first component is a driver strain that expresses Cre recombinase (Supplementary Fig. 3.2), an enzyme that excises DNA segments flanked by *loxP* recognition sequences (the 34 bp *loxP* sequence is absent from the *C. parvum* genome). Cre is driven by a constitutive promoter and detected in transgenics using a specific antibody. The second component is a strain carrying a *tdNeon* reporter in the *COWP1* locus linked by a 2A skip peptide. A terminator sequence flanked by *loxP* sites blocks expression, Cre-mediated excision will release the block (Fig. 3.4a). Mice were infected with each strain of parasite individually or with both in equal proportion. Only infection with both strains resulted in the shedding of green fluorescent oocysts (~10% of total oocysts from days 3–10 after infection; Fig. 3.4b–d). We next performed this assay in vitro and tested for tdNeon expression at 48 h and 72 h after infection. In contrast to mice, we did not detect expression of tdNeon in HCT-8 cells that were coinfected with Cre and floxed strains (Fig. 3.4b, c; $P = 0.0002$). Fluorescence was readily detected in our positive control, HCT-8 cells infected with oocysts obtained by Cre–loxP coinfection in mice. We conclude that gamete fusion occurs *in vivo* but not *in vitro*, and this block in fertilization prevents the formation of new oocysts and continued growth in culture.

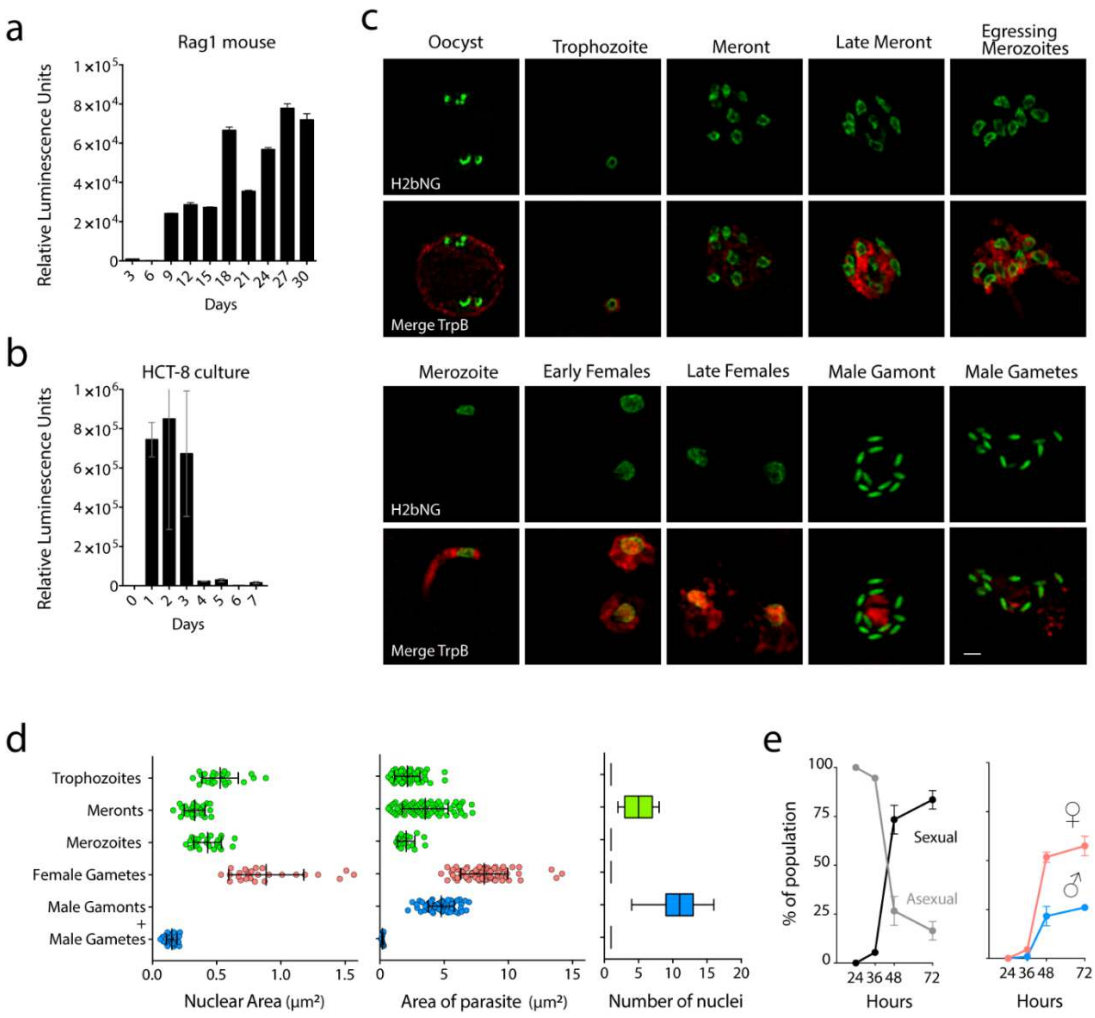


Fig. 3.1 *Cryptosporidium* life cycle stages revealed by the H2B–mNeon transgene. **a**,**b**, *C. parvum* infection was monitored by luciferase activity in mice lacking mature T and B cells (**a**; faeces were measured every 3 d) and HCT-8 cultures (**b**). Data are mean ± s.d. from three independent biological replicates. **c**, HCT-8 cultures were infected with H2B–mNeon transgenic parasites and fixed at 24 h ('Oocyst', 'Trophozoite', 'Meront', 'Late meront' and 'Egressing merozoites'), 36 h ('Merozoite') and 48 h ('Early females', 'Late females', 'Male gamont' and 'Male gametes') time intervals. Green, nuclei; red, cytoplasm (antibody against tryptophan synthase B (TrpB), cgd5_4560). This experiment was performed three times with similar results. Scale bar, 1 µm. **d**, Morphometric analyses of the size ($n = 25$) and number ($n = 100$) of nuclei and the area for each stage ($n = 75$) on the basis of the markers shown in **c**.The nuclear area (left) and total area (middle) of parasites stages are shown as mean ± s.d. of individual values represented as dots. The number of nuclei at particular parasite stages are represented as box plots (right). The box shows median and quartile range and whiskers represent extreme values. **e**, A time-course experiment in which stages were scored using the parameters defined in **d** revealed abrupt sexualization of cultures at 48 h into culture. Data are mean ± s.d. from three independent biological replicates.

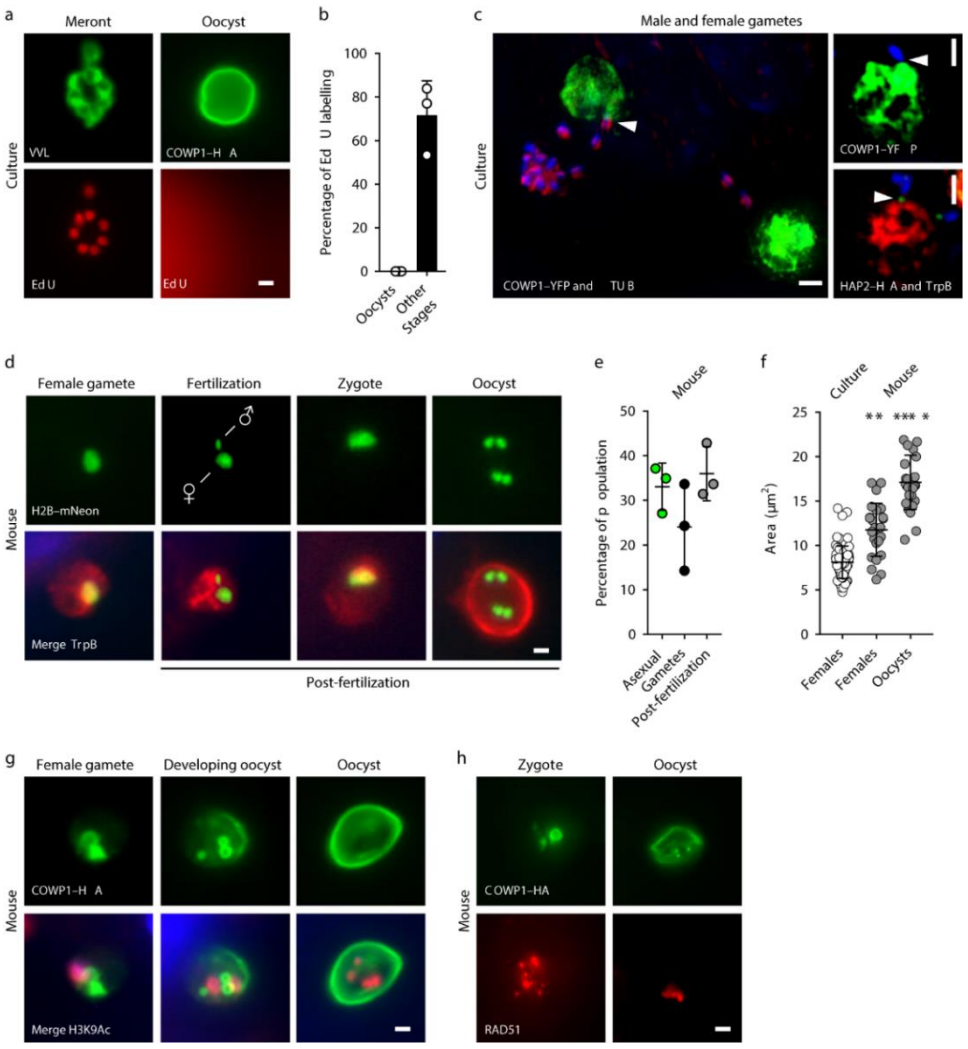


Fig. 3.2 *Cryptosporidium* males locate females in culture, but fertilization and meiosis only occur *in vivo*. **a,b**, HCT-8 cells were infected with COWP1–HA *C. parvum* and after 36 h of infection, the nucleotide analogue EdU was added to the medium. Then, 12 h later, cells were click labelled and counterstained with anti-HA antibodies or *Vicia villosa* lectin (VVL; **a**). Cells were scored for nuclear EdU labelling (**b**); 100 stages were quantified for three biological replicates, and the experiment was performed twice. Data are mean ± s.d. **c**, Representative images of encounters between male and female gametes in culture; gametes were identified using the indicated transgenes or antibodies, and attached males are highlighted by arrowheads. YFP, yellow fluorescent protein. **d**–**h**, *Ifng*−/− mice were infected with H2B–mNeon-expressing (**d**) or COWP1–HA-expressing (**g**,**h**) parasites, and intestines were sectioned and prepared for immunofluorescence assays and counterstained with anti-TrpB, anti-H3K9Ac or anti-RAD51 antibodies. Representative micrographs show progression of events following fertilization. Post-fertilization stages are abundant in vivo (**e**) and these stages were significantly larger than those found in vitro (**f**); each symbol represents a parasite, $n = 25$. For **e**, data are mean ± s.d. from three independent mice. For **f**, data are mean ± s.d.; the statistical analysis was performed using a two-sided Student's *t*-test comparing cultured females with in vivo females (**$P = 0.0023$) or with *in vivo* oocysts (****$P = 0.0001$). All of the microscopy experiments shown in **d**–**h** were performed twice with similar results. Scale bars=1 μm.

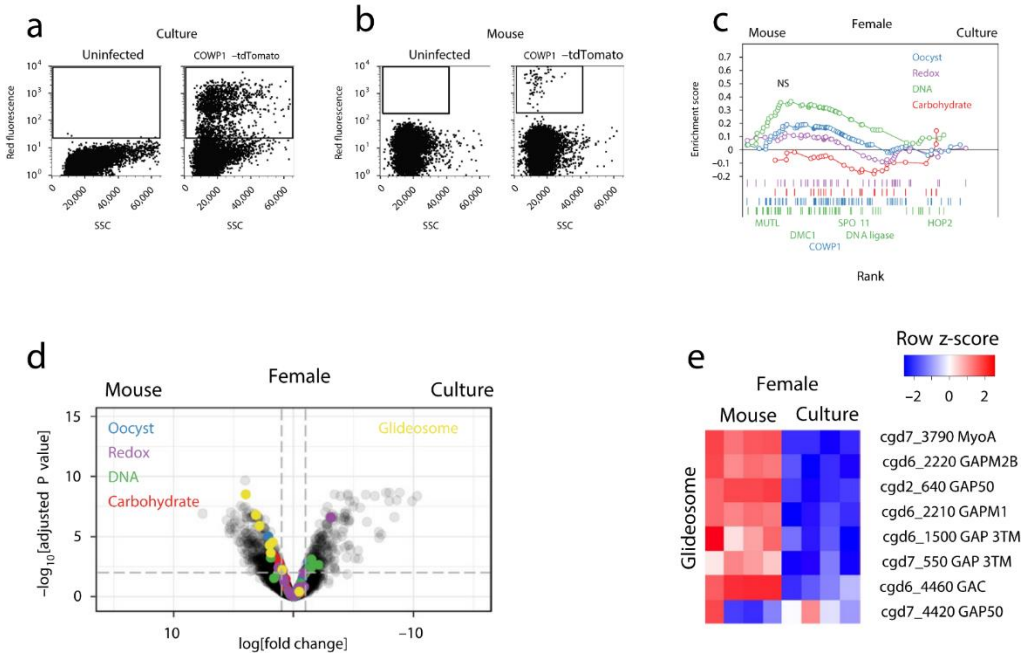


Fig 3.3 Isolation of *in vivo* and *in vitro* female stages by cell sorting and RNA sequencing. **a**,**b** Flow cytometry of infected cells with the indicated markers and origins. Gates used for sorting are shown as boxes. This experiment was performed twice. SSC, side scatter. **c**, Gene set enrichment analysis (GSEA) with multiple testing correction comparing cultured *in vitro* and *in vivo females*. Custom gene signatures were generated using Gene Ontology or community datasets available at CryptoDB. Processes annotated as 'oocysts', 'carbohydrates' 'redox' and 'DNA' are upregulated in females when compared to asexual stages (detailed in the figure 4.2 of the chapter 4). Note, that these female-specific processes do not show significant enrichment between *in vitro* and *in vivo* females (NS= not significant). *n* = 4 biological replicates per group. **d**, Volcano plots showing *C. parvum* genes that were differentially expressed between *in vitro* and *in vivo* females. *n* = 4 biological replicates per group. Each symbol represents a *C. parvum* gene, those genes representing the leading edge from **b** are indicated by the colour according to the pathway that they act in. The horizontal dashed line shows an FDR-adjusted *P* value of 0.01; the vertical dashed lines indicate a $\log_2$-transformed fold change of −1 and 1, respectively. **e**, A heat map of glideosome components, which are indicated in yellow in **d**. *n* = 4 biological replicates per group

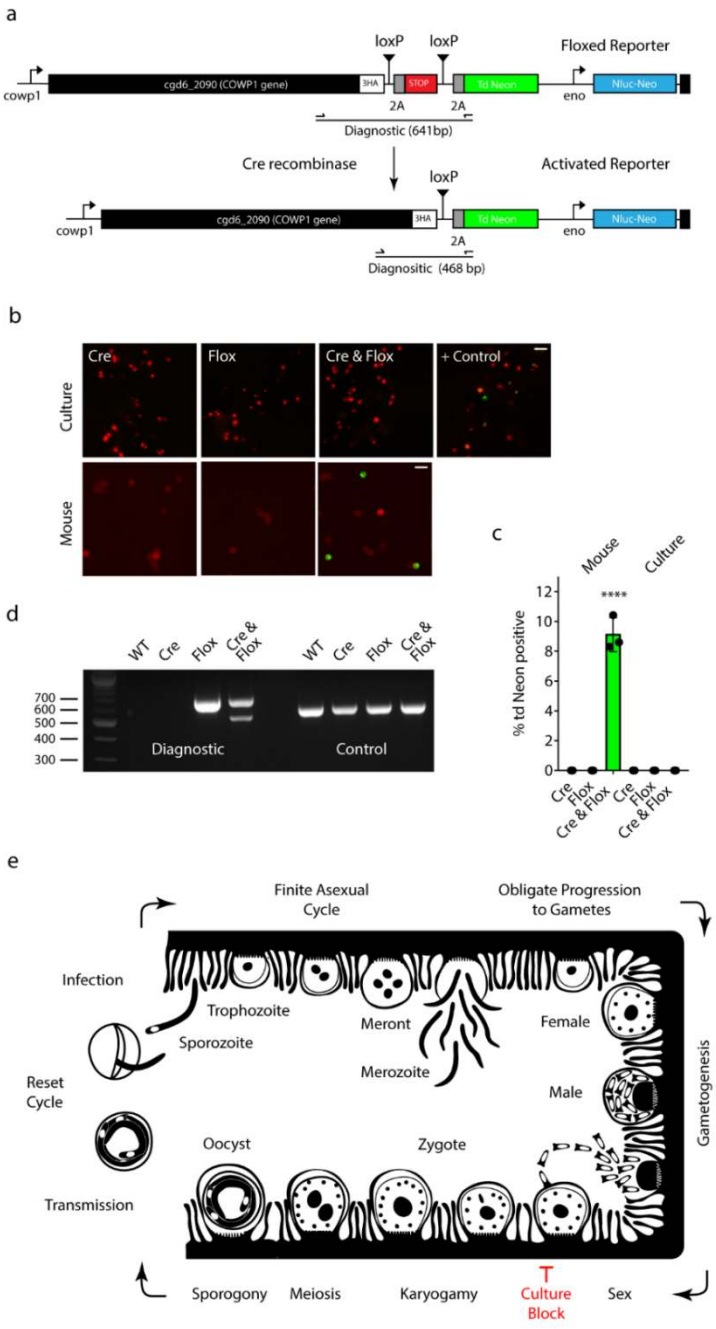
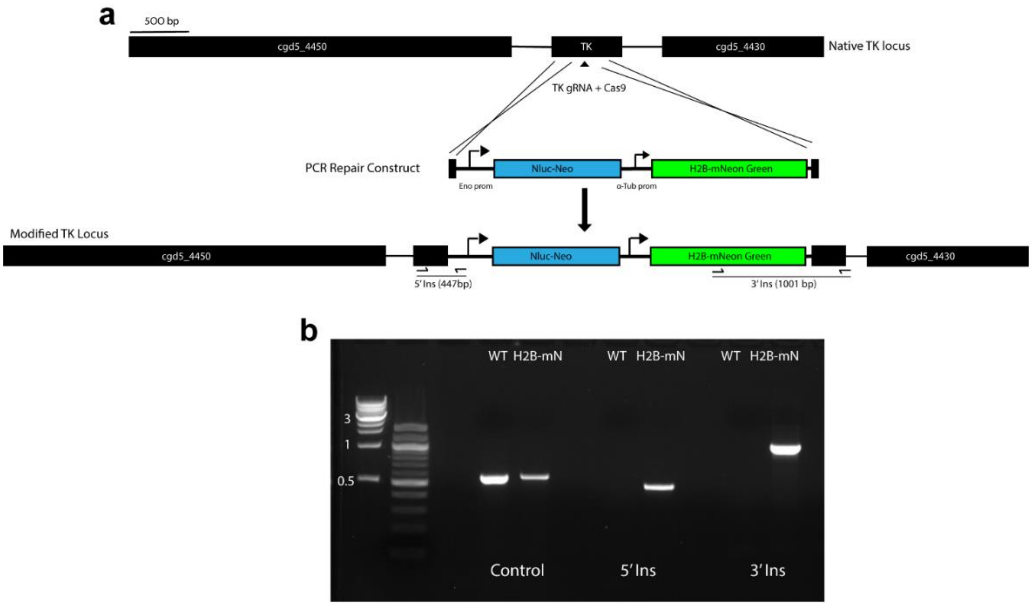
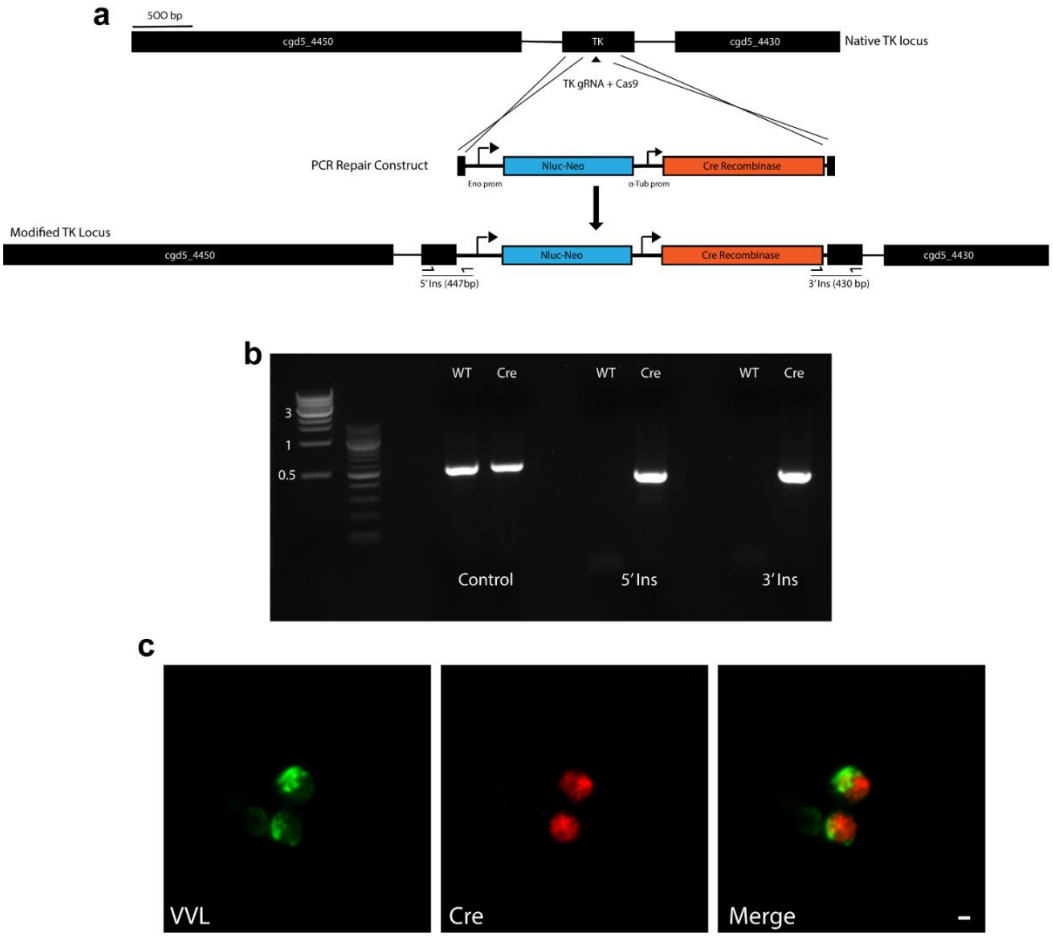


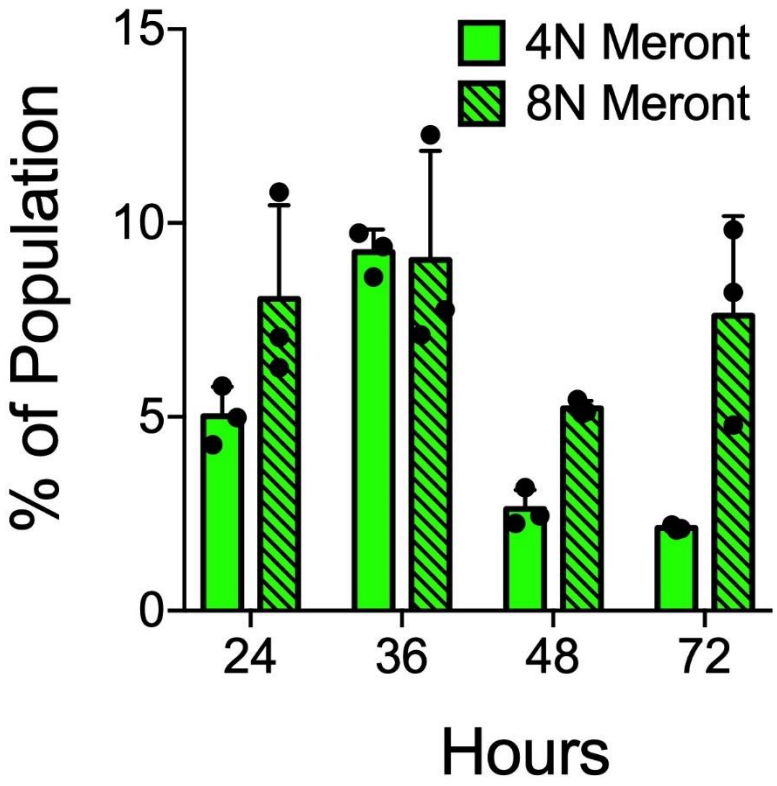
Fig. 3.4 A genetic fusion assay demonstrates fertilization *in vivo* but not *in vitro*. **a**, To detect gamete fusion, we engineered two *C. parvum* strains, one that constitutively expresses Cre recombinase (Supplementary Fig. 3.2) and a second that carries a *tdNeon* reporter flanked by *loxP* at the *COWP1* locus. **b**, Cre-mediated excision of a terminator results in reporter expression. HCT-8 cultures and *Ifng*−/− mice were infected with each strain individually or in combination. This experiment was performed twice. Cultured parasites were counterstained with anti-TrpB antibodies, oocysts with *Macula pomifera* agglutinin (both red) and scored for tdNeon expression. Scale bars, 10 μm. **c**, Three replicates were quantified for green fluorescence and 1,000 cells were counted for each replicate. Data are mean ± s.d. Green fluorescence was only observed after *in vivo* infection and only when both strains were present (****$P = 0.0002$, two-sided Student's *t*-test). As a positive control, cells were infected with parasites that were crossed in vivo (indicated in **b**). **d**, PCR mapping of the floxed (diagnostic) and α-tubulin (control) loci using the primer pair shown in **a**. Genomic DNA was isolated from wild-type parasites as well as oocysts from the mouse infection experiments. Crossing resulted in a new amplicon that was consistent with precise Cre excision. This experiment was performed twice with similar results. **e**, Schematic model of the C*. parvum* life cycle that highlights the model of obligate progression to sex and the fertilization block in HCT-8 culture. We do not show type II meronts here, which are often depicted as an obligate step towards gametes. Although we observed meronts with four and eight nuclei, we did not find a quantitative link between the meronts with four nuclei and gametes (Supplementary Fig. 3.3).



Supplementary Figure 3.1: Construction of a *C. parvum* strain expressing Histone H2B-mNeon. (**a**) Map of native *C. parvum* TK locus, the targeting construct and the modified locus indicating the CRISPR/Cas9 induced break and areas of homologous recombination. (**b**) PCR analysis demonstrating successful insertion into the TK locus mapping the regions of 5' and 3' cross over, see (**a**) for the respective sizes of the predicted amplicons. This experiment was performed two times with similar results.



Supplementary Figure 3.2: Construction of a *C. parvum* strain expressing Cre recombinase. (**a**) Maps of PCR repair construct for Cre expression, TK locus and modified TK locus post insertion. (**b**) PCR analysis for integration of the Cre cassette in the TK locus. (**c**) IFA of the transgenic strain emerging from drug selection with antibody to Cre confirms expression of Cre recombinase. Parasites were counterstained with VVL (Scale Bar= 1 μm). PCR mapping experiment and microscopy experiments each were performed twice.



Supplementary Figure 3.3: Abundance of different meront types in culture over time. HCT-8 cell cultures were infected with H2b mNeon parasites and fixed at 24, 36, 48 and 72 hours. Cultures were scored for meronts with either eight (type I) and four (type II) nuclei which are represented here as % of all observed parasite stages. Three independent biological replicates were used for this experiment and the data is represented as mean ± SD. Note that we do not find a temporal association between the emergence of meronts with four nuclei and the (subsequent) emergence of sexual stages.

3.4 Discussion

The complex life cycles of parasites are among the most fascinating aspects of their biology. *Cryptosporidium* is a minute protist with a highly reduced genome, and yet it continuously transforms itself into a menagerie of specialized stages that amplify asexually, transform into male and female gametes, undergo fertilization and build a resilient spore. Here we trace and analyze this life cycle, label and isolate specific stages, and discover the genes that define these stages to provide a road map for the molecular dissection of parasite sex. We rigorously demonstrate that *Cryptosporidium* undergoes sexual differentiation in HCT-8 culture, but a block in gamete fusion prevents the development of new oocysts and the parasite cultures ultimately arrest (Fig. 3.4e). The cause of this block remains to be elucidated but seems to be linked to the host rather than the physiology of the parasite. This may be overcome partially by culture modalities that provide structured environments to transformed cells(Heo et al., 2018; Morada et al., 2016) or by using stem-cell-derived models that self-organize into more complex organoids(Heo et al., 2018; Wilke et al., 2019). It is unclear whether this is due to differences in the infected host cells themselves or due to factors secreted by more complex assemblages. In all cases, improved growth is linked to appearance of oocysts. Overall, this is consistent with a model of obligate developmental progression and suggests that interventions targeting sex could potentially not only block transmission but also cure ongoing infection.

However, this proposed model of the lifecycle requires rigorous testing and validation in a mouse model. This can be achieved by specifically disrupting the transition phase from gametogenesis to sporogony to specifically block oocyst biogenesis. Amelioration of infection in mouse model because of the disruption of sexual development (and oocyst development) would strongly support the model of obligate requirement of sex to

maintain infection. Multiple processes like gametogenesis, male gamete motility, gamete interaction and post-fertilization development can be potentially targeted to test the proposed model. A comprehensive transcriptomics study of the sexual stages will provide us with a repository of gene targets that might be involved in different processes mentioned above. Such a study will also reveal potential drug and vaccine targets to specifically block sexual development and to potentially cure individuals.

Chapter 4: Defining the transcriptomes of the sexual stages of *Cryptosporidium*

Partial contents of this chapter are already published as: Tandel, J. et al. Life cycle progression and sexual development of the apicomplexan parasite Cryptosporidium parvum. Nat Microbiol 4, 2226–2236 (2019).

4.1 Abstract

Cryptosporidium is the leading cause of diarrhea-induced mortality in children after rotavirus. *Cryptosporidium* has a single-host lifecycle, and the parasite undergoes asexual replication and sexual development in the same host. However, the molecular mechanisms underlying this single-host lifecycle program are unknown. A comprehensive understanding of genes expressed in asexual and sexual stages of the parasite is required. The presence of a complex mixture of asexual and sexual populations of *Cryptosporidium* in infected culture and mice makes it technically challenging to delineate transcriptomes of asexual stages, males, and females. We thus identified stage-specific markers and developed reporter parasites that allowed us to isolate cells in a stage-specific fashion. Populations enriched in this way were analyzed by mRNA sequencing and we compared expression profiles between stages to define the transcriptome of asexual, male, and female parasites. The female transcriptome was found to be enriched for structural and enzymatic components required for oocyst wall synthesis, meiosis, and amylopectin (glycogen-like polysaccharide) synthesis. Males were enriched for uncharacterized transmembrane proteins, secreted and transmembrane proteases that might be involved in male-female interactions and gamete fusion. An evolutionarily conserved copper transporter was found to be enriched in males, and its *Plasmodium* ortholog is required for male exflagellation. Additionally, our transcriptional analyses have identified four sex-specific ApiAP2-DNA binding proteins that might be involved in regulating sexual stage-specific processes. Many of the sexual stage processes identified by our transcriptional analyses represent important drug and vaccine targets to block oocyst development.

4.2 Introduction

Cryptosporidium is one of the major contributors to global childhood mortality.(Kotloff et al., 2013; Liu et al., 2012) *Cryptosporidium* infection in its chronic and asymptomatic form contributes to poor developmental outcomes in children.(Agnew et al., 1998; Checkley et al., 1997, 1998) Drug(Amadi et al., 2009) and vaccines(Lemieux et al., 2018; Mead, 2010, 2014) against cryptosporidiosis are currently lacking and a detailed understanding of molecular profiles of different stages of the parasite will provide crucial therapeutic targets. A detailed molecular map of asexual and sexual stages will provide key insights into mechanisms of stage differentiation in *Cryptosporidium*. These mechanistic insights will help identify specific lifecycle stages that can be therapeutically targeted to block transmission and infection.

Our previous work has demonstrated that *Cryptosporidium* cannot grow continuously in HCT-8 cells due to a lack of parasite mating *in vitro*. We observed infection, asexual replication, and robust sexual development (~80%) but no fertilization and post-fertilization development was documented in HCT-8 cells.(Tandel et al., 2019) Contrastingly, fertilized and post-fertilized stages make up to ~35% of the total parasite population in infected mice.(Tandel et al., 2019) We have validated lack of parasite crossing in culture by using a Cre-reporter assay for sex.(Tandel et al., 2019) Our findings suggest that to grow continuously the parasite has to pass through a phase of obligate sexual differentiation, gamete mating, and sex, followed by meiosis and the formation of a new generation of the oocyst. We hypothesize that this progression which involves a regular reset of the lifecycle essentially every three days may also be an obligatory requirement for *Cryptosporidium* to maintain the continuous infection of a

host.(Tandel et al., 2019) As a result, disrupting sexual development or mating in *Cryptosporidium* break the cycle of infection.

A detailed understanding of sexual-stage specific processes is required for disrupting sex. We believe that stage-specific gene expression and the regulatory elements that control it would be most suitable as targets for such intervention. However, at this point, we know very little about these processes and the specific genes involved. Some insights have been obtained by following infected cultures longitudinally.(Mauzy et al., 2012) The simultaneous presence of multiple parasite stages in cultures and mice however complicates the isolation of pure populations of sexual stages for transcriptomics analyses. Here we define transcriptome of sexual stages by first identifying sex-specific molecular markers and then we use these markers to engineer sex-specific fluorescent reporter strains. We enrich sexual stages by flow cytometry and define their transcriptome by mRNA sequencing and comparative analyses.

4.3 Results

Cryptosporidium Oocyst Wall Protein-1 (COWP1) is a female-specific gene and its promoter drives female-specific expression of a reporter

The female gamete produces and stores components of the oocyst wall in wall-forming bodies that were previously described in related parasites, and earlier studies identified *Cryptosporidium* oocyst wall protein-1 (COWP1).(Spano et al., 1997) We tagged the COWP1 protein (cgd6_2090) by C-terminal insertion of either a fluorescent protein or a haemagglutinin (HA) epitope into the native locus (Supplementary Figs. 4.1a,b and 4.2). Transgenic parasites showed strong labeling of the oocyst wall (Fig. 4.1a). When infected cell cultures were examined, no expression was apparent at 24 h, but numerous fluorescent parasites were observed at 48 h. These parasites had a single large nucleus and multiple small foci of COWP1 consistent with wall-forming bodies (Fig. 4.1b). We next observed COWP1 expression in parasites throughout a detailed time course in vitro using the HA-tagged COWP1–HA strain. Parasites expressing COWP1–HA closely matched the stages that we identified for female gametes using H2B–mNeon in terms of morphology and the proportion of the overall parasite population at the observed time points (Fig. 4.1d, Supplementary Fig. 4.2). To study what controls the stage specificity of gene expression, we placed fluorescent protein reporters under the control of the presumptive COWP1 promoter region and ectopically expressed these constructs (Fig. 4.1 c,d, Supplementary Fig. 4.1c-e). Fluorescence (now cytoplasmic) was exclusively associated with female gametes and temporal expression matched that of the native locus, demonstrating that promoters, and thus probably transcription initiation, control stage specificity of gene expression (Fig. 4.1d).

Defining the *C. parvum* female transcriptome

To discover the genes associated with sex in *C. parvum*, we sought to isolate specific parasite stages. We developed flow cytometry protocols to sort infected cells based on the expression of fluorescent proteins by the parasite (Supplementary Fig. 4.3). Figure 4.2a shows sorts from cell culture and mice, in which infected and uninfected cells are readily discernible. Next, we conducted mRNA sequencing experiments using cells sorted for eno promoter-driven tdNeon (eno–tdNeon; Supplementary Fig. 4.4) and COWP1 promoter-driven tdTomato (COWP1–tdTomato) from 24 h or 48 h cultures to isolate asexual and female stages, respectively, as well as females from infected mice (Supplementary Fig. 4.5). We obtained between 5 million and 35 million reads for each sample, with 50,000 to 7,000,000 mapping to the *C. parvum* transcriptome, representing 2,500–3,400 of the 3,885 *C. parvum* genes (see Methods; Supplementary File 4.6). The analysis revealed robust transcriptional differences between asexual and female parasites. The transition to female gametes was accompanied by a two-fold or greater increase (false discovery rate (FDR)-adjusted $P < 0.01$) in the expression of 673 genes including *COWP1* (Fig. 4.2d, 451 genes are downregulated). We compared these genes with those that are associated with female gametogenesis in *Plasmodium berghei*(Yeoh et al., 2017) (this particular dataset was most comparable to our experiment) and found 72 shared orthologue groups that encompass 73 *C. parvum* genes and 81 *P. berghei* genes (~31% of the female *C. parvum* genes with an identifiable *P. berghei* homologue) as well as 595 *C. parvum*-specific genes. We also compared female-specific genes from *C. parvum* and *P. berghei* to gametocyte genes in *Eimeria tenella*(Walker et al., 2015) and identified a set of 41 orthologue groups that contained 42 *C. parvum* genes, 49 *P. berghei* genes, and 55 *E. tenella* genes.

The functional annotation of the *C. parvum* genome using Gene Ontology is very limited. We, therefore, assembled pathways using the Gene Ontology terms for DNA, carbohydrate, and oxidase and reductase metabolism, as well as a candidate oocyst wall proteome(Truong and Ferrari, 2006), to conduct enrichment analysis (Fig. 4.2b). We found significant enrichment when comparing females with asexual parasites (FDR-adjusted $P < 0.005$). Figure 4.3 shows additional clustering based on literature-based pathway annotation, those genes that were also found in the leading edge of the enrichment analysis are highlighted in red. *Cryptosporidium* is a haplont and meiosis is presumed to follow fertilization. Consistent with this view, we found that conserved eukaryotic factors of meiotic recombination— including DMC1, Spo11, HORMA, and HOP2—were preferentially expressed in females as well as proteins with a probable role in meiosis-associated DNA repair, including the mismatch repair protein MutL and DNA ligase (Figs. 4.2b and 4.3a). We also note chromosome segregation and cell-division factors, including condensins, cohesins, stage-specific cyclins, cyclin-dependent and NIMA kinases, and cytoskeletal proteins. Overall, we identified 37 genes in this meiosis category and many of these are shared among apicomplexan females (Supplementary Fig. 4.6).

Cryptosporidium oocysts remain infectious for months(Fayer et al., 1998) and female transcription provides candidate mechanisms of this resilience. Twenty-two enzymes that are required for the metabolism of amylopectin and trehalose are upregulated in females (Fig. 4.3c). Amylopectin is consumed(Fayer et al., 1998) by the sporozoites and the disaccharide trehalose may play a role in energy storage as well as serving to moderate osmotic stress(Elbein et al., 2003). *Cryptosporidium* oocysts are also highly resistant to chemical assault, including water chlorination(Korich et al., 1990), due to a complex multilayered wall made of proteins, carbohydrates, and lipids(Samuelson et al.,

2013). We identified 69 genes that are preferentially expressed in females and encode proteins with a probable role in oocyst wall synthesis, most of which have a predicted signal peptide. These include previously identified oocyst wall proteins and their homologues, numerous proteases (aspartic peptidases, serine proteases, and subtilases) and protein modifiers, such as amine oxidases(Walker et al., 2015), that serve to build or modify the proteinaceous components of the wall (Fig. 4.3b). Many protozoans have chitin and glucan cyst walls and the *Cryptosporidium* wall is labeled by various lectins, but no wall polysaccharide has been identified(Bushkin et al., 2012; Samuelson et al., 2013). Similarly, we did not find stereotypical chitin or glucan synthase; however, among female transcripts, there are numerous glycosyltransferases. Interestingly, this set contains two polysaccharide pyruvyl transferases (cgd7_2583 and cgd6_1450) and a UDP-glucose dehydrogenase (cgd8_920) that were all recently linked to capsule synthesis in pathogenic *Acinetobacter*(Kasimova et al., 2018), as well as proteins with lectin domains including chitin-binding proteins, which suggests a proteoglycan structure (Fig. 4.3b). Finally, we found that the two giant lipid synthases—polyketide synthase(Zhu et al., 2002) (cgd4_2900) and type I fatty acid synthase (cgd3_2180)—that were acquired by horizontal transfer from bacteria are specifically expressed in females. In *Mycobacterium tuberculosis*, these enzymes work in series to produce mycolic acids, which are key components of the mycobacterial wall. We chose two previously uncharacterized genes for experimental validation that were identified here as likely to be female-specific (cgd7_4810 and cgd7_5140) and used CRISPR–Cas9 to attach a c-Myc epitope tag. Transgenic oocysts reacted strongly with the anti-c-Myc antibodies, and in the culture, we noted exclusive staining of female gametes (Supplementary Fig. 9 of Tandel et. al.(Tandel et al., 2019)).

Among the genes for which expression is unique to females in vivo are also cgd5_2570 and cgd8_3130, which encode apetala2 (AP2)-domain proteins (Fig. 4.3d). AP2-type transcriptional regulators have been demonstrated in other apicomplexans to bind to specific genome features(De Silva et al., 2008; Kafsack et al., 2014), including promoters, and emerged as master regulators of life cycle progression. *Cryptosporidium* encodes a comparably small set of AP2s(Oberstaller et al., 2014) and many of these genes seem to be developmentally regulated, with expression patterns that differ greatly between asexual and sexual parasites and between *in vitro* and *in vivo* (Fig. 4.3d).

HAP2 is a male-specific maker but its promoter does not drive the expression of fluorescent reporters

Male gametes in most apicomplexans move with the aid of flagella, and the exclusive presence of these flagella in males provides numerous marker proteins. *Cryptosporidium* male gametes lack flagella but have a peculiar set of microtubules that are associated with and run along the length of their spindle-shaped nuclei(Ostrovska and Paperna, 1990) that we visualized using super-resolution microscopy (Fig. 4.4a). We also identified a *C. parvum* homologue of hapless2 (HAP2; Supplementary Fig. 4.7), a class II membrane fusion protein that is required for gamete fusion in a range of organisms including *Plasmodium falciparum* and *Chlamydomonas reinhardtii*, and is expressed by the male or minus gamete, respectively.(Liu et al., 2008) We epitope-tagged the C terminus of HAP2 and infected cell cultures with transgenic parasites. HAP2–HA labeling was found exclusively in

male gamonts (Fig. 4.4b) and gametes and was restricted to one end of the polarized male gamete (Fig. 4.4c). Time-course experiments demonstrated the appearance of males after 42 h of culture (Fig. 4.4d). We note that both sexes emerge at the same time and a male to female ratio of 1:2 for gamonts and 6:1 for gametes. We did not observe the expression of mCherry in males or any other stage when presumptive HAP2 promoter was used. This can be attributed to weak strength of the promoter or due to the presence of an unusually high number of introns (Supplementary Figs 4.7a-b and d) suggesting cryptic, post-transcriptional regulation of HAP2.

Identification of an 'early' male developmental stage and an ApiAP2 gene that is uniquely expressed in this stage

In addition to stages that we readily identify as male gamonts, we also noted HAP2 in a minor population of multinucleated parasites that become apparent in culture after 48 hours of infection (Figs. 4.5b and 4.4 b-c). The nuclei in these stages were round and closely clustered into a central rosette, while the nuclei of mature male gamonts are elongated and dispersed (Figs. 4.5b and 4.4 b-c). HAP2 appeared to surround the nuclei, a localization that might coincide with the endoplasmic reticulum. Overall, we concluded that these stages are likely early developmental forms of males. We identified an ApiAP2 gene with a similar temporal expression pattern to HAP2(Mauzy et al., 2012) (Supplementary fig 4.9a). We tagged this gene with an mCherry reporter (Supplementary fig 4.9b). The protein was specifically expressed in stages with nuclear morphology and timing resembling that of HAP2 'rosette' stages (Fig. 4.5a). AP2-14 (cgd6_2670) was localized to the nucleus. Note that DAPI staining pattern of the AP2-14 expressing stages resembles that of HAP2 'rosette' stages. We rarely observed the protein in mature male gamonts where it appeared cytoplasmic (Fig. 4.5a) and it is not

found in egressed male gametes (Fig. 4.5a). AP2-14-expressing stages had a median of 6 nuclei (ranging from 4-8 nuclei), and the total nuclear area of these stages (Fig. 4.5c; 1.829 ± 0.33 μm^2) was similar to that of HAP2 rosette stages (Fig. 4.5c; 1.46 ± 0.32 μm^2; $^{*}p< 0.03$) but significantly different to that of meronts (Fig. 4.5c; 4.12 ± 0.83 μm^2; $^{****}p< 0.0001$). Based on the morphological similarities between HAP2 rosette stages and AP2-14 expressing stages, we hypothesized that AP2-14 is expressed in early males, but its expression is lost in mature males.

To test whether AP2-14 is a gene specifically expressed in males, we engineered a strain in which we HA-tagged HAP2 and simultaneously introduced a tdNeon reporter under the transcriptional control of the presumptive promoter of the AP2-14 gene (Supplementary fig 4.9c; 382 bp of noncoding sequence 5' to the start codon). We observed tdNeon expression in male gamonts and male gametes (Fig. 4.5d). 97% of the tdNeon-expressing stages were found to be positive for HAP2 (Fig. 4.5c). Unlike the reporter, the AP2-14 protein is not observed in mature male gametes. This is most likely due to a post-translational mechanism that ensures the proteasomal decay of the AP2-14 protein once early males mature into gametes. However, experimental data are needed to confirm this hypothesis.

Defining the *C. parvum* male transcriptome

We infected HCT-8 cells with AP2-14 tdNeon parasite strain and sorted 10,000 male gamont-infected cells after 48 hours. We sequenced the RNA of the sorted male population (Fig 4.6a) and obtained between 18 million and 29 million reads per sample out of those 500,000 to 1.2 million aligning to the *C. parvum* genome (the remainder represents human transcripts as we sequence infected cells and not just parasites). We compared this male transcriptome to pre-existing transcriptomes of females (the *in vitro*

dataset) and asexual stages. We identified 546 and 1,271 genes being differentially expressed between males and females and males and asexual stages, respectively (false discovery rate (FDR)-adjusted $P < 0.01$, see Fig 4.6b and supplementary fig 4.10). 263 and 720 genes were found to be at least two-fold upregulated in males when male-female and male-asexual transcriptomes were compared, respectively (Fig 4.6b).

We identified genes that are commonly upregulated in males between male-female and male-asexual comparisons. This revealed a total of 116 genes unique to male gametes (Fig 4.6b). 51 genes (out of the 116 genes) were defined as uncharacterized proteins and only 4 of them had an ortholog in other apicomplexan parasites. Out of the 48 non-conserved uncharacterized proteins, 19 genes are predicted transmembrane proteins (Fig 4.6c) and 5 genes contain a signal peptide but no transmembrane domain (Fig 4.6d).

Molecular mechanisms of gamete fusion have been well studied in higher eukaryotes.(Rothmann and Bort, 2018) Mammalian sperm contain ‘acrosome’ secretory vesicles that contain key mediators like proteases, ion channels, transmembrane receptors, etc. which enable gamete fusion.(Rothmann and Bort, 2018) Sperm-specific proteases are needed to dissolve the extracellular zona pellucida proteinaceous layer to access female gametes in mammals.(Rothmann and Bort, 2018) We identified a total of six proteases that were upregulated in male gametes, and of these 4 proteases (cgd1_2240, cgd1_3690, cgd7_2850, and cgd2_3560) are either secreted or a membrane proteins (Fig 4.6e). These proteases might be involved in interacting with the female gamete. cgd1_2240 (aspartly peptidase A1 family protein is the homology of *Plasmodium* plasmepsin V protein (OG5_132039), which is involved in protein export to the host cell.(Boddey et al., 2010) Unlike the *Plasmodium falciparum* homolog(Boddey et al., 2010) (PF13_0133), cgd1_2240 lacks a transmembrane protein(Abrahamsen et al.,

2004). Contrastingly, cgd1_3690 is a signal-peptide containing aspartly protease that lacks apicomplexan orthologs (OG5_194978). This suggests that cgd1_2240 and cgd1_3690 are most likely to be secreted proteases with novel functions.

Cryptosporidium males express a signal peptide-containing kazal domain protein (Fig 4.6f; cgd5_3380) and its homologs are absent in other apicomplexan parasites (OG5_195310). Kazal domain-containing proteins are inhibitors of a variety of proteases(Thélie et al., 2019) and are one of the critical components of acrosome mixture to maintain sperm fertility by preventing premature activation of the acrosin protease(Rothmann and Bort, 2018; Thélie et al., 2019; Zheng et al., 1994). The *Cryptosporidium* kazal domain-containing protein might have a role in maintaining male fertility in a similar fashion.

Ions like Cu^{2+} and Ca^{2+} are important for the development of male gametes(Kenthirapalan et al., 2014; Ogórek et al., 2017) and are required for signaling during fertilization (Correia et al., 2015). Multiple ion transporters have been identified that play crucial roles in maintaining ion homeostasis in male gametes.(Correia et al., 2015; Kenthirapalan et al., 2014; Shukla et al., 2012) We identified a total of five genes (cgd1_700, cgd1_2550, cgd1_3200, cgd2_1310 and cgd7_670) upregulated in males that have been annotated as transporters (Fig 4.6g). Of these genes, cgd1_2550 is a broadly conserved copper transporter (OG5_139232). The *Plasmodium berghei* ortholog (PBANKA_130290) of cgd1_2550, a copper transporter, was found to be expressed in asexual blood stages and gametocytes, but its deletion severely affected male gametes exflagellation.(Kenthirapalan et al., 2014)

Overall, we have identified male-specific genes that might be required for interaction and fusing with female gametes.

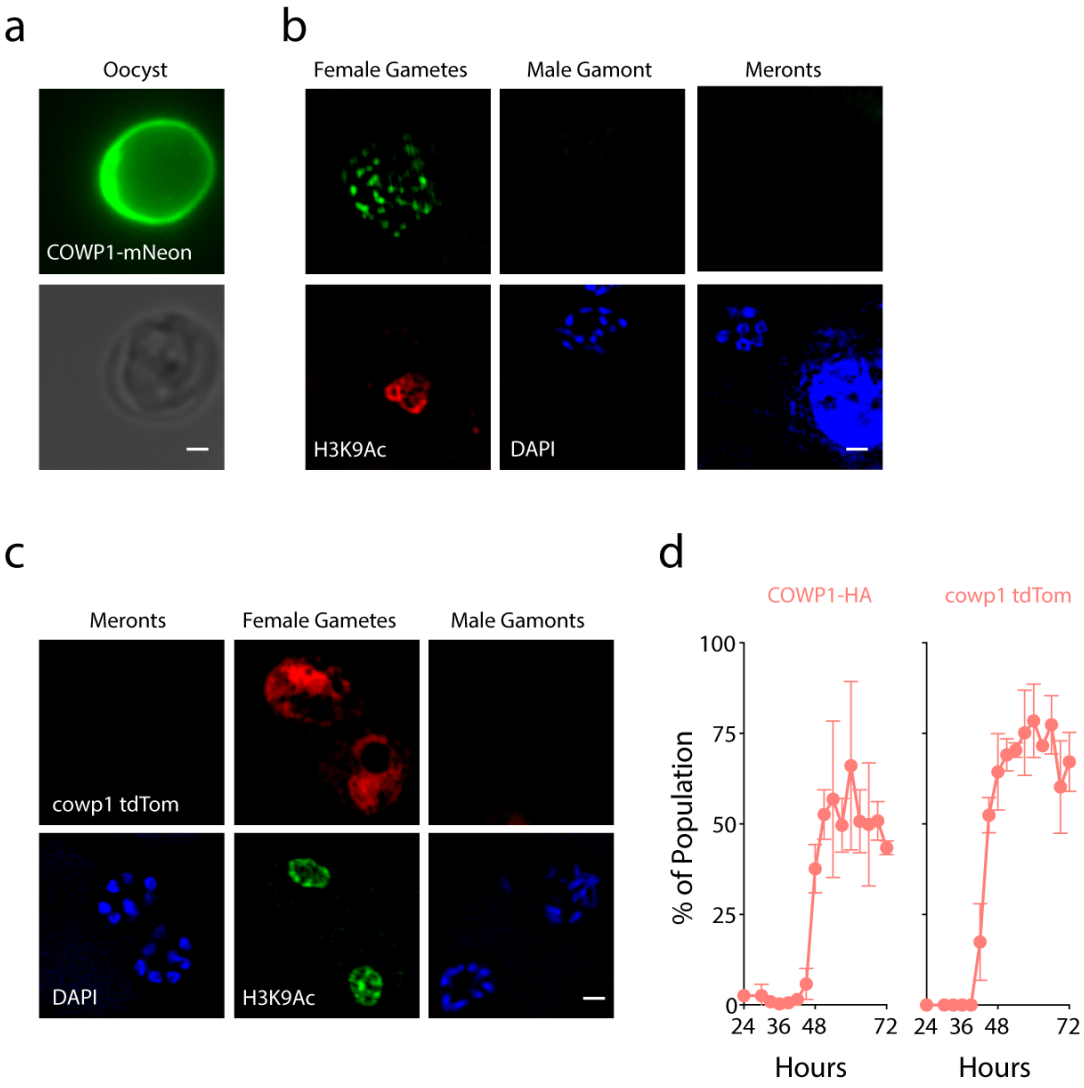


Fig. 4.1 Exclusive molecular marker for females of *C. parvum*. **a**–**d**, *C. parvum* were engineered to express COWP1–mNeon (**a**,**b**) and COWP1–HA (Supplementary Fig. 4.2) from the native locus or *COWP1*promoter-driven tdTomato from the ectopic *TK* locus (**c**). Note the mNeon labeling of the wall in oocysts purified from infected mice and punctate labeling in female gametes observed in infected HCT-8 cells. Labeling becomes apparent after 42 h of culture and is never observed in asexual meronts or male gametes (**b**,**d**). The *COWP1* promoter alone is sufficient to confer female-specific expression to a reporter protein (**c**,**d**). Anti-H3K9Ac antibodies were used to label the nuclei of females because they stain poorly with 4,6-diamidino-2-phenylindole (DAPI). For the time intervals in **d**, cultures were infected with the indicated transgenic strains and triplicate coverslips were fixed and processed for immunofluorescence assays. Parasite stages were scored for HA staining, the mean ± s.d. percentage of HA-positive stages among all of the parasites is shown for three independent biological replicates. All the scale bars are 1 μm in length.

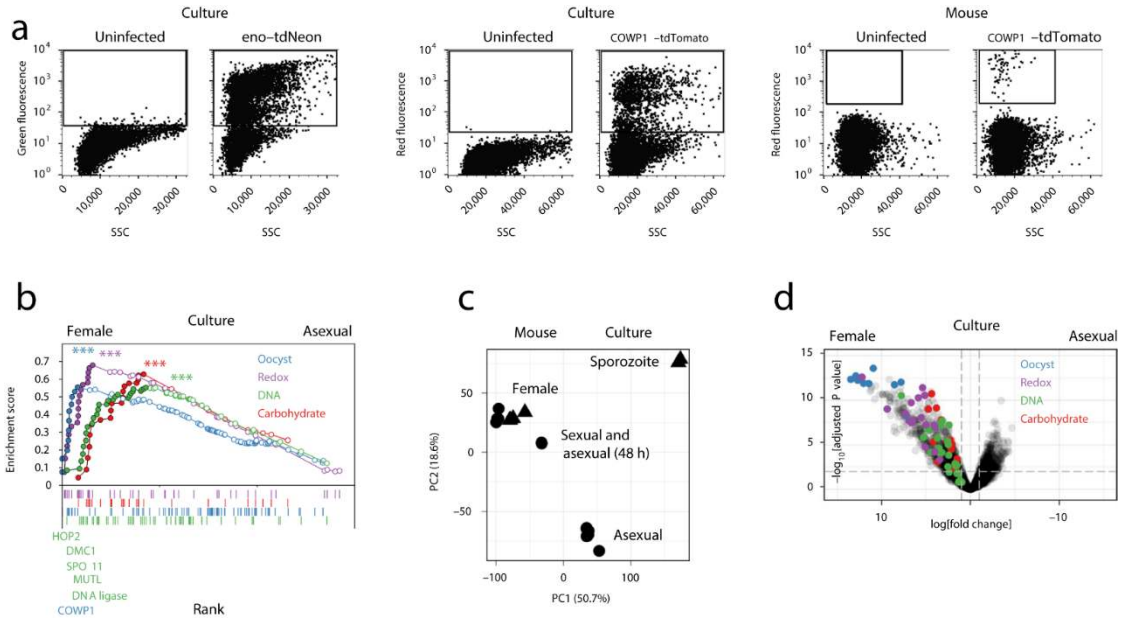


Fig. 4.2 Isolation of asexual and female stages by cell sorting and RNA sequencing. Flow cytometry of infected cells with the indicated markers and origins. Gates used for sorting are shown as boxes. This experiment was performed twice. SSC, side scatter. **b**, Gene set enrichment analysis (GSEA) with multiple testing correction comparing cultured asexual and female parasites. Custom gene signatures were generated using Gene Ontology or community datasets available at CryptoDB (**b**; carbohydrate (GO:0005975; normalized enrichment score (NES) = 1.67, FDR-adjusted $P = 0.004$), DNA (GO:0006259; NES = 1.68, FDR-adjusted $P = 0.005$), redox (GO:0055114; NES = 1.99, FDR-adjusted $P = 0$) and oocyst wall proteome(Truong and Ferrari, 2006) dataset (NES = 1.76, FDR-adjusted $P = 0.001$)). ***$P \leq 0.005$. $n = 4$ biological replicates per group. **c**, Principal component analysis of all RNA-sequencing datasets generated during this study (Supplementary Fig. 4.6). **d**, Volcano plots showing *C. parvum* genes that were differentially expressed between asexual and female parasites from culture. $n = 4$ biological replicates per group. Each symbol represents a *C. parvum* gene, those genes representing the leading edge from **b** are indicated by the color according to the pathway that they act in. The horizontal dashed line shows an FDR-adjusted *P*-value of 0.01; the vertical dashed lines indicate a $\log_2$-transformed fold change of −1 and 1, respectively.

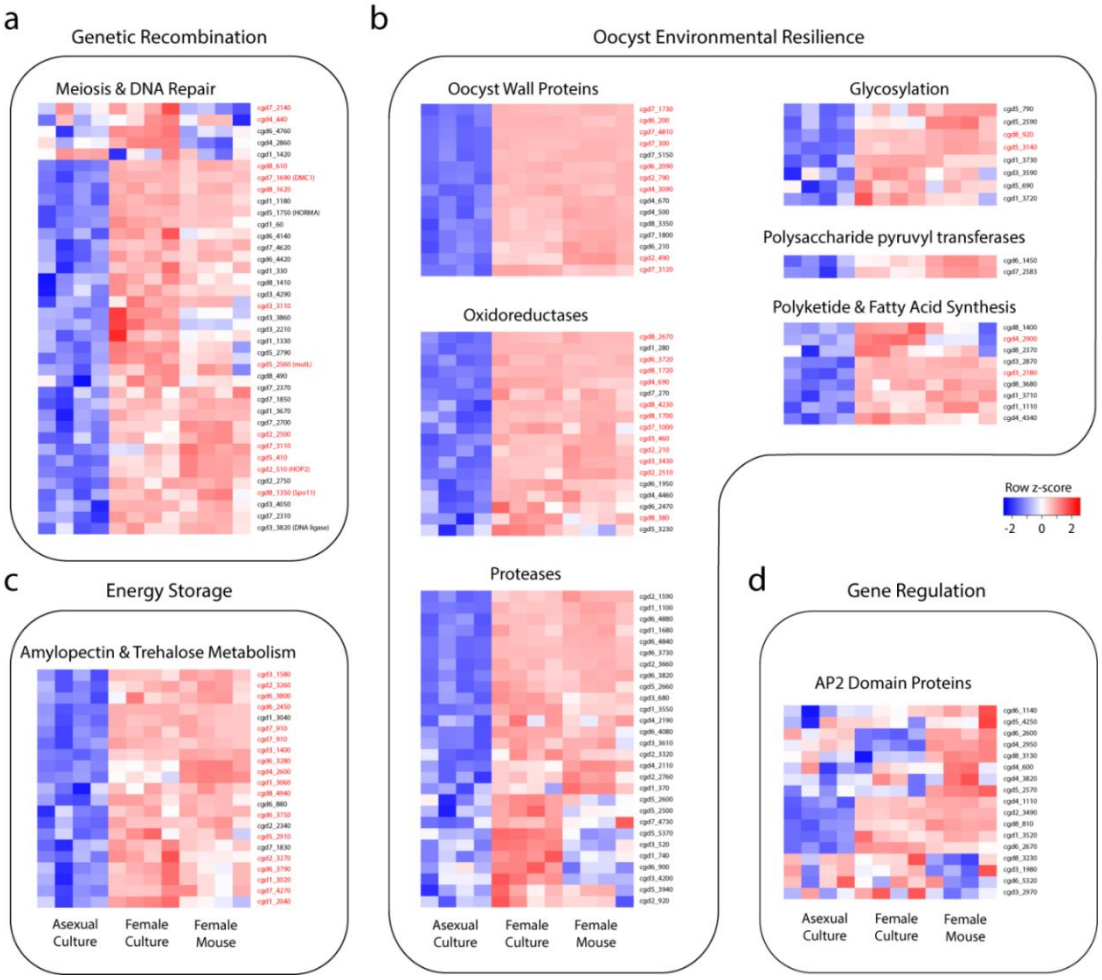


Fig. 4.3 Female gametes express genes that are required for genetic recombination and oocyst formation. **a**–**c**, Heat maps illustrating expression of genes associated with specific molecular functions that are upregulated in females (generally results from in vivo and in vitro females concur, although there are some exceptions). Genetic recombination (**a**), oocyst environmental resilience (**b**) and energy storage (**c**); *n* = 4 biological replicates per group. **d**, the Expression heat map for all *C. parvum* AP2 DNA-binding proteins. Note the pronounced difference identifying four genes upregulated in all females and two only *in vivo* females. As we were unable to sequence males, we cannot formally exclude that some genes that show high female-specific expression may be upregulated in all sexual stages. Expression values are given as row *z*-scores and annotated genes list are provided as Supplementary Files. Genes from the leading edge in Fig. 3b are highlighted in red text.

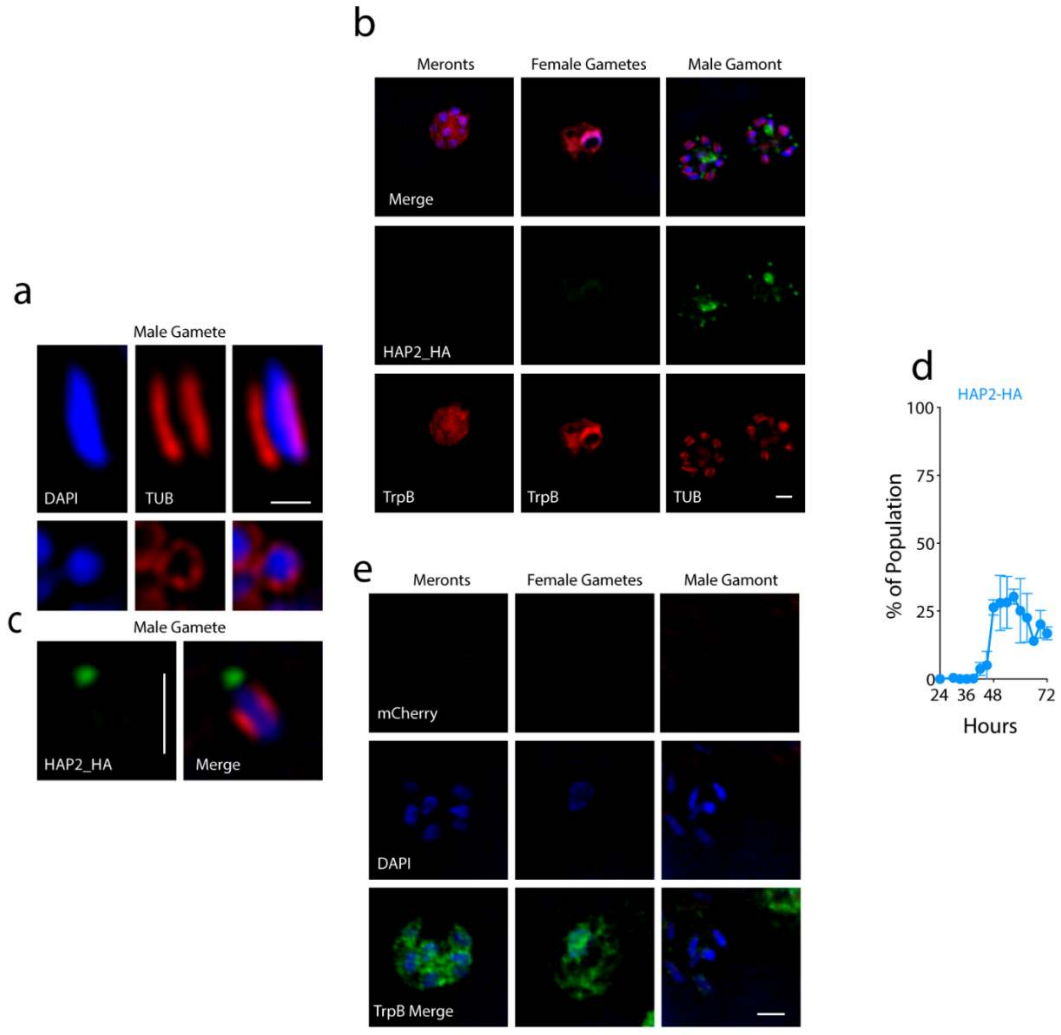


Fig. 4.4 HAP2 is a male-specific fusogen. **a.** Male gametes show a characteristic array of microtubules around the nucleus after staining with anti-tubulin antibodies. When parasites were engineered to express HAP2–HA from the native locus, antibody staining revealed exclusive labeling of free gametes (**c**) and male gamonts (**b**). HAP2 labels a single pole per mature gamete. This staining becomes apparent after 42 h of culture (**d**, blue). e. No expression of mCherry was observed in males or any other stages when 662 bp of HAP2 5'UTR was used. All of the microscopy experiments shown in this figure were performed independently three times. Scale bars in **b-c** and **e** is 1 μm; scale bar in **a** is 0.5 μm.

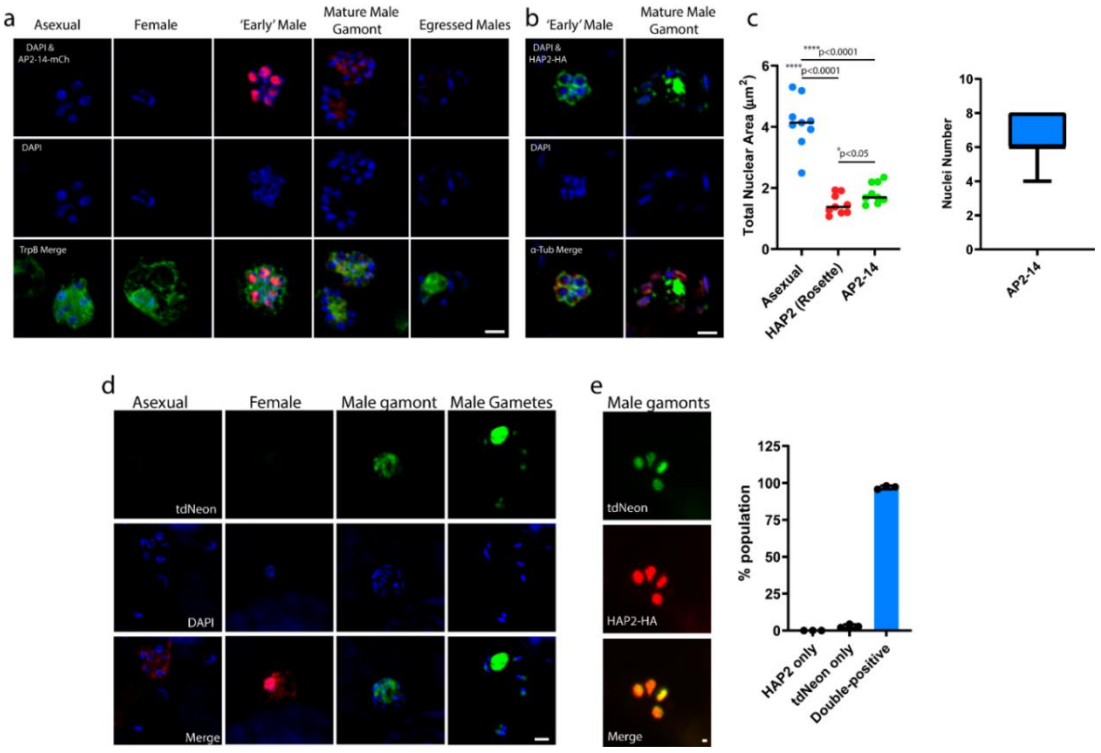


Fig. 4.5 AP2-14 (cgd6_2670) is an 'early' male-specific gene. **a**. Nuclear localization of AP2-14 has presumed 'early' males and cytosolic localization in rare male gamonts. Egressed male gametes lack the expression of AP2-14. **b**. Rosette and polar localization of HAP2-HA in 'early' and mature males, respectively. **c**. The number of nuclei in AP2-14 positive stages (*n*=30), and total nuclear area of asexual meronts, HAP2 'rosette' stages and AP2-14 stages were quantified (*n*=9 each). AP2-14 presumed promoter drives the expression of tdNeon specifically in males (**d**) and ~97% of the tdNeon-expressing population was positive for HAP2 (**e**). Scale bars in all the images are 1 μm in length.

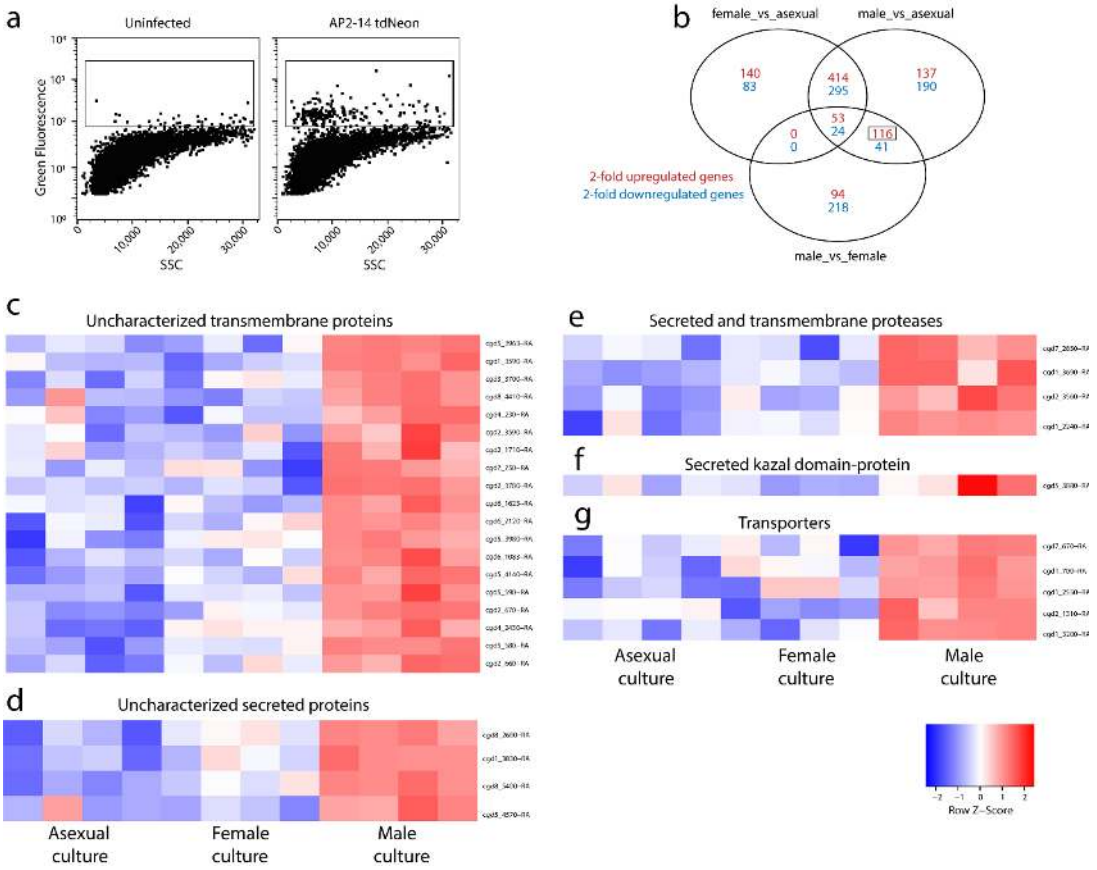
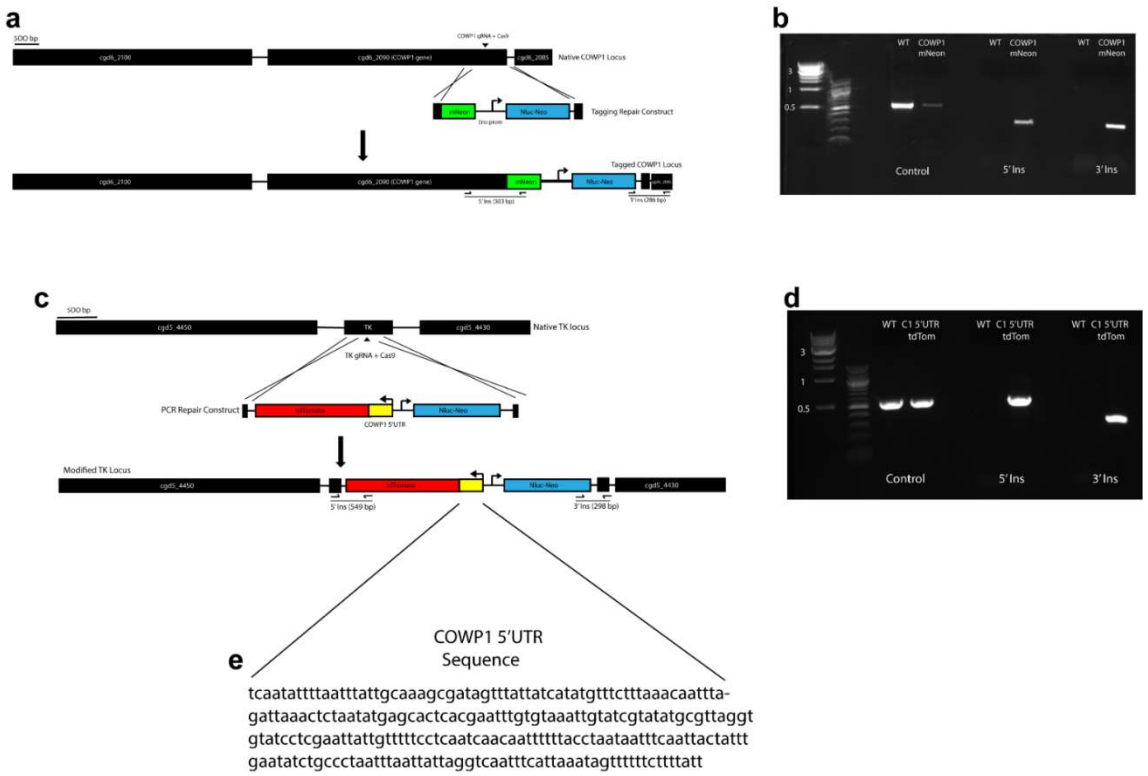
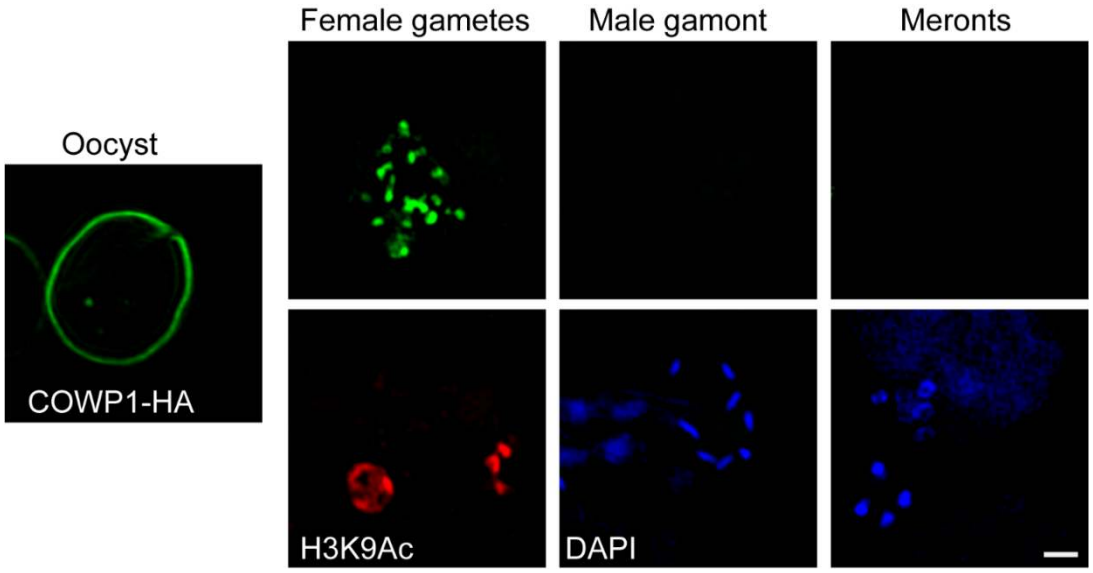


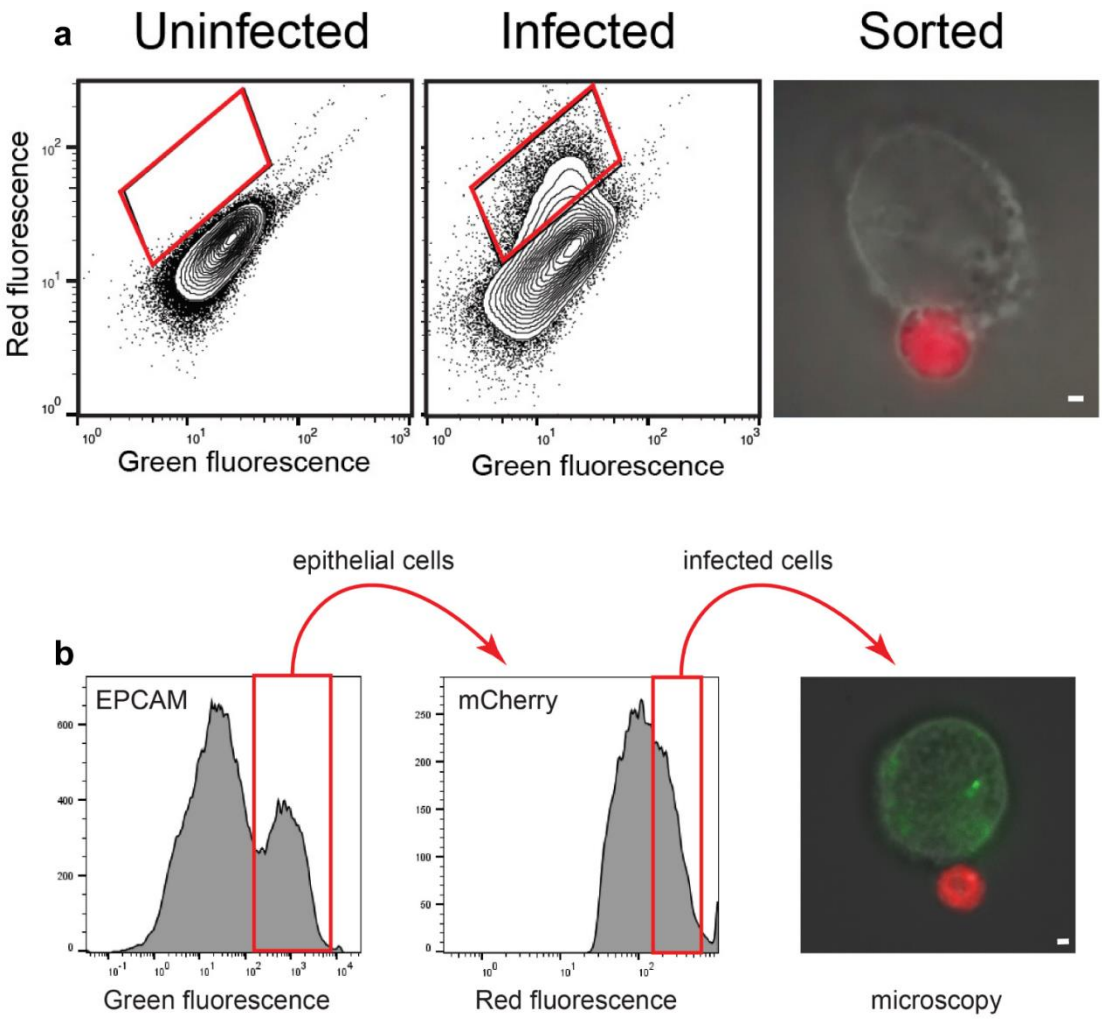
Fig. 4.6 Isolation of male gamonts by cell sorting and RNA sequencing. **a**. Flow cytometry of male gamonts from a 48-hour infected HCT-8 cultures with the indicated markers and origins. Gates used for sorting are shown as boxes. This experiment was performed twice. SSC, side scatter. **b**. 116 genes (highlighted by a black box) commonly upregulated (at least 2-fold) between *in vitro* male-*in vitro* female and *in v*itro male-*in vitro* asexual comparisons were selected to identify male-specific genes. Custom gene signatures (FDR $p < 0.01$) of 'uncharacterized transmembrane proteins' (**c**), 'uncharacterized secreted proteins' (**d**), 'secreted and transmembrane proteases' (**e**), 'secreted kazal domain protein' (**f**) and 'transporters' (**g**) were generated from 116 male-specific genes. The expression is given as row Z-score.



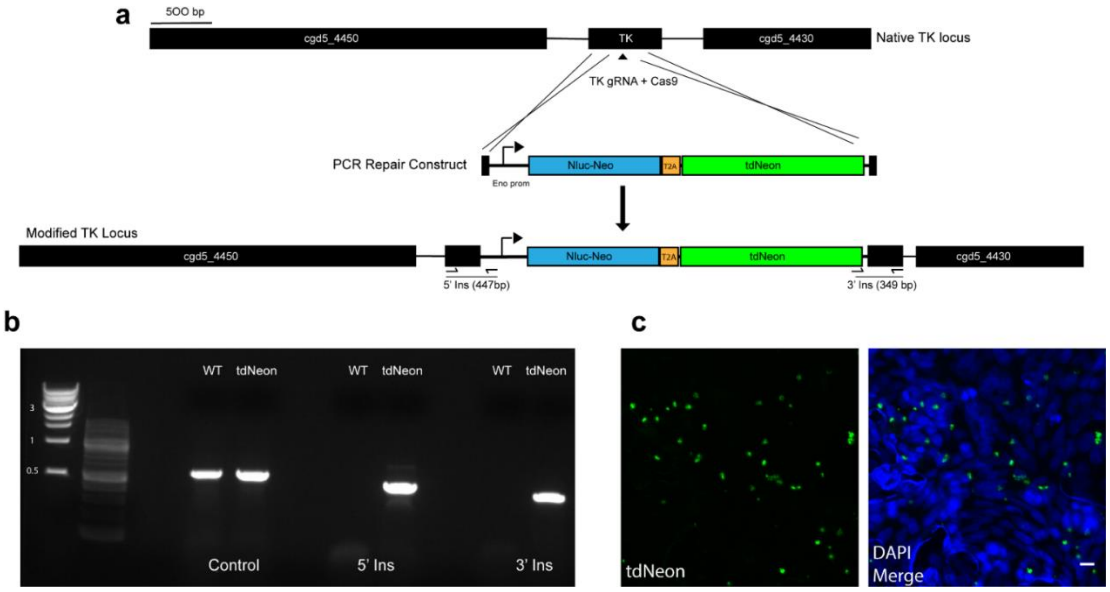
Supplementary Figure 4.1: Construction of *C. parvum* COWP1-mNeon and COWP1-tdTomato strains. (**a** and **c**) Maps depict the native loci, repair constructs, and modified loci for generating respective strains. (**b** and **d**) PCR analyses confirming successful insertions of repair constructs for COWP1-mNeon and COWP1 tdTomato respectively (see plasmid maps in (**a**) and (**c**)) for amplicons for the predicted 5' and 3' insertion sites. This experiment was performed two times with similar results. The presumptive promoter sequence used to drive the expression of tdTomato is represented in **e** (247 bp upstream of the 'start' codon).



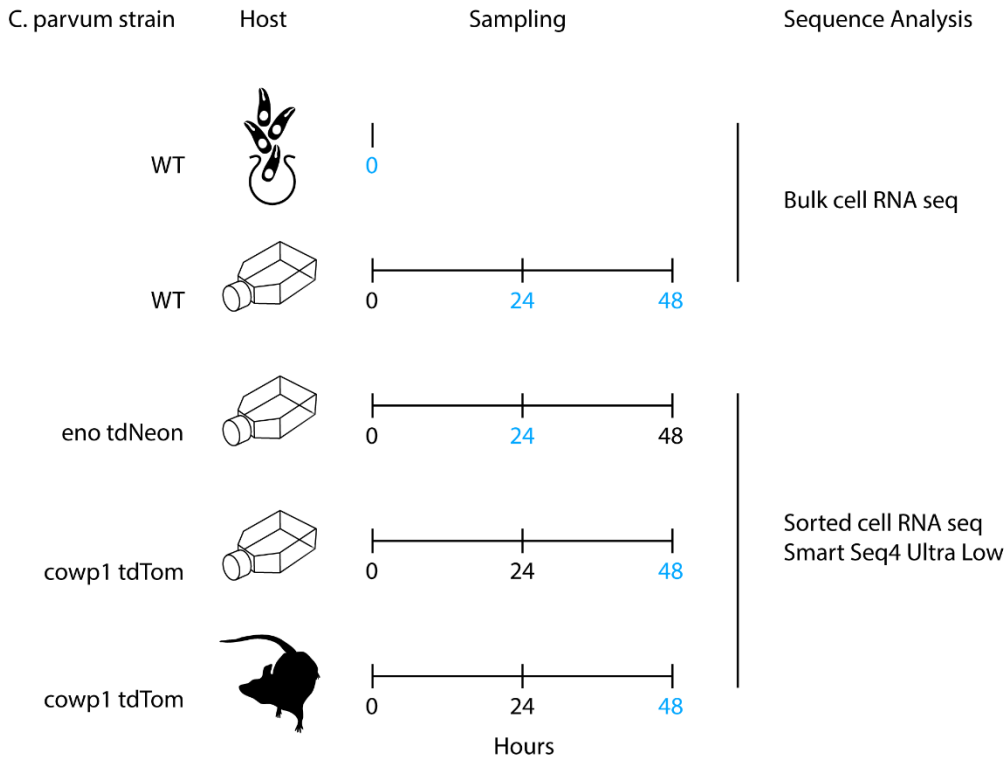
Supplementary Figure 4.2: Immunofluorescence analysis of female-specific expression of COWP1-HA. Note the localization of COWP1-HA to the wall of oocysts and punctate structures in female gametes. No staining is observed in asexual meronts or male gamonts. Female gametes were counterstained with an H3K9Ac antibody highlighting the nucleus. Males and meronts were identified by DAPI staining. Note that the large transcriptionally active nucleus of female gametes which stains poorly with DAPI (Scale Bar= 1 μm). This experiment was performed three times with similar results.



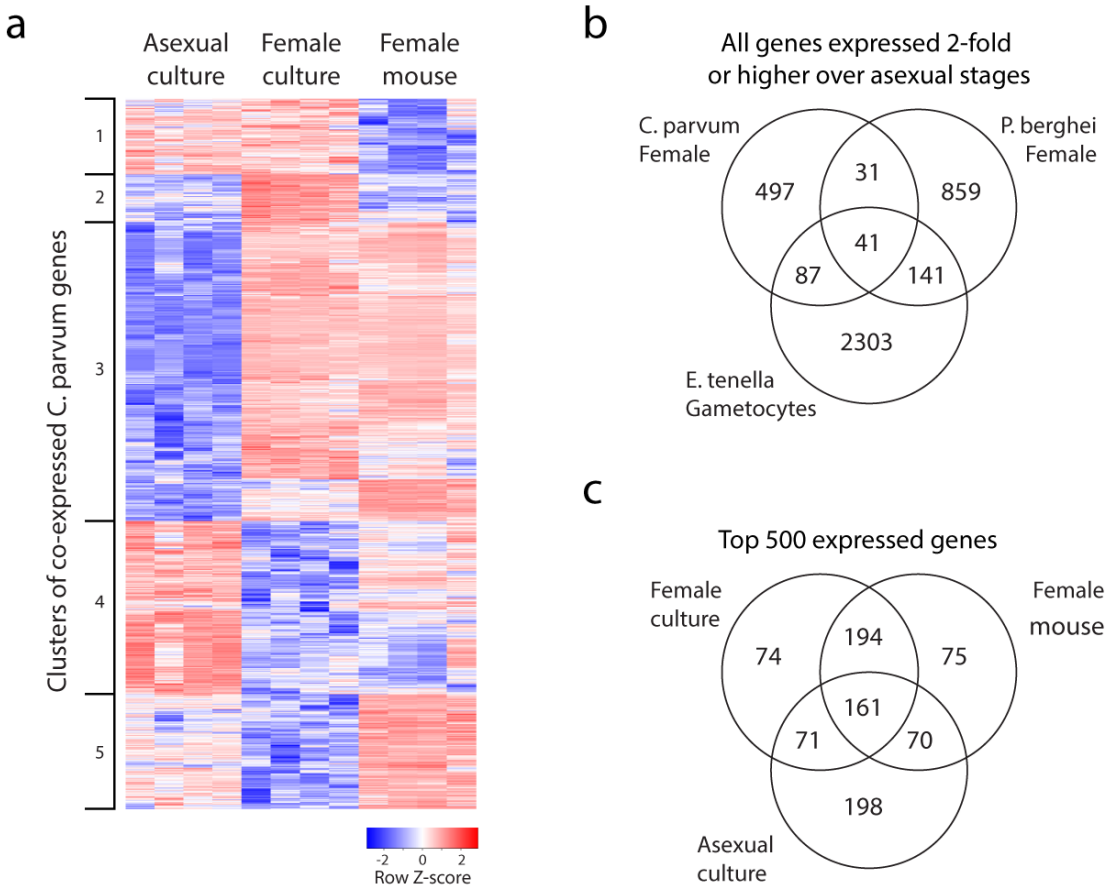
Supplementary Figure 4.3: Microscopic validation of the flow cytometry protocol to isolate parasite-infected cells. Initial experiments used parasites expressing mCherry under the tubulin promoter. Cells were sorted form infected cultures (**a**) and infected IFNg-/- mice (**b**). Gates used for sorting are shown in red. Sorted cells were imaged without further staining (cells from mice were stained with antibodies to EPCAM prior to sorting). The images show merge DIC and fluorescence channels (Scale Bar= 1 µm). We constructed numerous strains using different fluorescent protein genes (mCherry, GFP, YFP, mRuby, mScarlet, tdNeon and tdTomato) to identify those that produce the brightest fluorescence and the most robust sorting differential. We find tdNeon and tdTomato to yield the strongest green and red fluorescence, respectively. This experiment was performed three times with similar results.



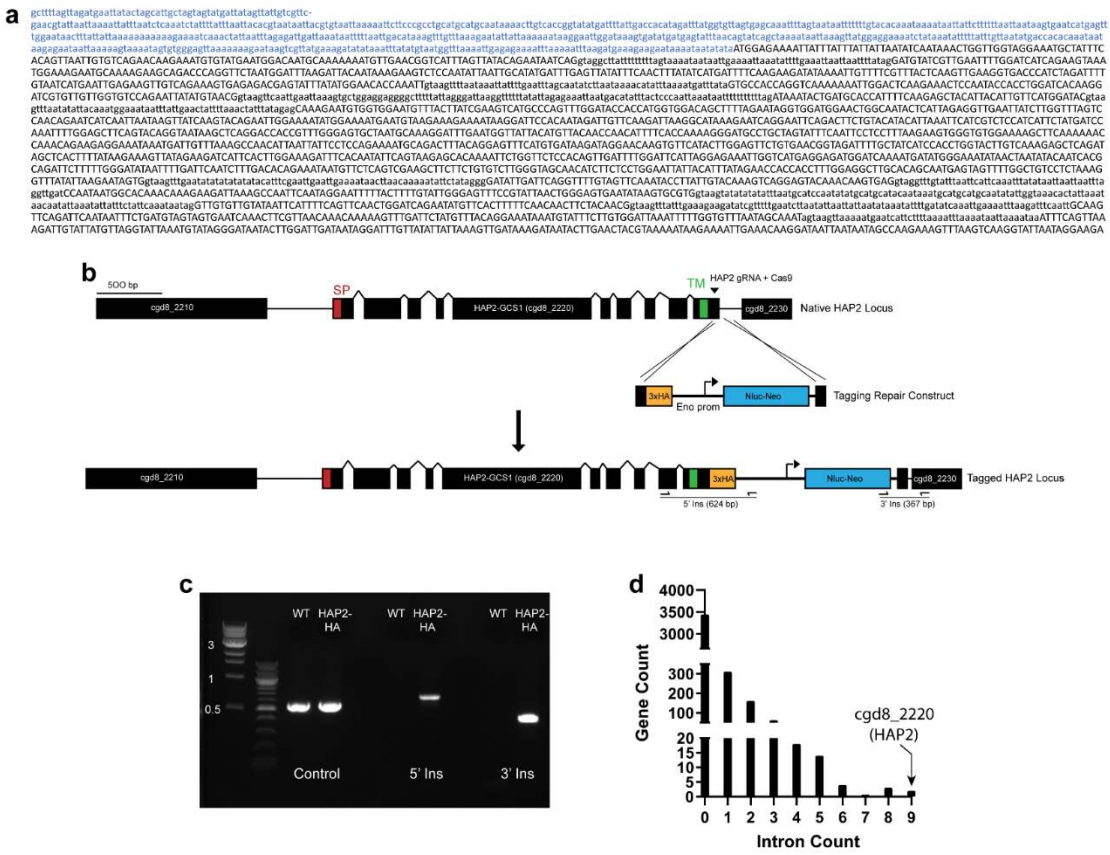
Supplementary Figure 4.4: Construction of eno tdNeon reporter strain. (a) The eno tdNeon constitutive reporter strain was constructed by linking the Nluc-Neo expression cassette with two copies of the mNeon Green fluorescent reporter gene in tandem. A viral T2A skip peptide linker was introduced between the tdNeon and Nluc-Neo cassettes. The expression construct was incorporated in the TK locus of the parasite and integration of the construct was validated by PCR **(b)**. **(c)** Expression of tdNeon reporter in the transgenic strain in a 24-hour infected culture (Scale Bar= 10 µm). This experiment was performed twice with similar results.



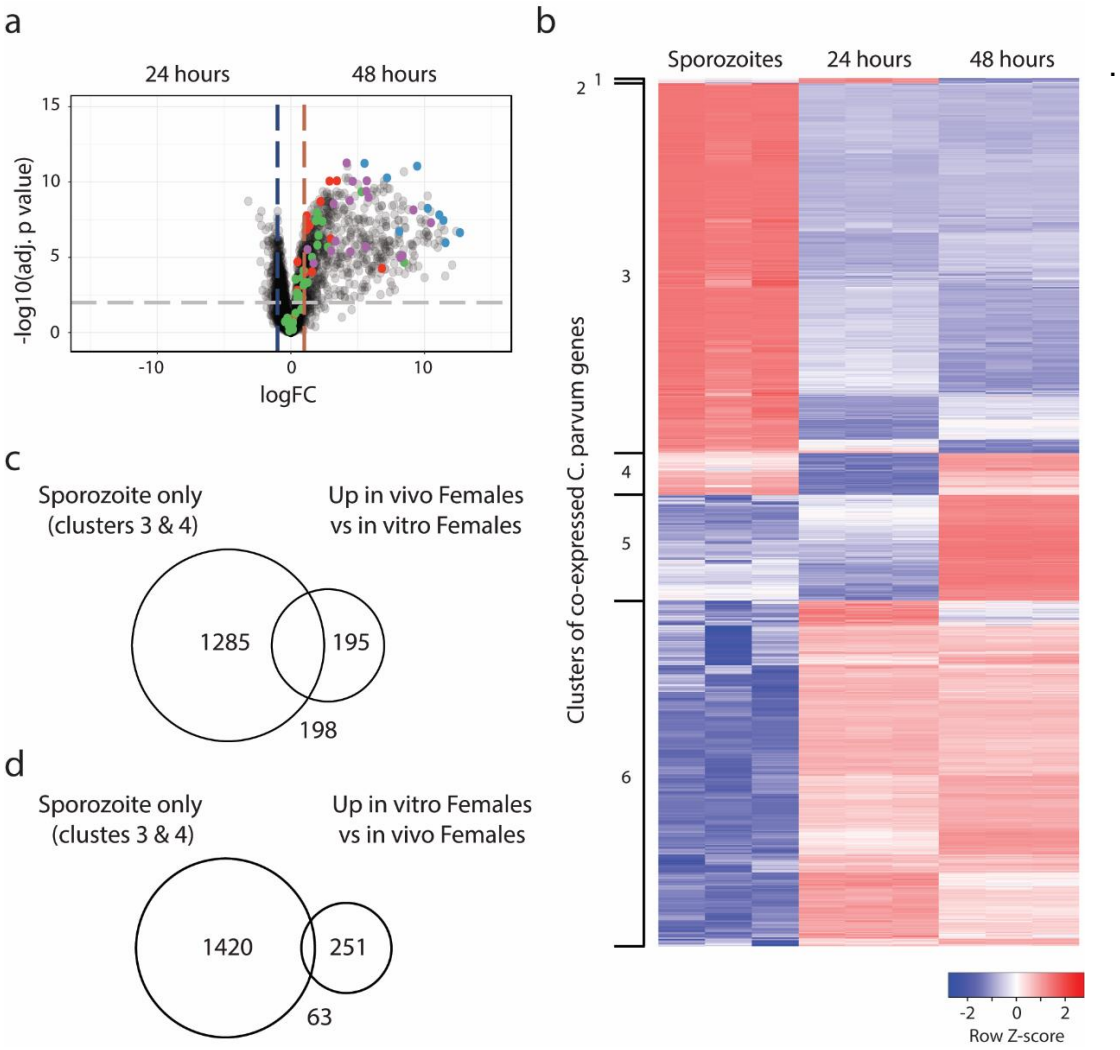
Supplementary Figure 4.5: Sampling of different parasite stages for RNA sequencing analysis. Schematic overview of RNA sequencing experiments conducted in this study indicating the *C.parvum* strain, the respective transgene, the source of parasites or infected cells (sporozoites freshly excysted from oocysts, infected HCT-8 cell cultures, or the resected small intestine of infected IFNg-/- mice), the time point of parasite harvest following infection (in hours highlighted in blue), whether or not cells were subjected to fluorescence-activated cell sorting.



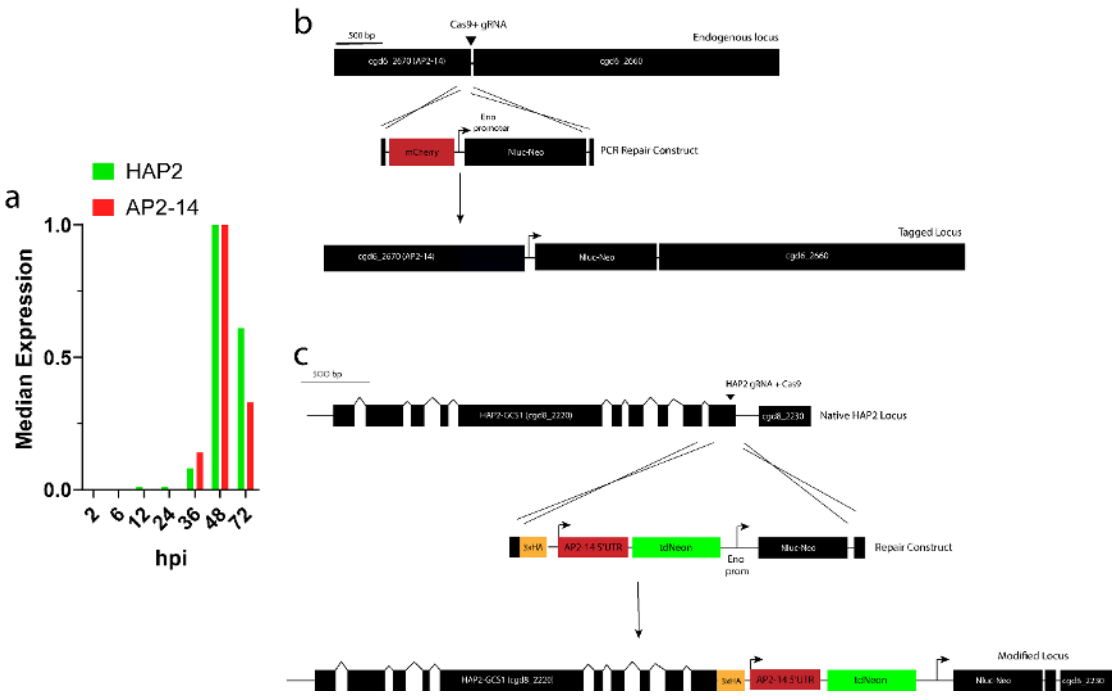
Supplementary Figure 4.6: Differential gene expression of asexual and female C. parvum sorted from HCT-8 cultures and infected mice. (**a**) A heatmap of cluster analysis of differentially expressed genes between the sorted cells (see Fig. 3a for further detail on sorted populations). Expression is given as a row z-score. Clusters are available for download as Supplementary File 3 (n= 4 biological replicates per group). (**b**) Venn diagram of all genes expressed 2-fold or higher in female (or in the case of *Eimeria tenella* gametocyte) stages over asexual stages. Numbers are given as ortholog groups containing one or more genes from the indicated species. See Supplementary File 4 for a complete list of ortholog groups and stage specific genes within each group for each species. (**c**) Venn diagram of the 500 most abundantly expressed genes for each parasite stage (n= 4 biological replicates per group). See Supplementary File 5 for complete lists of overlapping and unique genes.



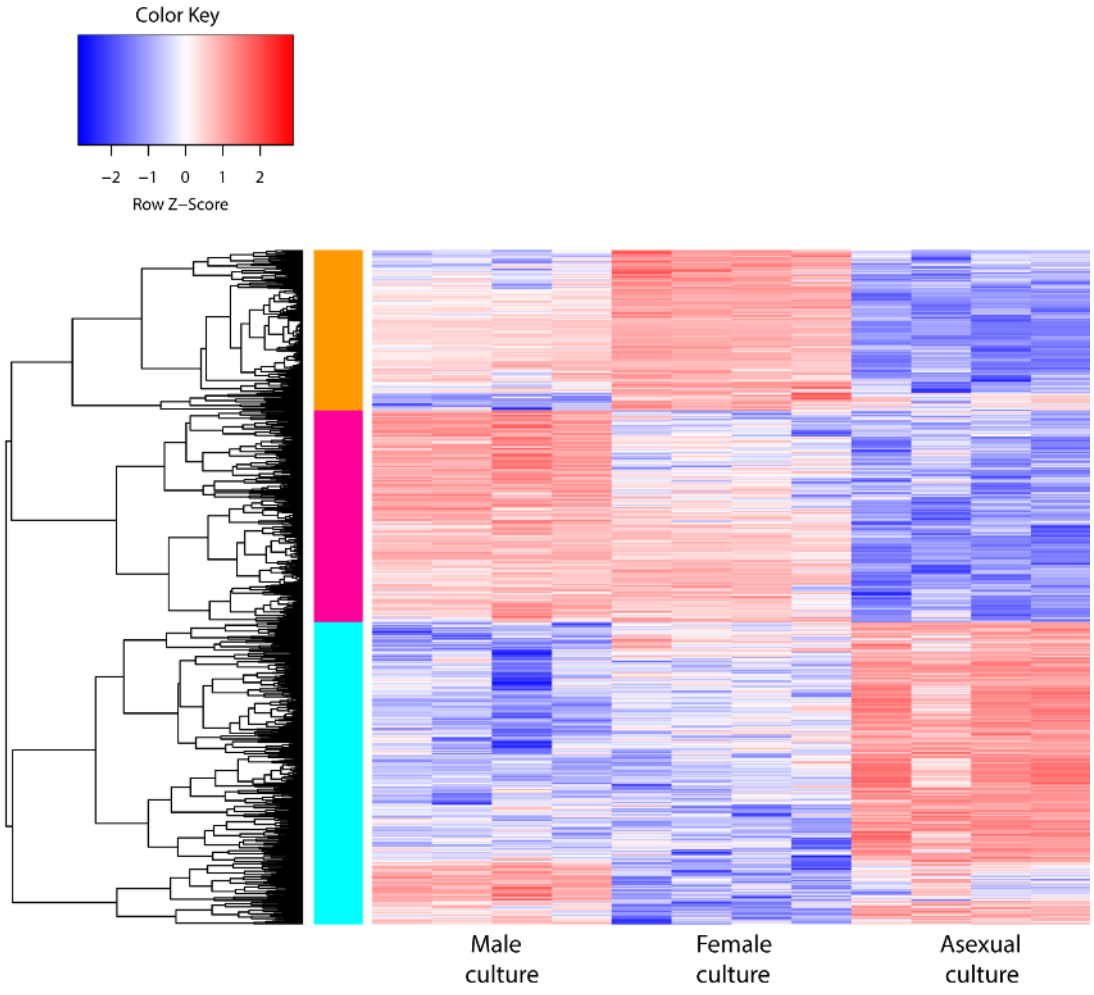
Supplementary Figure 4.7: Construction of an HA-tagged *C. parvum* HAP2. (**a**) Only a fragment of the HAP2 gene (cgd8_2220) is annotated in the publicly available *C. parvum* genome sequence (https://cryptodb.org/). We used RNA sequencing data and RT-PCR analysis to correct the gene model. While most *C.parvum* genes lack introns (**d** and highlighted in lower case in **a**), they are abundant in HAP2 as shown by the locus map in (**b**)**.** The protein was tagged with an HA epitope in the native locus. (**c**) PCR analysis confirmed successful epitope tagging of the HAP2 gene. This experiment was performed two times with similar results. (**d**) Graph depicting the distribution of annotated *C. parvum* Iowa II strain genes concerning the number of exons per gene (https://cryptodb.org/).



Supplementary Figure 4.8: Differential gene expression of excysted sporozoites and infected HCT-8 cultures. (**a**) Volcano plot of differentially expressed *C. parvum* genes between 24 and 48 hours *in vitro* (*n*=3 biological replicates per group). Each point represents a single *C. parvum* gene. The horizontal dashed line indicates an FDR of 0.01. Vertical dashed lines indicate $\log_2$ fold change of -1 and +1. Genes from the leading edge of gene enrichment analysis (Figs. 3.3c and 4.2b) are shown in red, carbohydrate (GO:0005975), green, DNA (GO:0006259), purple, redox (GO:0055114), and blue, oocyst wall proteome dataset(Truong and Ferrari, 2006). (**b**) Heatmap and clustering analysis of *C. parvum* genes differentially expressed between sporozoites and infected HCT-8 cultures at 24- and 48-hours post infection (n= 3 biological replicates per group). Expression is given as row z-scores. (**c** and **d**) Venn diagrams comparing genes expressed by sporozoites (found in clusters 3 and 4 in (**c**) to genes expressed 2-fold or higher by *in vivo* females over *in vitro* females (**c**) or expressed 2-fold or higher by in vitro females over in vivo females (**d**). Complete list of shared genes in Supplementary File 6. Note that 40% of genes of this category *in vivo* females are also upregulated in sporozoites. No such enrichment is obvious in *in vitro* females.



Supplementary Figure 4.9: Approach to validate the male-specific expression of AP2-14. a. Comparison of the temporal expression of cgd6_2670 (AP2-14) and cgd8_2220 (HAP2) transcripts in HCT-8 culture at different time points. The data has been graphed from Mauzy *et. al*..(Mauzy et al., 2012) Strategies to engineer AP2-14 mcherry tag (**b**) strain and AP2-14 tdneon male reporter line (**c**) have been represented.



Supplementary Figure 4.10: Differential gene expression between male gamonts, females, and asexual stages from HCT-8 cultures. Male gamonts were isolated from the 48-hour infected HCT-8 cells by flow cytometry for RNA-sequencing (fig 4.6a) and transcriptional datasets were compared to preexisting *in vitro* female and asexual transcriptomes (n=4). Differentially expressed genes between (2-fold change; FDR p< 0.01) *in vitro* males, *in vitro* females, and *in vitro* asexuals are represented by a heatmap. The expression is represented as a row Z-score.

4.4 Discussion

The single-host lifecycle of *Cryptosporidium* has been studied at an ultrastructural by electron microscopy in a variety of infected host species.(Current and Reese, 1986; Ostrovska and Paperna, 1990) Molecular mechanisms underlying complex transformations from asexual to sexual stages, fertilization, pots-fertilization development and sporulation in *Cryptosporidium* are uncharacterized. Comprehensive understanding of genes expressed in asexual stages, males and females will help identify molecular orchestrators involved in complex transformations form one stage to another. The presence of a complex mixture of *Cryptosporidium* intracellular stages in *in vivo*(Current and Reese, 1986; Ostrovska and Paperna, 1990) and culture(Tandel et al., 2019) makes it technically challenging to enrich a specific stage for a downstream molecular analysis. We overcame this challenge by designing stage-specific fluorescent reporter strains and flow sorting them for RNA sequencing.

Cryptosporidium Oocyst Wall Protein-1 (COWP1; cgd6_2090) gene has been confirmed as a female-specific gene(Spano et al., 1997), and its 247 bp of 5' UTR drove the expression of tdTomato specifically in females. We identified HAP2 (cgd8_2220) and AP2-14 (cgd6_2670) as male-specific genes. Our detailed analysis of male development revealed the presence of an early (rosette nuclei) and late (bullet-shaped nuclei) developmental stages of males. HAP2 was expressed in both early and late developmental stages with different localization patterns. However, AP2-14 was expressed in early male stages with nuclear localization but was either absent or present in the cytoplasm of late male gamonts. This implies that there exists a post-translational mechanism to ensure decay of AP2-14 in mature male gamonts. Only the promoter of AP2-14 (382 bp of the 5'UTR), but not that of HAP2 (647 bp of the 5'UTR), drove the expression of tdNeon reporter in males. HAP2 gene contains nine introns while ~85% of

the *Cryptosporidium* genes lack a single intron (Supplementary fig. 4.9d). This might suggest that additional post-transcriptional mechanisms guided by introns(Rose, 2019) regulate HAP2 expression, which cannot be recapitulated by its promoters alone. Genes required for meiosis, oocyst wall biogenesis and amylopectin metabolism were upregulated in females. The expression of meiosis genes like DMC1, HOP2, and Spo11 is only observed in females, and these unilaterally contribute to meiotic development after fertilization. Similar meiotic mechanisms have been observed in green algae(Triemer and Jr, 1977) and fungi(Sherwood and Bennett, 2009) that undergo zygotic meiosis.

Cryptosporidium female gametes express proteinaceous components (COWPs) of the oocyst wall. Oocyst wall proteins are further processed proteolytically and cross-linked.(Samuelson et al., 2013; Walker et al., 2015) Proteases and oxidoreductases required for proteolytic processing and crosslinking of oocyst wall proteins were also upregulated in female. Additionally, we observed expression of enzymes required for incorporating lipid and sugar components to the oocyst walls in females. Many of the enzymatic components identified in females required for oocyst wall biogenesis are absent in humans, which makes them potential drug targets.(Bakheet and Doig, 2009) Polyketide synthase enriched in female is well characterized biochemically(Weng and Noel, 2012) and many drugs have been designed against it(Aggarwal et al., 2017). Many of these PKS inhibitors can be tested against their anti-cryptosporidial activity. In a cyst-forming coccidia, *Eimeria*, the genes necessary for the oocyst wall biogenesis are expressed in the female stages.(Walker et al., 2015)

Cryptosporidium female gametes were enriched in genes required for synthesis and catabolism of glycogen-like amylopectin polysaccharide. Amylopectin might serve as an energy source for developing sporozoites.(Harris et al., 2004) Amylopectin might

additionally serve as a precursor for synthesis of trehalose required for tolerating osmotic stress.(Elbein et al., 2003)

Genes involved in gamete-gamete interactions and communications are potential vaccine and drug targets.(Chaturvedi et al., 2016) Robust sexual differentiation of *Cryptosporidium* in cultures makes it a convenient model system to study mechanisms by which male and female gametes communicate and initiate events leading to fertilization. *Cryptosporidium* male gametes might be guided to female gametes through pheromone or transmembrane receptors. Female gametes might deploy a receptor(s) on the host cell membrane to 'bait' male gametes. The mechanism of deploying a transmembrane protein on the host cell membrane already exists in apicomplexans.(Smith, 2014)

Cryptosporidium male gametes have to traverse host cell membranes, intracellular membrane, and female membrane to undergo fusion.(Ostrovska and Paperna, 1990) This suggests that *Cryptosporidium* male gametes might deploy additional sets of complex mechanisms to undergo karyogamy. Mammalian male gametes have an acrosomal compartment that releases proteases and membrane receptors to traverse the zona pellucida layer surrounding the egg.(Rothmann and Bort, 2018) We have identified multiple secreted and transmembrane proteases that are upregulated in males that might be involved in fusion with females. Kazal domain-containing proteins are required to maintain sperm fertility(Thélie et al., 2019) by preventing premature activation of acrosomal proteases(Zheng et al., 1994). *Cryptosporidium* kazal-domain protein (cgd5_3380) upregulated in male gametes might be involved in a synonymous process to prevent premature activation of male proteases. The presence of kazal-domain protein (absent in other apicomplexans) and secreted proteases in male gametes suggest that *Cryptosporidium* male gametes and mammalian

sperms(Rothmann and Bort, 2018) have evolved similar mechanisms to traverse multiple barriers to access female gametes.

The entrance of multiple sperm in eggs (polyspermy) results in abnormal zygotic development.(Gilbert, 2000) Proteases play an important role in the prevention of polyspermy by degrading membrane receptors on the surface of eggs.(Gilbert, 2000) Proteases from both sperm and eggs are known to remodel egg membranes to release any attached extra sperm to prevent polyspermy(Gilbert, 2000). *Cryptosporidium* male gametes outnumber females by a ratio of 6:1 and hence it is not hard to envision that *Cryptosporidium* might have evolved similar mechanisms to block polyspermy. Some of the identified male- and female-specific proteases might be involved in the remodeling membrane of female gametes to block secondary fertilization. Other than proteases, oxidases remodel fertilized mammalian eggs by crosslinking the proteinaceous components.(Gilbert, 2000)

Copper has been identified as an important trace element required for exflagellation in *Plasmodium* male gametes(Kenthirapalan et al., 2014) and mammalian spermatogenesis(Ogórek et al., 2017). Impairment of copper homeostasis might cause dysfunction of copper-dependent enzymes and production of free radicals that damage DNA.(Ogórek et al., 2017) cgd1_2550 copper transporter might be involved in maintaining copper homeostasis in *Cryptosporidium* male gametes.

Expression of male- and female-specific genes identified are regulated by the promoter regions. ApiAP2 DNA-binding proteins might be involved in regulating the expression by binding the promoter regions of stage-specific genes.(Kafsack et al., 2014; Kaneko et al., 2015; Sinha et al., 2014) We have identified three female-specific (cgd8_810, cgd2_3490 and cgd4_1110) and one male-specific (cgd6_2670) ApiAP2 genes in

Cryptosporidium. Sex-specific ApiAP2s might be involved in regulating expression of many of the sex-specific genes that we have identified.

Our transcriptional analyses of male and female gametes have revealed multiple genes that might be involved in sexual stage development, mating, gamete fusion, meiosis, post-fertilization development, sporulation, and oocyst wall biogenesis. Mechanistic studies of these genes will reveal their potential roles in sexual development and sex. These genes represent a repository of drug and vaccine targets to block oocyst biogenesis to prevent transmission and autoinfection.

Our previous work has shown that *Cryptosporidium* cannot be maintained in transformed cell line, HCT-8 cells, because parasites fail to mate.(Tandel et al., 2019) We hypothesize that sex, and subsequently renewed production of oocysts is required to maintain infection in a host. As a result, disruption of sexual development or sex should break the cycle of infection in an infected animal model. Many of the identified sex-specific genes represent candidates to test essentiality of sex to maintain infection in mice.

Chapter 5: Conditional gene knockout in *Cryptosporidium* enables ablation of an essential transcriptional regulator expressed in female gametes

5.1 Abstract

Cryptosporidium has emerged as one of the leading causes of childhood mortality. Drugs and vaccines against the parasite are lacking due to the scant understanding of the biology of the parasite. Understanding the biology of the lifecycle of the parasite will be important to identify key stages that can be targeted to block infection and transmission. We have demonstrated that *Cryptosporidium* cannot be maintained continuously in transformed cell lines because they fail to undergo fertilization.(Tandel et al., 2019) Consequently, we hypothesize that sex and renewed generation of oocyst are required to continuously maintain infection in a host. Selective perturbation of sexual development or sex should break the cycle of infection in a host. We aim to genetically disrupt sexual development by targeting sex-specific ApiAP2 genes which have been identified as master regulators of stage-specific development in apicomplexans. We have identified a female-specific ApiAP2 gene, cgd4_1110 (termed as AP2-F), which is essential for the growth of the parasite. We further developed a rapamycin inducible DiCre recombinase system to conditionally delete essential genes in *Cryptosporidium*. We have successfully demonstrated the utility of the DiCre recombinase system to conditionally ablate the expression of the AP2-F gene by excising the floxed 5' UTR and the 'start' codon. We further aim to use this engineered strain to demonstrate the role of AP2-F in female development and maintaining infection *in vivo*.

5.2 Introduction

Cryptosporidium undergoes asexual replication and sexual development in a single host.(Current and Reese, 1986; Tandel et al., 2019; Tyzzer, 1907) Parasite sex results in the production of meiotic spores called oocysts. In most apicomplexan parasites that feature fecal-oral transmission, oocysts shed with the feces are not immediately infective but undergo meiosis and sporogony in the environment.(del Cacho et al., 2010; Dubey et al., 2011) *Cryptosporidium* is unique in that its oocysts mature within the host cell, which enables autoinfection through sexual development.(Current and Reese, 1986; Tyzzer, 1907) This could suggest two plausible models of the lifecycle of this parasite. *Cryptosporidium*, like *Plasmodium*(Phillips et al., 2017), might undergo asexual replication and sexual differentiation simultaneously. Contrastingly, *Cryptosporidium* might undergo limited rounds of asexual replication followed by obligatory gametogenesis like *Eimeria*.(Mesfin and Bellamy, 1978) Our work has shown that when grown in HCT-8 tissue culture *Cryptosporidium* undergoes asexual replication for a limited number of generations followed by robust sexual differentiation.(Tandel et al., 2019) *Cryptosporidium* gametes form but fail to have productive sex in culture and do not sporulate, the inability to form new oocysts may thus explain the lack of continuous infection of transformed cell lines.(Tandel et al., 2019) In contrast, *Cryptosporidium* parasites mate and undergo sporogony in mice and, in the absence of effective host immunity, grows without limit.(Tandel et al., 2019) Based on these observations, we wonder whether sex might be an obligatory requirement to sustain infection. To test this hypothesis, we aim to selectively perturb sexual development, sex, or sporogony to block the development of oocysts and evaluate the consequence of such treatment in a mouse model of infection. Our transcriptomic studies of sexual stages have revealed key

genes that are likely involved in gametogenesis and the formation, survival, and infectiousness of the oocysts. These genes can be targeted to specifically disrupt sexual development, fertilization, and oocyst development to test the hypothesis of the essentiality of sex to maintain infection in a host.

Recently, ApiAP2 DNA binding proteins(De Silva et al., 2008) have been identified as stage-specific transcriptional modulators involved in the regulation of development of gametocytes(Kafsack et al., 2014; Sinha et al., 2014), liver stages(Iwanaga et al., 2012), ookinetes(Kaneko et al., 2015; Yuda et al., 2009) and sporozoites(Yuda et al., 2010) in *Plasmodium*; and tissue cyst-stage(Radke et al., 2013) in *Toxoplasma*. The *Cryptosporidium* genome encodes 17 putative ApiAP2 proteins. Several of these proteins were found to specifically bind to DNA motifs present in the promoter regions of co-expressed genes(Oberstaller et al., 2014), suggesting that *Cryptosporidium* ApiAP2 genes might have a similar role in transcriptional modulation of stage-specific development. We have identified a set of four ApiAP2 genes that are upregulated in sexual stages. Perturbation of these factors might disrupt gene expression in a fashion specific to sexual stages and could thus ablate the sexual part of the lifecycle while leaving the asexual portion intact. This suggests sex specific ApiAP2s as strong candidates to test the importance of sex for lifecycle progression and infection.

Here we have identified an ApiAP2 gene (cgd4_1110) that is expressed only in female gametes. In multiple attempts, we have been unable to disrupt its locus, and thus presume is essential. To be able to rigorously test the function of essential genes we designed, engineered, and validated a conditional gene ablation model using an inducible version of Cre-recombinase. Importantly, this new system established gene regulation in a single transfection. Using this system, we engineered a mutant parasite in

which cgd4_1110 here is regulatable and test the impact of the loss of this protein on parasite development *in vitro* and *in vivo*.

5.3 Results

AP2-F is a female-specific ApiAP2 gene and is essential for the parasite.

To specifically block the sexual development, we decided to disrupt sex specific ApiAP2 transcriptional developmental modulators. We have identified a set of four ApiAP2 genes (cgd4_1110, cgd2_3490, cgd8_810, and cgd6_2670) that are transcriptionally upregulated in females (Tandel et al., 2019) (Note of these genes, cgd6_2670 has been demonstrated to be male-specific in the chapter. 4). Of these genes, we tagged cgd4_1110 with a mNeon-Green reporter (or 3XHA tag) and infected HCT-8 cells with this strain. Parasites were counterstained with an antibody to tryptophan synthase B(Tandel et al., 2019) (TrpB) and DAPI and were imaged by Structured Illumination Super-Resolution Microscopy (SR-SIM). We detected the expression of cgd4_1110 exclusively in females and localized the protein to the nucleus (Fig. 5.1a). We further confirmed the female-specific expression of cgd4_1110 by demonstrating its co-expression with the female marker DMC1 (Fig. 5.1a).(Jumani et al., 2019)
Sequence analyses predict an N-terminal (D1, aa 150-198) and a C-terminal AP2 DNA binding domain (D2, aa 1000-1053) for AP2-F. To test whether AP2-F is essential for parasite propagation, we set out to engineer three different strains. One in which we inserted the selection marker into the N-terminus D1 domain resulting in the disruption of the entire gene, a second strain in which we targeted only the C-terminus D2 domain and left remainder of the gene intact, and third, a strain in which we attached 3XHA tag at the C-terminal end without any deletion. We were only able to retrieve transgenic parasites in which we disrupted D2 or appended the 3XHA tag. We attempted to ablate the D1 domain five times using three different guide RNA sequences but failed consistently. We conclude that the locus can be modified (confirmed by PCR insertion mapping; Fig. 5.1d) and that D2 appears dispensable for the function of AP2-F and the

growth of the parasites (Fig 5.1b). In contrast, failure to achieve full ablation of the gene suggests that AP2-F is required for growth (Fig 5.1c).

An inducible DiCre recombinase system for gene ablation in *Cryptosporidium*

While failure to ablate a gene is consistent with an essential function of the encoded protein it is not proof of such a function. To test this relationship more rigorously, we sought to develop an experimental model to conditionally ablate gene function in *Cryptosporidium*. Several approaches have been applied to the related apicomplexans *T. gondii* and *P. falciparum* to modulate transcription initiation(Meissner et al., 2001), mRNA(Ganesan et al., 2016; Goldfless et al., 2014; K et al., 2020) or protein stability(Armstrong and Goldberg, 2007; Brown et al., 2018; Nishimura et al., 2009), or to excise genes using recombinase genes(Combe et al., 2009; Knuepfer et al., 2017). Here we describe our efforts to use an inducible Cre-recombinase to excise a sequence flanked by LoxP recognition site, often referred to as a floxed sequence. Two aspects of genetic engineering in *C. parvum* imposed significant technical limitations. Only a single selection marker is currently available, target gene modification and introduction of Cre thus must occur in a single transfection. Secondly, recombination frequency drops off sharply for inserts larger than 5000 bp. We thus designed a miniaturized single insertion modification system. The 'single-hit' approach for simultaneous floxing of a gene and expression of DiCre has been described in detail in the supplementary figure 5.1.

To be able to introduce LoxP sites into the coding sequence without disrupting translation we explored the use introns. We searched the *C. parvum* genome for introns and shortlisted several small introns prior to settling on a 73 bp sequence from gene cgd1_1320. This gene is shows robust constitutive expression in different lifecycle stages(Mauzy et al., 2012; Tandel et al., 2019) and the intron features a readily

identifiable the 5' donor (Ggtaag), a 3' acceptor consisting of a polypyrimidine stretch.(Abrahamsen et al., 2004) We engineered a plasmid in which this intron was inserted into the middle of the coding region of the nanoluciferase (Nluc) gene. Transient transfection of this reporter into sporozoites followed by infection of HCT-8 cells resulted in a level of luciferase activity (Fig. 5.2a) that was indistinguishable ($p > 0.05$) from that of the Nluc gene without any introns (positive control). We did not detect luciferase activity when engineering a Nluc gene containing an intron lacking the 5' donor site (Fig. 5.2a; $p < 0.0001$). We next introduced loxP sequences at various positions of this intron and found most of them not to disrupt luciferase activity (Fig. 5.2b; $p > 0.05$ when comparing insertion at 53 bp from the 5' end with loxP free control). We conclude that the chosen intron is faithfully spliced when introduced in trans and that it tolerates a well-placed loxP site.

We next developed a plasmid to overexpress an inducible version of Cre recombinase (DiCre)(Mesén-Ramírez et al., 2019), in this model the enzyme is split into two inactive fragments that dimerize and regain activity in the presence of the drug rapamycin. The detailed arrangement of this construct is shown in Supplementary Fig. 5.1. To test whether DiCre is rapamycin inducible when expressed in *C. parvum*, we designed a transient assay in which the expression of the nanoluciferase gene is disrupted by a stop sequence containing a YFP reporter gene floxed by artificial introns. The transfection of either floxed or DiCre plasmid alone did not result in luciferase activity. However, when parasites were co-transfected with both constructs and rapamycin was present in the culture medium nanoluciferase was detected (Fig 5.2c). No luciferase activity was observed in the absence of rapamycin (Fig 5.2c; $p < 0.0001$). Note that the concentration of rapamycin used for induction here (100 nM) is well within the range tolerated by the parasite (Supplementary fig 5.2a).

DiCre triggers rapamycin-inducible knockout of a dispensable gene

Next wanted to test the ability of the DiCre system to excise a floxed sequence from the *C. parvum* genome in a stably transformed parasite. We targeted the well-characterized and dispensable thymidine kinase gene (TK)(Vinayak et al., 2015), replacing the last 222 bp of the native sequence with a recodonized version preceded by an artificial intron carrying a loxP site (Fig 5.3a). We also appended a 3XHA epitope tag to be able to detect the protein as well as cassettes for expression of DiCre and Nluc-Neo. (Fig 5.3a-b). When these parasites were used to infect HCT-8 cells expression of the HA tagged TK protein was detected (Supplementary fig 5.2c). Suggesting that the placement of the artificial intron within the coding sequence of a gene did not affect its expression. PCR mapping confirmed successful modification of the TK gene, a product of 1280 bp was confirmed by Sanger sequencing as the targeted flanked by loxP sites (Fig 5.3b). A smaller band consistent with the 743 bp expected after Cre meditated excision was also detectable. This suggests that stable expression of DiCre recombinase results in some 'leaky' activity even in the absence of rapamycin (Supplementary fig 5.2b).

We next infected HCT-8 cells with the TK DiCre strain and cultured them in the presence and absence of rapamycin. Cultures were harvested after 12, 24, 36, and 48 hours, and genomic DNA was isolated and analyzed by PCR. In control samples, we detected both the full-length floxed and the smaller excised band (Fig 5.3c). Rapamycin treatment resulted in the progressive loss of the full-length band beginning at 24 hpi. We also scored parasites for the presence of the protein and conducted IFA experiments using the anti-HA antibody to detect TK expressed from the modified locus and *Vicia villosa* lectin (VVL) to visualize all parasites. As expected from the PCR mapping, we detect both tagged and untagged parasites in cultures grown in the absence of rapamycin suggesting some background Cre activity. When rapamycin was added to cultures

during growth we detected robust inducible loss of HA staining at 48 hpi (Fig 5.3d-e; Control versus Rapamycin= 40.72 ± 6.5% v/s 12.53 ± 4.67%; $p< 0.01$), differences at 24 hpi were modest but detectable (Figs 5.3d-e; $p< 0.05$).

To independently assess DiCre mediated excision we measured TK activity, using a previously established EdU incorporation assay(Pawlowic et al., 2019; Vinayak et al., 2015) with and without rapamycin induction (see supplementary figure 5.2b). WT parasites and a previously established TK KO(Vinayak et al., 2015) strain were used as positive and negative controls, respectively. 53.64% ± 6.5% of the WT parasites were EdU-positive, no staining was observed in the TK KO (Figs 5.3g-f). In the TK DiCre strain 24.98 ±, 8.7% of the untreated sample was found to be EdU-positive, this number dropped to 4.7 ± 5.94% upon rapamycin treatment (Figs 5.3g-f; $p< 0.05$). We conclude that in *C. parvum* genes can be floxed and maintain activity, that there is some backrgound DiCre excision that results in accumulation of the modified locus over time, and that treatment with rapamycin produces inducible loss of the targeted gene and the activity of its product.

DiCre Conditionally ablates the expression of AP2-F by excision of its promoter

We next sought to impose rapamycin inducible ablation on the AP2-F gene. However, our initial attempts to flox both DNA-binding domains and introduce DiCre at the 3' end of the genes failed. AP2-F is a significantly larger gene than TK (3,480 bp) requiring a more expansive repair construct. We have experienced difficulty using inserts larger than 5 kb in many independent genes targeting attempts. We decided to disrupt the expression of the gene by floxing the much smaller promoter and the start codon, which should disrupt transcription and translation of the gene (Figs 5.4a-b). Modifying the endogenous promoter of the AP2-F gene is technically challenging because we cannot

prevent undue recombination through recoding, an approach typically used for coding sequences. Therefore we replaced the endogenous promoter with a floxed promoter of *Cryptosporidium* oocyst wall protein 1 (COWP1) a promoter that we have established to drive transcription specifically in females gametes.(Tandel et al., 2019) Note that we positioned the downstream intron with a loxP site downstream of the COWP1 promoter and the 'start' codon (Fig 5.4b). We engineered two strains with floxed AP2-F, one containing the 3XHA epitope tag and one lacking it. Transfection with these constructs resulted in a successful selection of drug-resistant parasites. PCR and Sanger sequencing confirmed modification of the AP2-F gene (Figs 5.4b-c) and we focused on the epitope-tagged strain in a subsequent experiment. (Fig 5.4e).

We infected HCT-8 cells with the AP2-F floxed strains and incubated for 24, 36, and 48 hours with and without 100 nM rapamycin. PCR analysis using primers spanning both loxP sites we found the promoter fully intact when parasites were grown in the absence of rapamycin (Figs 5.4b, d, and f). Rapamycin resulted in promoter excision that increased over time.

To assess the impact of rapamycin treatment on the AP2-F protein, HCT-8 cells were infected with the floxed parasites and stained with antibodies to HA (AP2-F) and DMC1 as a marker of female gametes.(Jumani et al., 2019) Replacement of the endogenous promoter with the floxed COWP1 5'UTR still resulted in female-specific expression of AP2-F when compared to AP2-F 3XHA strains. Note, that only 60% of the females in the AP2-F flox strain expressed AP2-F compared to 100% of the females in the AP2-F 3XHA strain (Supplementary fig 5.1c). However, under rapamycin, the percentage of female gametes staining for AP2-F dropped to 7.62 ± 3.38% from 60.22 ± 18% in untreated controls (Fig 5.4g*; p*< 0.01). We conclude that rapamycin treatment results in promoter excision and loss of AP2-F expression.

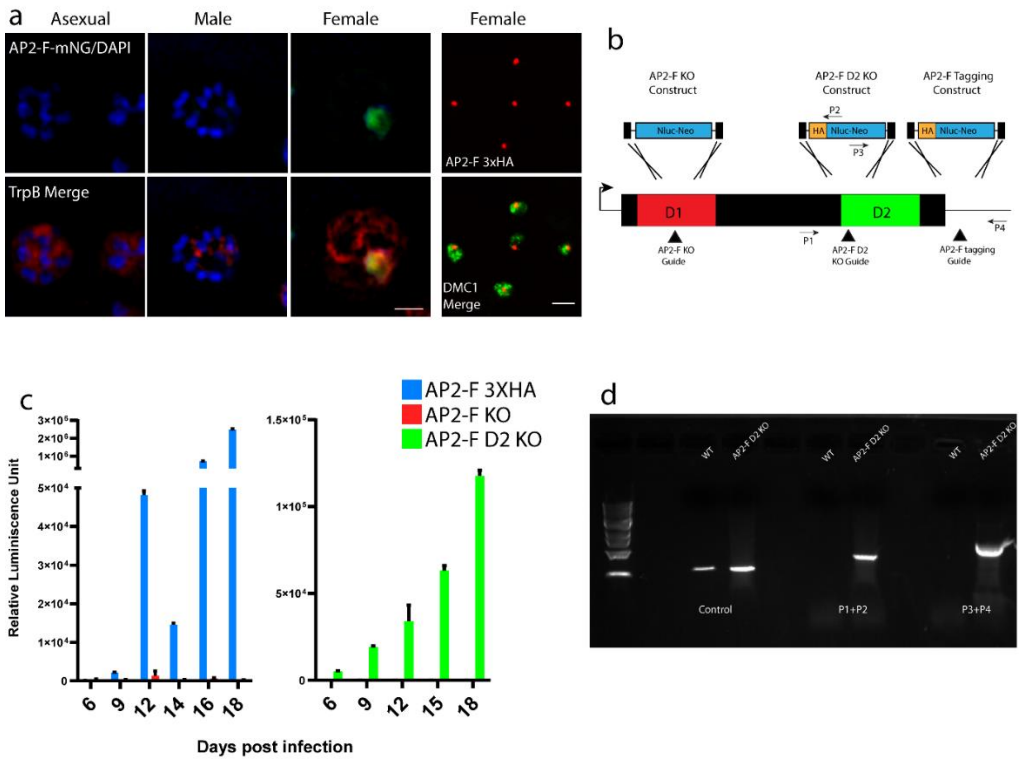


Figure 5.1 AP2-F is an essential gene and it localizes to the nucleus of female gametes. **a**. AP2-F-mNeon Green (cgd4_1110) is specifically expressed in females and localizes to the nucleus. Parasites were counterstained with TrpB antibody (red) and DAPI (Scale bar= 1 µm). Female-specific expression of 3XHA-tagged AP2-F (red) was confirmed with DMC1 staining (green) of female stages (Scale bar= 5 µm). **b**. PCR repair constructs and guides used to for AP2-F KO, AP2-F C-terminus DNA binding domain (D2) KO, and 3XHA-tagging of AP2-F. Recovery of transgenic parasites from drug selection was estimated by measuring the fecal nanoluciferase activity (Mean ± S.D.; *n*= 3). **d**. PCR map representing the successful deletion of the C-terminus DNA binding domain (D2) of the AP2-F gene. The primers used in mapping 5' and 3' insertions have been represented in **b**.

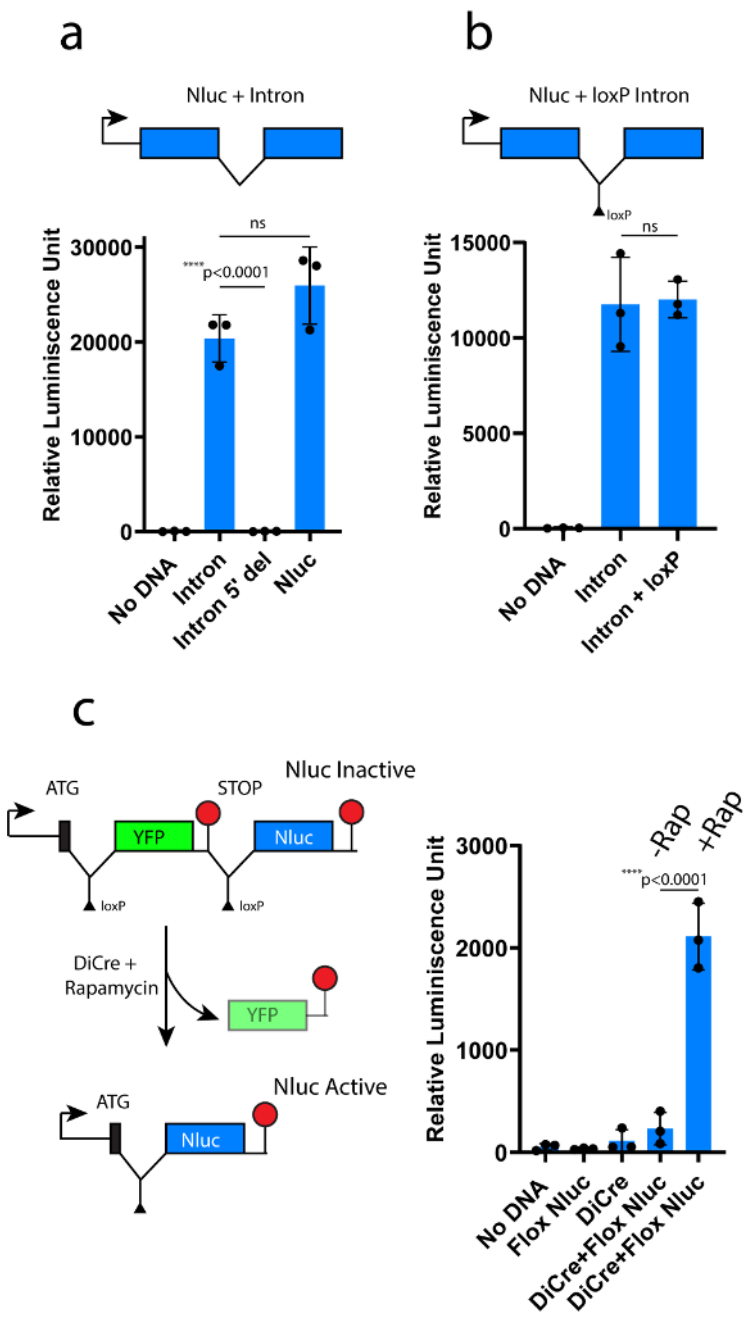


Figure 5.2 Reporter assays to test the functionality of the artificial intron and DiCre recombinase tool. Nanoluciferase-based transient reporter assay was used to determine the normal functioning of cgd1_1320 intron (**a**), intron lacking 5' donor site (**a**) and intron with a loxP site (**b**). Nanoluciferase gene lacking the intron was used as the positive control (**a**). **c**. The regulated activity of DiCre was measured by transfecting parasites with either DiCre or floxed plasmids or in combination (with and without 100 nM rapamycin). The mean of three replicates and S.D. has been represented in the graph. One-way ANOVA was used to measure the difference between the groups followed by Tukey's multiple comparison test to measure differences between different pairs of groups.

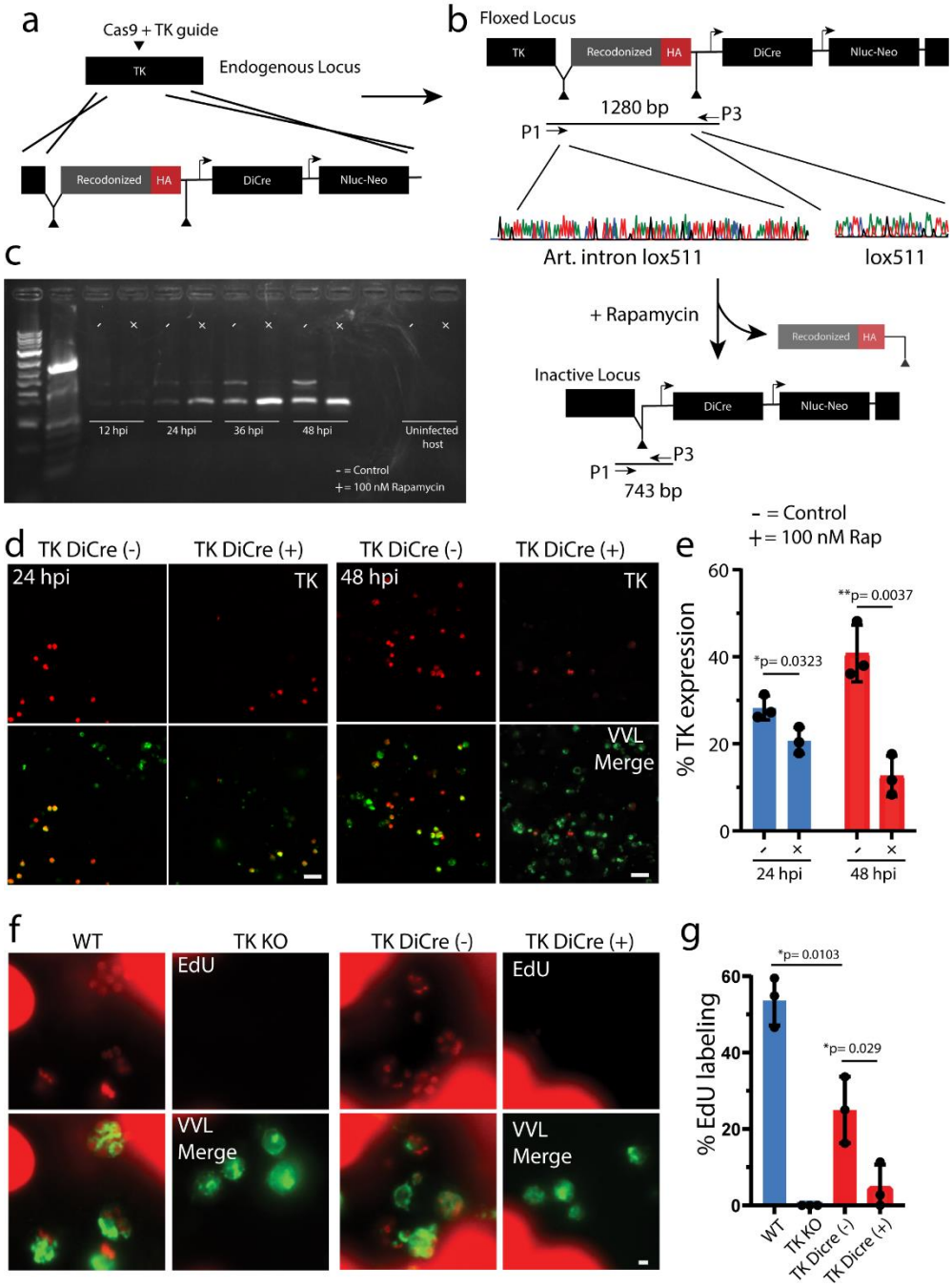


Figure 5.3. DiCre-mediated functional KO of the thymidine kinase gene. a. The C-terminal region of the thymidine kinase gene was floxed with the artificial intron. DiCre recombinase and Nluc-Neo drug resistance marker was expressed ectopically via α-tubulin and enolase promoters, respectively. Successful floxing of the gene was confirmed by Sanger DNA sequencing (**b**). Rapamycin inducible KO of the TK gene was measured by PCR *in vitro* at different timepoints (**b-c**). Functional loss of TK was measured by IFA (**d-e**) and EdU-labelling assay (**f-g**). Parasites were counterstained with VVL. Experiments were performed in triplicates and student's t-test used for the statistical analysis. The schematic of the EdU experiment is represented as supplementary figure 5.2b.

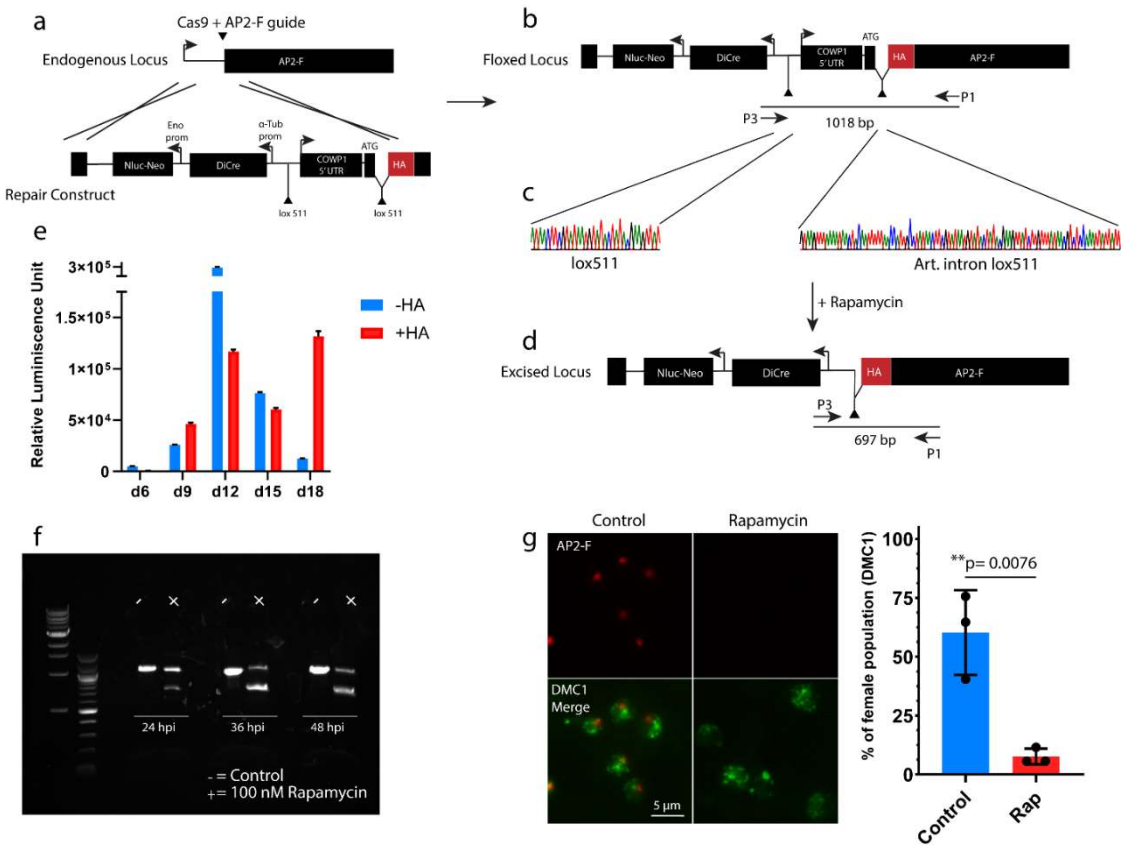
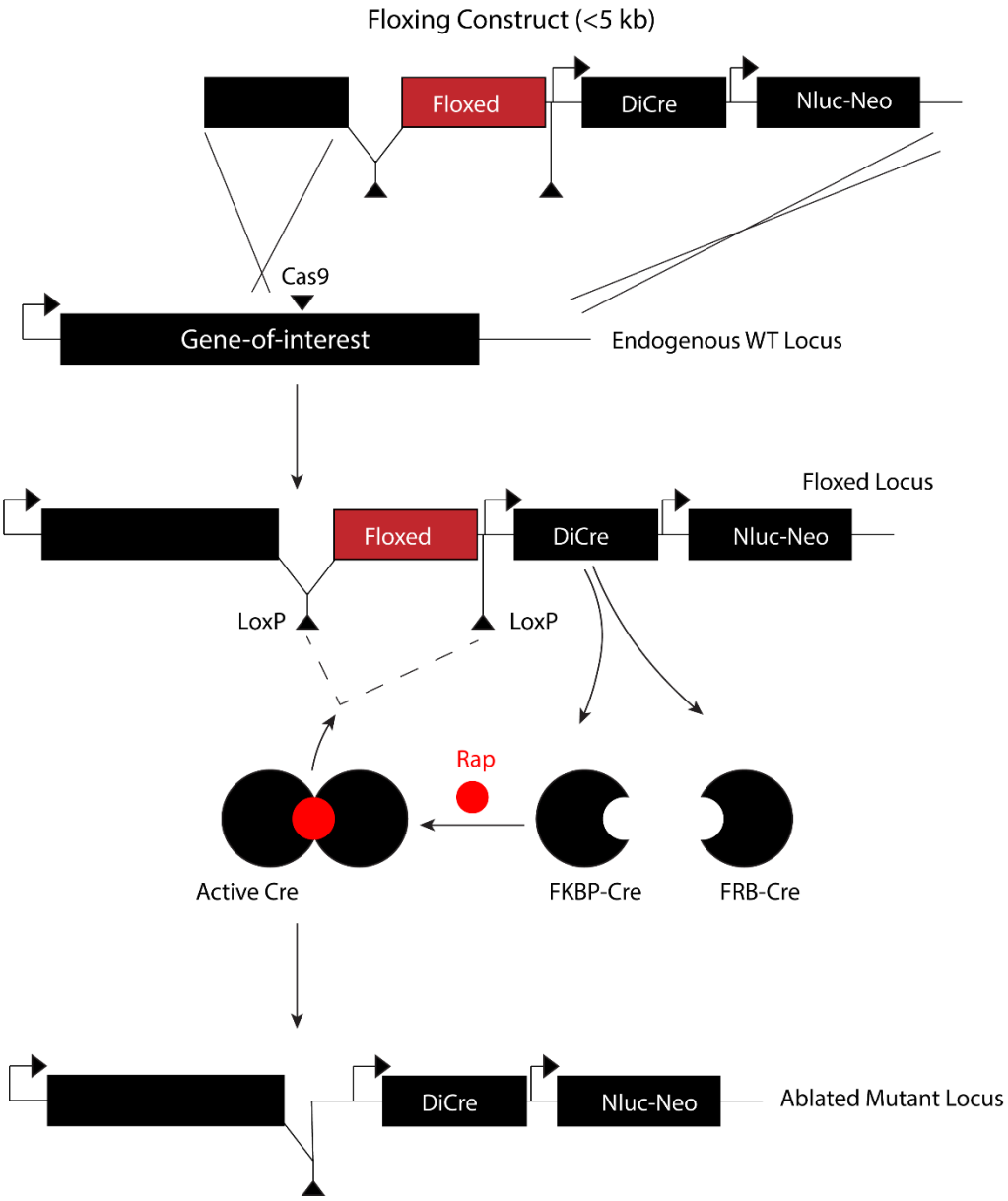
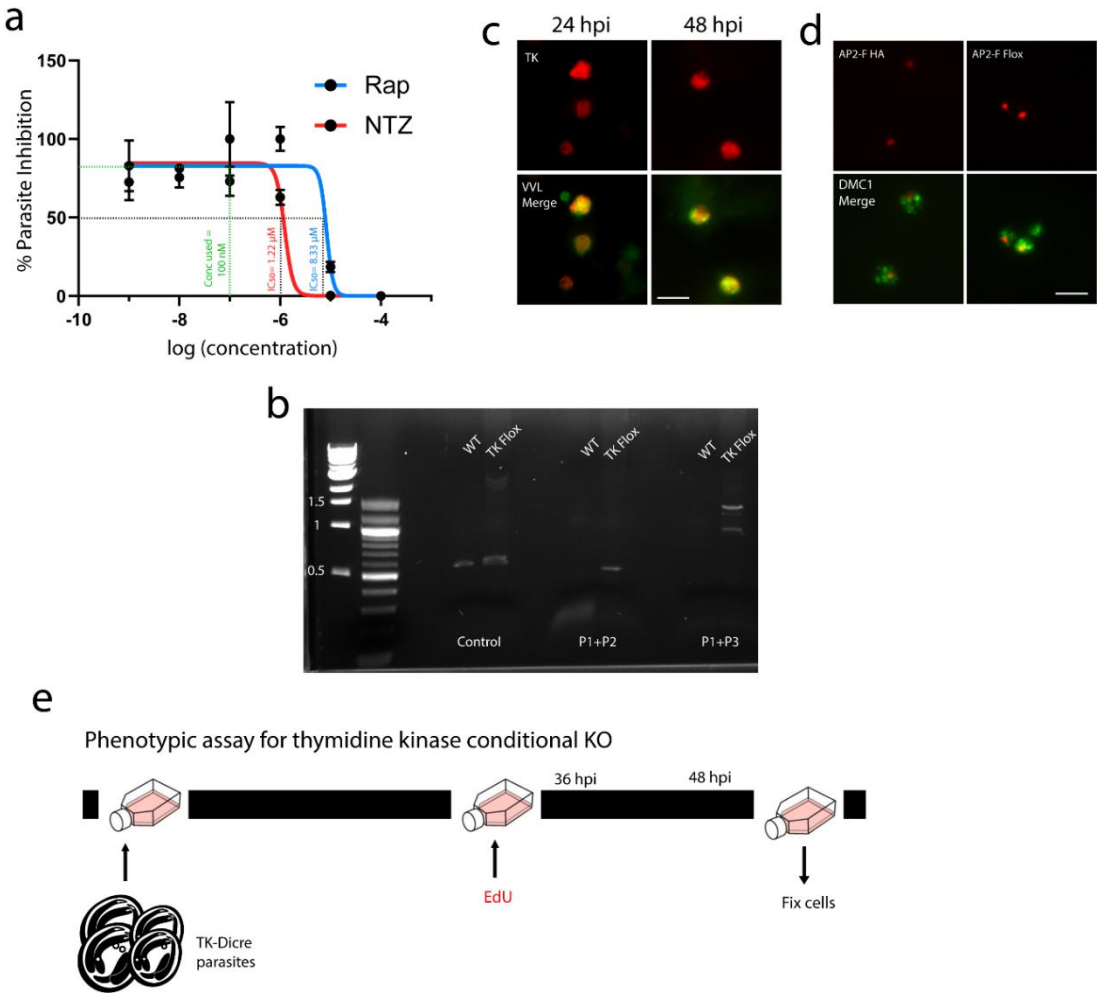


Figure 5.4. DiCre-mediated conditional KO of the AP2-F gene. a. The endogenous promoter of the AP2-F gene was replaced by a floxed COWP1 5'UTR. The artificial intron was inserted after the 'start codon' followed by a 3XHA epitope tag. Successful transfection was assessed by measuring fecal nanoluciferase activity to score for transgenic parasites (**e**). Sanger DNA sequencing confirmed the successful floxing of the AP2-F gene (**c**). PCR analysis confirmed rapamycin-induced loss of the floxed segment *in vitro* at respective timepoints (**b,d and f**). Loss of AP2-F expression was measured by IFA after 48-hour induction. Females were counterstained with the DMC1 antibody. Experiments were performed in triplicates and student's t-test used for the statistical analysis.



Supplementary Figure 5.1. 'One-hit' strategy for simultaneous floxing of a gene and overexpression of DiCre in *Cryptosporidium*. *Cryptosporidium* currently has a single drug selection marker. Hence, we designed a single-delivery strategy that will simultaneously flox the gene-of-interest and overexpress DiCre recombinase. A single targeting construct containing the floxed segment, DiCre, and drug selection cassette is incorporated in the desired region by using the CRISPR/Cas9 system. The targeted region is floxed by an intron-containing a LoxP one side and another LoxP on the other side. Two physically separate Cre-FRB and Cre-FKB components are expressed from one DiCre cassette. The DiCre components remain inactive in the absence of the rapamycin, thus do not disrupt the floxed locus. Rapamycin triggers the dimerization of the DiCre, resulting in recombination and disruption of the floxed region.



Supplementary Figure 5.2. Auxiliary figures for the development of the DiCre-based knockout system to study the AP2-F gene. a. IC_{50} of rapamycin (Rap) was measured by treating HCT-8 cells infected with Nluc-expressing *C. parvum*. Cells were treated with rapamycin concentrations ranging from 1 nM- 100 µM. Nitazoxanide (NTZ) was used as a positive control. The experiment was performed in triplicates. The IC_{50} was found to be 8.33 µM which was a log higher than that of nitazoxanide (IC_{50}= 1.22 µM). **b**. PCR map confirming the floxing of the C-terminal 222bp of the thymidine kinase gene. **c**. Expression of 3XHA tagged thymidine kinase (red) in the TK flox strain at 24- and 48-hours post-infection. Parasites were counterstained with VVL (green). **d**. Female-specific expression of AP2-F in a strain with C-terminus 3XHA tag on AP2-F (AP2-F HA) and AP2-F HA DiCre strain (AP2-F Flox). The scale bars in all the micrographs are 5 µm. **e**. Graphical depiction of the EdU-labelling experiment to assess the rapamycin-induced loss of the thymidine kinase activity.

5.4 Discussion

ApiAP2 DNA binding proteins have been identified as key transcriptional regulators of stage differentiation(Kafsack et al., 2014; Radke et al., 2013; Sinha et al., 2014) and development(Iwanaga et al., 2012; Kaneko et al., 2015; Yuda et al., 2010) in apicomplexans. ApiAP2 proteins modulate the expression of stage-specific genes and their disruption results in the misregulation of stage-specific processes.(Kafsack et al., 2014; Kaneko et al., 2015; Sinha et al., 2014) Instead of targeting individual sexual stage-specific processes, we aimed to disrupt ApiAP2 genes to specifically disrupt sexual or post-fertilization development to test whether sex is essential to maintain infection in a host. We identified an ApiAP2 gene, cgd4_1110 (AP2-F), as a female-specific ApiAP2, and its disruption was not tolerated by the parasite. In *Plasmodium falciparum* knockout of ApiAP2 genes regulating the development of gametocytes(Kafsack et al., 2014; Sinha et al., 2014), liver stages(Iwanaga et al., 2012), sporozoites(Yuda et al., 2010), and ookinetes(Kaneko et al., 2015) are technically possible because disruption of these stage does not affect the asexual blood cultures. However, a similar approach cannot be taken to study sexual-stage specific AP2s in *Cryptosporidium*, as it would disrupt the development of oocyst (oocyst are the stages that can be recovered from mice and much of our experimentation relies oocyst isolation and manipulation(Vinayak et al., 2015)).

We developed a DiCre conditional knockout system to allow for controlled disruption of the AP2-F gene stimulated by the addition of a small molecule.(Knuepfer et al., 2017) Typically such systems are built in multiple steps to introduce the recombinase and to flank the target gene with loxP sites. The limitations of the *C. parvum* transfection system forces us to deliver a complex regulatory system in a single insertional event and

imposed size restrictions forced us to miniaturize all aspects of the single hit cassette. Our strategy relies on short floxed introns and we show that those are well tolerated and efficiently excised using two genes, TK, and AP2-F, as examples. In our first approach, we floxed a portion of the TK gene from the 3' end. This is feasible for genes in which the encoded protein harbors a domain essential to activity close to the C-terminus (100 aa or less). For AP2-F conditional KO, we rendered its expression conditional by replacing the endogenous promoter with a floxed promoter. Intergenic regions are typically very small in the *C. parvum* genome offering short promoters as targets for excision. Importantly, this allows modulation irrespective of the size of the gene or the position of critical elements within the gene. When implementing this strategy, it is important though to use a surrogate promoter appropriate for the timing and strength of expression of the native gene. Expanding transcriptomic data of all lifecycle stages now provide a repository of matching genes and promoters.

As other authors before(St-Onge et al., 1996; Utomo et al., 1999) us we observed 'leaky' DiCre activity when targeting a dispensable gene, we documented some excision in the TK gene even in the absence of rapamycin. We did not conduct extended serial passage experiments, but it is likely that the floxed segment is successively lost over time. In contrast, we did not observe such a sub-population of floxed AP2-F parasites prior to rapamycin induction. This difference may be linked to differences in the expression of DiCre or the relative accessibility of the LoxP sites in these two genes. A third, and maybe most likely explanation is that AP2-F is an essential gene and undue excision of the promoter will result in a strong counter selection eliminating mutant parasites from the population.

When rapamycin was added to cultures infected with mutant parasites, we observed robust induction of excision in both strains with similar kinetics. DiCre activity was detectable after 24 hours and reached a plateau at 48 hours. Rapamycin induction of the floxed TK strain *in vitro* resulted in the complete transition to the excised population. However, for AP2-F we observed a small population that retained the floxed promoter after 48 hours. A similar phenomenon was observed for the excision of an essential, blood-stage gene in *P. falciparum*.(Knuepfer et al., 2017) There DiCre activity was detected as early as 4 hours and plateaued at 36 hours after 36 hours leaving around 20% of the population unchanged.(Knuepfer et al., 2017) The cause of recalcitrance to recombination in a small population is unknown. In future experiments, we plan to sequence the unexcised fragments to detect potential mutations in lox sites that might prevent recombination.

AP2-F expression in the floxed strain was observed in ~60% of females compared to 100% in C-terminus 3XHA-tagged AP2-F strain. This can be explained by utilization of COWP1 promoter or the loxP intron in the floxed strain which might be result in delay in AP2-F protein expression in females.

Rapamycin-induced DiCre excision reduced the percentage AP2-F expression in females by ~90%. The loss of AP2-F expression did not affect the number and overall morphological appearance of female gametes. This demonstrates that AP2-F is not required for the growth of asexual stages or the commitment to develop into female gametes. Typically, ApiAP2 genes that commit parasites to differentiation are expressed in the stage preceding that event.(Kafsack et al., 2014; Radke et al., 2013; Sinha et al., 2014) We, therefore, hypothesize that AP2-F has a role in the later maturation of female gametes or is required for events that unfold during or following fertilization. As

fertilization does not occur in culture(Tandel et al., 2019), we cannot observe the phenotypic consequence of loss of AP2-F in HCT-8 cells. We further aim to test the effect of loss of AP2-F on infection in mice. Treatment of mice providing orally or intraperitoneal has been shown to activate DiCre activity *in vivo*. Our next aim is to measure the effect of loss of AP2-F on post-fertilization development and oocyst biogenesis in infected mice. We further plan to test whether AP2-F disruption affects the overall infection in mice. These experiments will test whether sex is essential in driving infection in a host.

Overall, we have demonstrated the functionality of DiCre system to conditionally disrupt essential genes. This system will be important to validate potential drug targets. Alternately, DiCre system can be used to conditionally overexpress transcription factors(Kent et al., 2018) involved in male/female determination and asexual fate maintenance. For example, conditional overexpression of a potential repressor of sexual differentiation provides an opportunity to engineer a strain that can be maintained asexually in HCT-8 cultures perpetually. DiCre expressed under specific promoters can be used for lineage tracing to identify certain asexual populations that are primed to differentiate into males or females.(He et al., 2017)

DiCre system has a significant use studying functionality of genes, testing potential drug targets, manipulating the parasite lifecycle and for lineage tracing.

Chapter 6: Conclusion and Future Directions

Apicomplexan parasites have evolved complex lifecycles to replicate(Auld and Tinsley, 2015; Meissner, 2013), disseminate(Brancucci et al., 2017; Lyons et al., 2002; Yuda et al., 2009), escape immune recognition(Casares and Richie, 2009; Lyons et al., 2002), and to diversify by mating with different genotypes(Brown et al., 2001). The developmental mechanisms that underlie these complex morphological and molecular transformations between different life stages have been intriguing to me. The molecular mechanisms underlying the differentiation of pluripotent stem cells into a wide variety of different fates have been well characterized in higher eukaryotes.(Hwang et al., 2008) Most of the well-known mammalian developmental transcription factor-like homeo(Duverger and Morasso, 2008), bZip(Lin et al., 1997), bHLH(Dennis et al., 2019), and forkhead domain(Hannenhalli and Kaestner, 2009) are absent in apicomplexans.(Balaji et al., 2005) This implies that apicomplexans have developed unique modulators for stage differentiation.(Balaji et al., 2005) Differentiated cells originating from stem cells maintain their fates permanently(Hwang et al., 2008) with only a few exceptions(Kamada et al., 2016). However, as single cell organisms, apicomplexan parasites must adopt their different life stages iteratively. This suggests that apicomplexans have evolved molecular mechanisms to ensure reversible plasticity. Many of these intriguing questions about the developmental biology of apicomplexans remain to be answered.

In the last ten years, AP2 DNA binding proteins have been identified as regulators of stage-specific development of apicomplexan parasites.(Iwanaga et al., 2012; Kafsack et al., 2014; Radke et al., 2013; Sinha et al., 2014; Yuda et al., 2009, 2010) The only exception has been a Myb-1 gene that modulates the development of the *Toxoplasma*

cyst stage.(Waldman et al., 2020) In addition to the identification of developmental regulators, molecular cues that help parasite sense host species(Dubey et al., 2011; Phillips et al., 2017), host nutrient status(Price et al., 1999), immune response(Brancucci et al., 2017; Skariah et al., 2010), and tissue location(Dubey et al., 2011; Genova et al., 2019) have been identified. A sampling of the host environment to control differentiation is an intuitive feature of *Toxoplasma* and *Plasmodium* that develop into different stages depending upon the host species. But are these mechanisms of environmental sampling for differentiation relevant in a single-host apicomplexan like *Cryptosporidium*? Or are single-host parasites programmed for stage-differentiation that follows internal cues (like the number of asexual cycles) rather than environmental ones? These broad developmental questions led me to study the lifecycle of a single-host parasite like *Cryptosporidium*.

No experimentally tractable model of a single-host lifecycle apicomplexan is currently available, and I was particularly drawn to develop such a system in *Cryptosporidium* because of its importance in infecting humans. *Eimeria* remains the closest single-host lifecycle apicomplexan analog for *Cryptosporidium*. However,ability of the *Cryptosporidium* to sporulate within the host sets it apart from *Eimeria* (where sporulation outside the host), leading to different consequences of sexual development on parasite amplification in both these parasites. The preliminary discovery of the inability of *Cryptosporidium* to mate in HCT-8 cells provided empirical support to the hypothesis that sex might be essential for the parasite to maintain infection. This suggests that a therapeutic strategy targeting sex would block transmission and infection in a host. To a molecular biologist like me the pursuit of these questions was intellectually stimulating. To be able to test my hypotheses on the critical importance of

sex for infection with this parasite, I required more in-depth knowledge of processes that control the lifecycle and a genetic strategy to conditionally disrupt them. To this end I identified sex-specific markers, engineered sex-specific fluorescent reporter strains, defined transcriptomes of sexual stages, and designed a DiCre conditional KO tool for *Cryptosporidium*. I believe that these resources will allow me to test my central hypothesis in a mouse model. I have proposed immediate experimental plans in the following section. I will also discuss further questions regarding the possible mechanisms of sexual differentiation, sex determination and mating in *Cryptosporidium* that might be intellectually stimulating for the *Cryptosporidium* research community. I believe that solutions to the challenges of developing anti-cryptosporidial therapeutics and vaccines lie in answering questions about the basic biology of *Cryptosporidium*.

Conclusion

Overall, this work has increased our understanding of the potential model of the lifecycle of the parasite. *In vivo* and *in vitro* comparison of lifecycle progression of *Cryptosporidium* has revealed that sex might be an obligatory step in maintaining infection in a host. Understanding the effect of conditional perturbation of AP2-F (cgd4_1110) on oocyst development and infection in a mouse model will be crucial in testing the such a model (Fig. 6).

Our work has further provided insights into molecular processes that are unique to sexual stages. Studying these processes will further contribute to the understanding of sexual development, fertilization, and post-fertilization development. Furthermore, many of these processes represent key drug and vaccine targets to block transmission and autoinfection. This work has demonstrated successful conditional ablation of an essential gene in *Cryptosporidium*. Conditional gene knockout can be further leveraged together with sexual stage transcriptomics to develop a mechanistic understanding of sexual development and to validate drug and vaccine targets.

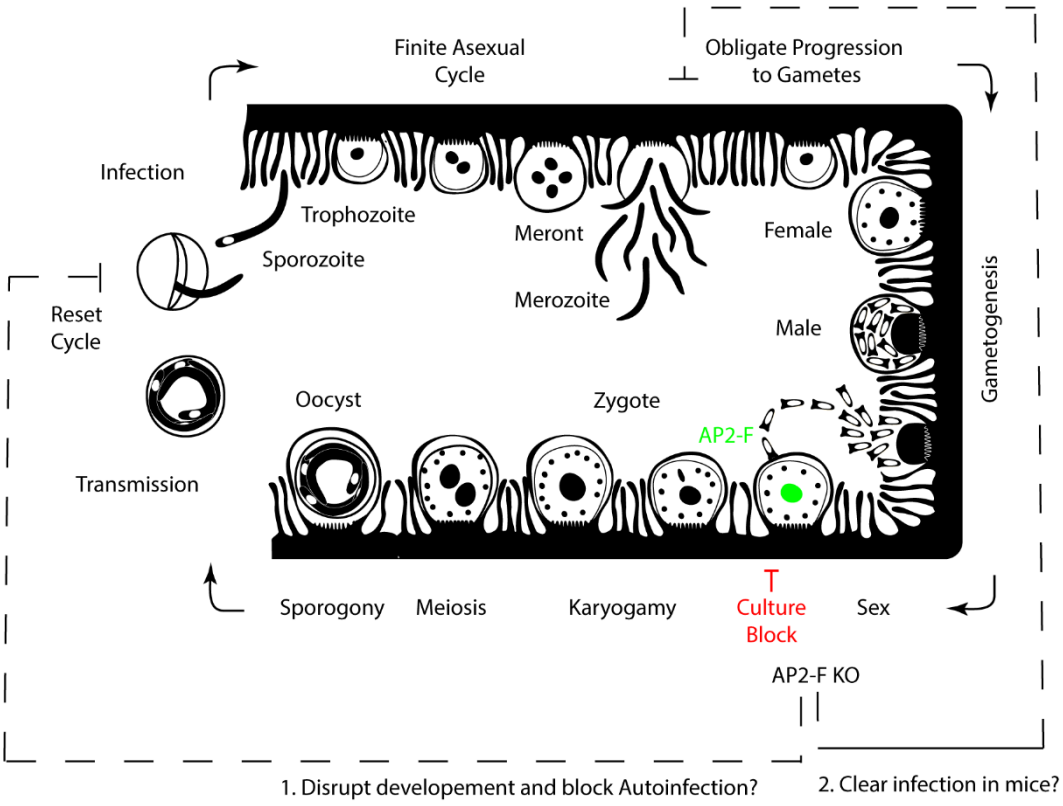


Figure 6. Proposed model of the lifecycle of *Cryptosporidium parvum*. *Cryptosporidium parvum* infects HCT-8 cells, replicate asexually, and undergo robust sexual differentiation. However, the parasites fail to fertilize in HCT-8 cells, thus blocking oocyst development. Contrastingly, parasites fertilize in mice and undergo oocyst development. Hence, a lack of continuous amplification in HCT-8 cells can be explained by the failure to fertilize and produce oocyst. These observations hint towards a model of an obligatory sexual development to maintain infection in a host. An approach to specifically disrupt sexual development (conditional KO of AP2-F) and testing its effect on infection will be needed.

Future Directions 6.1 Investigate the role of AP2-F in the *Cryptosporidium* lifecycle and chronicity of infection in mice

Our experiments so far suggest that AP2-F is essential for the continued propagation of the parasite and that it is required for the sexual part of the lifecycle. However, conditional disruption of AP2-F *in vitro* did not affect female development in a fashion that we were able to detect. AP2-F thus most likely is required for later developmental steps that follow fertilization. We aim to mechanistically understand the role of AP2-F in oocyst development and maintenance of infection in an animal host by pursuing the following lines of investigation:

6.1.1 Identify the processes that are regulated by AP2-F

Conditional disruption of AP2-F in culture did not affect the development of asexual stages or female development. This implies that AP2-F might be involved in modulating post-fertilization processes like meiosis and sporulation. AP2-F might also be involved in producing females that are competent for fertilization. Alternatively, AP2-F might also be a repressor acting to prevent the undue expression of asexual of male genes in female stages. In *Plasmodium*, AP2-G2 has been identified as a repressor that downregulates the expression of asexual phase genes in gametocytes.(Sinha et al., 2014; Yuda et al., 2015) Disruption of AP2-G2 results in abnormal gametocyte development.(Sinha et al., 2014; Yuda et al., 2015) Pulldown of AP2-F 3XHA and ChIP sequencing(Kaneko et al., 2015; Waldman et al., 2020) of the AP2-F bound DNA sequence will help identify target genes. Similarly, we aim to induce disruption of AP2-F in HCT-8 cells infected with the floxed AP2-F strain to identify genes that show expressions change upon AP2-F depletion. AP2-F might also be involved in the maturation of females by modulating

genes that are needed for successful fertilization and post-fertilization development. We have already proven that genes required for meiosis, amylopectin synthesis, and oocyst wall biogenesis are transcribed prior to fertilization. While the phenotypic effect of disruption of these processes on fertilization and sporulation cannot be gauged in HCT-8 cells, RNA-sequencing after disruption could be informative in identifying pre-fertilization female-specific processes regulated by AP2-F. It is also possible that AP2-F might be fully or partially functional only after fertilization. Since fertilization is not observed in HCT-8 cells (chapter 3), mice or organoid systems(Heo et al., 2018; Wilke et al., 2019) will have to be used to address this issue. The murine model system is technically challenging as it requires the flow-sorting of intracellular stages from mice. The current AP2-F floxed strain lacks a fluorescent marker, and we cannot reengineer the same strain with an extra expression cassette for a fluorescent reporter (maximal 5 kb repair limit). Intestinal organoids can be infected with the AP2-F floxed strain and can be transcriptionally profiled in bulk. Another advantage of using the organoid host system is that any effect of AP2-F disruption on post-fertilization or oocyst development can be captured by transcriptional profiling. Comparison of transcriptomes of *in vitro* and *in vivo* female stages have helped us identify gene signature that is upregulated uniquely in post-fertilization stages (chapter 3). Misregulation of genes unique to the post-transcriptional gene-signature would suggest a role of AP2-F in post-fertilization development.

6.1.2 Investigate possible roles of AP2-F in the fertilization, post-fertilization development, and oocyst biogenesis

For this study, we aim to use organoid systems as they sustain the post-fertilization development of *Cryptosporidium*.(Heo et al., 2018; Wilke et al., 2019) It is possible to

quantify females and different post-fertilization stages by microscopy in organoids. It is thus an ideal host system to quantify the developmental effect of AP2-F conditional depletion on females to oocyst transition. Antibody reagents that can discriminate between females, post-fertilization stages, and oocysts can be used to quantify different stages. We have already demonstrated that staining the nucleus of the parasites can distinguish between females, zygotes, and oocysts. Alternatively, staining with the COWP1 antibody will help in distinguishing females (vesicular) and oocyst (wall).(Spano et al., 1997; Tandel et al., 2019) We hypothesize that AP2-F depletion would result in reduced oocyst development.

6.1.3 Investigate the effect of AP2-F disruption on oocyst shedding and autoinfection in mice

The experiments outlined above will help us understand the role of AP2-F in modulating the transition from female to oocyst. Should we find such disruption of development, we would expect that conditional disruption of AP2-F in infected mice would block oocyst shedding. This would afford us to test whether sex and formation of oocysts are required to maintain infection in mice. To test these hypotheses, we aim to infect mice with AP2-F floxed strain and treat mice with or without rapamycin. We aim to measure changes in oocyst shedding and intracellular parasitic burden upon AP2-F disruption. We expect that AP2-F disruption should reduce the shedding of the oocysts, which in turn should decrease the infection in mice.

Both intraperitoneal and oral administration of rapamycin in mice results in the distribution of rapamycin in a variety of organs including the small intestine.(Komarova et al., 2012; Leontieva et al., 2014) This suggests that rapamycin administered to in

infected mice is likely available to parasites in the intestine. Similarly, rapamycin has been successfully used in DiCre-mediated activation of reporters in mice.(Jullien et al., 2003, 2007) We will optimize the rapamycin-induced DiCre activity in parasites *in vivo* by infecting mice with the TK floxed strain and measuring excision of the floxed segment after rapamycin treatment.

Future Directions 6.2: Understanding male-female gamete interactions in *Cryptosporidium*

6.2.1 Mechanisms involving surface protein-protein interactions between male and female gametes

In Chapter 3 we have demonstrated that *Cryptosporidium* undergoes robust gamete production in HCT-8 cells and IFN-γ KO mice and in mice gametes undergo fertilization *in vivo*. We have also show that HAP2 is a male-specific transmembrane protein in *Cryptosporidium*. The role of HAP2 in fusion of male and female gametes has been demonstrated rigorously in *Chlamydomonas* and *Plasmodium*.(Liu et al., 2008) Immunization of individuals with a *P. falciparum* HAP2 synthetic, subunit vaccine resulted in the generation of anti-HAP2 antibodies that blocked fertilization in *Plasmodium*.(Angrisano et al., 2017) The role of HAP2 in mediating fusion of males with females still needs to be investigated in *Cryptosporidium*.

Interaction of gametes is a multi-step process involving activation, binding, and fusion.(Mori et al., 2015) HAP2 is only required for the final fusion step as *Chlamydomonas* HAP2 mutants remain attached without undergoing fusion(Liu et al., 2008) This suggests that additional proteins remain to be identified in *Cryptosporidium*

that are responsible for activation and attachment. *Cryptosporidium* female gametes are intracellular, which requires the male gamete to cross the host cell membrane to access the female.(Ostrovska and Paperna, 1990; Tandel et al., 2019) *Cryptosporidium* female gametes might export a unique transmembrane protein onto the host cell membrane to 'bait' male gametes. Protein machinery to translocate membrane proteins on the host cell surface already exists in other apicomplexans.(Smith, 2014) My colleague, Jennie Dumaine, has identified a protein that is exported by intracellular *Cryptosporidium* parasites into the host cells (unpublished observations). This implies that protein-export machinery exists in *Cryptosporidium*. Further studies in the *Cryptosporidium* model with an intracellular female gamete, could thus provide insight into a novel aspect of parasite biology.

Targeting proteins involved in male-female interactions through active or passive immunization could produce neutralizing antibodies and might be exploited to prevent transmission (as in *Plasmodium*(Chaturvedi et al., 2016)) and block auto-infection. Our transcriptional analyses detailed in Chapter. 4 revealed multiple male- and female-specific transmembrane proteins providing candidates for the molecules that enable gamete interactions. Alternatively, we can use our reporter assay for sex detailed in Chapter. 3 to screen for peptides that physically block gamete interactions.(Saw and Song, 2019) Candidate peptides conjugated with affinity tags can be used in a pull-down experiment to identify gamete-specific interacting partners.(Wysocka, 2006) Candidates identified in these experiments can be rigorously tested using our DiCre conditional gene KO tool.

Passive immunization of children younger than 2 years old through maternal vaccination might be an attractive approach for the delivery of such a vaccine.(Vojtek et al., 2018)

Colostrum obtained from cows immunized with *C. parvum* p23 antigen when administered to newborn calves reduced oocyst shedding by 99.8% compared to calves given colostrum from cows that were not immunized.(Perryman et al., 1999) This result highlights the importance of studying genes involved in *Cryptosporidium* gamete interactions from a biological and translational perspective.

6.2.2 Mechanisms involving priming of male gametes prior to fusion with females

Vertebrate and invertebrate sperm undergo an acrosomal reaction (AR) after encountering eggs.(Rothmann and Bort, 2018; Yanagimachi, 2011) During the AR, the outer membranous component (acrosomes) of the sperm head undergoes Ca^{+2}-dependent exocytosis to release their enzymatic contents that include glycosidases and proteases that degrade the extensive glycocalyx of the egg.(Rothmann and Bort, 2018; Yanagimachi, 2011) This lytic process results in an opening in the protective coat of the egg, providing an access route for the sperm cell.(Rothmann and Bort, 2018; Yanagimachi, 2011) This process is followed by the exposure of sperm receptors on the inner acrosomal membrane which interact with egg membrane receptors to undergo membrane fusion.(Rothmann and Bort, 2018; Yanagimachi, 2011)

Cryptosporidium male gametes have a polarized structure with an electron-dense ‘basal body’ at its apical end.(Ostrovska and Paperna, 1990) The apical end is surrounded by membrane projections called ‘adhesive zones’.(Ostrovska and Paperna, 1990) As it is this end of the gamete that undergoes fusion with the female,(Ostrovska and Paperna, 1990) it might be functionally analogous to the sperm acrosome. The apical end might contain the male-specific proteases that we describe in chapter. 4 as well as other enzymes involved in attacking proteins and glycoproteins(Pelaseyed et al., 2014) on the

surface of the host cell membrane or the female membrane in preparation of fusion. *Cryptosporidium* male gametes express a unique, secreted kazal-domain protein (cgd5_3380). Kazal domain-containing proteins are protease inhibitors and some of them are known to prevent premature activation of sperm-specific proteases.(Rothmann and Bort, 2018; Thélie et al., 2019; Zheng et al., 1994) cgd5_3380 kazal domain protein might be prepackaged with male-specific proteases in vesicles to prevent untimely activation of male proteases. These ideas are immediately testable.

Plasmodium male gametes are elongated and lack a *Cryptosporidium*-like 'basal body' structure.(Straschil et al., 2010) and fertilize female gametes that are free of their host cell in the mosquito midgut.(Aly et al., 2009) These differences suggest that *Cryptosporidium* male gametes have evolved distinct structural and molecular mechanisms to fertilize with an intracellular female, which requires them to overcome multiple membranous barriers to access females.

6.2.3 Mechanisms to prevent fertilization by multiple male gametes (polyspermy)

In addition to mechanisms enabling physical interaction with sperm, eggs have evolved strategies to avoid fertilization with multiple male gametes. Abnormal fertilization of an egg with multiple sperm (polyspermy) can impair the development of an embryo.(Gilbert, 2000) Mammalian eggs avoid polyspermy via two successive mechanisms: 'fast block' and 'slow block'. 'Fast block' occurs 0.1s after the initial sperm-egg contact. During this process, Na^+ ion channels in the eggs open to depolarize the egg membrane from its resting potential of -70 mV.(Gilbert, 2000) 'Slow block' occurs 10-60 seconds after the sperm has entered the egg. During this process, proteases and oxidases stored in the egg in vesicles are secreted to strip away membrane receptors required for sperm

recognition and fusion.(Gilbert, 2000) The sperm receptors-containing vitelline membrane is further removed by pumping water into the space between the egg membrane and the vitelline membrane.(Gilbert, 2000) Lastly, the egg membrane is rendered impenetrable by crosslinking membrane proteins by secreted oxidases.(Gilbert, 2000)

Cryptosporidium male gametes outnumber females by 6:1. This might ensure the fertilization of most of the female gametes. However, an encounter of a female gamete with a cluster of male gametes could lead to polyspermy and the parasite might have evolved mechanisms to avoid polyspermy. Secreted proteases and oxidases expressed by females might be involved in remodeling the female membrane to prevent a secondary fertilization event. In addition, secretion of oocyst wall proteins from wall forming bodies(Spano et al., 1997) immediately after fertilization might provide a structural barrier that prevents further access of male gametes.

Future Directions 6.3: Understanding potential mechanisms of chemotaxis between *Cryptosporidium* gametes

Chemotaxis of gametes towards each other through the release of diffusible chemoattractants termed pheromones has been observed in a wide variety of organisms including algae, fungi, worms, insects and mammals.(Gomez-Diaz and Benton, 2013; Kochert, 1978) The mechanisms of chemotactic movement of gametes are still uncharacterized in the apicomplexans including the well-studied *Plasmodium* and *Toxoplasma*. However, the molecular basis of chemotaxis in algal(Kochert, 1978), fungal(Kochert, 1978), and mammalian gametes(Sun et al., 2017) can provide a

blueprint to investigate mechanisms of chemotaxis in apicomplexans. We have documented robust sexual differentiation of *Cryptosporidium* in infected HCT-8 cells (chapter. 3), which is followed egress of male gametes, and we frequently observed females surrounded by male gametes. This makes *Cryptosporidium* an ideal apicomplexan model to study the chemotactic motility of male gametes towards females.

The chemistry of pheromones has been extensively studied in algal and fungal systems.(Kochert, 1978) A wide variety of chemicals like cAMP, lipids, peptides, and hydrocarbons have been identified as chemoattractants for male gametes.(Kochert, 1978) However, most of the algal and fungal pheromones are cyclic, conjugated hydrocarbons, and steroids.(Kochert, 1978) Since the nature of metabolites and genes involved in the chemotaxis of gametes is unknown, an unbiased approach is likely required to identify them. We have already developed a variety of stage-specific fluorescent reporter strains that can be used to measure chemotaxis in response to chemical cues via live microscopy. Pheromones derived from sexual stages should be secreted in the extracellular environment and can be identified by comparing 'secretomes' of asexual and sexual cultures. Fractionated secretomes derived from the sexual culture can be further tested for activity on male egress and motility. Alternatively, a library of synthetic chemical compounds can be tested for their effects on male egress and motility. A similar approach has been used to measure chemotaxis in the nematode *Strongyloides* in response to multiple host-derived cues like heat and odorants.(Safer et al., 2007)

Chemotaxis in apicomplexan gametes has not been studied yet but it has been observed in *Plasmodium* blood stages(Mantel et al., 2013; Regev-Rudzki et al., 2013) and hemolymph- and oocyst-derived *Plasmodium* sporozoites(Akaki and Dvorak, 2005).

Exosomes and exosome-like vesicles released from *Plasmodium*-infected RBCs contain parasite-derived proteins that communicate survival and transmission cues between parasites.(Mantel et al., 2013; Regev-Rudzki et al., 2013) Exosomes derived from *Cryptosporidium* sexual cultures can be tested for their chemotactic activity and protein content.

Molecular mechanisms of sensing chemotactic signals by male gametes have been well characterized in the mammalian system. CatSpers (cation ion channels of sperm) Ca^{+2} ion channels in the mammalian sperm are activated by a variety of inducers including progesterone (female hormone), egg-specific membrane receptors, and cyclic nucleotides (cAMP and cGMP).(Sun et al., 2017) Influx of Ca^{+2} through CatSper results in the activation of a variety of signaling cascades that affect sperm hypermotility, acrosome reaction, and egg penetration.(Sun et al., 2017) *Cryptosporidium* male-specific ion channels described in chapter. 4 can be investigated for their potential role in sensing female-derived pheromones. Male-specific ion channels can be expressed in a heterologous *Xenopus* oocyte model system and then be stimulated by 'secretome' from a sexual culture to test their activity to transport ions.(Papke and Smith-Maxwell, 2009)

Future Directions 6.4: Identifying the barrier(s) that prevent fertilization in culture

Identifying factors or their lack thereof that prevent fertilization in HCT-8 cells will be crucial in developing an 'easy' culturing platform for *Cryptosporidium*. Currently, only intestinal organoids-based culture systems can be used to maintain parasites continuously *in vitro*.(Heo et al., 2018; Wilke et al., 2019) However, organoids systems

require a lot of labor to isolate crypts from mice and establishing them in culture(Heo et al., 2018; Wilke et al., 2019), making it an inconvenient system for routine passaging of the parasite.

The lack of fertilization in culture can be attributed to *Cryptosporidium* sexual stage developmental competency or host-derived factors. For example, it is possible that sexual stages in HCT-8 cells develop abnormally, which makes them fertilization incompetent. Transcriptomes of sexual stages derived from HCT-8 and mice can be compared to pinpoint potential fertilization defects. In chapter. 4 we have compared the transcriptomes of female gametes derived from HCT-8 and mice. Most of the genes were commonly shared between *in vitro* and *in vivo* females. However. genes required for post-fertilization (like glideosome genes) development are upregulated in *in vivo* female datasets, which confounds identification of genes that might be involved in making females fertilization competent. We still must compare transcriptomes of males from culture and mice. We intend to infect mice with AP2-14 tdNeon male reporter strain and isolate males by flow cytometry for transcriptional analyses.

Other than comparing transcriptomes, gametes from mice can tested for their fertilization competency in HCT-8 cells. Females isolated from mice are challenging for this experiment because isolated female-infected enterocyte might not survive when transplanted in culture. Secondly, we do not have female reporter strains that distinguish between unfertilized and fertilized females. Accidental isolation of fertilized females will not help distinguishing whether fertilization happened in mice or culture. Male gametes from mice can be added in a 48-hour infected HCT-8 cells to test for their ability to fertilize females *in vitro*. AP2-14 tdNeon male reporter strain can be used to isolate male gametes from infected mice.

Host- or microbiome-derived metabolites, proteins, etc. in the intestinal environment might be required for fertilization. Parasite fertilization happens in mouse and human-derived intestinal organoids.(Heo et al., 2018; Wilke et al., 2019) This suggests that microbiome- and extra-intestinal tissue derived factors (like pancreatic proteases) have no role in parasite fertilization. 'Secretomes' of uninfected organoids can be tested for promoting fertilization in infected HCT-8 cells. Intestinal organoid 'secretomes' can be further fractionated to identify an active component that might promote fertilization in HCT-8 cultures. The reporter assay for sex described in chapter.3 can be used to screen potential factors that trigger fertilization in HCT-8 cells.

Besides secreted factors, enterocytes membrane receptors might be involved in mediating fusion between *Cryptosporidium* gametes. This potential receptor might be a primary recognition factor for males to fuse with females. Such a receptor must be lacking in HCT-8 cells. Ectopic expression of such a receptor in HCT-8 cells might promote *Cryptosporidium* sex in culture. McConnell et. al.(McConnell et al., 2011) have defined the proteome of murine enterocyte brush border. This work provides a repository of potential transmembrane protein that might aid *Cryptosporidium* sex. Candidates can be further shortlisted by applying following criteria: 1. Genes should be conserved between human and mice. 2. Gene must be only expressed in enterocytes but not in HCT-8 cells. 3. Gene should be a potential transmembrane protein. A genetic screen can be devised to identify gene candidates (from the filtered list) that permit *Cryptosporidium* fertilization in HCT-8 cells. Selected candidates can be overexpressed using an overexpression library in HCT-8 cells.(Parekh et al., 2018) The overexpression plasmid consists of a drug selection marker and a barcode that is unique to the gene being overexpressed.(Parekh et al., 2018) HCT-8 cells overexpressing this library can

be then coinfected with Cre and floxed tdNeon strains described in chapter. 3. An HCT-8 clone that is permissive for parasite sex will be infected with a tdNeon reporter-positive (fertilized) parasite. Such a permissive clone can be flow sorted and sequenced(Tandel et al., 2019) for its candidate barcode.(Parekh et al., 2018) Quantifying barcodes from the sorted population with unsorted population should identify gene candidates that are enriched in HCT-8 population that is permissive for sex. A multiplexed approach of screening candidates will be useful in identifying a membrane receptor that is required for *Cryptosporidium* fertilization.

Future Directions 6.5: Understanding the mechanisms of sexual commitment in *Cryptosporidium*

In recent years, tremendous progress has been made in deciphering mechanisms of stage differentiation in apicomplexans.(Kafsack et al., 2014; Sinha et al., 2014)·(Iwanaga et al., 2012)·(Radke et al., 2013) ApiAP2 DNA binding proteins have emerged as master regulators of stage differentiation(Kafsack et al., 2014; Sinha et al., 2014)·(Iwanaga et al., 2012)·(Radke et al., 2013), but additional factors include the Myb-1-like transcriptional regulator of bradyzoite development in *Toxoplasma*(Waldman et al., 2020).

AP2-G is a transcriptional activator required for gametocyte development in *Plasmodium*.(Kafsack et al., 2014; Sinha et al., 2014) *Plasmodium* blood-stage parasites replicate asexually as a default state with stochastic differentiation (1- 10% of the total population) into sexual stages. Gametocyte development is affected by a variety of hosts and parasite-specific factors including host anemia status(Price et al., 1999), parasite

density(Brancucci et al., 2017; Mantel et al., 2013; Regev-Rudzki et al., 2013), anti-malarial drugs(Buckling et al., 1999), ER stress(Chaubey et al., 2014), host cell age(Peatey et al., 2013), and extracellular vesicles[153,154]. The *Plasmodium* parasite has evolved a strategy to modulate gametocyte development frequency by gauging the external host environment. This has been corroborated by the identification of host-derived lysophosphatidylcholine (LysoPC) as a repressor of gametogenesis in *Plasmodium*.(Brancucci et al., 2017) Levels of LysoPC in the blood and tissue microenvironment act as a molecular indicator for immune status(Drobnik et al., 2003; Ollero et al., 2011) and parasitemia(Lakshmanan et al., 2012; Orikiiriza et al., 2017) for *Plasmodium* parasites, providing the environmental molecular cue for the parasite to escape the host. LysoPC was found to repress *Plasmodium* gametogenesis by reducing the expression of the AP2-G transcript.(Brancucci et al., 2017) However, the molecular pathway that links LysoPC induction with AP2-G transcript repression remains to be deciphered. It is thus assumed that the *Plasmodium* parasite is programmed to undergo sexual differentiation by sampling its external environment.

Unlike *Plasmodium*, *Cryptosporidium* parasites undergo robust sexual differentiation (~80%) after three rounds of merogony. Our unpublished data suggest that a single round of merogony requires ~12 hours. We observe sexual stages somewhere between 42- 48 hours post-infection. Hence it can be assumed that by 36 hours the parasite undergoes three rounds of merogony. The ability of *Cryptosporidium* to sexualize in response to external cues like stress, host immune status, etc. remain to be fully investigated. However, based on our observations, we hypothesize that *Cryptosporidium* is programmed by an internal ‘molecular clock’ that triggers sexual differentiation after sensing the completion of three rounds of merogony.(Smith et al., 2002) Such a

‘molecular clock’ mechanism could be achieved by the expression of a repressor of gametogenesis during the first merogony. Subsequent rounds of merogony will result in dilution of the repressor, and after the third round of merogony, the levels of repressor should fall to a level that will trigger gametogenesis. A similar ‘clock mechanism’ has been identified in budding yeast that gauges cell size as a proxy for time to initiate the G1 cell cycle.(Schmoller et al., 2015) Whi5, a transcriptional repressor of the G1 phase, is synthesized in the preceding G2/M phase and inhibits G1 activation.(Schmoller et al., 2015) Increase in the cell size results in the dilution of Whi5, thus relieving the cells from transcriptional repression to activate the G1 state.(Schmoller et al., 2015) Contrasting to the Whi5 model of dilution at the behest of an increase in cell size, the dilution of the *Cryptosporidium* gametogenesis repressor must happen by asexual replication.

A forward genetic approach can be implemented to identify the regulator(s) of sexual commitment in *Cryptosporidium*. Since the HCT-8 cells only support asexual replication but not mating of *Cryptosporidium*, it can be used to select for a ‘vegetative strain’ that has lost its ability to sexualize. Such a vegetative strain should continuously persist in an asexual state. Serial passaging(Sinha et al., 2014) of parasites in HCT-8 cells or chemical mutagenesis of parasites may help in selecting for such a mutant. Alternatively, a more biased approach investigating ApiAP2 proteins specifically expressed in asexual stages can be taken to identify a potential repressor of sexual development. Our work has demonstrated the role of AP2-F in oocyst development and its potential involvement(Oberstaller et al., 2014) in regulating genes required for post-fertilization (chapter. 5). We have already identified three ApiAP2 proteins (cgd5_4250, cgd8_3230, and cgd8_3130) that are exclusively expressed in asexual stages by transcriptomics studies (chapter.4) and by gene tagging approach (data not shown). A DiCre-based

conditional overexpression of such candidates should result in repression of sexual development in *Cryptosporidium*.

Future Directions 6.6: Understanding the mechanisms of sex determination in *Cryptosporidium*

The ability of *Cryptosporidium* to undergo sexual differentiation in HCT-8 cells makes it a convenient apicomplexan model system to study the mechanisms of sex determination. Sex determination occurs using a diverse set of mechanisms and varies among organisms, sex can be intrinsically determined by inheritance, inherited but changing over the lifetime, or driven by the environment. Sex in mammals and birds is determined by the composition of sex chromosomes. Sex-determining genes like SRY(Koopman et al., 1991) (mammals) and DMRT1(Ca and Ah, 2004) (birds) are located on sex chromosomes that activate a developmental program of a specific sex. For example, mammalian males and females have a XY or XX sex chromosome configuration, respectively. The SRY gene is present only on the Y chromosome and encodes a transcription factor that stimulates testosterone production, which in turn triggers the development of male sexual organs.(McLaren, 1991) In females, who lack SRY genes, the default ovary-forming pathway is activated.(McLaren, 1991)

Contrary to the mammalian sex-determination system that is genetically hardwired, the sex determination mechanism in reptiles is dependent on environmental cues like temperature. In the case of the European pond turtle, *Trachemys scripta*, egg incubation temperature below 25°C produces only males, while a temperature above 30°C produces only females.(Crews et al., 1995) This environmental mechanism of sex

determination has been deciphered in the European turtles, *Emys orbicularis*.(Pieau et al., 1994) An aromatase enzyme in this organism catalyzes the conversion of testosterone into estrogens.(Pieau et al., 1994) The aromatase activity is enhanced at higher temperature thus producing more estrogen, resulting in higher frequency of female offspring.(Pieau et al., 1994)

Since both male and female gametes of apicomplexan parasites are derived from the same clone, the presence or absence of sex chromosomes cannot explain sex determination in apicomplexans. Both male and female gametes of apicomplexan parasites carry genetic cargo for sex determination of both sexes but they these are differentially activated in respective gametes. I therefore propose that the mechanisms of sex determination in apicomplexan parasites should have the two following features: 1. Activation of a genetic program to commit an asexual clone to one of the two sexes. 2. Concomitant repression of the molecular program required for the differentiation into the opposite sex. Many molecular mechanisms of concomitant activation and repression of two different genetic programs to adopt two different cell fates exist in viruses(Griffiths et al., 2000), bacteria(Griffiths et al., 1999), and in higher eukaryotes(Zaret, 2008). For example, lambda bacteriophage after infecting its bacterial host can replicate to produce and release more infective phages (lytic cycle) or can integrate itself in the host genome to persist in a dormant state (lysogeny phase).(Griffiths et al., 2000) Lytic cycle is maintained by the phage Cro repressor protein that represses the expression of cI repressor required for the onset of the lysogeny phase.(Griffiths et al., 2000) Similarly, the cI repressor downregulates the expression of Cro during the lysogeny phase.(Griffiths et al., 2000)

Beyond a mechanism of concomitant activation and repression of genetic programs, an additional mechanism may be required to communicate fates between cells differentiating into the opposite sex. In certain species of fungi and green algae, vegetative cells differentiate into one of the sexes followed by the release of a diffusible 'developmental pheromone' that triggers the differentiation of asexual cells in the vicinity into the opposite sex.(Kochert, 1978) Other than the diffusion of signaling molecules, cell fates can also be communicated by cell-cell contact. For example, the Notch signaling pathway in mammals is used to convey fates between two different cells that are in physical contact with each other through transmembrane Notch receptors and ligands.(Andersson et al., 2011) Notch signaling e.g. ensures that two brain stem cells in contact with each other adopt neuronal and glial fates, respectively.(Andersson et al., 2011) A similar mechanism can be envisioned in the case of terminally differentiated merozoites in a meront that communicate their sexual fates with each other through physical contact.

Identifying sex determination factors in *Cryptosporidium* will require molecular analysis at the level of an individual cell. Single-cell RNA sequencing has been successfully implemented to identify molecular profiles of individual cells in a heterogeneous population.(Buenrostro et al., 2018) A similar technique can be used to sort individual meronts or merozoites to identify the molecular determinants of sex in *Cryptosporidium* at a single cell level.

6.7 Concluding Remarks

The work described in this dissertation lays the foundation for understanding the relationship between the programming of the lifecycle of *Cryptosporidium* pathogen and infection in a host. The insights we gained into stage specific gene expression combined with the new genetic tools we have developed for *Cryptosporidium* can now be used to unravel the biology of sex in *Cryptosporidium*.

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