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Impact of external stimuli on life cycle progression in the intestinal parasites *Eimeria falciformis* and *Giardia duodenalis*

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Zusammenfassung

Der Lebenszyklus einer Vielzahl von eukaryotischen Parasiten beinhaltet zwei oder mehrere morphologisch verschiedene Stadien in unterschiedlichen ökologischen Nischen. Dabei ist es für den Parasiten in gewissen Fällen von Vorteil, den Übergang zwischen diesen Stadien zeitlich zu koordinieren. Bei vielen Parasiten ist unbekannt, welche Faktoren die Progression des Lebenszyklus beeinflussen, bzw. welche Gene daran teilhaben. Darüber hinaus kann der zeitliche Ablauf der Entwicklung entweder genetisch prädeterminiert (kanalisiert) sein oder von äußeren Einflüssen abhängen; die Entwicklung des Parasiten kann demnach als Fall phänotypischer Plastizität betrachtet werden.

In dieser Dissertation wurde die Progression der Lebenszyklen zweier einzelliger Darmparasiten in Abhängigkeit von wechselnden Umwelteinflüssen untersucht. Mit Mäusen als Modellorganismen wurden die Replikation der Parasiten *in vivo*, sowie die Progression des Lebenszyklus der Parasiten studiert. Bei den intrazellulären *Eimeria falciformis* wurden Parasiten im Oozysten-Stadium in den Fäzes als Maß für die Reproduktion quantifiziert und die Transkriptome von Parasit und Wirt wurden bei Infektion von Wirten unterschiedlicher Immunkompetenz, insbesondere auch bei Mäusen mit geschwächtem adaptivem Immunsystem (Rag1-Mutanten die keine reifen T- und B-Zellen besitzen), analysiert. Man geht im Allgemeinen davon aus, dass der hier betrachtete externe Stimulus, die Immunantwort des Wirtes, für das Pathogen eine Stresssituation darstellt. Die andere hier untersuchte Spezies ist *Giardia duodenalis*, ein extrazellulärer Darmparasit. Es wurde untersucht, inwiefern das Wachstum von *G. duodenalis in vivo* von der Verfügbarkeit der Aminosäure Arginin abhängt. Arginin fördert zwar die Replikation des Trophozoitenstadiums *in vitro*, die Relevanz *in vivo* ist jedoch nicht geklärt. Auch für die Infektion mit *G. duodenalis* wurden Wirtsorganismen verwendet, die sich in ihrer Reaktion auf den Stimulus – in diesem Fall die Verfügbarkeit von Arginin – unterscheiden. Mäuse mit einer Defizienz im mTOR-Signalweg (mechanistic Target Of Rapamycin) wurden infiziert. Dieser Signalweg ist u.a. essenziell für die Wahrnehmung der Aminosäureverfügbarkeit und damit die zelluläre Wachstumsregulation. Bei *G. duodenalis* wurden das Wachstum innerhalb des Wirtes (Replikation der Trophozoiten) sowie die Ausscheidung von Zysten (Reproduktion) in Wirten mit argininhaltiger bzw. argininfreier Ernährung untersucht.

Bei *E. falciformis* hatten Unterschiede in der Immunkompetenz des Wirts weder Auswirkungen auf den Zeitpunkt der Oozystenausscheidung noch auf das Transkriptomprofil des Parasiten. Entgegen der gängigen Erwartung konnte *E. falciformis* keinen Nutzen aus der Immunschwäche seines Wirtes ziehen, d.h.in den Fäzes geschwächter Wirtsorganismen fand sich keine erhöhte Anzahl an Oozysten. Im Fall von *G. duodenalis* war, anders als frühere *in vitro* Studien hätten vermuten lassen, war die Replikation des Trophozoitenstadiums innerhalb des Wirts nicht auf Arginin aus der Nahrung angewiesen. Es wurden jedoch bei argininfrei ernährten Mäusen weniger, der für die Übertragung – und somit die Vollendung des Lebenszyklus - wichtigen, infektiösen Zysten in den Fäzes gefunden als bei Mäusen mit

argininhaltiger Kost. Im Gegensatz zu *E. falciformis* konnte *G. duodenalis* auch von der Infektion eines „geschwächten“ Wirts profitieren.

Aufgrund dieser Daten handelt es sich bei *E. falciformis* um einen, hinsichtlich des Verhältnisses von Immunantwort des Wirts und der Progression seines Lebenszyklus, genetisch kanalisierten Parasiten. Seine mangelnde Empfänglichkeit für diesen Stimulus könnte in der relativen Vorhersehbarkeit der Immunantwort von Säugern begründet liegen. Da es aufwendiger ist Plastizität zu erhalten, als ein kanalisiertes Programm durchzuführen, das bei vorhersehbaren Stimuli eine Reaktion überflüssig macht, sollte die genetische Kanalisierung im Laufe der Evolution begünstigt werden. *G. duodenalis* hingegen wies, messbar durch die geringere Produktion von Zysten unter argininarmen Bedingungen, phänotypische Plastizität gegenüber dem untersuchten Reiz, Arginin, auf. Die Ausscheidung von Zysten war bei mTOR-defizienten Wirten gegenüber dem Wildtyp erhöht, während die Replikation der Trophozoiten nicht beeinflusst wurde. In infizierten mTOR-defizienten Epithelien ist das zelluläre Wachstum (beispielsweise bei der Wundheilung) beeinträchtigt. Durch Probleme bei der regelmäßigen Erneuerung der Epithelien oder Schädigung im Zuge einer Infektion kann Arginin vom Wirt ins Darmlumen austreten und somit für den Parasiten verfügbar werden. Die erhöhte Reproduktion in geschwächten Wirten sowie die verringerte Replikation unter Arginin-armen Bedingungen lässt sich unter Beachtung der Stoffwechselwege von *G. duodenalis* mechanistisch erklären. In *G. duodenalis* kann durch den Arginindihydrolase (ADH) Stoffwechselweg unter Verwendung von Arginin ATP – ein zellulärer Energieträger – erzeugt werden. ADH stellt somit eine Alternative zur Gewinnung von ATP aus der Verstoffwechselung von Glukose, der Glykolyse, dar. Schon früher wurde gezeigt, dass ein Zwischenprodukt der Glykolyse, D-Fruktose-6-Phosphat, für die Bildung von N-Acetylgalactosamin, einem Baustein für die Wände der Zysten von *G. duodenalis*, genutzt wird. Die Vernetzung von Glykolyse, Argininstoffwechsel und Zystenwand-Biosynthese könnte somit Arginin-abhängig die synchrone Progression im Lebenszyklus vom Trophozoiten- zum Zystenstadium in vielen *G. duodenalis* Zellen herbeiführen. Eine derartige Koordination ist von adaptivem Wert, z.B. wenn dadurch erreicht wird, dass eine gewisse infektiöse Dosis (Zystenzahl) den nächsten Wirt erreicht. Der Arginingehalt in der Nahrung variiert zwischen jeder Mahlzeit für ein Individuum und zwischen Individuen. Mit phänotypischer Plastizität auf den unvorhersehbaren und nicht konstanten Zufluss von Arginin zu reagieren, ist eine mögliche Strategie um die Progression des Lebenszyklus zu synchronisieren.

Die Ergebnisse dieser Dissertation zeigen, dass Arginin ein relevanter Stimulus für die Progression des Lebenszyklus von *G. duodenalis* ist. Es wird angeregt, dass aus der unvorhersehbaren Argininaufnahme durch den Wirt ein Selektionsdruck für phänotypische Plastizität (Reaktionsfähigkeit auf Arginin) folgt. Des Weiteren machen unterschiedliche Ernährungsweisen jeden individuellen Wirt zu einer einzigartigen ökologischen Nische. Dadurch kann in jedem individuellen Wirt ein anderer Argininverwertungs-Genotyp des Parasiten bevorzugt werden. Durch dieses Phänomen entstünde genetische Variation zwischen den Zysten die von verschiedenen Wirten ausgeschieden werden. Somit würde nicht nur phänotypische Plastizität begünstigt und selektiert; es ist auf Populationsebene auch genetische Variation (Polymorphismus) in Genen mit Bezug zum Argininstoffwechsel zu erwarten.

Insbesondere wird erwartet, dass natürliche Auslese auf die Gene des ADH Stoffwechselwegs, z.B. auf dessen kritisches erstes Enzym, die Arginindeiminase (ADI), wirkt und Polymorphismus in diesen Genen befördert.

Abstract

Many eukaryotic parasites have life cycles with two or more morphologically distinct stages in different ecological niches. Timing the conversion from one stage into another is in some cases beneficial for successful transmission into a new host. For many parasite species, little is known about determinants for such life cycle progression or the identities of the genes involved. In addition, the developmental timing can either be genetically pre-determined (canalized) or can depend on the exposure to an external stimulus, i.e., parasite development can be seen as a case of phenotypic plasticity.

In this thesis, life cycle progression of two unicellular intestinal parasites was investigated in response to external stimuli. Using mice as infection models, in vivo parasite replication and life cycle progression was studied. For intracellular *Eimeria falciformis*, oocyst stage parasites in feces (reproduction) was quantified, and parasite and host transcriptomes were analyzed in differently immune competent hosts, including hosts with a poor adaptive immune response (Rag1-mutants lacking mature T- and B-cells). The external stimulus – a host immune response – is generally expected to induce stress to the pathogen. The other parasite species investigated here is *Giardia duodenalis*, an extracellular parasite of the intestine. *G. duodenalis*' dependence on availability of the amino acid arginine for in vivo growth was investigated. Arginine supports parasite replication in its trophozoite life stage in vitro, although the in vivo relevance is unknown. A weakened host model was also used for the *G. duodenalis* infection. Mice deficient in a signaling network known as the mTOR-pathway (mechanistic Target of Rapamycin) were infected. The pathway is, among other things, central for sensing amino acid availability and thereby the regulation of cell growth, e.g. in intestinal epithelial cells. For *G. duodenalis*, within-host growth (trophozoite replication) and cyst shedding (reproduction) were assessed in hosts fed arginine-sufficient and arginine-free diets.

In *E. falciformis*, different host immune competence did not change the timing of oocyst shedding or influence parasite transcriptome profiles. Counterintuitively, *E. falciformis* was unable to benefit from hosts with weakened immune responses, i.e. there were not more oocysts in feces of weakened hosts. In contrast to previous in vitro studies, *G. duodenalis* did not depend on dietary arginine for replication of the trophozoite life stage within its host. However, infective cysts, which are important for transmission and thus the completion of the life cycle, were less abundant in feces in arginine-poor conditions, compared to arginine-sufficient ones. Contrary to *E. falciformis*, *G. duodenalis* was also able to benefit from infecting a weaker host and generate more infective cysts there than in infections in wild type hosts.

Based on these data, *E. falciformis* is an example of a genetically canalized parasite with regards to host immune stimulus and life cycle progression. Its unresponsiveness to the stimulus may be explained by the relative predictability of mammalian immune responses. Maintaining plastic responses is more costly than a canalized program, and predictable stimuli would obviate the need to react. Selection would thus favor the "cheaper" genetic canalization. In *G. duodenalis*, shedding of fewer cysts under arginine-poor conditions instead revealed phenotypic plasticity in response to the investigated stimulus, arginine. In addition, cyst shedding in mTOR-deficient hosts was higher than in WT. Trophozoite replication was unaffected. In

infected mTOR-deficient epithelia, cellular growth (e.g. wound healing) is hampered. If epithelia cannot renew normally or are harmed by the infection, arginine may leak from the host into the lumen and become available to the parasite. The observed higher reproduction in weakened hosts and *G. duodenalis*' reduced replication in arginine-poor conditions can be explained mechanistically by considering the parasite's metabolic pathways. In *G. duodenalis*, an arginine dihydrolase, ADH, pathway utilizes arginine to generate ATP –the cellular energy-carrier. ADH is therefore an alternative to glucose-dependent glycolysis to generate ATP. Glycolysis was previously shown to shunt an intermediate, D-fructose-6P, into the N-acetyl-galactosamine synthesis pathway. N-acetyl galactosamine is an important component for the *G. duodenalis* cyst wall. Thus, glycolysis, arginine metabolism, and cyst wall biosynthesis could, synchronize life cycle progression from trophozoite to cyst stages among many *G. duodenalis* cells simultaneously in an arginine-dependent manner. Such coordination can have an adaptive value, e.g. by ensuring that a certain infective dose (number of cysts) reaches the next host. Arginine content varies between food sources and arginine concentrations in meals vary both for one individual and between individuals. Sensing the unpredictable and non-constant arginine-influx by phenotypic plasticity could be a strategy to synchronize life cycle progression.

Based on the results of this thesis, arginine is a relevant stimulus for life cycle progression in *G. duodenalis*. Unpredictable host arginine intake is suggested to result in selection for phenotypic plasticity (responsiveness to arginine stimulation). In addition, differences in diets *between* hosts make each host individual a unique ecological niche. Therefore, different parasite arginine-utilizing genotypes may be favored in different host individuals. Such a phenomenon would generate genetic variation between cysts shed from different hosts. Therefore, not only would plastic capacity as observed here be beneficial and selected for, but genetic variation (polymorphism) in arginine-utilizing genes is expected at the population level. Specifically, natural selection is expected to act on genes of the ADH pathway, for instance its important first enzyme arginine deiminase (ADI), and to promote polymorphism in this gene set.

Schlagwörter:
Eimeria
Giardia
Protozoa
Lebenszyklus
Zyste
Oozyste
Darm
Infektion
Wachstum
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Keywords:
Eimeria
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mouse model
in vivo

Table of Content

List of abbreviations

• Antimicrobial peptide	AMP
• Arginine dihydrolase	ADH
• Arginine deiminase	ADI
• Cre-recombinase	Cre
• Cycle threshold	ct
• Day(s) post infection	dpi
• Dendritic cells	DCs
• *Eimeria falciformis*	Ef
• Epidermal growth factor	EGF
• False discovery rate	FDR
• Gene ontology	GO
• Generalized linear model	GLM
• Immunoglobulin	Ig
• interferon gamma	IFN
• Interleukin	IL
• Major histocompatibility complex	MHC
• Mechanistic/mammalian Target of Rapamycin	mTOR
• Multidimensional scaling	MDS
• *Mus musculus*	Mm
• Nitric oxide	NO
• Ornithine carbamoyltransferase	OCT
• Phosphate buffer saline	PBS
• Recombination activating gene	Rag
• Rhoptry kinase	RopK
• Small subunit	ssu
• specific pathogen-free	SPF
• Starting quantity	SQ
• Surface antigen	SAG
• T helper	Th
• Tumor necrosis factor	TNF
• Wild-type	WT
• (Quantitative) polymerase chain reaction	qPCR/PCR

Definitions

Phenotypic plasticity refers to phenotypic differences which are a result only of responses to external stimuli, i.e. without contribution of genetic variation. By this definition the contribution of phenotypic plasticity to a given phenotype can only be observed and measured in clonal organisms. In any polymorphic (see polymorphism) population with variations in phenotypes, both genetic diversity and phenotypic plasticity may contribute to the phenotype.

Polymorphism is the presence of different alleles in a population, i.e. genetic variation. In some definitions, polymorphism is used to describe the total expression of a phenotype, however, since this also includes contributions from phenotypic plasticity this definition is *not* applied here and instead *strictly genetic variation* is intended.

Replication (of *Giardia* spp.) is here defined exclusively as trophozoite division

Reproduction (of *Giardia* spp.) is here defined as trophozoite encystation and successful transport of the cyst into shed feces. Without reproduction no transmission will take place, whereas G. duodenalis with these definitions can *replicate* without achieving transmission.

Preface

This thesis is based on work carried out at Humboldt-Universität zu Berlin, Germany, and Robert Koch-Institute, Berlin, Germany.

All data on the organism *Eimeria falciformis* reported here has been previously published (Ehret, Spork, et al. 2017). This data is presented with Results&Discussion in the same section, since this best fits the nature of that project. For all data produced in the *Giardia duodenalis*-project: data is original, and was produced and analyzed by the author of this thesis, Totta Ehret Kasemo, unless otherwise specified (see specifically for histology data in Figure 9). *G. duodenalis*-project data was generated under the direct supervision of Dr. Anton Aebischer and with extensive support from Dr. Christian Klotz, Robert Koch-Institute, Berlin, Germany.

Citations from own published work are indicated with an indentation in the text and are initiated with the following sentence:

The following section is a quote from my published work (Ehret, Spork, et al. 2017).

Additions or changes to the published work are indicated by underlining the text. References were formatted to this thesis. Edits of typing or spelling-errors are not indicated.

1 Introduction

Many eukaryotic parasites have life cycles with two or more morphologically distinct stages in different ecological niches. Timing the conversion from one stage into another may in some cases be beneficial for successful transmission into a new host (Reece, Ramiro, and Nussey 2009), as shown e.g. for parasitic trypanosomes (Reuner et al. 1997; Dean et al. 2009; Rojas et al. 2019) and the malaria parasite (Paul and Brey 2003). However, for most parasite species, little is known about determinants for such life cycle progression and the genes involved in regulating it. The two projects presented in this thesis investigate protozoan parasite life cycle progression and if or how differentiation from one life cycle stage into another may be influenced by external stimuli. I study two unicellular parasites: *Giardia duodenalis* and *Eimeria falciformis*, of the gastrointestinal tract of mammals.

1.1 Phenotypic plasticity

Evolutionary biology asks fundamental questions about living organisms. It may address why a pathogen sometimes causes severe disease, resist treatment, or form dormant stages, whereas they in other cases behave differently. Variation may be explained by either parasite traits, or hosts being different, or by interactions between parasite and host. As drug resistance is an increasing problem, understanding the evolution of pathogens will help predict effects of treatments on the pathogen, such as likelihood of development of drug resistance. (Mideo and Reece 2012) I therefore find it both interesting and meaningful to place my parasitology data and findings on life cycle progression in the context of evolutionary concepts such as **phenotypic plasticity.** It is the phenomenon of genetically identical individuals displaying different phenotypes as a result of variations in their environment (Stearns 1989). Such differences can be achieved by, e.g., variation in gene expression or by developmental programs becoming for instance differently timed in response to stimuli, and/or can be constrained by the chemical and physical environment (temperature, pH, etc.). (Stearns 1989). Non-constitutive immune functions, i.e. responses to an assault such as a pathogen, are an example of phenotypic plasticity in e.g. humans (Pancer and Cooper 2006).

1.1.1 Phenotypic plasticity in parasites

Eukaryotic pathogens, parasites, are ubiquitous. Both multicellular worms and unicellular protozoan parasites cause human and animal disease, leading to loss of working capacity (~disability-adjusted life years, DALYs), deaths, and economic loss due to animal fitness losses. Examples of relatively well-known human unicellular parasites, which are the focus of this thesis, are the malaria parasite *Plasmodium falciparum;* the causative agents of toxoplasmosis (*Toxoplasma gondii*); cryptosporidiosis (*Cryptosporidium* spp.); various forms of leishmaniasis (*Leishmania* spp.); and African sleeping sickness (*Trypanosoma* spp.). *Eimeria* is a large genus (see details below) of protozoa which receive attention for causing diarrheal disease and economic loss in livestock (Clark, Tomley, and Blake 2017). *G. duodenalis* (syn.

Giardia lamblia or *Giardia intestinalis*) is an intestinal parasite which is one of the major infectious agents causing diarrhea in humans worldwide. *G. duodenalis* coinfections occur with protozoa such as *Cryptosporidium* spp. and *Entamoeba histolytica* (reviewed in Baldursson and Karanis 2011). Despite *G. duodenalis*' distant relationship to *Cryptosporidium* spp. the two are often associated since both are spread via contaminated water. This illustrates both that distantly related protozoan species may have a similar niche and infection route, and that they experience partly similar challenges and external stimuli despite their differences regarding inter-host lifestyle (e.g. extra- and intracellular). In contrast, their niche specificity (e.g. within or outside a host cell) also expose them to highly specific stressors, but they may nevertheless possess similar strategies to cope with those different challenges (Vonlaufen et al. 2008). In our attempts to understand infections and develop interventions it may be useful to keep a broad perspective and learn from insights in distantly related species. *E. falciformis* and *G. duodenalis* are the parasites of study in this thesis. The former is an intracellular apicomplexan parasite (such as *Plasmodium* spp. and *T. gondii*) of mice (*Mus musculus*); the latter is an extracellular metamonad of humans and other mammals, more closely related to e.g. *Trypanosoma* spp..

In parasites, life cycles can but do not have to be examples of phenotypic plasticity. Life cycles with morphologically and ecologically different stages are exhibitions of the competence of a genome to produce different phenotypes. However, these can only be defined to exhibit phenotypic *plasticity* if the differentiation from one stage into another occurs in response to *external* stimuli (i.e. is not genetically programmed or canalized) (general definitions of phenotypic plasticity can be found e.g. in Stearns 1989; DeWitt and Scheiner 2004; Fusco and Minelli 2010). Any parasite can certainly be exposed to extreme conditions (external stimuli) which will alter the life cycle progression (e.g. halt it completely upon exposure to toxins). However, I consider biologically relevant stimuli most useful to distinguish genetically canalized progression from that externally induced, i.e. the phenotypic plasticity cases. Both *Eimeria* spp. and *Giardia* spp. have asexual replicative, motile stages in their hosts and an environmentally, non-motile stable stage (oocyst/cyst; see life cycles below for each species). For both species it is poorly understood how life cycle progression and differentiation into another stage are induced.

The following section is a quote from my published work (Ehret, Spork, et al. 2017).

> "For many parasite species it <u>also</u> remains unclear whether differences in pathology are due to parasites' genotypic or phenotypic (plastic) differences, the latter <u>often</u> resulting from host-parasite interactions, e.g., host immune responses. An exception are Nematode infections (reviewed by (Viney and Diaz 2012)), in which for example worm length and other aspects of morphology (Weclawski et al. 2014), or developmental timing (Weclawski et al. 2013) has been shown to vary with host genotype. However, it is unclear to which extent such differences a) are passively imposed on the parasite, or b) an adaptive response of the parasite. Such adaptive plasticity might be a determinant of the extent of host specialization, the likelihood of host-switches and ultimately the degree to

which co-speciation and co-adaptation (together defining co-evolution) are observed."

Even a passively imposed effect of certain stimuli could be adaptive, in that the parasite has "declined" to counteract the effect of the external stimuli, if counteracting it was less beneficial than accepting or otherwise adapting to the effect. A possible example would be a certain stressor inducing encystation. Instead of investing in neutralizing the stress, the adaptation could be to optimize the phenotypic change (in this example encystation) to e.g. increase speed or synchronize encystation in the infecting colony. Synchronization for a life stage switch has been demonstrated in African *Trypanosoma* spp.. They are extracellular, motile protozoa of blood and cause African sleeping sickness in humans or nagana in animals (Matthews, McCulloch, and Morrison 2015). *Trypanosoma brucei* differentiates from the so-called *slender* form into its transmissive *stumpy* stage (which is taken up by the tsetse vector) in a density-dependent manner (Reuner et al. 1997). A detailed mechanism was only recently described and depends on a G-protein coupled receptor in slender parasites and release of oligopeptides, which induce stage-switching in the other infecting cells (Rojas et al. 2019). This is an example of external stimuli inducing life cycle progression and the mechanistic understanding is suggested by the authors to inspire development of new drugs.

For the parasites under study here, much less is known about mechanisms governing life stage switching. For *E. falciformis*, the complete life cycle cannot be reproduced in vitro and no protocol exists (to my knowledge) for in vitro oocyst generation. However, in vivo *E. falciformis* life cycle progression is predictable in laboratory models with highly reproducible timing of oocyst shedding within the same mouse strain (Schmid et al. 2013; Ehret, Spork, et al. 2017). Whether parasite-generated signals for synchronization exist is not known and whether there is a molecular trigger for encystation in *G. duodenalis* is not known, although lipid starvation and an increase in pH are thought to contribute (Einarsson and Svärd 2015). Whether *G. duodenalis* coordinates encystation is also an open question. In vitro encystation is not complete, meaning that varying degrees but never 100% of a culture encysts and spontaneous encystation in normal growth medium is also reported (Einarsson and Svärd 2015). Pham et al. observed that an encystation marker (a cyst wall protein) was induced in densely colonized foci in a mouse infection model (Pham et al. 2017). The incomplete encystation in vitro could suggest a lack of synchronizing factors, whereas the expression of cyst wall components specifically detected in densely colonized areas could suggest the opposite (although the sensitivity would be intrinsically lower where less parasites reside, and non-dense areas were not analyzed separately in that study). For my discussion, I will use "plastic capacity", "plasticity", and "plastic response" to refer to phenotypic plasticity or phenotypic plastic capacity, or specify if anything else is intended.

In order to test plastic capacity, one approach is to expose the organisms to extreme stress using e.g. high doses of a known drug against the parasite. Whereas such an approach is likely to induce a strong response and ensure an experimental readout, there is a risk that the outcome is artificial and does not reflect true parasite responses during infection. Such results could generate misleading interpretations. In this thesis I focus on biologically relevant stimuli for

each parasite, i.e., a potential stressor which is likely to occur during untreated infection (host immune responses) and nutritional stress (host dietary restriction). These stimuli are described and motivated separately for each parasite below.

1.2 Two model protozoa for plasticity studies

1.2.1 *Eimeria falciformis* – a natural parasite of mice

The following introductory section is a quote from my published work (Ehret, Spork, et al. 2017).

> "*E. falciformis* is an intracellular parasite in the phylum Apicomplexa, which comprises more than 4000 described species (Duszynski 2011). Prominent pathogens of humans are found in this phylum, such as *Toxoplasma gondii*, the causative agent of toxoplasmosis, *Plasmodium* spp., causing malaria, and *Cryptosporidium* spp., which cause cryptosporidiosis. Coccidiosis is a disease of livestock and wildlife caused by coccidian parasites which are dominated by >1800 species of *Eimeria* (Duszynski 2011). The genus is best known for several species which are problematic for the poultry industry (Chapman et al. 2013). *E. falciformis* naturally infects wild and laboratory *Mus musculus*, and its genome is sequenced and annotated making it a useful model for studying *Eimeria* spp. (Heitlinger et al. 2014). The parasite has its niche in the cecum and upper part of colon, mainly in the cells of the crypts (Haberkorn 1970; Schmid et al. 2013)
>
> ### *E. falciformis* life cycle
>
> This monoxenous parasite goes through asexual (schizogony) and sexual reproduction, which results in the host releasing high numbers of oocysts approximately between day six and 14 after infection. When a mouse ingests *E. falciformis* oocysts, one sporulated oocyst releases eight infective sporozoites inside the host, which infect epithelial crypt cells. Within the epithelium, merozoite stages form in several rounds of asexual reproduction, followed by gamete formation and sexual reproduction, within the same host. Schizogony takes place approximately until day six and then gametes form and sexual reproduction takes place, resulting in unsporulated oocyst shedding. Schizogony is not completely synchronous; the exact number of schizogony cycles is unclear and could vary naturally (Haberkorn 1970; Mesfin and Bellamy 1979). There is evidence for a genetic predisposition of *Eimeria* spp. to perform different numbers of schizogony cycles, as parasites can be selected to become

"precocious", completing the life cycle faster with a reduced number of schizogony cycles (Montes et al. 1998; Pakandl 2005). Additionally, it has been shown that *Eimeria vermiformis*, also a parasite of *M. musculus* intestines, displays prolonged patency (period of oocyst shedding) but an unaltered length of prepatency periods in mice of different immune status (Rose, Owen, and Hesketh 1984; Rose and Hesketh 1986; Rose, Wakelin, and Hesketh 1985; Rose 1974). Whether this developmental plasticity in *E. vermiformis* is reflected on the transcriptional level of that parasite has not been investigated. Timing of both patency and prepatency was shown to be non-plastic in *E. falciformis* var. pragensis (Rose and Hesketh 1986). Beyond developmental timing it is not known whether parasite strategies – i.e. processes optimizing host exploitation – are plastic and can be triggered by exogenous stimuli, such as host immune responses.

Eimeria spp. induce strong immune responses

Eimeria spp. generally induce host protection against re-infection (Rose 1974; Blagburn and Todd 1984; Rose, Hesketh, and Wakelin 1992; Gadde et al. 2009; Sühwold et al. 2010; A. L. Smith and Hayday 1998; L. Smith and Hayday 2000) and T-cells play a major role (Rose, Hesketh, and Wakelin 1992; Sühwold et al. 2010; A. L. Smith and Hayday 1998b). In response to *E. falciformis* infection of laboratory mice, interferon gamma (IFNγ) is upregulated (Stange et al. 2012; Schmid et al. 2013). In an IFNγ-deficient mouse host model which displays larger weight losses and intestinal pathology but also lower oocyst output for *E. falciformis*, the wild-type (WT) phenotype was recovered by blocking IL-17A and IL-22 signaling (Stange et al. 2012). Also in *E. vermiformis,* IFNγ, interleukin-6 (IL-6), and major histocompatibility complex (MHC) class I and II have been shown to be required for protective immune reactions in mice (A. L. Smith and Hayday 1998a). These studies demonstrate that adaptive immunity clearly plays a role in limiting the reproductive success of *Eimeria* spp. infection, but effects on the parasite, apart from reproductive output, remain poorly understood. It is an open question whether the parasite is passively impacted or responds, e.g., via changes in its transcriptome, to changes in the host immune response."

1.2.2 Goals of *E. falciformis* research project

Eimeria spp. are described as extremely niche specific. Intuitively, this suggests a rather limited requirement for phenotypic plasticity and capacity to survive variations in its ecological niche, simply since the niche can be considered relatively predictable. However, a recent study in wild

mice calls for a modification of the high niche-specificity (unpublished data, Victor H. Jarquin and Emanuel Heitlinger, Humboldt-Universität zu Berlin). This instead implies higher plastic capacity in *E. falciformis*, since even within laboratory mouse models e.g. bacterial composition varies (reviewed in T. Ehret et al. 2017), and between wild mouse species it will even more. Other determinants of the parasite niche such as food intake and other pathogens (coinfections) in the gastrointestinal tract are likely to vary more too, suggesting that plastic capacity would be beneficial. The strong immune responses induced by *E. falciformis* can be assumed to shape the microenvironment during infections and are therefore likely to have contributed to adaptations for survival, e.g. phenotypic plasticity. If the parasite possesses plastic capacity in response to immune defense stimuli, the prediction is that variations in host immune defenses would cause different parasite phenotypes. In a genetically canalized parasite, the phenotype under study would remain the same even in hosts with drastic differences in their immune response to the parasite. Plasticity would allow opportunism in weak hosts, seen e.g. by increased oocyst shedding, whereas genetically canalized parasites would not be able to profit from a weaker host. As described above, other *Eimeria* spp. display a capacity to become precocious which suggests plasticity in those species. In this thesis, I have investigated *E. falciformis* transcriptional responses to varying host immune conditions. A strong immune response is part of the ecological niche of *E. falciformis* and was therefore selected as the biologically relevant stimulus to evaluate. Using naïve as well as challenge infected hosts of wild type (WT), and of T- and B- cell impaired mice provided an experimental setup with variations in the intensity of host immune stress elicited on the parasite. I have studied the course of infection under which parasites enter epithelial cells, undergo schizogony, and reproduce sexually to generate oocysts in seven days. Parasite transcriptional responses to the stimulus were contrasted with reproductive success (oocyst shedding) as well as evaluated in parallel with the host transcriptomes in a dual RNA-seq analysis. These data provide a first description of *E. falciformis* transcriptional plasticity in response to a biologically relevant stimulus.

1.2.3 *Giardia duodenalis* – an intestinal pathogen of mammals

G. duodenalis is a unicellular eukaryotic pathogen. It is unusual in its (trophozoite stage) morphology and some cellular functions, e.g. the lack of mitochondria (a feature shared with other metamonads), the presence of two symmetrical nuclei, four flagellar pairs, and a ventral disc of tubulin which is used for attachment. The extracellular parasite colonizes the intestine of mammals and causes diarrheal disease. For survival outside a host it forms infective cysts which survive but do not replicate. Below I present selected aspects of *G. duodenalis* biology and epidemiology which I consider relevant for this thesis. For additional background I refer to a number of reviews on *Giardia* spp. immunology (Eckmann 2003; Klotz and Aebischer 2015; Singer 2016; Allain et al. 2017), genomics (R.C. Andrew Thompson and Monis 2012), taxonomy (R. C.A. Thompson and Ash 2019), and basic cell biology (Adam 2001).

G. duodenalis *life cycle*

Giardia spp. have two major life cycle stages: the trophozoite stage and the cyst stage (Kulda and Nohynkova 1995). Trophozoites have two nuclei, each with a diploid genome. Trophozoites colonize the intestinal epithelium of a host and may cause disease. To replicate, a trophozoite duplicates its genome and undergoes cytokinesis to generate two trophozoites. As

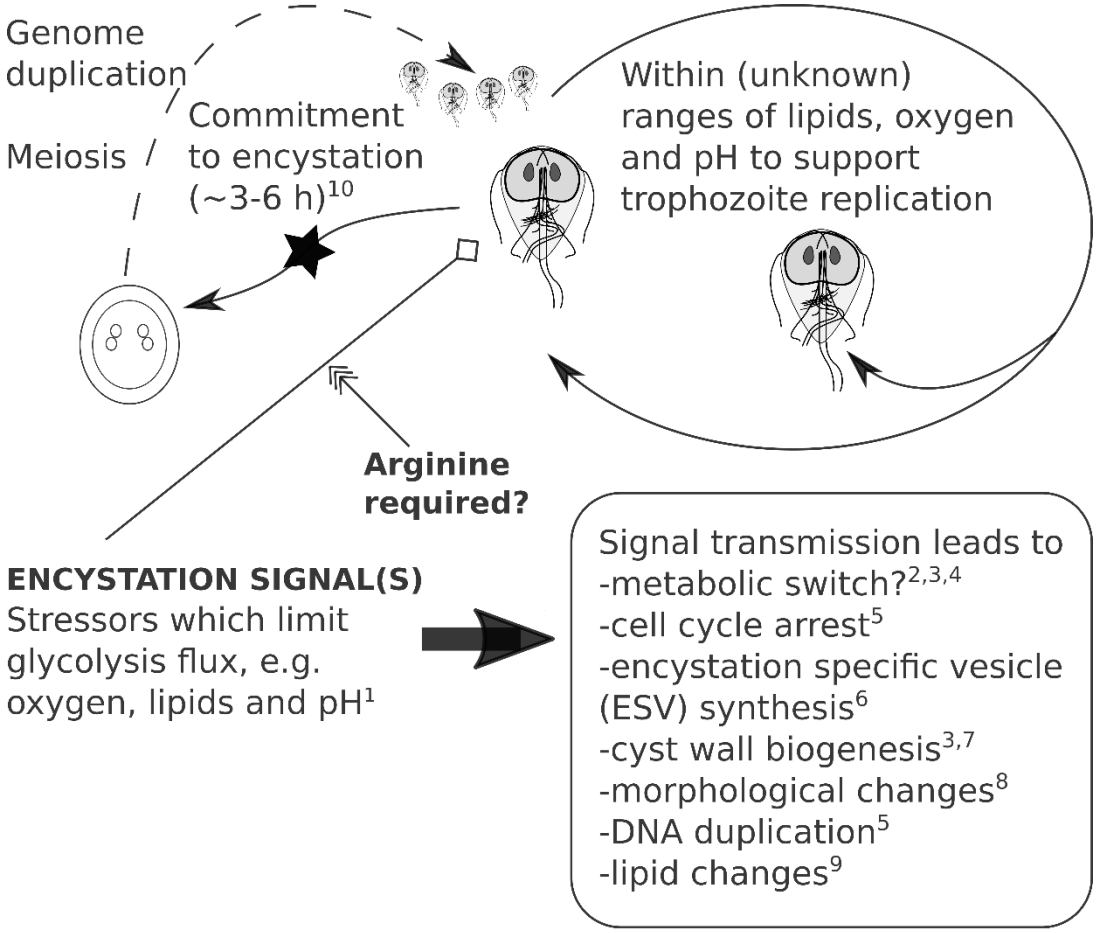

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Figure 1. G. duodenalis *life cycle indicating potential encystation signals and subsequent cellular changes upon encystation. Two stages are shown: the replicating trophozoite, which generates two new trophozoites, and the encysting trophozoite which generates a cyst with four nuclei, which upon transmission to a new host will release four trophozoites.*

I will use the term here, *Giardia* spp. *replicate* when they divide as trophozoites and *reproduce* when a trophozoite encysts and a viable cyst successfully leaves the host with the feces Figure 1. During encystation, one nuclear division and two genome duplications take place, which generates a cyst with four tetraploid nuclei (Bernander, Palm, and Svärd 2001). During encystation, nuclear fusion and probably homologous recombination occur (demonstrated in assemblage A) (Poxleitner et al. 2008) and genetic data support the existence of meiotic pathways and genes in *G. duodenalis* (Ramesh, Malik, and Logsdon 2005; Cooper et al. 2007; Melo et al. 2008). When a cyst enters a new host, commonly via contaminated water or food (Cacciò and Ryan 2008), it is exposed to the gastrointestinal environment with e.g. low pH and bile, and each cyst generates four trophozoites. These trophozoites are capable of either replication as trophozoites or reproduction, i.e. may generate a new cyst and complete the life cycle.

Initiation of encystation

Patterns for cyst shedding in *G. duodenalis* mouse models are not as distinct (Bartelt et al. 2013) as those seen for e.g. *E. falciformis* (see above and Ehret et al., 2017). However, *G. duodenalis* cyst shedding peaks occur and can be a result of peaking trophozoite growth, synchronized encystation, or both. Coordinated life cycle progression has been well studied in e.g. *Trypanosoma* spp. (Matthews, McCulloch, and Morrison 2015; Rojas et al. 2019) but also in free-living *Dictyostelium*, which respond to nutrient stimuli to synchronize life stage switches (Loomis 2014). In *G. duodenalis*, encystation has been studied in vitro (Lauwaet et al. 2007; Morf et al. 2010; Faso and Hehl 2011; Einarsson et al. 2016) which implies that there is no absolute control of medium conditions, since no defined growth medium is available for *G. duodenalis*. For encystation, protocols of different efficiency have long been available. For instance, Luján et al. listed ten reported protocols in 1997 (Luján, Mowatt, and Nash 1997). The major variations are increasing pH in addition to 1) a bile-free incubation followed by addition of porcine bile with lactic acid in medium (Gillin et al. 1987), or 2) incubation with cholesterol-starvation and fetal calf serum addition (Luján et al. 1996), or the most recent protocol 3) with adult bovine serum and high bovine bile (of varying concentration) in medium (Einarsson et al. 2016). Transcriptional changes upon in vitro (Morf et al. 2010; Einarsson et al. 2016) and in vivo (Pham et al. 2017) encystation confirm upregulation of cyst wall proteins (CWP) which are also used as molecular markers for encystation. Encystation specific vesicles (ESV) containing those proteins are detected upon in vitro encystation and thought to transport material to the cyst wall (Lauwaet et al. 2007; Morf et al. 2010; Faso and Hehl 2011; Einarsson et al. 2016). After induction, *G. duodenalis* appears to go into an encystation program which reaches a point of no return after which they proceed to encyst even if the inducing conditions are removed. In vitro this was determined to ~3-6h post induction. (Sulemana, Paget, and Jarroll 2014; Einarsson et al. 2016). Taken together, current in vitro-generated data suggests that a drastic change in growth conditions and especially in lipid content and pH contribute to initiating encystation in *G. duodenalis,* and that parasites commit to the life stage switch.

The disease giardiasis and its epidemiology

Humans commonly experience symptoms of *G. duodenalis* infection within seven to 10 days and spontaneous clearance of symptomatic infection is common within two to three weeks. In areas with good sanitation the prevalence is estimated to 0.4-7.5% and in poor sanitation areas higher, between 8 and 30% (U. Ryan and Cacciò 2013) but it is generally recognized that probably there are at least as many asymptomatic as symptomatic cases of *G. duodenalis* infections world-wide (Einarsson, Ma'ayeh, and Svärd 2016). The approximation for symptomatic cases is ~200 million persons per year, globally (U. Ryan and Cacciò 2013). Symptoms include nausea, bloating, severe diarrhea and abdominal cramps. Descriptions of symptoms vary greatly and correlate poorly with parasite data, i.e. its genotype. (Klotz and Aebischer 2015) Therefore, it is still unclear whether certain parasite genotypes (see below) are more pathogenic than others. Differences in host susceptibility and symptoms can also be caused by e.g. variations in diet, microbiota (Singer and Nash 2000; Barash et al. 2017), immune competence (Eckmann 2003; Buret et al. 2015; Einarsson, Ma'ayeh, and Svärd 2016), and probably other infections. Being a globally distributed disease, one can also expect discrepancies in how medical personnel and patients perceive symptoms and report them, a challenge for *G. duodenalis* surveillance. WHO included giardiasis as a neglected tropical disease (NTD) in 2006 (Savioli, Smith, and Thompson 2006), and unfortunately the lack of studies for many aspects of *G. duodenalis* infections still reflects this decision. Part of the explanation for discrepancies in epidemiological data is also likely methodological, i.e. inconsistencies in parasite typing (e.g. number of genes and the method used (R. C.A. Thompson and Ash 2019), as well as interpretation of sequence data which may be inconsistent and is not standardized for this tetraploid organism. In short, giardiasis is a NTD and many aspects of the disease are poorly understood and data is often contradictory.

Giardia *spp. classification*

G. duodenalis assemblages (~genotypes) A-H infect a broad range of mammals. Assemblages A and B are responsible for disease in humans, but also infect other primates and mammalian wildlife. A dataset of infections in Germany contained ~30% assemblage A parasites, 70% assemblage B parasites, and of all cases, ~20% carried both assemblage A and B (unpublished data, Christian Klotz, Robert-Koch Institute, Germany). When assemblages A and B occur in animals it is unclear whether they are the same sub-types which infect humans.(Cacciò and Ryan 2008) Determining the zoonotic potential is complicated by the lack of culturing methods for other genotypes than *G. duodenalis* assemblage A and B (and not all of these can be cultured either as mentioned above); coinfections with more than one *G. duodenalis* genotype in samples; and technical challenges for genotyping described above. Thompson and Ash (R. C.A. Thompson and Ash 2019) recently proposed 10 different to-date identified *Giardia (*prev. *duodenalis)* spp. to replace the assemblage classification system. They suggested to classify assemblage A as *G. duodenalis* and assemblage B as *Giardia enterica.* These two species share 77% nucleotide/78% amino acid sequence homology (Franzén et al. 2009). In this thesis I use "*G. duodenalis*" as species name for the eight assemblages. My work was carried out using assemblage B, partly motivated by the indications for higher prevalence of this assemblage in

humans. I use subtype GS/H7 and in descriptions and discussions of my data referring to *G. duodenalis*, this isolate is intended.

Host responses to G. duodenalis

The mechanisms by which *G. duodenalis* (and other *Giardia* spp.) induce immune responses are not well understood. Some parasite proteins have been shown to activate immune responses (importantly here arginine deiminase (ADI) is among them, but also e.g. fructose-1,6-bisphosphate aldolase, ornithine carbamoyl transferase and α-giardins (Klotz and Aebischer 2015)) but we do not know why some individuals elicit stronger immune responses, develop inflammation and e.g. leaky epithelia, whereas others are asymptomatic. Of relevance in this thesis (see below), mechanistic Target of Rapamycin (mTOR) has been identified as sensitive to the presence of *G. duodenalis* or derived proteins. Arginine depletion by *G. duodenalis* ADI induces cytokine profile changes in dendritic cells, DC, from human donors. Those changes coincided with changes in phosphorylations which could be linked, not only to the mTOR kinase S6, but also to one of its targets, CD83 (Banik et al. 2013). This suggests that DCs would sense *G. duodenalis* induced arginine depletion (given that it takes place) through the mTOR signaling network and initiate a response. Arginine dependent changes in the mTOR associated p70S6 kinase (one of two variants of S6) have also been reported (M. J. Rhoads et al. 2006), further supporting the relevance of this pathway in response to arginine, another focus of this thesis. The latter changes were additionally associated with enterocyte migration in the intestine. If *G. duodenalis* consumes arginine and induces mTOR signaling also in intestinal cells, one would expect mTOR to induce cellular and potentially even systemic responses. The association of p70S6 kinase with cellular migration proposes a mechanism for e.g. observed morphological changes in intestine upon *G. duodenalis* infections.

Immune defenses which can be expected upon host responses to *G. duodenalis* include T helper (Th) cell 1, Th2, and Th17 responses, i.e. no distinct branch of the adaptive immune response (Klotz and Aebischer 2015). Several studies support that immunoglobulin (Ig) A is important to clear *Giardia* spp. infections. In the mouse model, IgA does not appear necessary for clearance though, and depending on infection dose ($5x10^5$ or 10^7 *G. duodenalis* assemblage B) and time for measurements, different results were acquired. (Singer and Nash 2000; Langford et al. 2002) Pre-activated DCs stimulated in vitro by *G. duodenalis* lysates produce increased levels of e.g. tumor necrosis factor (TNF) α, interleukin (IL) 6, and IL-12 – all pro-inflammatory cytokines (Banik et al. 2013). Mouse experiments have also supported that DCs are an important source of IL-6 to protect against *G. duodenalis* (assemblage B) (Kamda, Nash, and Singer 2012). Other innate immune responses such as antimicrobial peptides (AMP), nitric oxide (NO) production and intestinal proteases have been implicated in protections against *Giardia* spp. In vitro killing by e.g. defensins (example of AMP) has been demonstrated and the molecules are secreted by intestinal epithelium Paneth cells, and found in mucus. Mucus itself is also suggested to have a protective function as a physical barrier. (Klotz and Aebischer 2015)

The long-suggested importance of arginine during G. duodenalis *infection*

Arginine utilization is reported among many pathogens (e.g. Gogoi et al., 2016) and the enzymes responsible for its metabolism are considered virulence factors (e.g. McGraw et al., 1999). An example is the bacterium *Pseudomonas aeruginosa,* a lung-pathogen in humans, which utilizes arginine as an energy source. In addition, a secondary metabolite (agmatine) of arginine catabolism in an arginine decarboxylase-pathway is not only a source for ATP generation in the bacterium. Its extracellular presence (from a e.g. human host *or* the bacterium itself) promotes inflammation and bacterial biofilm formation (Paulson et al. 2014 and reviewed in Gogoi et al. 2016). Such interactions which involve arginine both as energy source and signaling molecule, and a role in both pathogen and host, have been suggested also for *G. duodenalis* (Jarroll, E.L., and Paget 1995; Eckmann 2003; Pham et al. 2017)).

G. duodenalis increases in vitro growth for ~24h upon addition of 5 or 10 mM arginine to growth medium (Edwards et al., 1992), levels comparable to biologically relevant concentrations in humans (Adibi and Mercer, 1973). The effect reported by Edwards et al. was seen also in the absence of glucose in the growth medium. The arginine dihydrolase (ADH) pathway, which is not present in humans, was described for *G. duodenalis* in 1990 (P. J. Schofield et al. 1990). Its presence in *G. duodenalis* has been confirmed by the published genomes (Morrison et al. 2007; Franzén et al. 2009 for assemblages A and B, respectively) and its activity by gene expression experiments in vitro (Birkeland et al. 2010; Morf et al. 2010; Stadelmann et al. 2013) and in vivo (Pham et al. 2017), although in the latter only one pathway enzyme (ornithine carbamoyl transferase, OCT) was among the top differentially regulated ones. ADI upregulation was e.g. demonstrated at 6h when parasite mRNA was compared at different time-points after exposure to a model of intestinal epithelial cells, the Caco2 cell-line (Stadelmann et al. 2013). With the ADH pathway, *G. duodenalis* can efficiently utilize arginine as an alternative energy source to glucose (Philip J. Schofield et al. 1992). Therefore, poor access to arginine is expected to hamper parasite growth, although to my knowledge no information on the in vivo relevance for this capability has so far been available.

It has also been suggested that arginine is important for encystation and coping with oxidative stress (Jarroll, E.L., and Paget 1995; Pham et al. 2017). *G. duodenalis* may consume or degrade arginine to hamper host responses during infection. However, arginine also appears to support growth in the trophozoite stage and possibly arginine improves encystation independently of a potential effect on the host. If that function is significant for the parasite, effects on hosts could be neutral, positive for the parasite (impaired host signaling or NO production) or even negative (a warning signal to the host). This work investigates total replication and reproduction effects on the parasite when arginine access is varied.

Aspects of arginine in mammalian hosts

Arginine is perhaps best known in humans for being the substrate for nitric oxide synthase (NOS), which generates nitric oxide (NO), an important innate immune defense molecule. However, in mammals arginine has several other functions and is for instance a substrate for arginase to generate ornithine which can feed into polyamine synthesis (Morris 2006) which is also the case in *Giardia* spp. (Maia et al. 2008). Arginine is a semi-essential amino acid in humans, meaning it is generally synthesized to sufficient levels, but it can become limiting under certain extreme conditions such as diseases and infections (Morris 2006). Arginine is regulated in the body, demonstrated by varying levels in different body fluids compared to e.g. leucine or glutamine. Its concentration in food also varies greatly and it is for instance quite low in concentration in human milk, compared to other amino acids (J. M. Rhoads and Wu 2009). Humans can convert ornithine, a metabolite of arginine catabolism, via citrulline back to arginine on a systemic level in the so called intestinal-renal axis. Arginine generated via this axis is mainly synthesized in the kidneys. (Morris 2006) About 40% of dietary arginine is in adults catabolized in the intestine and does not reach the circulation (J. M. Rhoads and Wu 2009). Indications for a local effect in the intestine of arginine deprivation come from experiments demonstrating growth defects in a Caco2 model of intestinal epithelial cells (Stadelmann et al. 2012). Stadelmann et al. report reduced proliferation of those cells if they are grown in the absence of arginine, and a smaller effect when medium was complemented with citrulline. Therefore, although arginine concentrations probably are reduced in intestine by dietary restriction and have effects locally on intestinal epithelial cells (as suggested by Stadelmann et al., 2012), mammals compensate lost intake systemically. In blood such compensation comes to ~15% from de novo synthesis and ~85% from protein ((Morf et al. 2010). Therefore, short-term dietary restriction of arginine is not expected to have dramatic overall effects on mammals.

A weakened host model to investigate G. duodenalis *plastic capacity*

As described above, arginine manipulation experiments in this thesis aim to investigate the in vivo relevance of arginine availability for parasite replication and reproduction. In addition, I include a host model in which I expect beneficial conditions for the parasite. This serves to provide further evidence for or against phenotypic plastic capacity in this parasite. The model of choice is a mechanistic Target of Rapamycin (mTOR)-deletion mutant specific for intestinal epithelial cells (IECs, determined by the villin-promotor; see Sampson et al. 2016 and Materials and Methods for details). The central role and broad effects of sensing and signaling in the mTOR network in regulating cellular growth in response to external cues, including nutrients, can hardly be exaggerated (Laplante and Sabatini 2012). Functions of mTOR signaling and the mTOR gene in intestinal epithelium were thoroughly evaluated by Sampson et al. (2016). Specific cells of intestinal epithelium were affected by tissue-specific mTOR disruption: absorptive cells, Paneth cells and Goblet cells regenerated to a lesser extent after irradiation damage in mTOR-deficient mice. Arginine is a potent stimulator for mTOR signaling (e.g. Morris 2006; J. M. Rhoads and Wu 2009). Arginine supplementation during rotavirus-infection activated mTOR signaling seen by phosphorylation of one of its targets, p70S6 kinase, and as

expected increased protein synthesis. Oral treatment with the mTOR-signaling inhibitor Rapamycin (and simultaneous arginine stimulation) partially decreased protein biosynthesis and decreased epithelial electrical resistance (Corl et al. 2008), a measure of "leakiness".

In human endothelial cells, Rapamycin mTOR-signaling inhibition stimulated arginine uptake by increasing protein expression of CAT2, an arginine transporter (Visigalli et al. 2007). This suggests that an mTOR mutant would behave similarly and increase its capacity for arginine uptake. However, since mTOR has broad effects also on tissue regeneration after injury, the total effect of a mTOR-mutant could also be a slowly healing, and possibly leaky, phenotype.

Paneth cells, shown to regenerate poorly in the Sampson et al. (2016) mTOR epithelium mutant mouse, support tissue regeneration but also secrete antimicrobial peptides (Porter et al. 2002), which may influence *Giardia* spp. (Eckmann 2003). Goblet cells produce and secrete mucins, the major components of intestinal mucus (Birchenough et al. 2015) and a defect in this function during infection could also be beneficial for *Giardia* spp.. After evaluating several genes involved in mTOR-signaling, Sampson et al. conclude that mTOR gene disruption in intestinal epithelium causes atrophy upon radiation-induced injury. Taken together, the literature suggests that tissue-specific mTOR-disruption can be used as a model which causes localized benefits for an intestinal extracellular parasite without drastic overall effects on the host (at least not for the time-frame of interest here; 7 days, see Methods). Establishing the *G. duodenalis* infection model in tissue-specific mTOR-deletion mice in this thesis serves to introduce a host in which phenotypic plasticity could be revealed. Parasite sensitivity and responsiveness measured in phenotypic readouts such as trophozoite or cyst numbers could support the presence of phenotypic plasticity in *G. duodenalis*. In addition, the previously described implications for mTOR-sensing of arginine in DCs (Banik et al. 2013) and intestinal epithelium (M. J. Rhoads et al. 2006) suggest that this infection model may be valuable in future studies of the role of arginine during *G. duodenalis* infections.

1.2.4 Goals for *G. duodenalis* research project

G. duodenalis utilizes glucose in axenic culture but in one study grew better on arginine (Edwards et al. 1992). This suggests plastic capacity in *G. duodenalis* with regards to arginine utilization. Attempts to culture parasites isolated from infected humans are challenging (unpublished data, Christian Klotz, Robert Koch Institute, Berlin, Germany) and indicate plasticity since they sometimes succeed given enough time (see also Nash 2019) or fail (indicating limits of plasticity or lack thereof). Although it appears clear that arginine does offer growth benefits for *G. duodenalis* (in vitro), its relevance in vivo, e.g. through access via host diets, could be different since the metabolic context is more complex. Here, I investigate *G. duodenalis* trophozoite replication and encystation success under different arginine availability in vivo. I thereby address a long-standing question of the overall importance of arginine access for *G. duodenalis* replication and reproduction in vivo. I analyze the relevance of my findings both in the context of *G. duodenalis* biology/infections, and in an evolutionary context by applying the concept of phenotypic plasticity to discuss my observations.

1.3 Aim of this thesis

This project has three major aims. The overall aim is to generate data on protozoan parasite phenotypes in response to biologically relevant external stimuli and apply theoretical concepts of phenotypic plasticity to evaluate those findings in two distantly related protozoan parasites. To that aim, I 1) assess *E. falciformis'* transcriptional plasticity in response to host immune defenses; and 2) determine the impact of sufficient versus poor arginine availability on in vivo growth of *G. duodenalis* trophozoite replication and reproductive success: cyst generation and shedding.

2 Materials and Methods

2.1 Project I*: E. falciformis*

The following Materials and Methods section is a quote from my published work (Ehret, Spork, et al. 2017)

2.1.1 Mice, infection procedure and infection analysis

Three strains of mice were used in our experiments: NMRI, C57BL/6 (Charles River Laboratories, Sulzfeld, Germany), and Rag1−/− on C57BL/6 background (obtained from German Rheumatism Research Centre, Berlin). Rag1−/− −mice are deficient in T- and B-cell maturation. Animals where infected as described by Schmid et al. (Schmid et al. 2012), but tap-water was used instead of PBS for administration of oocysts. Briefly, NMRI mice were infected two times, which will be referred to as naïve and challenge infection. For the naïve infection, 150 sporulated oocysts were administered in 100 µL water by oral gavage. During the naïve infection of 52 mice, all animals were weighed every day. On day zero, before infection, as well as on 3 dpi, 5 dpi and 7 dpi, caeca from 3 to 4 sacrificed mice per timepoint were collected. Epithelial cells were isolated as described in (Schmid et al. 2012), using a protocol which generated epithelial cells with 90% purity. For challenge infection, mice recovered spontaneously and after 4 weeks they were challenge infected. Recovery was monitored by weighing and visual inspection of fur. For the challenge infection, 1500 sporulated oocysts were applied by oral gavage in 100 µL water (a higher dose was necessary to establish a challenge infection). Tissue from three to four mice per replicate was pooled for both non-reinfection control (referred to as day 0 of challenge infection) and for all other samples. Rag1$^{-/-}$ mice and the background C57BL/6 strain control mice were also subjected to naïve and challenge infections with 10 sporulated oocysts in 100µL water in both cases. Samples were taken on day 0 (pre-infection control) and 5dpi in both naïve and challenge infections of these mice and were otherwise treated as described above for NMRI mice. Oocyst shedding was determined from eight NMRI mice in naïve infection and four challenge infected ones; from 15 naïve Rag1$^{-/-}$mice and C57BL/6 mice respectively, and from nine challenge infected Rag1$^{-/-}$ mice and C57BL/6 mice, respectively. Overall oocyst output was compared using Mann-Whitney U-test in R (“R Development Core Team” 2008).

2.1.2 Oocyst purification for infection, sequencing and quantification

Oocysts for infection were purified by NaOCl flotation of mouse feces stored in potassium dichromate, in which oocysts for infection were allowed to sporulate at room temperature for at least 5 days. During the patency phase, feces of mice were collected and oocysts were flotated using saturated NaCl-solution. The oocyst output was quantified using the McMaster chamber. For sequencing, unsporulated oocysts were purified twice per day from feces of NMRI mice on 8–10 dpi, and immediately subjected to RNA purification. The strain "*E. falciformis* Bayer Haberkorn 1970" was used for all infections and parasite samples. It is maintained through passage in NMRI mice in our facilities as described previously (Schmid et al. 2012).

2.1.3 Sporozoite isolation

Sporocysts were isolated according to the method of (Kowalik and Zahner 1999) with slight modifications. Briefly, not more than 5 million sporulated oocysts were resuspended in 0.4% pepsin solution (Applichem), pH 3, and incubated at 37 °C for 1 h. Subsequently, sporocysts were isolated by mechanical shearing using glass beads (diameter 0.5 mm) and a vortex mixer, washed and separated from oocyst cell wall components by centrifugation at 1800 g for 10 min. Sporozoites were isolated from sporocysts by in vitro excystation. For this, sporocysts were incubated at 37 °C in DMEM containing 0.04% tauroglycocholate (MP Biomedicals) and 0.25% trypsin (Applichem) for 30 min. Released sporozoites were purified in cellulose columns as described in (Schmatz, Crane, and Murray 1984).

2.1.4 RNA extraction and quantification

For RNA-seq, total RNA was isolated either from infected epithelial cells, sporozoites, or unsporulated oocysts using Trizol according to the manufacturer's protocol (Invitrogen). In addition, unsporulated oocysts in Trizol were treated by mechanical shearing using glass beads for at least 20 min under frequent microscopic inspection. Purified RNA was used to produce an mRNA library using Illumina's TruSeq RNA Sample Preparation guide. This kit uses poly-T priming and we thus do not assess non-polyadenylated transcripts like those derived from the apicoplast genome. For qPCR, uninfected and infected epithelial cells from 3, 5 and 7 dpi were isolated as described above and cells were stored in 1 mL Trizol at −80°C. Total RNA was isolated using the PureLink RNA Mini Kit (Invitrogen) and immediately reverse

transcribed into cDNA using the Superscript III Platinum Two Step qRT-PCR Kit (Thermo Fisher Scientific). These RNA preparations were used for RT-qPCR of Eimeria 18S and creation of a mouse gene reference index. For the reference index, the mouse genes cytochrome c-1 (Cyc), peptidylprolyl isomerase A (Ppia) and peptidylprolyl isomerase B (Ppib) were amplified using the primers Cyc1_qPCR_f (5′- CAGC TACCATGTCACAAGTAGC-3′) and Cyc1_qPCR_r (5′-ACCACTTATGCCGCTTCATG -3′); Ppib_qPCR_f (CA AAGACACCAATGGCTCAC) and Ppib_ qPCR_r (5′-T GACATCCTTCAGTGGCTTG-3′); Ppia_qPCR_f (5′-AC CGTGTTCTTCGACATCAC-3′) and Ppia_qPCR_r (5′-ATGGCGTGTAAAGTCACCAC-3′), respectively. The *E. falciformis* 18S gene was amplified using the primers Ef18s_for (5′-ACAATTGGAGGGCAAGTCTG-3′)and Ef18s_rev (5′-AAACACCAACAGACGCAGTG-3′). After initialization at 50°C for 2 min followed by activation of enzymes at 95°C, 40 amplification cycles consisting of denaturation at 95°C for 15s and combined annealing and elongation at 60°C for 60s were performed. After each cycle the fluorescent signal was measured. A reference index was constructed taking the cube route of the multiplied cycle threshold (ct)-values for the three mouse genes. This composite "index ct-value" was used to calculate the ct difference (delta-ct) of the *E. falciformis* 18S gene. The lowest of these values was set as reference for calculation of how much more *E. falciformis* 18S RNA was detected compared to the level of background noise in the sample with the lowest value leading to delta-delta ct, or "noise normalized" ct-values. The number of transcripts above noise level was calculated taking these values as exponents to the base two. The procedure was performed in triplicate for each experimental group. A linear model was constructed in R ("R Development Core Team" 2008) to predict these noise normalized delta-ct values by day post infection (dpi) and type of infection (naïve or challenge infected). This model excludes measurements at 0 dpi infection as background noise.

2.1.5 Sequencing and quality assessment

cDNA libraries were sequenced on either GAIIX (13 samples) or Illumina Hiseq 2000 (14 samples) platforms after preparation in a total of four experimental batches as specified in Table 3. A fastq_quality_filter (FASTQ- toolkit, version 0.0.14, available at https://github.com/ agordon/fastx_toolkit) was applied to Illumina Hiseq 2000 samples using a phred score of 10. We intentionally did not use a stringent trimming before mapping to genome assemblies as the

mapping process itself has been shown to be a superior quality control (MacManes 2014).

2.1.6 Alignment and reference genomes

The *M. musculus* mm10 assembly (Genome Reference Consortium Mouse Build 38, GCA_000001635.2) was used as reference genome for mapping and corresponding annotations were used for downstream analyses. The *E. falciformis* genome (Heitlinger et al. 2014) was downloaded from ToxoDB (Gajria et al. 2007). For mapping, mouse and parasite genome files were merged into a combined reference genome, and files including mRNA sequences from both species were aligned against this reference using TopHat2, version 2.0.14, (Trapnell, Pachter, and Salzberg 2009) with the option –G specified, and Bowtie2, version 1.1.2, (Langmead and Salzberg 2012). This was done to avoid spurious mapping in ultra- conserved genomic regions. Single-end and pair-end sequence samples were aligned separately with library type 'fr-unstranded' specified for pair-end samples. Bam files were used as input for the function "featureCounts" from of the R package "Rsubread" (Liao, Smyth, and Shi 2014). All subsequent analyses were performed in R ("R Development Core Team" 2008).

2.1.7 Differential mRNA abundance, data normalization and sample exclusions

After import of data to R, mouse and parasite data was separated using transcript IDs and analyzed, including normalization, separately. For each species, count data was normalized using the R-package edgeR version 3.16.2 (Robinson, McCarthy, and Smyth 2010) with the upperquartile normalization method. This raw data underlying our study is available as supplementary data S1. Briefly, genes with below an overall of 3000 reads (mouse) and 100 reads (*E. falciformis*) summed over all samples (libraries) were removed and normalization factors were calculated for the 75% quantile for each library. This normalization is suitable for densities of mapping read counts which follow a negative binomial distribution. We excluded samples NMRI_2nd_3dpi _rep1 and NMRI_2nd_5dpi_rep2 due to low parasite contribution (0.012% and 0.023%) to the overall transcriptome. Technically, this exclusion made it possible to obtain parasite read counts in agreement with a negative binomial distribution. Both excluded samples are from challenge infection and it is likely that the infected mice were immune to re-infection. One additional sample (NMRI_1stInf_0d- pi_rep1) was excluded because the uninfected

control showed unexpected mapping of reads to the *E. falciformis* genome (0.033%). As samples and individual replicates were sequenced in batches to different depth and using different instrumentation (Table 3) we performed multidimensional scaling of samples as quality controls using "plotMDS". We also plotted mean expression vs. difference (MA) plots using "plotSmear". Both functions are provided in the R package edgeR v 3.16.2(Robinson, McCarthy, and Smyth 2010).

2.1.8 Testing of differentially abundant mRNAs and hierarchical clustering

We also used edgeR v 3.16.2 (Robinson, McCarthy, and Smyth 2010) further to fit generalized linear models (GLMs with a negative binomial link function) for each gene (glmFit) and to perform likelihood ratio tests for models with or without a focal factor (glmLRT) using the "alternate design matrix" approach specifying focal contrasts individually. Tested contrasts comprised for the mouse a) infections at each time-point versus uninfected controls, b) corresponding timepoints between different mouse strains and c) corresponding timepoints and mouse strains for naïve and challenge infection. Since the control sample for infection in naïve NMRI mice was removed from the analysis (see above), the two uninfected replicates from challenge infection were used as uninfected controls in all NMRI mouse analyses. For the parasite, contrasts were set between a) all different stages of the life cycle, as well as b) and c) as above (see also results in Table 4). Mouse mRNAs which responded to infection or were differently abundant at different dpi (0 vs "any dpi" or "any dpi" vs "any dpi"; see Table 4) and *E. falciformis* genes showing differences between any life cycle stage (oocysts versus sporozoites, or either of those versus "any dpi" or "any dpi" versus "any dpi") were selected and used for hierarchical clustering. Hierarchical clustering was performed using the complete linkage method based on Euclidean distances between Z-scores (mRNA abundance values scaled for differences from mean over all samples of each gene in units of standard deviations).

2.1.9 Enrichment tests and evolutionary conservation test

Gene Ontology (GO) enrichment analysis was performed using the R package topGO with the "weight01" algorithm and Fisher's exact tests. We additionally performed a correction for multiple testing on the returned p-values (function "p.adjust" using the BH-method (Benjamini and Hochberg 1995)). Similarly, a Fisher's exact test and

corrections for multiple testing were used to test for overrepresentation of transcripts with a signal sequence for entering the secretory pathway or containing transmembrane domains (as inferred using Signal P) which are predicted for the *E. falciformis* genome. Evolutionary conservation of gene families was analyzed based on categories from (Heitlinger et al. 2014) which are as follows: i) *E. falciformis* specific, ii) specific to the genus *Eimeria*, compiled by an analysis of *E. falciformis*, *E. maxima* and *E. tenella*, iii) Coccidia: *Eimeria* plus *T. gondii* and *Neospora caninum*, iv) Coccidia plus *Babesia microti*, *Theileria annulata*, *Plasmodium falciparum* and *Plasmodium vivax* v) the same apicomplexan parasites as in iv plus *Cryptosporidium hominis*, vi) universally conserved in the eukaryote super-kingdom inferred from an analysis of *Saccharomyces cerevisiae* and *Arabidopsis thaliana*. These categories were tested for overrepresentation in parasite gene clusters with particular patterns described in the text using Fisher's exact-tests. Resulting p-values were corrected for multiple testing using the procedure of Benjamini and Hochberg (Benjamini and Hochberg 1995) and reported as false discovery rates (FDR).

2.1.10 Correlation analysis of apicomplexan transcriptomes

Transcriptome datasets from (Reid et al. 2014; Walker et al. 2015) and (Hehl et al. 2015) were downloaded from ToxoDB (Gajria et al. 2007). Orthologues between *E. falciformis*, *E. tenella* and *T. gondii* were compiled as in (Heitlinger et al. 2014) and only 1:1:1 orthologue triplets were retained for analysis, as multi-paralog gene-families might contain members showing divergent evolution of gene-expression due to neo/sub functionalization. Mean mRNA abundances per life cycle stage were used for samples from our study. Spearman's correlation coefficients for expression over different samples in all studies and over different species represented by their orthologues were determined. Hierarchical clustering with complete linkage was used to cluster resulting correlations coefficients.

2.2 Project II: *G. duodenalis*

2.2.1 Infection experiments

One major readout of interest and expected limiting (low sensitivity) read-out in my experiments was cyst-shedding. The animal experiment allowance would determine the number of animals allowed to use in experiments. Therefore, cyst shedding data from Shukla et al (Shukla and Sidhu 2011) were applied in a power analysis (Appendix 1) to determine necessary animal group size. The requirement for sensitivity (difference with p-value ≤ 0.05) was set to 106 cysts/gram feces or more between any groups. This difference in average value between days post infection (dpi) was reported in the same paper and therefore thought to be a realistic difference to detect.

Determining parameters of infection

When applied, antibiotics (1.4g/L Neomycin (Cayman-Chemicals/Biomol GmbH, Germany, Item No. 14287), 1g/L Ampicillin (Sigma-Aldrich (now Merck), Germany) and 1g/L Vancomycin (a kind gift from M. Heimesaat, Charite, Berlin) were supplied in the drinking water and changed every three days in pilot experiment 2 and 1-2 times per week in the following experiments. All experiments were performed with ~50/50% female/male mice no older than 11 weeks at the start of experiments.

If nothing else is indicated, mice were housed in groups of 2-5 mice per cage, unless males were fighting in which case they were housed separately. The facilities used are SPF (specific pathogen-free) certified (Appendix 2). Animals had access to water ad libitum, and to food during the 12h dark cycle (6pm to 6am). During pilot infections, feces were collected in antibiotics-containing water (250µL milli-Q water with erythromycin, 200µg/mL, chloramphenicol, 400µg/mL, amicazin µg/mL, tetracycline 200 µg/mL, rifampicin 400 µg/mL, and phosphomycin 100 µg/mL) from individual mice at 6am, 12pm, and 6pm and stored at 4°C until analysis. At 6pm, mice were moved to a new cage and the following day, at 6am, mice were again transferred to a new cage and the group total ("night feces") feces were collected from each cage in a 50mL tube with 35mL antibiotics-containing water as above. In total, four different pilot infection experiments were carried out over approximately seven months as summarized in

2.2.2 Experimental infections of mTOR-deletion mice and diet manipulation

Mouse strains

Mice with intestinal epithelium-specific mTOR-gene knock-out were generated from breeding of strains previously established (Sampson et al., 2016). Briefly, two male and two female mice

which are heterozygote for Cre-recombinase under the intestinal-epithelium specific villin promoter (B6.Cg-Tg(Vil1-cre)997Gum/J) were purchased (The Jackson Laboratory, USA). Mice were kept for seven days in our facility and then used for breeding. Similarly, two male and two female mice with loxP-regions flanking both copies of the gene of interest, mechanistic Target of Rapamycin, mTOR, (B6.129S4-Mtortm1.2Koz/J: here referred to as $mTOR^{flox/flox}$ and WT) were purchased from the same company at the same time. Mice were bred in-house by our facility (Robert Koch-Institute, Berlin, Germany). F1 offspring which were positive for Cre *and* heterozygote (always heterozygote due to the cross with B6.Cg-Tg(Vil1-cre)997Gum/J) for loxP-mTOR; "$mTOR^{flox}$" were used to generate F2-mice. These were positive for Cre-recombinase and homozygote for loxP-mTOR, i.e. $mTOR^{\Delta IEC}$ lacking the mTOR gene in cells expressing villin (ΔmTOR in intestinal epithelial cells). Mice were genotyped using primers and protocols from the provider (primer order-numbers: Vil1-Cre forward: 14506; Vil1-Cre reverse: 18960; Vil1-Cre internal control forward: oIMR7338; Vil1-Cre internal control reverse: oIMR7339; loxP-mTOR forward: 11649; and loxP-mTOR reverse: 11650). As determined by pilot experiments, seven days prior to infection approximately equal numbers of 6-10 weeks old males and females were randomly assigned to experimental groups and placed accordingly in new cages. Antibiotics were provided in the drinking water and mice were housed as in pilot experiment number 4.

Table 1. Primers for genotyping loxP-mTOR (also $^{mTORflox/flox}$ and WT) and Villin-Cre (see text). For complete PCR and genotyping-protocols, see Appendix 3.

Name by Jackson Laboratory	Sequence	Comment
34368	GAT AAT TGC AGT TTT GGC TAG CAG	loxP-mTOR Forward
34369	CTC CTT CTG TGA CAT ACA TTT CCT	loxP-mTOR reverse
18960	TTC TCC TCT AGG CTC GTC CA	Villin-Cre forward
14506	CAT GTC CAT CAG GTT CTT GC	Villin-Cre reverse
oIMR7338	CTA GGC CAC AGA ATT GAA AGA TCT	Villin-Cre internal ctrl forward
oIMR7339	GTA GGT GGA AAT TCT AGC ATCATC C	Villin-Cre internal ctrl reverse

Arginine manipulation in mouse diets

Seven days prior to infection, the experimental diet ("arginine-free": EF Crystalline AA Arginine free, purified diet, 10mm, order number S1039-E010, ssniff Spezialdiäten, GmbH, Arnsberg, Germany) or control diet ("normal diet", EF Crystalline AA, Control diet, 10mm, order number S1039-E005, ssniff Spezialdiäten, GmbH, Arnsberg, Germany) were introduced. Diets are crystalline, i.e. each amino-acid is added separately (as opposed to addition of whole protein), ensuring a 0% L-arginine experimental diet. In order to keep the concentrations of all other amino-acids constant in the control diet, minor differences in protein (1.6kcal%), fat (0.4kcal%) and carbohydrate (1.2 kcal%) content were accepted, with a total difference in energy content of 0.1MJ/kg (total Atwater energy content 16.6 or 16.7 MJ/kg. See additional details in Appendix 4).

Parasites for infection

G. duodenalis GS clone H7 (ATCC 50581; ATCC, Germany) were purchased and cultured. Stocks of passage eight were frozen in liquid nitrogen. For in vitro culture and amplification, TYI-S-33 medium with bovine bile as previously described (Keister, 1983), and Amphotericin B (2µg/mL final concentration, Biochrom, Germany) and Gentamicin (100µg/mL final concentration, Sigma-Aldrich/Merck, Germany) was used. Parasites were cultured in 11mL volume medium and one confluent culture tube typically contained 5-8*10^6 trophozoites (parasites appear to "squeeze" if cultures are left untouched, hence the large variation in numbers). For infections, trophozoites were quickly thawed in RT and added to 5mL pre-warmed medium (37°C) followed by centrifugation at 900g for five minutes at 4°C. The supernatant was carefully removed, and 1mL warm medium added. The ~1.5mL parasite suspension was added to the culture tube containing 9.5mL warm medium. Tubes were visually inspected for viable (motile and/or attached with beating flagella) trophozoites. The medium was changed one day after thawing and then every 3-4 days until infections. New medium was prepared at least every 5 days. 2-3h prior to infection, taking tubes directly from incubator the medium was removed and tubes were refilled with fresh RT medium. This served to remove unattached (possibly dead) parasites. Tubes with fresh medium were placed on ice for 30 minutes to detach parasites and hit against the palm 3-4 times to render more parasites to detach. Cultures were poured into a 50mL tube containing 35mL ice cold PBS and centrifuged at 900g for 5 minutes at 4°C. The supernatant was removed, and parasites counted twice in at least two different dilutions. Trophozoites were diluted with ice cold PBS to 5x10^6 +/- 5x10^5 trophozoites per 100µL. At least 2mL more suspension than required was prepared to improve handling and avoid bubbles during oral infections. Parasites were stored on ice up to 40 minutes prior to infection. After infections, remaining parasites were added to normal culture tubes with warm medium and in all cases grew normally for at least seven days.

Animal sacrifice and sample collection

Animals were moved to a dissection room and placed in a large measurement glass cylinder with paper and anaesthetized in Isoflurane (CP-Pharma, Germany). Blood was collected through retro-orbital bleeding and collected in EDTA-coated 1.5mL tubes and placed on ice. If necessary, mice were replaced in Isoflurane prior to sacrifice by cervical dislocation.

The mouse was then placed on an ice-cold metal plate for dissection. A three cm piece of small intestine, starting at 2 cm from pylorus, was measured and placed on a cool petri dish. Luminal content was removed by gently rolling a pipet tip over the tissue and luminal content was collected in 0.5mL RNALater. The three cm tissue for DNA and RNA extraction was placed in 1mL RNALater. Both samples were placed on ice within 3 minutes and stored on ice during dissections up to 3.5h and then frozen at -20°C until further processing. The following one cm small intestine was measured and placed in empty tubes which were immediately placed in liquid nitrogen and later stored in -70°C until further processing. The remaining small intestine was flushed with cold PBS, rolled ("Swiss roll") in cassettes, and placed in RT 4%

formaldehyde for histological analysis. Cecum was cut one to two times, placed in 10mL 4% formaldehyde and shaken. Caeca were kept in RT for 24h and then stored at 4°C until further processing.

Homogenization of small intestinal tissue for RNA/DNA extractions

Tissue samples for RNA and DNA extraction were thawed in a water bath at 37°C for 3-5 minutes and thereafter kept at RT. Samples were weighed and 60mg (+/-5mg) tissue was transferred into homogenization tubes; 1.5mL RNase free tubes were filled with 1mm bead-mixture (50/50% 1mm and 0.1mm sharp-edged RNase free silicon carbide beads from Bio Spec Products Inc, pre-treated at 180°C for 2.5h) and 600µL Buffer RLT from Qiagen RNeasy Mini kit (Qiagen GmbH, Germany) with 1% RNase free 2-mercaptoethanol (Aldrich, now Merck, Germany). Homogenization was performed immediately with a Pecellys 24 tissue homogenizer (Bertin Instruments, France) at 5500xg for 60 seconds. Homogenates were centrifuged for 3 minutes at 21 500xg at RT. Supernatants (~300-400µL, without foam which forms on top) were collected and stored in RNase free 1.5mL tubes at -70°C until RNA/DNA extraction.

Parasite quantification in small intestinal tissue by qPCR

Supernatants from homogenized tissue were thawed in a water bath at 37°C for 1-2 minutes. 100µL supernatant, corresponding to 17mg tissue, was used for DNA extraction. DNA was extracted using the NucleoSpin Tissue kit (Machery-Nagel, Düren, Germany), protocol 5 according to manufacturer's instructions with the first proteinase K-incubation at 56°C for 1h and 100µL elution volume. Yields were between 82 and 218ng DNA/µL. *G. duodenalis* quantification by qPCR was performed based on protocols and primers in (Verweij et al. 2003). A multiplex reaction which amplifies the *Giardia* small subunit (ssu) ribosomal RNA gene and an internal amplification control (IAC) in the same reaction was carried out using a BioRad cycler (Bio-Rad Laboratories, GmbH, Germany). Primers used are listed in Table 2. Reagents used were 12.5µL Maxima qPCR MasterMix (ThermoScientific), 2.0µL each of ddIAC forward and reverse primers, 0.5µL ddIAC-Cy5 probe, 2.5µL IAC plasmid, 1.0µL each of Giardia-80F/Giardia-127R forward and reverse primers, 0.5µL Giardia-105T probe, 1µL DEPC-treated water, and 2uL sample DNA in a total reaction volume of 25µL. The internal amplification plasmid from Deer et al. was used (Deer, Lampel, and González-Escalona 2010). The cycle program was as follows: 10 minutes initial denaturation at 95°C (1 cycle) followed by 40 cycles of: 15 seconds denaturation at 95°C, 30 seconds annealing at 60°C, 30 seconds of extension at 72°C. Standard curves were produced in every experiment using quantified DNA extracted by the protocol above from in vitro grown trophozoites as follows: *G. duodenalis* GS/H7 (ATCC 50581) trophozoites were cultured to passage 8 confluent cultures (3-4 days after passaging), detached on ice for 30 minutes, washed in 1:5 units PBS by centrifugation at 5000xg for 5 minutes. Pellets were then resuspended in 110µL PBS (and further diluted as appropriate) for counting. $50x10^6$ trophozoites were used for DNA extraction on five separate columns and the eluates were pooled and frozen at -20°C in DNA low binding tubes as DNA stock. All qPCR measurements were performed in 2 technical replicates. Raw data was

processed using the commercial CFX Maestro 1.1 software (version 4.1.2433.1219, Bio-Rad Laboratories, GmbH, Germany). Thresholds were set manually for each run. It was set as low as possible, with the requirement of generating no cycle threshold (ct)-signal for clearly negative samples (no exponential increase in fluorescence). Inhibition was defined as a sample having a ct value >2 ct over IAC average ct. No samples were inhibited. All non-template controls were negative. Two technical replicates were analyzed and not allowed to differ by >2ct (in which case the analysis was repeated). The CFX Maestro software computes DNA SQ based on the standard curves in each analysis. The average SQ of the two replicates was used to calculate *G. duodenalis* genome equivalents per gram tissue assuming 0.1pg DNA per trophozoite (0.05pg DNA per genome; Erlandssen & Rasch, 1994) and the known input amount of tissue (equivalent of 0.34mg/qPCR reaction). Seven dpi samples were compared using a Kruskal-Wallis rank sum test in R. Comparisons were done for WT diets, WT versus $mTOR^{\Delta IEC}$ (normal diet), and $mTOR^{\Delta IEC}$ diets.

Table 2. Primers and target plasmid for G. duodenalis *small subunit RNA (ssu) qPCR (Verweij et al. 2003) and internal amplification control for inhibition in samples.*

Name and stock concentration	Sequence	Comment
dd-IAC_f (10 pmol/µl)	5'- CTAACCTTCGTGATGAGCAATCG -3'	Forward control primer
dd-IAC_r (10 pmol/µl)	5'- GATCAGCTACGTGAGGTCCTAC -3'	Reverse control primer
dd-IAC-Cy5 (10 pmol/µl)	5'-Cy5-AGCTAGTCGATGCACTCCAGTCCTCCT-BBQ-3*	Probe and quencher
IAC target plasmid (10^6 copies/ µl)	See Deer, Lampel, and González-Escalona 2010	Amplification control plasmid
Giardia-80F (3.3 µM)	5'-GACGGCTCAGGACAACGGTT-3'	ssu forward primer
Giardia-127R (3.3 µM)	5'-TTGCCAGCGGTGTCCG-3'	ssu reverse primer
Giardia-105T (3.3 µM)	FAM-5'-CCCGCGGCGGTCCCTGCTAG-3'-BHQ1**	Probe and quencher

Quantification of parasites in feces: cyst analysis by microscopy

Fecal samples were collected daily from 4-7 dpi in main experiments (with mouse genotype and diet manipulation). In one separate experiment, feces were collected from 0-14 dpi to determine shedding post the timepoint for RNA-seq. Samples were taken from individual mice between 6am and 7am. Each pellet was collected in 500 µL water during infection establishment and 250µL water in subsequent experiments. Water contained antibiotics (as described above) to avoid bacterial growth and stored at 4°C until processing. For microscopy of samples from dietary and mouse genotype manipulation experiments, each fecal pellet was weighted, and water volume adjusted to 100mg feces per mL liquid. In both infection establishment experiments and manipulation experiments, pellets were disrupted by vortexing or (if necessary) manually with a pipet tip followed by vortexing. Large particles were allowed to settle for 2-5 minutes and then 50µL liquid was carefully resuspended and collected for staining. Samples were stained with a commercial anti-Giardia cyst antibody (Giardi-a-Glo, Waterborne INC, New Orleans, USA; 1µL per sample) and 1% 1,4-diazabicyclo[2.2.2]octane (DABCO; Carl Roth GmbH, Germany) to slow down bleaching. Samples were incubated in the dark at RT for 30 minutes and 6µL sample was analyzed in KOVA Glasstic Slide 10 counting chambers (KOVA International, California, USA). The detection limit for infection

establishments was 20 000 cysts/gram feces, and 1666 cysts/gram in the diet- and mTOR-manipulation experiments. The difference was due to the volume of sample analyzed in the respective experiments. A representative image from such analysis is shown in Figure 2.

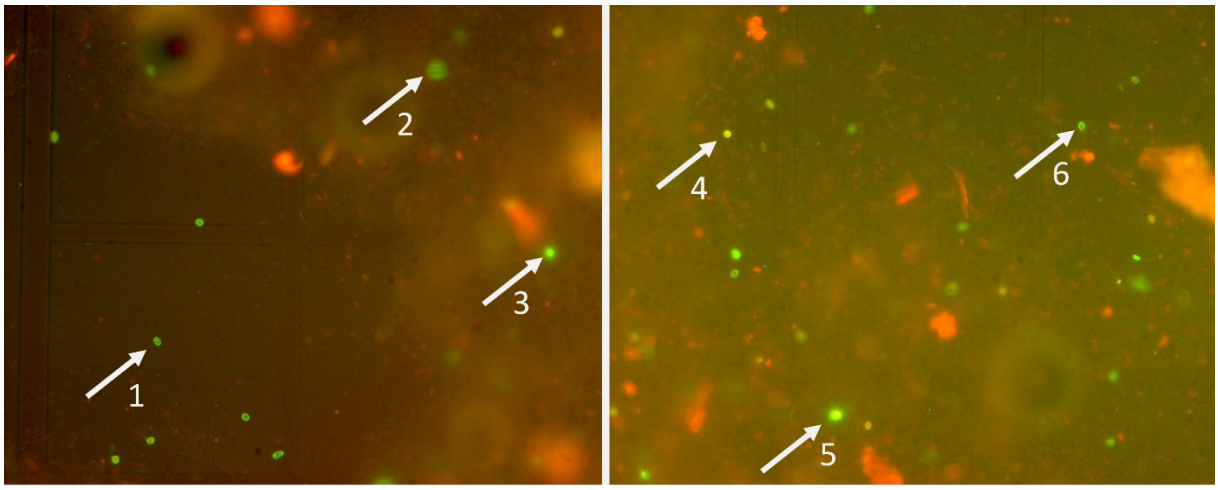

Figure 2. Two representative images of the microscope view for counting cysts (green). Propidium iodide (PI) stains DNA. Arrows and numbers exemplify the following: 1 and 6: typical G. duodenalis *cysts not staining for PI; 2: green autofluorescence of fecal material; 3 and 5: typical* G. duodenalis *cyst out of focus; 4: typical* G. duodenalis *cyst stained with PI*

Analysis of cyst counts

G. duodenalis cysts counts were analyzed for significant differences between experimental groups. All analyses were performed in R ("R Development Core Team" 2008). First, all counts from four to seven dpi were summarized ("pooled") per experimental group (genotype+diet combination) and pairwise tested for differences by a Kruskal-Wallis rank sum test. Seven dpi samples were further compared using a Kruskal-Wallis rank sum test. Pooled and 7dpi comparisons were done for WT diets and WT versus mTOR$^{\Delta IEC}$ (normal diet), and for pooled samples the comparison for mTOR$^{\Delta IEC}$ diets was also performed. See data in Appendix 5.

Parasite genome equivalents in feces: DNA extraction and qPCR

The samples used for microscopy were also used to extract DNA to quantify total parasite genome equivalents in fecal samples. Samples were resuspended using cut pipet tips (1000µL) and 100µL (corresponding to 10mg feces) was collected in new tubes. DNA was extracted following the protocol from the bead-based Maxwell 16 FFPE Plus LEV DNA Purification Kit on a Maxwell 16 Instrument (Promega Corporation, Wisconsin, USA) with the following exceptions: 14uL proteinase K and 126uL "Incubation buffer", and 500uL lysis buffer was used per sample. DNA was eluted in 70µL nuclease-free water and stored at 4°C. qPCR for *G. duodenalis* ssu with IAC inhibition control was performed as described above for small intestinal samples but with 3uL DNA (equivalent of 0.4mg feces) and no added water. Starting

quantities (SQ) of *G. duodenalis* DNA was estimated by the CFX Maestro software using standard curves as described above. Total genome equivalents (cysts + trophozoites) per gram feces was calculated as (SQ in 0.4 mg feces / 0.05pg DNA per genome)*2500. Seven dpi samples were compared using a Kruskal-Wallis rank sum test in R. Comparisons were done for WT diets, WT versus $mTOR^{\Delta IEC}$ (normal diet), and $mTOR^{\Delta IEC}$ diets.

Histological analysis of small intestinal tissues

Small intestinal samples were rinsed with cold PBS, rolled and stored 24-36h in 4% paraformaldehyde at RT followed by storage at 4°C until further processing. Histology was performed at iPATH.Berlin, Core Unit of Charité - Universitätsmedizin Berlin, Germany by Dr. Anja Kühl. Paraffin sections (1-2 µm) of small intestinal swiss rolls were dewaxed and subjected to periodic acid-Schiff reaction (PAS; Sigma-Aldrich). Sections were coverslipped with corbit balsam (Hecht, Germany). For immunohistochemical detection of mTOR protein, 1-2 µm paraffin sections were dewaxed and subjected to a heat-induced epitope retrieval step prior to incubation with anti-mTOR (clone 7C10, Cell Signaling). This was followed by incubation with biotinylated secondary antibody (Dianova). For detection, alkaline phosphatase-labelled streptavidin and chromogen RED (both Agilent) were employed. Nuclei were counterstained with hematoxylin (Merck) and sections were coverslipped with glycerol gelatin (Merck). Images were acquired using the AxioImager Z1 microscope (Carl Zeiss MicroImaging, Inc.).

3 Results

The results from the two research projects are divided into two sections. The first reports my findings from *E. falciformis* infections in mice and has been published (Ehret, Spork, et al. 2017). That section is a quote from the published work, in which results and discussion were integrated in one section. Additional aspects of my *E. falciformis* data which as relevant for this thesis are included in the Discussion. For the *G. duodenalis* project, results and discussion are reported separately.

3.1 Project I: *E. falciformis*

The following Results and Discussion section is a quote from my published work (Ehret, Spork, et al. 2017)

3.1.1 Immune competent hosts induce protective immunity against *E. falciformis* infection

To investigate *E. falciformis* development throughout the life cycle in a natural mouse host (NMRI mice) dual transcriptomes were produced at 3, 5, and 7 days post infection (dpi), which are suitable time points to assess asexual and sexual developmental stages of the parasite in its host (Haberkorn 1970; Mesfin and Bellamy 1979). We also investigated parasite development and transcriptomes in a mouse strain which is severely limited in adaptive immune responses (Rag1−/−; "immunocompromised" hereafter) with Rag1−/− and the respective isogenic background strain (C57BL/6 as control) at day 5 post infection. To further elucidate host immune responses and variation between the host genotypes and parasite sensitivity to host immunity, we also challenge infected all mouse groups (i.e. infected after recovery of a first infection; see Methods) and sampled at the same time-points as in naïve mice. Infections showed drastically decreased oocyst output (Fig. 1a and b) in immune competent hosts undergoing a second, challenge infection compared to naïve animals infected for the first time (Mann–Whitney test, in NMRI, n = 12, U = 32, p = 0.004; in C57BL/6, n = 24, U = 111, p = 0.008). Similarly, a strong reduction of parasite 18S rRNA (in cecum epithelia) in the challenge infection down to 3.5% of the amount measured in naïve hosts was detected in reverse transcription quantitative PCR (RTqPCR) in NMRI hosts (Fig. 1c). The model inferring this had a good fit (R2 = 0.94) and the change of the intercept for challenged compared to naïve hosts was highly significant (t = −6.71; p < 0.001). Differences in the slope were not significant (t = −1.522; p = 0.15), indicating that the amount of parasite material on

3 dpi is sufficient to explain a linear increase until 7 dpi. Overall this data is in line with the strong reduction of oocyst shedding seen in challenge infected immune competent mice, but also suggests that the host immune defense disturbs the parasite already at an early stage of infection, possibly even before 3 dpi. In contrast, in immune deficient mice no significant difference in parasite reproductive success (Fig. 1a) was observed between naïve and challenge infection (Mann–Whitney test; $n = 24$, $U = 96$, $p = 0.10$). Both in the immunocompromised and immune competent animals, however, all mice had cleared the infection by day 14. We thereby note that *E. falciformis* infection is self-limiting also in mice without mature T- and B-cells, however with a delayed peak of oocyst shedding in immune deficient hosts (Fig. 1b). *E. vermiformis*, in contrast, has been shown to display prolonged patency (shedding of oocysts up to 23 instead of 16 dpi) in immunocompromised hosts (Rose, Owen, and Hesketh 1984; Rose and Hesketh 1986; Rose, Wakelin, and Hesketh 1985). In comparison, the delayed peak of shedding we observe for *E. falciformis* in immunocompromised hosts does not affect pre-patency and patency periods (beginning and end of oocyst shedding), confirming earlier reports of largely lacking developmental plasticity in *E. falciformis* (Rose and Hesketh 1986). We take advantage of the presence of the same life cycle stages in hosts of varying immune competence to assess whether *E. falciformis* optimizes its host exploitation strategies in response to varying host defenses.

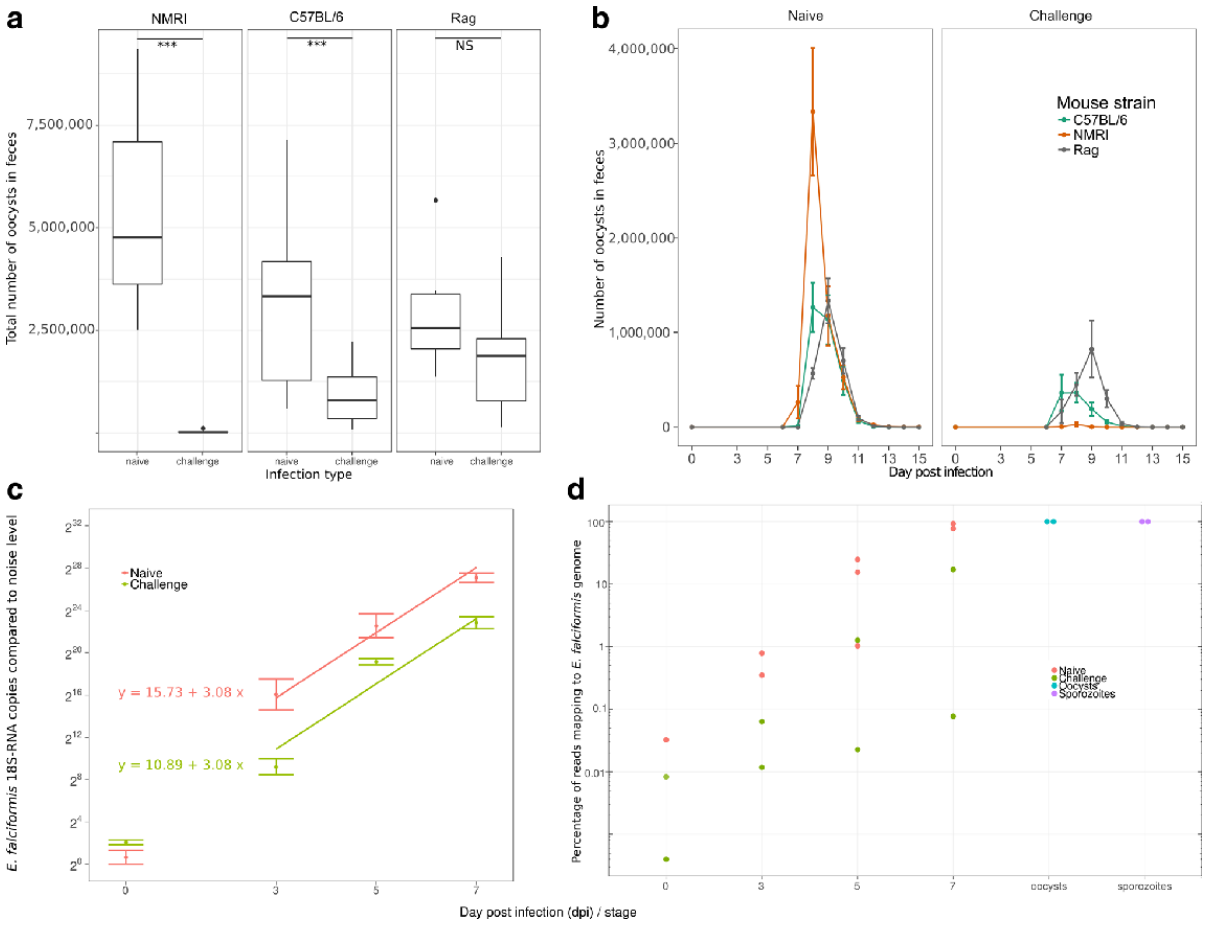

Figure 3. Oocyst output and changes in intensity of E. falciformis *infection in mouse. Oocyst counts in naïve and challenge infection are shown for three different mouse strains. For infection of naïve NMRI 150 oocysts were used, for challenge infection 1500 oocysts. For C57BL/6 and Rag1$^{-/-}$ mice 10 oocysts were used in each infection. a) Overall output of shed oocysts and (b) shedding kinetics are depicted. c) RT-qPCR data of* E. falciformis *18S in NMRI mice displays an increase in parasite mRNA over the course of infection. Significantly less parasite 18S transcripts (normalized against host transcripts of house-keeping genes) were detected in challenge infected mice. Formulas and prediction lines are given for linear models. d) The percentage of parasite mRNA detected by RNA-seq increases during infection (shown for NMRI). More mRNA is detected in naïve mice compared to challenge infected mice. Sporozoites and oocysts contained ~100% parasite material.*

3.1.2 Parasite and host dual transcriptomes can be assessed in parallel

We found the increase in parasite numbers over time after infection to also be reflected by the proportion of *E. falciformis* mRNAs sequenced in the combined pool of transcripts from host and parasite (for NRMI mice in Figure 3d). Using mRNA from infected cecum epithelium we demonstrate that even early in infection (3 dpi, during early asexual reproduction) there is sufficient parasite material to detect parasite

mRNAs in the pool including host mRNAs, and to quantify individual host and parasite mRNA abundance (Table 4). The number of total (host + parasite) read mappings for individual replicates ranged from 25,362,739 (sample Rag_1stInf_0dpi_rep1) to 230,773,955 (NMRI_2ndInf_5dpi_rep1). Similar to qPCR, a minimal level of background noise in the abundance estimates of *E. falciformis* transcripts is detected in RNA-seq for uninfected mice at 0 dpi. We did not detect bias in overall mRNA abundance patterns induced by, e.g., use of different sequencing platforms (and resulting differences in overall depth of sequencing), or by groups of samples processed in parallel (experimental batches) using a multivariate technique (multidimensional scaling, MDS; Appendix 6: Figure S1). Efficient normalization was confirmed in that samples with large differences in parasite read proportions show similar transcriptome signatures. This normalization also resulted in unimodal distributions of read numbers (Appendix 7: Figure S2) in agreement with negative binomial distributions assumed for statistical modeling and testing. Remarkably, at 7 dpi before oocyst shedding peaks, samples from infected naïve mouse epithelium contained 77% and 92% parasite mRNA, i.e., drastically more mRNA from the parasite than from the host (Figure 3d and Table 3). Our transcriptomes for these late infection samples are in agreement with previously published microarray data from mice infected with *E. falciformis* (Schmid et al. 2013), as log2 fold-changes at our 7 dpi versus controls correlated strongly – for given mRNAs – with log2 fold changes at 6 dpi versus controls in that study (Spearman's $\sigma = 0.72$, $n = 9017$, $p < 0.001$; Appendix 8: Figure S3). Considering both biological differences in the experiments, such as exact time-points for sampling, and technical differences between the two methods, this correlation confirms the adequacy of using dual RNA-seq for assessing the host transcriptome in the presence of large proportions of parasite mRNA. Below, we first describe changes in the mouse transcriptome and suggest possible mechanisms at play. Variance in host transcriptome changes upon infection constitutes a potential environmental stimulus or stress for parasites to react on, as addressed later.

Table 3 Summary of data per sample, sorted according to number of reads mapping to the E. falciformis *genome.*

Sample[a]	Sequencing method	Batch	Total reads	Reads mapping mouse	Reads mapping *E. falciformis*	Percentage *E. falciformis*	# *E. falciformis* genes[b]
NMRI_2ndInf_0dpi_rep1	GAII	2	108,937,797	70,489,674	247	0.0004	1
Rag_1stInf_0dpi_rep1	hiseq	3	25,362,793	18,853,850	443	0.0023	2
C57BL/6_1stInf_0dpi_rep1	hiseq	3	35,731,249	25,119,348	457	0.0018	2
C57BL/6_1stInf_0dpi_rep2	hiseq	3	47,085,959	34,377,133	608	0.0018	2
Rag_1stInf_0dpi_rep2	hiseq	3	46,556,156	35,233,327	676	0.0019	2
NMRI_2ndInf_0dpi_rep2	hiseq	3	58,122,244	40,794,245	3406	0.0083	51
NMRI_2ndInf_3dpi_rep1[c]	hiseq	3	57,934,016	40,544,287	4803	0.0118	95
NMRI_2ndInf_5dpi_rep2[c]	hiseq	3	63,965,539	48,289,181	10,941	0.0227	407
NMRI_1stInf_0dpi_rep1[c]	GAII	1	82,364,585	55,176,243	17,954	0.0325	701
NMRI_2ndInf_3dpi_rep2	hiseq	3	65,548,826	46,171,909	29,548	0.0640	1580
NMRI_2ndInf_7dpi_rep2	hiseq	3	67,487,466	51,722,265	40,091	0.0775	1836
Rag_1stInf_5dpi_rep1	hiseq	3	38,651,359	29,982,453	63,024	0.2098	2548
Rag_1stInf_5dpi_rep2	hiseq	3	34,779,832	25,297,803	99,000	0.3898	2828
C57BL/6_1stInf_5dpi_rep1	hiseq	3	40,904,388	29,319,604	185,969	0.6303	4173
Rag_2ndInf_5dpi_rep1	hiseq	3	50,049,848	37,093,621	192,856	0.5172	4167
C57BL/6_1stInf_5dpi_rep2	hiseq	3	29,511,368	18,062,349	215,696	1.1801	3823
C57BL/6_2ndInf_5dpi_rep1	hiseq	3	35,148,432	25,660,184	262,909	1.0142	4563
NMRI_1stInf_3dpi_rep1	GAII	1	73,236,430	49,993,358	394,384	0.7827	5220
NMRI_1stInf_3dpi_rep2	GAII	2	160,709,694	117,791,044	413,051	0.3494	4862
NMRI_1stInf_5dpi_rep2	GAII	2	119,902,722	76,419,774	794,570	1.0290	5333
NMRI_2ndInf_5dpi_rep1	GAII	2	230,773,955	143,186,486	1,846,840	1.2734	5533
NMRI_2ndInf_7dpi_rep1	hiseq	3	70,366,762	41,467,146	8,634,201	17.2335	5875
NMRI_1stInf_5dpi_rep1	GAII	2	76,702,168	47,037,087	8,669,701	15.5631	5700
Sporozoites_rep2	GAII	0	19,551,681	8656	11,470,604	99.9246	5513
NMRI_1stInf_5dpi_rep3	GAII	0	191,099,180	83,735,624	27,839,458	24.9513	5784
NMRI_1stInf_7dpi_rep1	GAII	1	66,505,514	3,310,666	39,400,884	92.2488	5932
Sporozoites_rep1	GAII	1	67,325,397	4334	43,774,401	99.9901	5825
Oocysts_rep1	GAII	1	68,859,802	3805	49,653,065	99.9923	5695
Oocysts_rep2	GAII	0	151,090,783	18,524	71,019,860	99.9739	5777
NMRI_1stInf_7dpi_rep2	GAII	1	139,749,046	21,699,324	73,539,445	77.2159	5943

[a]Sample names are given with information separated by underscore as follows: 1) mouse strain, 2) naïve (1st) or challenge (2nd) infection, 3) dpi (days post infection), and 4) replicate number
[b]Number of expressed *E. falciformis* genes (read counts >5)
[c]These samples were removed from downstream analyses because of uncertain infection status

The mouse transcriptome undergoes large changes upon E. falciformis *infection*

We here show that upon infection with *E. falciformis*, which induces weight loss (Appendix 9: Figure S4) and intestinal pathology in mice, the host transcriptome undergoes drastic changes affecting more than 3000 individual mRNA profiles significantly (edgeR; glm likelihood-ratio tests corrected for multiple testing, false discovery rate [FDR] < 0.01). Statistical testing for differential abundance between infected and uninfected mice revealed that differences in mRNA abundance were more pronounced (both in magnitude and number of genes affected) at the two later time-points post infection (Table 4, Figure 4a, Appendix 10: Figure S5). 325 mRNAs were differently abundant (FDR

< 0.01) between controls and 3 dpi, 1804 mRNAs between controls and 5 dpi, and 2711 mRNAs between controls and 7 dpi. This leads to a combined set of 3453 transcripts responding to infection. Differentially abundant mRNAs early in infection (3 and 5 dpi) were not a mere subset of genes differentially abundant later in infection (7 dpi; Figure 4a), which would be the case if the same genes were regulated throughout infection. Instead, the transcriptional profile of the mouse changes more fundamentally with different genes varying in abundance late compared to early in infection. This is in line with expression of cytokines as major regulators of immune responses (Stange et al. 2012; Ovington, Alleva, and Kerr 1995) against *E. falciformis* and with extended regulation of the mouse transcriptome upon infection (Schmid et al. 2013). To further analyze the distinct responses early and late in infection, we performed hierarchical clustering on transcript abundance patterns at different time-points post infection (Figure 4b). Three main sample clusters formed (dendrogram indicating similarities between columns at top of Figure 4b). Immune deficient Rag1−/− mice, including infected Rag1−/− samples, show an expression pattern most similar to uninfected samples. This similarity between infected and non-infected Rag1−/− samples confirms the immune deficiency phenotype; a failure to react to infection in these mice, and suggests a strong influence of the adaptive immune system on overall transcriptional responses. Surprisingly, these patterns indicate that innate immune responses and other B- and T-cell independent processes play detectable though relatively small roles (mouse gene cluster 4; Mm-cluster hereafter, Figure 4b) in shaping the mouse transcriptome upon *E. falciformis* infection.

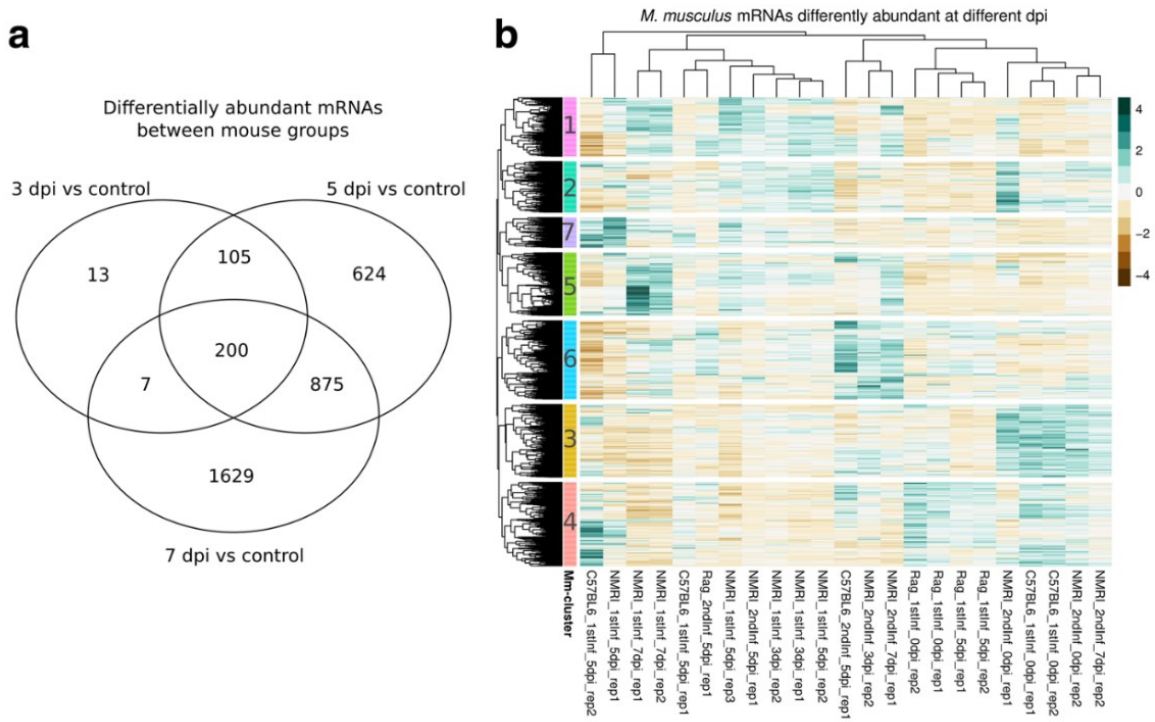

Figure 4. Differentially abundant mouse mRNAs and clustering thereof. a Venn diagram visualizes the overlap between genes showing differential abundance (FDR < 0.01; edgeR glm likelihood-ratio tests) between i) uninfected controls and different time-points post infection and ii) between different time-points and the sum of all genes reacting to infection. Controls from challenge infection were used. b Hierarchical clustering of differentially abundant mRNAs performed on Euclidean distances using complete linkage. Cluster cut-offs (dendrogram resolution) were set to identify gene-sets with profiles interpretable in relation to the parasite

Table 4. Number of mouse and E. falciformis *mRNAs significantly differentially abundant in different comparisons (Contrasts).*

Contrast	Number of *E. falciformis* mRNAs with FDR < 0.01	Number of mouse mRNAs with FDR < 0.01
NMRI 7 dpi vs. uninfected control		2711
NMRI 5 dpi vs. uninfected control		1804
NMRI 3 dpi vs. NMRI 7 dpi	1399	1322
C57BL/6 5 dpi vs. uninfected control		919
NMRI 7 dpi naïve vs NMRI 7 dpi challenge	0	857
NMRI 5 dpi vs. NMRI 7 dpi	2084	732
Rag1$^{-/-}$ vs C57BL/6		362
NMRI 3 dpi vs ctrl		325
C57BL/6 5 dpi naïve vs C57BL/6 5 dpi challenge	0	175
Rag1$^{-/-}$ 5 dpi vs control		42
NMRI 3 dpi naïve vs NMRI 3 challenge	1	18
NMRI 3 dpi vs. NMRI 5 dpi	103	0
NMRI 5 dpi vs. oocysts	3691	
Sporozoites vs. oocysts	3532	
NMRI 3 dpi vs. oocysts	3303	
NMRI 7 dpi vs. oocysts	3202	
NMRI 7 dpi vs. sporozoites	2663	
NMRI 5 dpi vs. sporozoites	1726	
NMRI 3 dpi vs. sporozoites	1705	
NMRI control vs. C57BL/6 control	13	

Empty cells indicate that comparison is not applicable

Responses to parasite infection differ between immunocompromised and immune competent mice

The self-limiting nature of *E. falciformis* infection and host resistance to reinfection ((Ovington, Alleva, and Kerr 1995) and Figure 3a) makes it interesting to analyze transcriptomes of immune competent hosts in depth. On 3 and 5 dpi, mRNAs of two gene clusters have overall high abundance in samples of all immune competent infected animals (Mm-

clusters 1 and 2). Other mRNAs (Mm-clusters 3 and 4) show lowered abundance in all those infected samples. Gene Ontology (GO) terms enriched among the mRNAs which become more abundant only early in infection (Mm-clusters 1 and 2) are, e.g., "stem cell population maintenance", "mRNA processing", and "cell cycle G2/M transition", indicating tissue remodeling in the epithelium. This is expected in an infection which damages epithelial tissue (Blagburn and Todd 1984; Stange et al. 2012; Stange 2012), but the early onset of these reactions is noteworthy. In addition, terms such as "regulation of response to food" are enriched (Appendix 11: Table S1). This is interesting since weight losses and malnutrition are generally common during parasitic infections (Stephenson, Latham, and Ottesen 2000; Aloisio et al. 2006), also in *Eimeria* spp. infections (Stange 2012; Preston-Mafham and Sykes 1970; Sharman et al. 2010), and weight loss was also seen in the present study (Appendix 9: Figure S4). Genes whose mRNA levels decreased in abundance upon infection (Mm-clusters 3 and 4) indicate induction of IL-1 and IL-6, which are involved in inflammation, including T- and B-cell recruitment and maturation, and broad acute phase immune responses (Appendix 11: Table S1). IL-6 has also been shown to support tissue repair and inhibit apoptosis after epithelial wounding (Kuhn et al. 2014). In addition, IL-6 is linked to Th17 responses (Park et al. 2005) which are known to play an important role in responses to *E.falciformis* (Schmid et al. 2013; Stange et al. 2012). It is therefore surprising to see these cytokines being downregulated, and it might indicate host regulatory functions to limit e.g. tissue damage due to inflammation Further terms indicate a regulation of transforming growth factor-β (TGFβ) which is important for wound healing in intestinal epithelium (Beck et al. 2003), epidermal growth factor (EGF) and tumor necrosis factor (TNF), which regulate proliferation of epithelial cells and inhibit apoptosis in epithelial cells (Suzuki et al. 2010; Kaiser and Polk 1997). Inhibition of Notch signaling, which is also highlighted by GO terms, has been shown to alter the composition of cell-types in the epithelium towards Paneth and Goblet-like cells (VanDussen et al. 2012). Although speculative, several of the GO terms (e.g. "calcineurin-NFAT signaling cascade", "Inositol-phosphate mediated signaling", "Notch receptor processing" in addition to those mentioned above) annotated to genes whose mRNA levels change in abundance upon early infection (Mm-cluster 3 and 4) can be linked to explain fundamental mechanisms. Inositol signaling can lead to release of calcium and calcineurin-dependent translocation of NFAT to the nucleus; and there to activation of NFAT target genes in T-cells, but also many other cell types (Macian 2005). In addition, changes in the host epithelium do take place when cells are invaded by, e.g., *E. falciformis*, but also generally by pathogens, and this is reflected

in the stem-cell and cell cycle-related GO terms described above for Mm-clusters 1 and 2. Further investigation of the role of the processes and molecules highlighted here will contribute to better understanding of epithelial responses to intestinal intracellular parasitic infection. Interestingly, in T- and B- cell deficient hosts, the same four groups of genes described above (Mm-clusters 1–4, Figure 4b), which are responsible for these dominating responses in immune competent hosts show no differences between infected and non-infected immune deficient animals.

Adaptive immune responses characterize late infection

Pronounced transcriptional changes in the mouse host occur late in infection in immune competent animals (Table 4 and Mm-cluster 5 in Figure 4b). Annotated processes and functions (GO terms) for genes with increased abundance at 7 dpi reflect the expected onset of an adaptive immune response (Appendix 11: Table S1). As late as 5 dpi, genes responsible for these enrichments are still low on mRNA abundance. This confirms a strong induction of immune responses, particularly adaptive immune responses, between 5 and 7 dpi. This result is well in line with previously described immune responses to infection with *Eimeria* spp. (Rose 1974; Blagburn and Todd 1984; Rose, Hesketh, and Wakelin 1992; Gadde et al. 2009; Sühwold et al. 2010; A. L. Smith and Hayday 1998b; a. L. Smith and Hayday 2000).

Protective responses occur earlier in challenge infected than in naïve hosts

Transcriptomes from three samples from early and late challenge infection show the same distinct profile of elevated mRNA abundance at 3, 5 and 7 dpi (Mm-cluster 6, Figure 4b). The underlying mRNAs are highly enriched for GO terms for RNA processing, e.g., splicing, which indicates post-transcriptional regulation. In addition, terms for histone and chromatin modification are enriched (Appendix 11: Table S1). This, along with less oocyst shedding during challenge infection, suggests that protective immune responses in challenge infected animals are regulated both at the transcriptional and post-transcriptional level. The high abundance of these mRNAs at different time-points post infection in wild type hosts (NMRI) further indicates that protective immunity is similar at these time-points. Possibly, induction and chronologic differences in challenge infected animals occur before 3 dpi. The completely cleared infection in some samples (Table 3; and unexpected clustering of e.g. NMRI_2ndInf_7dpi_rep2), apart from clearly demonstrating protection, also supports an early

timing of this response upon challenge infection. However, the distinct shared profile at the investigated timepoints (days 3, 5, and 7) does show that the protective response is still detectable at the transcriptional level several days after the challenge.

3.1.3 A framework to interpret *E. falciformis* transcriptomes is provided by orthologues in the Coccidia *E. tenella* and *T. gondii*

To establish *E. falciformis* as a model for coccidian parasites, transcriptome profiles of orthologue genes from closely related parasites can help to draw parallels between life cycle stages. This can be informative in predicting gene function and in analyzing evolutionary forces acting on the different life cycle stages. Therefore, we performed correlation analysis between our *E. falciformis* transcriptome and RNA-seq transcriptomes from closely related parasites at corresponding stages of their life cycles. Two datasets for the economically important chicken parasite *E. tenella* (Reid et al. 2014; Walker et al. 2015) and one dataset of the model apicomplexan parasite *T. gondii* (Hehl et al. 2015) were included. The latter was used because it is to date the only available dataset for the life cycle of *T. gondii* for multiple stages within and outside of both an intermediate host and the definitive (cat) host, and it is therefore comparable with our data. For all samples from these studies and our data, abundances of orthologous genes were correlated and Spearman's coefficient was compared (Figure 5).

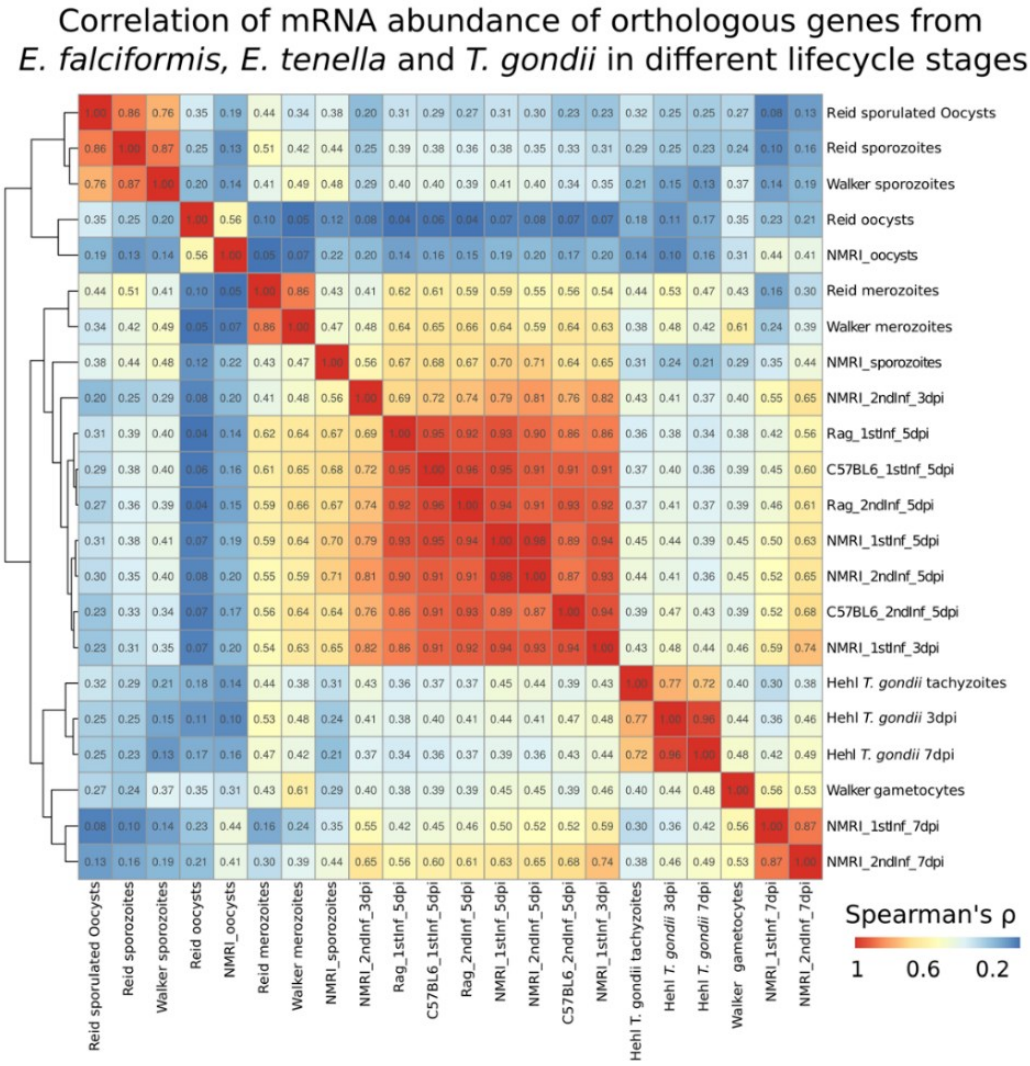

Figure 5. Correlations of E. falciformis *mRNA abundance with orthologues from other Coccidia.* E.falciformis *mRNA abundance was compared to that of orthologous genes of* E. tenella *(Reid et al. 2014; Walker et al. 2015) and* T. gondii *(Hehl et al. 2015). Correlation coefficients (Spearman's ρ) were clustered using complete linkage.* T. gondii *and* Eimeria *spp. "late infection" samples cluster together.* E. falciformis *early infection samples cluster with* E. tenella *merozoites.* E. falciformis *sporozoites cluster with* E. falciformis *early infection, whereas unsporulated oocysts cluster with* E. tenella *unsporulated oocysts.*

With the exception of sporozoites (see below), transcriptomes tend to be more strongly correlated (similar) between corresponding life cycle stages of different parasite species than between stages in the same parasite species. Orthologues in *E. tenella* and *E. falciformis* gamete stages (purified gametocytes and 7 dpi intestinal samples, respectively) are highly correlated in their expression across the two species, indicating conserved gene sets orchestrating sexual replication of the two parasites. Similarly, transcriptomes of *E. tenella* merozoites from both independent studies of that parasite are most similar to early *E. falciformis* samples, indicating similarity also during asexual

reproduction. *E. falciformis* unsporulated oocyst transcriptomes share the highest similarity with those of unsporulated *E. tenella* oocysts. *E. falciformis* sporozoites transcriptome profiles are more similar to *E. falciformis* early infection samples than to sporozoite transcriptomes of *E. tenella* orthologues. Similarities between sporozoites and early infection stages could be explained by similar biological processes, especially host cell invasion (and reinvasion by merozoites), being prepared or performed. Sporozoites are the only life cycle stages in which orthologue mRNA abundance patterns show such dissimilarities to *E. tenella* and this might indicate a higher species specificity of the genes and processes in this invasive stage. This could be a result of virulence factors being expressed in this stage, which are known to undergo rapid gene family expansion, as seen in surface antigens (SAGs) in *E. falciformis* (Heitlinger et al. 2014), *T. gondii* (Gajria et al. 2007), *Neospora caninum* (Reid et al. 2012), and other *Eimeria* spp. (Reid et al. 2014), or var. genes in *Plasmodium falciparum* (Gardner et al. 2002). Below we provide a detailed description of the *E. falciformis* transcriptome, including a discussion of genes which have been shown to be important in closely related parasites such as *E. tenella* and *T. gondii*.

3.1.4 Overall transcriptional changes in the life cycle of *E. falciformis*

Similar to the host transcriptome, differences in parasite mRNA abundance were mostly observed between late and early infection. Between 3 and 5 dpi 103 mRNAs were differently abundant (edgeR likelihood ratio tests on glms; FDR < 0.01), whereas between 3 and 7 dpi 1399 mRNAs, and between 5 and 7 dpi 2084 mRNAs were differentially abundant (Figure 6a, Table 4, Appendix 10: Figure S5). We therefore define transcriptomes as distinct at a threshold of >1000 parasite genes being differently expressed given the statistical power of our experiment (and i.e. regard the ~100 genes in 3 dpi versus 5 dpi less relevant for our analysis). Hierarchical clustering resulted in seven different gene clusters, with differently pronounced profiles in different life cycle stages (sample clusters). Confirming the analysis based on significant thresholds (differential abundance), clustering did not separate samples from 3 and 5 dpi and we thus refer to these as “early infection” and 7 dpi as “late infection”.

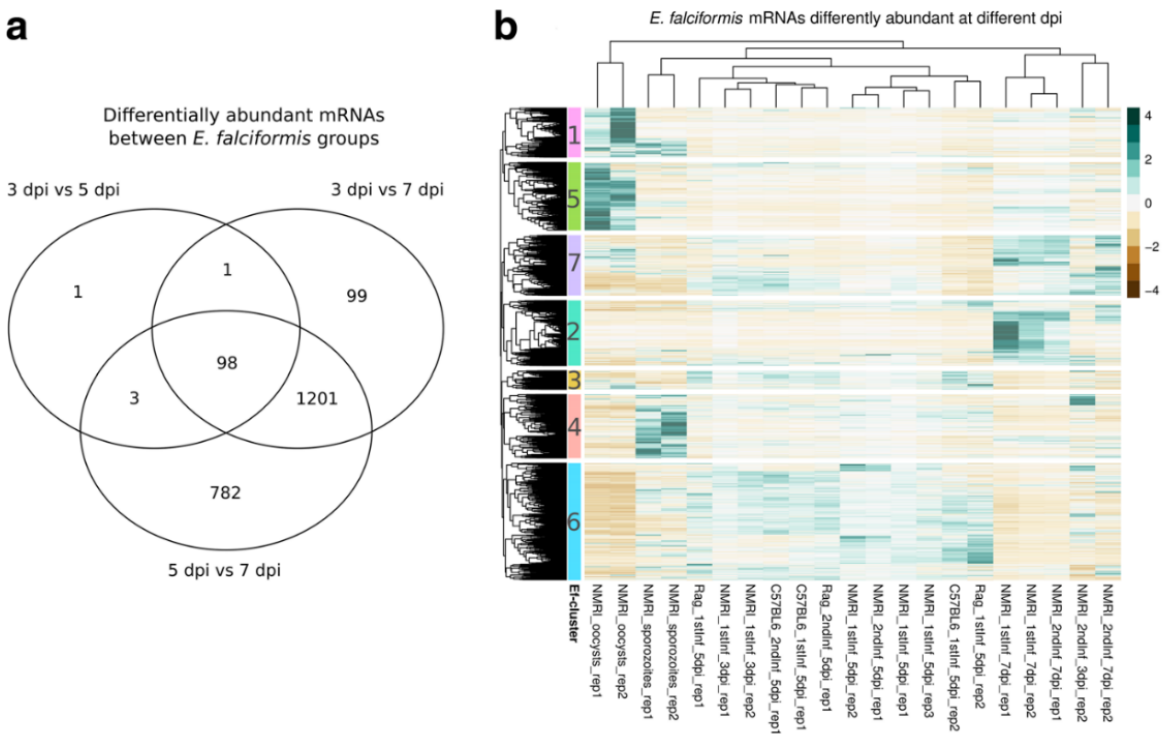

Figure 6. Differentially abundant E. falciformis *mRNAs and clustering thereof. a Venn diagram visualizes the overlap between genes showing differential abundance (FDR < 0.01; edgeR glm likelihood-ratio tests) between intracellular stages at 3 dpi, 5 dpi and 7 dpi. b Hierarchical clustering of abundance profiles for differentially abundant mRNAs performed on Euclidean distances using complete linkage. Cluster cut-offs (dendrogram resolution) were set to identify gene-sets with profiles interpretable in relation to the parasite life cycle. Clusters are represented with color on the left-hand side of rows and additional numbering is used to refer to clusters (right).*

Distinct abundance differences (>1000 genes differentially expressed and separated by sample clustering) define early infection with a single cluster of genes (parasite gene cluster 6, "Ef-cluster 6" hereafter, Figure 6b). At those time-points asexual reproduction takes place (Haberkorn 1970; Mesfin and Bellamy 1979). Two separate gene clusters define late infection (7 dpi, Ef-clusters 2 and 7). The separation of these genes into two gene clusters was driven by slightly different expression profiles during other life cycle stages while being mainly characterized by very strong expression at 7 dpi. In these samples we assume gametocytes to be present due to the peak of oocyst shedding 1 day later (Figure 3a) (Mesfin and Bellamy 1979) and similarity of these transcriptomes with purified *E. tenella* gametocytes (Fig. 3). The extracellular stages, sporozoites (Ef-cluster 4) and unsporulated oocysts (Ef-clusters 1 and 5) are clearly distinguished by high mRNA abundance. In order to assess the biological relevance of these patterns, we applied enrichment analyses for GO terms and "gene family conservation profiles" based on earlier annotations (Heitlinger et al. 2014).

Sporozoites express genes which are evolutionarily unique to E. falciformis

Sporozoites are in our study released from oocysts in vitro, after which they are capable of invading host cells. We suggest that the requirement for proteins which mediate motility and other invasion processes are reflected by their mRNA levels in the transcriptome. Due to the "host-mRNA free" nature of transcriptomes generated from sporozoites raised in vitro and deep sequencing it was possible to assess those transcripts even at relatively low mRNA expression levels observed for some of them (R. Ryan, Shirley, and Tomley 2000) (Appendix 10: Figure S5). We find that *E. falciformis* sporozoites are defined by a group of genes (Ef-cluster 4, Figure 6b) that is largely specific to *E. falciformis* (Table 4). This indicates that *E. falciformis* does not share with other species many of the abundant sporozoite genes so far described for those Coccidia. Interestingly, five out of 12 SAG gene transcripts predicted for *E. falciformis* (Heitlinger et al. 2014) are typical for sporozoites. SAG proteins, divergent or unrelated between species, are thought to be involved in host cell attachment and invasion, and possibly in induction of immune responses in other apicomplexan species (Reid et al. 2014, 2012; Mineo and Kasper 1994; Grimwood and Smith 1996; Cowman and Crabb 2006; Carruthers and Boothroyd 2007; Chow et al. 2011). In total, mRNAs encoding ten SAGs were detected as differentially abundant in our data, but in other life cycle stages than sporozoites. Such expression of particular SAGs in stages other than sporozoites has been reported for *E. tenella* (Tabarés et al. 2004). Genes also receiving attention as potential virulence factors in *E. tenella* are rhoptry kinases (RopKs) (Talevich and Kannan 2013). Transcripts of two out of ten *E. falciformis* orthologues of RopKs are highly abundant in sporozoites (Ef_cluster 4). Also in *E. tenella,* some RopKs are expressed predominantly in sporozoites and have been shown to be differentially expressed compared to *E. tenella* intracellular merozoite stages (Oakes et al. 2013). For genes with orthologues known to be important in other Coccidia, e.g., SAGs and RopKs, orthologues indicate a molecular function, but the biological relevance of their expression in *E. falciformis* remains unclear. For the overall biological functions of sporozoite genes (Ef-cluster 4), GO enrichment data suggests ATP production and biosynthesis processes as dominant features (Appendix 12: Table S2). In addition, this invasive stage is characterized by "maintenance of protein location in cell" and GO terms which indicate similar biological functions. Possibly, this reflects control of microneme or rhoptry protein localization as sporozoites prepare for invasion. The genes driving the enrichment of those processes (annotations) would be good candidates

for mechanistic studies to investigate apicomplexan and *E. falciformis* specific invasion processes. Sporozoites therefore display a transcriptome indicative of large requirements for ATP and production of known virulence factors such as SAG and RopKs and are characterized by expression of species-specific genes. Genes typical for the sporozoite stage displayed a species-specific profile with the respective gene families absent outside *E. falciformis* (Table 4). This mirrors our analysis of orthologous genes, in which sporozoites were the only life cycle stage not displaying strong cross-species correlation in their transcriptome. This suggests that traits involved in host cell invasion may have evolved quickly, and rapidly became specific for a parasite in its respective host species or target organ niche.

Growth processes dominate the transcriptome during asexual reproduction

Invasion of epithelial cells by sporozoites is followed by asexual reproduction leading to a massive increase in parasite numbers between 3 and 5 dpi, when several rounds of schizogony take place in a somewhat unsynchronized fashion (Haberkorn 1970; Mesfin and Bellamy 1979). In early infection, and similar to sporozoites, mRNAs annotated for biosynthetic activity are enriched, but different genes/mRNAs are contributing to enrichment of similar GO terms compared to sporozoites (Appendix 12: Table S2). Enrichment of terms referring to replication and growth-related processes (biosynthesis) highlights the parasite's expansion during schizogony. Amongst early infection high abundance mRNAs, we found four out of ten RopKs which are predicted in *E. falciformis* (Heitlinger et al. 2014). This is the largest number of RopKs in any one group of differentially abundant mRNAs in our analysis and they constitute a statistically significant enrichment (Fisher's exact test; $p < 0.001$). Three of these have orthologues in *T. gondii*: ROP41, ROP35 and ROP21 (Taylor et al. 2006; Saeij et al. 2007; Fleckenstein et al. 2012; Fox et al. 2016). Our data gives a first overview of expression patterns for *E. falciformis* RopKs and offer a good starting point for functional analysis of these virulence factors in *Eimeria* spp.

Gametocyte motility dominates the transcriptome late in infection

Two *E. falciformis* gene clusters show a distinct profile characterized by high mRNA abundance on 7 dpi (Ef-clusters 2 and 7; Figure 6b). Both clusters display low mRNA abundance in other life cycle stages, especially in oocysts and sporozoites. Enriched GO terms such as "movement of cell or subcellular component" and "microtubule-based

movement" along with terms suggesting ATP production (e.g. "ATP generation from ADP") indicate the presence of motile and energy demanding gametocytes in these samples. Peptide and nitrogen compound biosynthetic processes along with "chitin metabolic process" (Appendix 12: Table S2) also suggest that the parasite produces building blocks for oocysts and their walls in this stage. Our data confirms findings of Walker et al. (2015) in *E. tenella* gametocytes: these authors also identified cytoskeleton related and transport processes as upregulated in gametocytes compared to merozoites or sporozoites (Walker et al. 2015).

Oocysts are characterized by cell differentiation and DNA replication processes

Oocysts are the infective stage in the life cycle of Coccidia. They are shed with feces as unsporulated, "immature", capsules, and in the environment they undergo sporulation – meiotic and mitotic divisions (Duszynski 2011) – and become infective. Our oocysts were purified in the unsporulated stage from passage in lab mice. Two expression clusters of mRNA are highly abundant in this stage (Ef-clusters 1 and 5; Figure 6b). One of these oocyst gene sets (Ef-cluster 5) is enriched for apicomplexan-shared orthologues (Table 5) and for GO terms such as "DNA repair", "protein modification process" and "cell differentiation", supporting that expected sporulation processes have been initiated. The same cluster is also the only cluster which is enriched for transmembrane domains (Fisher's exact test, FDR < 0.001).

Table 5. Enrichments and underrepresentation of species or species-group orthologues in E. falciformis *gene clusters.*

E. falciformis cluster	Conservation category	Odds ratio	*p*-value	FDR
Ef-cluster 2 (up at 7 dpi)	Conserved	0.67	9.03E-06	1.90E-04
Ef-cluster 4 (up in sporozoites)	Conserved	0.72	2.44E-04	1.71E-03
Ef-cluster 7 (up at 7 dpi)	Conserved	1.72	1.11E-10	4.65E-09
Ef-cluster 2 (up at 7 dpi)	ApicomplexaC	0.45	1.84E-04	1.71E-03
Ef-cluster 5 (up in oocysts)	ApicomplexaC	1.86	3.76E-05	5.26E-04
Ef-cluster 4 (up in sporozoites)	*E. falciformis*	3.05	2.38E-04	1.71E-03
Ef-cluster 1 (up in oocysts)	*Eimeria*	0.68	1.83E-03	9.59E-03
Ef-cluster 6 (up in early inf)	Apicomplexa	1.46	1.11E-03	6.64E-03

Odds ratios higher than one indicate enrichment and smaller than one indicate underrepresentation. Conservation categories were chosen as previously described [10]. Only significant results (FDR < 0.05) are shown

E. falciformis *does not respond plastically to differences in the host transcriptome*

We show that infections with *E. falciformis* in its natural host, the house mouse, display a chronological pattern independent of the immune status of the host. This suggests genetic canalization of the number and timing of asexual reproductive cycles during schizogony. Similar observations have been reported before for a closely related parasite strain (Rose and Hesketh 1986). Beyond developmental timing, parasites appear to lack strategies for most efficient host-interactions in response to the host's immune status. This is supported by the lack of differences in parasite transcriptomes from immune competent and immune deficient hosts, or from naïve and challenge infected hosts (Figure 6b). In its core our finding of a lack of transcriptional plasticity is a negative result: we can – given our experimental design and statistical power – not reject our null hypothesis, which is the absence of differences. It is impossible to prove a negative (Popper 1980). However, using the changes across the parasite life cycle as a benchmark we can state that any change in the parasite transcriptome would be so minute to be very unlikely to correspond to an altered "infection program" or strategy. Only recently have transcriptomes been used to investigate plasticity in "infection programs", which parasites induce as a response to host signals. Since gene expression is orchestrated by the genetic makeup of an organism, plasticity in transcription – when it occurs – is likely to be an adaptation which allows the parasite to react on host stimuli and to produce an altered phenotype. We here suggest that it is useful to distinguish between such plastic (responsive) transcription programs and more "passive" forms of phenotypic change imposed on the parasite without being controlled at the transcriptional level. In our case, the extent of oocyst shedding – probably an important component of parasite fitness – appears to be attributable to "unbuffered" host impact. In a Nematode, the presence of phenotypic plasticity has for example been shown to lack a transcriptional basis (Weclawski et al. 2014), and could therefore be regarded "passive" or "unbuffered". In contrast, unicellular *Entamoeba* spp. infections of variable pathogenicity (i.e. phenotypic plasticity) did indeed manifest in transcriptional differences between the parasites under various in vitro conditions (Weber et al. 2016). Among apicomplexan parasites, different infection programs with distinct transcriptional profiles have been proposed: in *Plasmodium* spp., the parasite's transcriptome is distinct in different mouse genotypes (BALB/c and C57BL/6) and tissues within one genotype (Lovegrove et al. 2006), hence demonstrating the capability for – likely adaptive – plasticity in this parasite. Similarly, and even more closely related to

Eimeria spp., the coccidian *T. gondii* forms dormant tissue cysts (bradyzoites), a process induced by and depending on the host environment (Ferreira da Silva et al. 2008), and involving large changes in parasite transcriptomes (Buchholz et al. 2011). In addition, *T. gondii* is capable of infecting all studied warm-blooded vertebrates and all nucleated cells in those animals (David Sibley 2011) suggesting parasite plasticity in different host environments also in the tachyzoite stage. A switch from epithelial remodeling and innate immune processes to adaptive immune responses in the immune competent host, between 5 and 7 dpi, is paralleled by a switch from asexual to sexual reproduction of *E. falciformis* irrespective of host immune status. This contemporaneity might be an evolutionary adaptation of the parasite to host responses in order to complete its life cycle before the host environment becomes hostile. Such a response could be based on genetically canalized developmental timing or the parasite sensing an immune challenge and establishing a reaction plastically. Our results on parasite development support a genetically canalized developmental timing. Beyond this developmental timing, the severity of *E. falciformis* infection (measured as the extent of oocyst shedding) varies between hosts of different immune competence. We propose that adaptive plasticity would be identified as a transcriptional response. Since the parasite's transcriptome in an immune deficient host cannot be distinguished from the one in an immune competent host, we suggest that *E. falciformis* follows a non-plastic, and instead genetically canalized program in the mouse host. We therefore conclude that *E. falciformis* cannot plastically adjust infection strategies to optimize exploitation of hosts which vary in susceptibility.

Quote from (Ehret, Spork, et al. 2017) *ends here.*

3.2 Project II: *G. duodenalis*

3.2.1 Establishment of *G. duodenalis* infection in a mouse model

In order to optimize conditions for experimental *G. duodenalis* GS/H7 infections in my model animals in our facilities, experiments were carried out using the $mTOR^{flox/flox}$ (WT control; see Methods). Pilot infections served to ensure a reproducible readout of one of my most important parameters, cyst shedding, in our setting. To that end, we performed pilot infections for a number of reasons. We needed to 1) evaluate the necessity to pre-treat animals with a previously published antibiotics-mix (e.g. Barash, Maloney, et al. 2017; Barash, Nosala, et al. 2017 as well as personal communication with Steven Singer) as commonly required to establish *G. duodenalis* infection in mice. We also 2) evaluated two previously published infection doses (10^6 and $5x10^6$ trophozoites) which generated cyst shedding; 3) evaluated the possibility to use both males and females without introducing too high variance for the above specified sensitivity; 4) sampled at different time-points (6 am, 12 am and 6 pm) during the day to determine whether certain time-points generated lower variance and/or higher sensitivity for cyst detection; and finally 5) to identify the peak cyst shedding day in order to sacrifice hosts around this timepoint.

In order to reduce, replace and refine experiments, I aimed to have as low an infection dose as possible for the desired readout. I also had a preference to avoid unnecessary manipulation (antibiotics treatment), and a preference to use both males and females, to both generate sex-independent data and to reduce the number of animals required in the breeding process. For practical reasons and the relatively minor drawbacks (Adell et al. 2014), trophozoites were chosen for infection. Doses applied in two previous publications were evaluated; one was from Shukla and Sidhu (Shukla and Sidhu 2011), which used assemblage A, Portland strain I parasites, BALB/c mice (sex not reported), no antibiotics, and an infection dose of $5x10^6$ trophozoites. The other by Barash et al. (Barash, Maloney, et al. 2017) used assemblage B, GS/M/H7 trophozoites in C57BL/6J female mice, with antibiotics pre-treatment and 10^6 trophozoites. The first infection without antibiotics and the low infection dose (

Table 6) did not generate detectable cysts. We therefore proceeded with both antibiotics-treatment and the higher dose to ensure a readout and cysts were detected in all animals (Figure 7). In initial experiments, antibiotics treatment correlated with a temporary decline in weight-gain or weight-loss, and a worsened general appearance (e.g. fur and posture) in some mice for 3-4 days as a result of antibiotics treatment. Therefore, instead of pre-treating with antibiotics for 3 days as in Barash et al. (2017), I pre-treated for 7 days prior to infection since all mice had returned to their weight curve 5-6 days after introduction of antibiotics. With this protocol, no mice had fitness-scores on the day of infection.

Table 6. Pilot infection experiments overview.

Infection establishment experiment number	Mouse sexes	Antibiotics pre-treatment	Infection dose	Housing condition	Comment
1	Male and female	No	10^6	2-5 mice per cage	No cysts detected by immunofluorescence labelling and microscopy
2	Male and female	Yes, 3 days prior to infection	$5x10^6$	2-5 mice per cage	Cysts were detected in all individuals
3	Male and female	Yes, 7 days prior to infection	$5x10^6$	1 mouse per cage	Cysts were detected in all individuals
4	Male and female	Yes, 7 days prior to infection	$5x10^6$	2-5 mice per cage	Cysts were only evaluated as present or non-detectable; not quantified. Cysts were detected in all individuals

The minimum requirement here was to achieve detection of a difference of (sensitivity) 10^6 cysts/gram feces between days or groups (Appendix 1).

Antibiotics were required in our setting

An initial experiment was performed with a low infection dose and without antibiotics pre-treatment of mice. A commercial anti-*Giardia* cyst antibody was used for detection. Commercially available cysts spiked into mouse feces were included as positive controls. No cysts were detected from mice infected in this experiment.

Established cyst shedding and time of day for sampling

Based on the previously published requirement to pre-treat some mice with antibiotics in order to establish infection (Singer and Nash 2000) I now introduced pre-treatment with antibiotics. In infection establishment experiment number one and two (

Table 6) mice were pre-treated for three days prior to infection. Due to temporary observed weight losses, this time was increased to seven days prior to infection for infection establishment experiment number three and four. A meta-analysis of *Giardia* spp. infections and cysts as one readout parameter (Adell et al. 2014) had reported drastic differences in cyst shedding numbers in mice only when the infection dose was increased from 10^7 to 10^8 trophozoites. Although providing an indication for the dose change sensitivity, that study compared different experiments from different facilities, different *G. duodenalis* assemblages, and did not consider antibiotics treatments. I reasoned that a slight increase from 10^6 to $5*10^6$ could have an impact in our specific setup and increase chances to establish infection without the drawback of introducing animal suffering. I therefore simultaneously increased infection dose and introduced antibiotics pre-treatment. Cyst shedding was confirmed by microscopy in all infected WT individuals, and the kinetic was within the expected range (Figure 7 and in the literature e.g. (Shukla and Sidhu 2011).

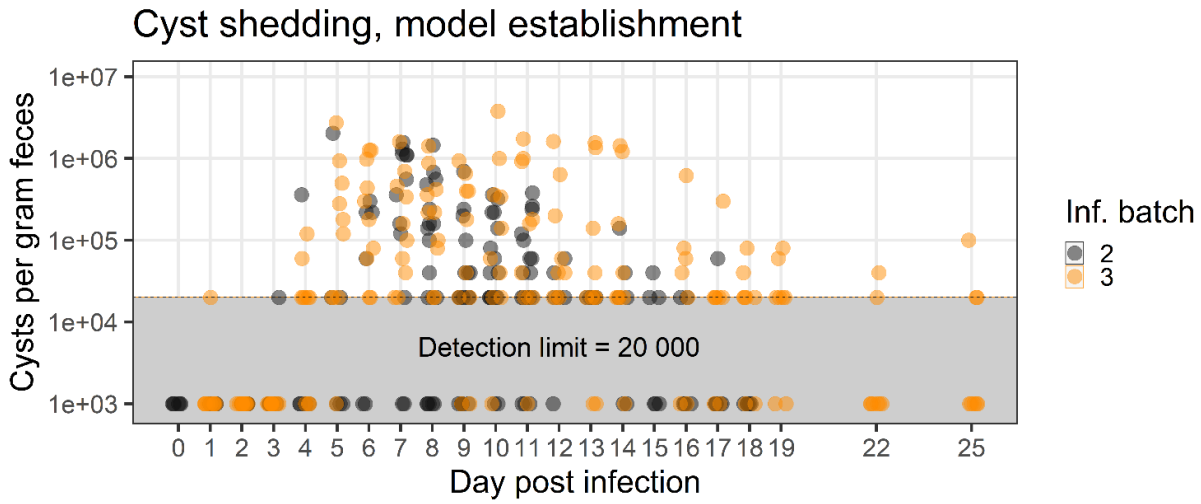

Figure 7. Pilot experiment cyst shedding in WT mice with standard facility diet. Cyst numbers were determined by microscopy of labelled cysts. Results are shown from experiments using antibiotics in mouse drinking-water, and $5x10^6$ trophozoites for oral infection. In experiment (batch) No. 2, mice were housed in groups of 2-5 mice and the experiment ended on 18 dpi, and in experiment (batch) No. 3 all mice were housed in individual cages. Cysts were detected in all individuals on at least one examined time-point in both experiments. Datapoints shown at 10^3 are zero-counts. n for batch 2 = 6 and n for batch 3 = 10.

Pooled feces collected after the 12h dark cycle with access to food were also analyzed and had slightly higher cyst counts (data not shown) but based on the large variation in numbers between individuals that sensitivity was considered to be driven by a few individuals and not informative for the desired downstream analyses.

Infection was established by oral gavage in all individuals

The cyst shedding data showed that individuals had different peak-shedding days post oral gavage infection. Although such patterns might be present also in published datasets, those generally visualize means/medians and variance/standard deviations/s.e.m., but seldomly show individual datapoints. I therefore wanted to exclude that initial infection by oral gavage had failed in some individuals, and that shedding in those was starting (and peaking) later due to infection by cysts by coprophagy from infected cage-mates. Therefore, an additional experiment was carried out with the established protocol and with individual housing of all mice. All individuals shed cysts in this experiment. Although re-infection by cysts cannot be excluded there were two major reasons to co-house the mice. One reason is to reduce animal suffering and additional stress which may also influence experimental outcomes. The other reason was to enable simultaneous infection batches of all four experimental groups (due to space limitations in the facility). Therefore, mice were onwards housed in groups of 2-5 mice per cage (with exceptions of biting/fighting males which in a few cases were housed in single cages).

Cyst shedding peaked on day eight post infection

In order to select the day for analysis of intestinal parasite loads by qPCR, cecum analysis and RNA-seq transcriptome analysis of host epithelial responses, a high-parasite load day was desired. Cyst shedding data for all pilot infections carried out with the same infections dose and antibiotics treatment were pooled and day 8 post infection determined as the highest median cyst shedding day, on which all animals shed cysts. This peak was not as distinct as can be seen in some published datasets (Shukla and Sidhu 2011), but the pattern is commonly seen in experimental infections of mice with *G. duodenalis* as well as *Giardia muris* (e.g. Roberts-Thomson et al. 1976 and personal communication Steven Singer, Georgetown University, USA, and Ivet Yordanova, Free University, Germany).

3.2.2 Manipulation of diet and mTOR signaling in host epithelia

To test the impact of arginine availability on *G. duodenalis* replication and reproduction in vivo, I infected mice with *G. duodenalis* assemblage B trophozoites (GS/H7). Mice were fed normal food (containing 1% L-arginine) or arginine-free food. Since I am interested in the role of arginine, which is sensed in the host by the mTOR network, mice with reduced mTOR expression levels in intestinal epithelia were used (applying the Cre-loxP system for mTOR-floxed animals; (Sampson et al. 2016), see Methods). See Figure 8 for an overview of the experimental design.

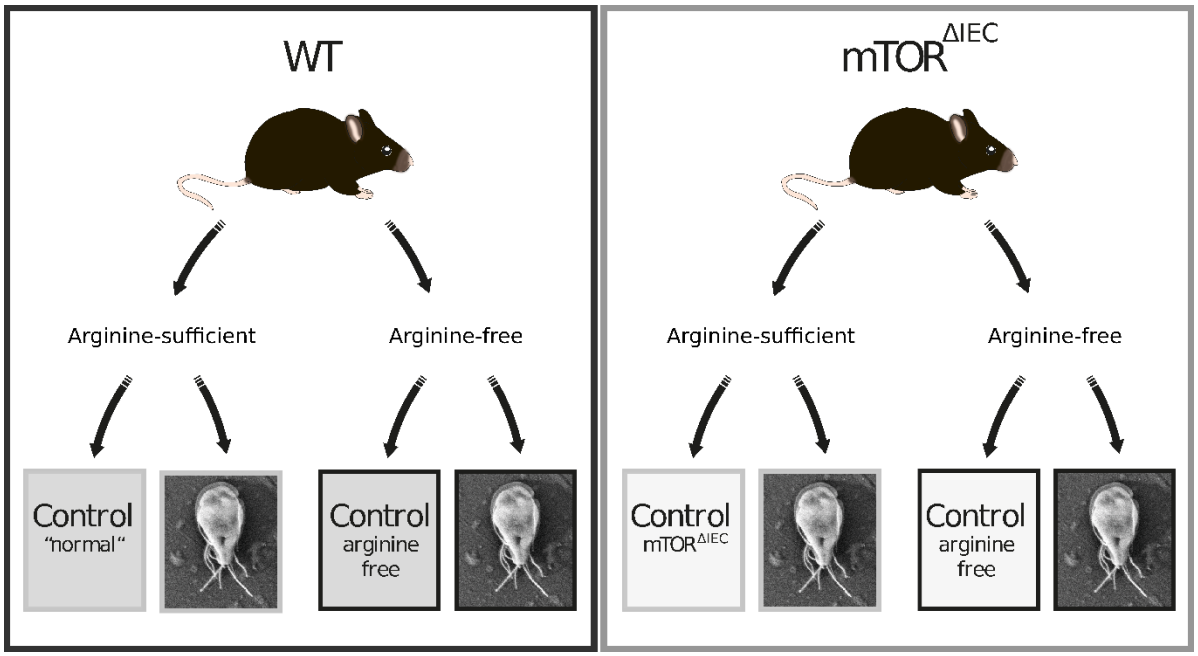

Figure 8. Overview of experimental groups. Bottom boxes represent the eight experimental groups. WT = mouse expressing mTOR flanked by loxP, mTOR$^{\Delta IEC}$ = Cre-induced mTOR deletion in intestinal epithelial cells. "Sufficient arginine" = 1% L-arginine present in food, "Arginine-free" = food lacking L-arginine, Control = uninfected, parasite image (courtesy of Scott C. Dawson, UC Davies) = group was infected with G. duodenalis *trophozoites. Illustration by Estefania Delgado Betancourt, Robert Koch-Institute.*

Mice expressing Cre-recombinase under the epithelium-specific villin-promoter were crossed with mice with a loxP-sequence flanking the mTOR region, targeting mTOR for Cre-recombinase. Genotypes of all mice were confirmed by PCR and gel electrophoresis. mTOR deletion was evaluated by histology and staining of the mTOR protein in randomly selected male and female animals. An incomplete deletion was confirmed in small intestine, with the general pattern that some villi appeared to express the protein whereas others do not (Figure 9). As expected, non-epithelial cells (not expressing villin and therefore not expressing Cre-recombinase) display normal mTOR protein expression. These phenotypes as well as the growth phenotype are comparable with the original publication of the model by Sampson et al. (2016).

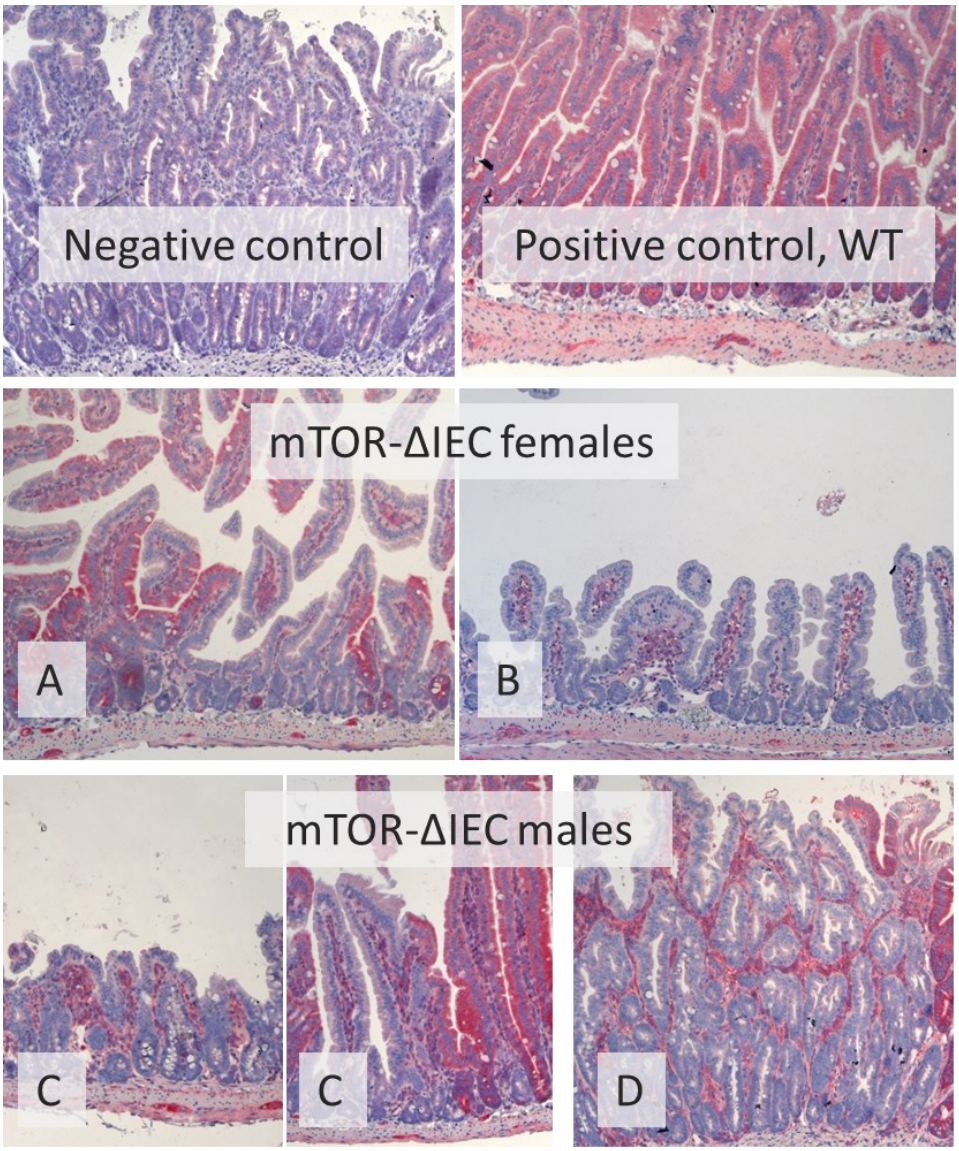

Figure 9. mTOR protein expression in in-house bred WT (upper left) with (positive control) and without (negative control) applying the primary anti-mTOR antibody, mTOR$^{\Delta IEC}$ females (middle), and males (bottom). Letters indicate individuals. This data was generated from tissue samples provided by the author which were processed by Dr. Anja Kühl, iPATH.Berlin, Core Unit of Charité - Universitätsmedizin Berlin.

Fecal analysis confirms infection in all individuals

Seven days post infection was the day before the most pronounced cyst shedding peak in the pilot experiments described above. Hosts were sacrificed on day seven post infection for analysis of small intestinal parasite load by qPCR, RNA-seq transcriptome analysis of small intestine, and analysis of parasites in cecum. Infection in all mice was confirmed on at least one timepoint between four and seven dpi by qPCR of individual fecal samples (Figure 10).

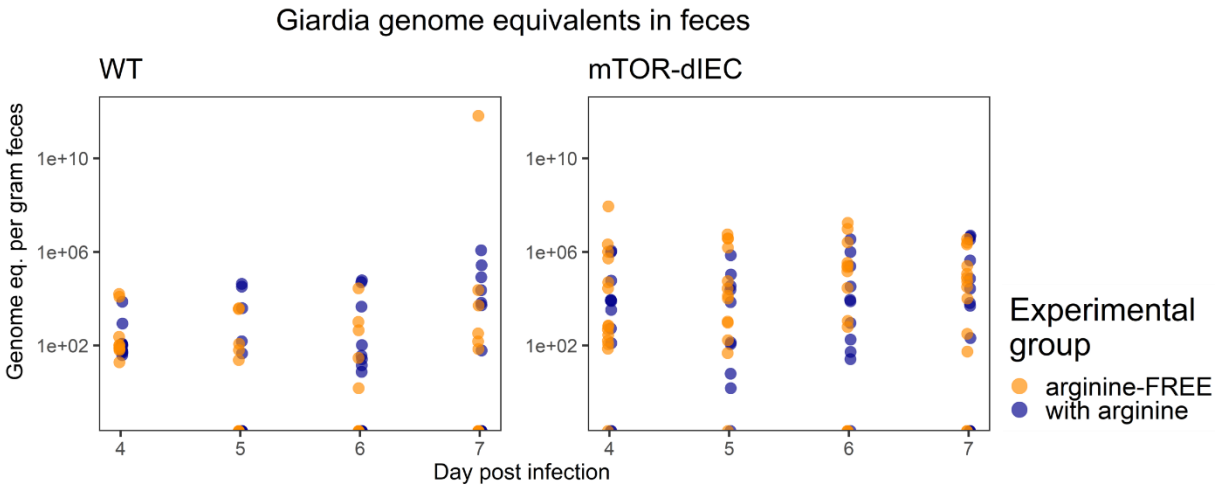

Figure 10. Genome equivalents of G. duodenalis *ssu DNA from fecal samples from individual mice. All individuals were positive on at least one analyzed timepoint, confirming parasite colonization in all mice (see raw-data in Appendix 12). Number of samples (n) vary slightly between days but are between 8 and 13 for WT on normal diet; 9 and 12 for WT on arginine-free diet; 10 and 12 for mTOR$^{\Delta IEC}$ on normal diet; 13 and 14 for mTOR$^{\Delta IEC}$ on arginine-free diet, in all groups (see raw-data). A Kruskal-Wallis rank sum test was applied on seven dpi with the comparison between WT normal diet versus WT arginine free diet rendering p=0.47 (n_{norm}=12; $n_{arg\text{-}free}$ = 12), WT and mTOR$^{\Delta IEC}$ on normal diet rendering p = 0.25 (n_{WT}=12, n_{mTOR}=12), and mTOR$^{\Delta IEC}$ on normal diet versus mTOR$^{\Delta IEC}$ on arginine free diet p = 0.83 (n_{WT}=12, n_{mTOR}=13).*

Cyst shedding does not reflect total parasite abundance

The same individual fecal samples analyzed by qPCR were used to assess cyst shedding from 4-7 dpi. A commercial *Giardia* cyst-specific detection antibody was used and manual counting was performed (Figure 11). In contrast to the detection of *G. duodenalis* DNA in feces, cysts were only detected (i.e. shedding above the detection limit of 1666 cysts/gram feces) in some of the infected individuals. In WT mice fed a normal diet (Figure 11), cyst shedding was detected on seven dpi in 32% (7/22) of the individuals. Under arginine depleted conditions (Figure 11), cysts were only detected on six dpi and in 10% (2/20) of all individuals. Statistical tests for the pooled total cyst count between WT mice on normal and arginine-free diets (Figure 13) were not significantly different, which was expected considering the high number of samples below the detection limit.

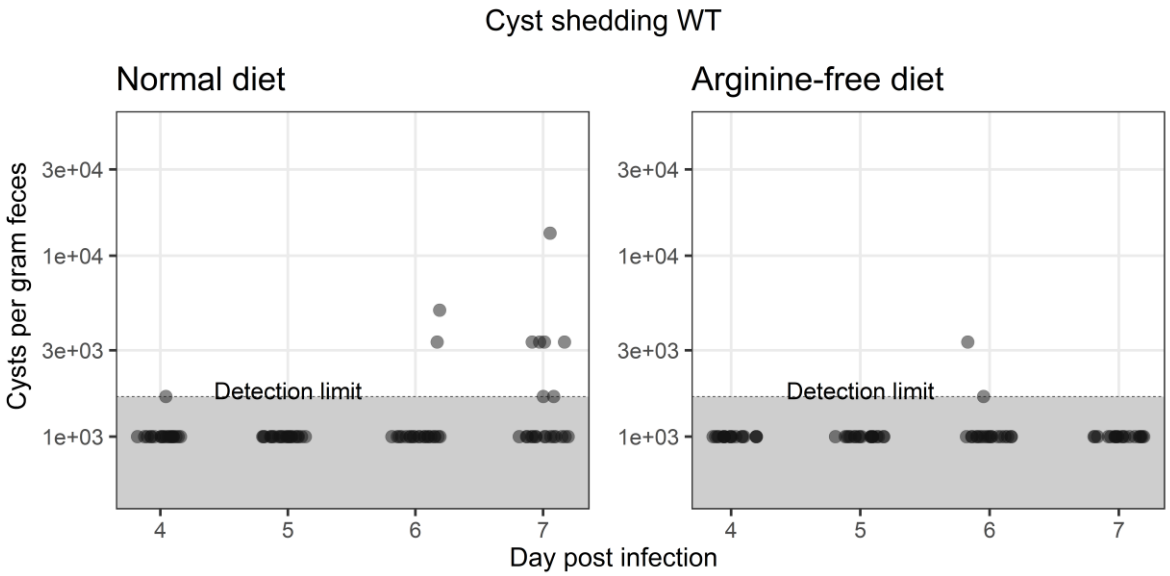

Figure 11. Cyst shedding as determined by microscopy of feces from individual WT mice. WT mice with normal food: n=22, and WT mice with arginine-free food: n=20. Data is from three independent experimental batches. WT cyst counts on seven dpi were compared with a Kruskal-Wallis rank sum test with p=0.007.

Cyst shedding is higher in mTOR$^{\Delta IEC}$ hosts

In vivo, *G. duodenalis* interacts directly with small intestinal epithelial cells. The plasticity of *G. duodenalis* could partly be assessed by investigating the parasites' capacity to benefit from weakened hosts. The epithelium-specific mTOR (incomplete) deletion model was chosen (see Introduction and Methods for more background). As for WT hosts, I analyzed cyst shedding in intestinal epithelium-specific mTOR deletion mutant mice (mTOR$^{\Delta IEC)}$. These hosts shed significantly more cysts than WT between 4 and 7 dpi (Figure 11, middle, Kruskal-Wallis rank sum test, p=0.013). There was no significant difference between the normal versus arginine-free diets in mTOR$^{\Delta IEC}$ hosts (p=1). In mTOR$^{\Delta IEC}$ mice, cyst shedding was detected on all timepoints (4-7dpi, Figure 12). On seven dpi, 42% (10/24) of the mTOR$^{\Delta IEC}$ mice shed detectable levels of cysts, compared to 32% in WT mice. In spite of the incomplete knock-out, significantly more cysts were shed in mTOR$^{\Delta IEC}$ mice fed a normal diet than in the WT control group (Figure 13).

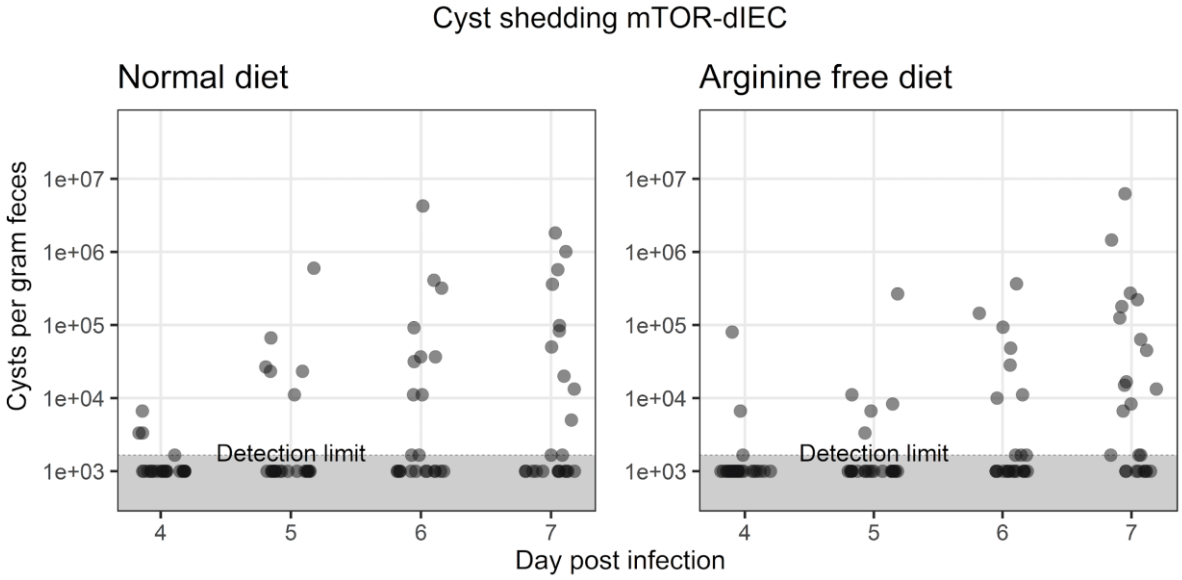

Figure 12. Cyst shedding from individual mice deficient in intestinal epithelial mTOR expression ($mTOR^{\Delta IEC}$). Data shown for $mTOR^{\Delta IEC}$ mice with normal food, n=24; and $mTOR^{\Delta IEC}$ with arginine-free food, n=25. n is one infected mouse which was sampled every day. $mTOR^{\Delta IEC}$ cyst counts between normal and arginine-free diets on seven dpi were compared with a Kruskal-Wallis rank sum test with p=0.41. The detection limit (1667 cysts/gram) indicates the theoretically lowest cysts/gram feces for which one cyst would be detected. A zero data-point should be considered to have a value between zero and the detection limit minus one (1666 cysts/gram). Data is from three independent experiments.

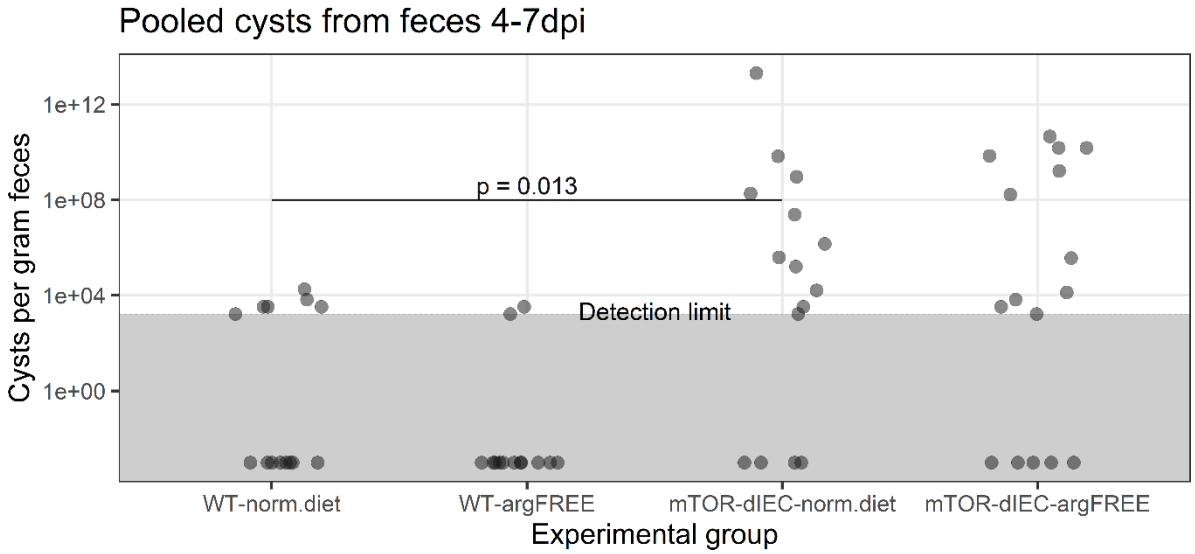

Figure 13. Pooled cyst counts from feces from 4-7dpi in all experimental groups. One data-point represents the 4-7dpi pool for one mouse. A zero data-point should be considered to lie anywhere within the grey area (see Methods). The difference between WT and $mTOR^{\Delta IEC}$ is

significant (p=0.013) whereas the difference between different diets in WT (p=0.092) and mTOR$^{\Delta IEC}$ (p=1.0) are not (Kruskal-Wallis rank sum tests).

No difference in intestinal parasite load between groups on seven dpi

Since I was interested in assessing the effect of arginine-lack on parasite replication as well as reproduction, I analyzed *G. duodenalis* genome equivalents in small intestine as a proxy for mainly trophozoite numbers. Hosts from the four infected experimental groups were sacrificed on seven dpi and DNA extracted for qPCR measurements. Except for two individuals, all samples were positive for *G. duodenalis* in small intestine on seven dpi (Figure 14). There were no significant differences between experimental groups (Kruskal-Wallis rank sum test).

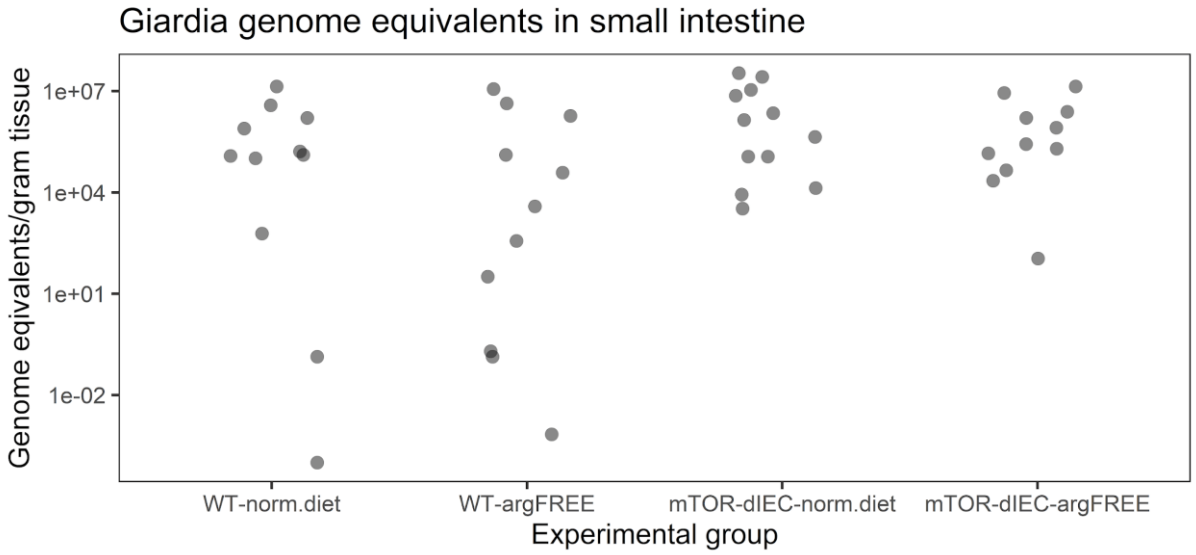

Figure 14. G. duodenalis *genome equivalents in small intestine on seven dpi. Two individuals (WT on normal and arginine-free diets, respectively) had no signal (not visualized). Tests were performed between diets in WT, diets in mTOR$^{\Delta IEC}$ respectively and WT versus mTOR$^{\Delta IEC}$ on normal diets. No differences were significant (p=0.49 and p=0.76, and p=0.30, respectively by Kruskal-Wallis rank sum test). WT, normal diet: n=11; WT arginine-free diet: n=11; mTOR$^{\Delta IEC}$ normal diet: n=11; and mTOR$^{\Delta IEC}$ arginine-free diet: n=12.*

Dietary arginine has an impact on encystation in WT but not in mTOR$^{\Delta IEC}$

On seven dpi, WT hosts on normal diets shed significantly more cysts than those on arginine-free diets (in the latter group, no cysts were detected in any individual. Kruskal-Wallis rank sum test, p=0.007, n_{normal}=22 and $n_{arg\text{-}free}$=20) on seven dpi. In contrast, parasite loads in small intestine on seven dpi are not different between any experimental groups. Compared to WT (normal diet), cyst shedding was tendentially but not significantly higher in mTOR$^{\Delta IEC}$ on seven dpi only (p=0.06, n_{WT}=22 and $n_{mTOR\Delta IEC}$= 24, data in Figure 11 and Figure 12). However, as noted previously overall (pooled) cyst shedding was indeed higher in mTOR$^{\Delta IEC}$ than in WT.

Therefore, both host epithelial mTOR and dietary arginine have an impact on *G. duodenalis* cyst shedding.

4 Discussion

The overall findings in this thesis contribute insights into the biology of two distantly related protozoan parasites, as well as an evaluation of those separate projects in the theoretical framework of phenotypic plasticity. Data interpretation and the *E. falciformis*-specific discussion for that project is included in that result section.

4.1 *G. duodenalis* responds plastically to dietary arginine

With the data presented here, I for the first time demonstrate in vivo that low or no access to arginine hampers *G. duodenalis* cyst generation and reproduction, but not trophozoite growth. It has previously been proposed that *G. duodenalis* shunts D-fructose-6P from glycolysis into cyst wall polysaccharide production during encystation, and that ADH and glycolysis both contribute to ATP synthesis during this process (Jarroll et al. 2011; Pham et al. 2017). In Figure 15, I propose an extension of this model, with the support of the data presented in my thesis. I show in vivo that access (or no access) to arginine does not appear to impact the number of intestinal stage parasites (mainly trophozoites), but arginine limitation indeed reduced the number of cysts detected in feces. Similar to my results, in vitro encystation efficiency of assemblage A WB6 parasites decreased from 4-8% to 0.1% when ADI, the first arginine catabolizing enzyme of the ADH pathway, was overexpressed. (Touz et al. 2008) This suggests that excess arginine catabolism, as in the overexpression experiment by Touz et al., is hampering encystation presumably by too rapid removal of arginine from the encystation medium. Therefore, both external (e.g. dietary) and internal (parasite catabolism) arginine restriction or depletion appear to impact *G. duodenalis* assemblage A (Touz et al. 2008) and B (this study) encystation success negatively. Supporting the hypothesis that arginine only becomes limiting during encystation are the initial experiments which characterized ADH and ADI in *G. duodenalis* Portland-1 strain, assemblage A (Schofield et al. 1992). They determined glycolysis flux and ADH-enzyme activities (ADI, ornithine transcarbamoylase (OTC), and carbamate kinase (CK)) and ADH flux in PBS by using labelled L-arginine and glucose, respectively. Interestingly here, the same pathway fluxes were determined for both pathways while adding either up to 10mM L-arginine (for glycolysis flux) or 10mM glucose (for ADH flux) with minor effects on the two pathways. The authors concluded that there does not appear to be any crosstalk between the pathways, but the alternative model presented here – crosstalk between glycolysis and ADH arginine-metabolism exclusively during encystation – is not contradictory to Schofield's et al. (1992) findings since they did not investigate the life stage switch. In addition, their data was generated using undefined growth-medium which makes it difficult to draw general conclusions.

The model presented here proposes that *G. duodenalis* is exposed to one or a number of stressors, e.g. those which have previously been shown to influence encystation: lipid starvation and/or an increase in pH (Gillin et al. 1987; Luján et al. 1996; Einarsson et al. 2016). Common

for in vitro encystation protocols is also a drastic change in growth medium (e.g. bile-free to bile-containing). In addition to the requirement for a stressor, based on the data in this thesis I propose a requirement for a minimum (and not yet defined) amount of arginine and ADH metabolism. If glucose is not a limiting resource, but there is nevertheless a need for ADH-generated ATP during encystation, it seems likely that encystation coincides with an inability of the parasite to perform glycolysis (or the downstream pyruvate metabolism, which also generates ATP). Therefore, to identify the stressor or stressors which can induce encystation, requirements for glycolysis and pyruvate metabolism, such as oxygen pressure, can be considered. In order to perform controlled experiments to identify triggers and requirements for encystation, and considering the evidence for metabolic control of encystation, a defined growth medium for in vitro cultures is most likely necessary. Experiments which add defined amounts of e.g. arginine to defined culture conditions should ideally also be carried out using a chemostat to control e.g. nutrient and oxygen levels prior to perturbing the *G. duodenalis* culture and attempting to induce encystation in a controlled manner.

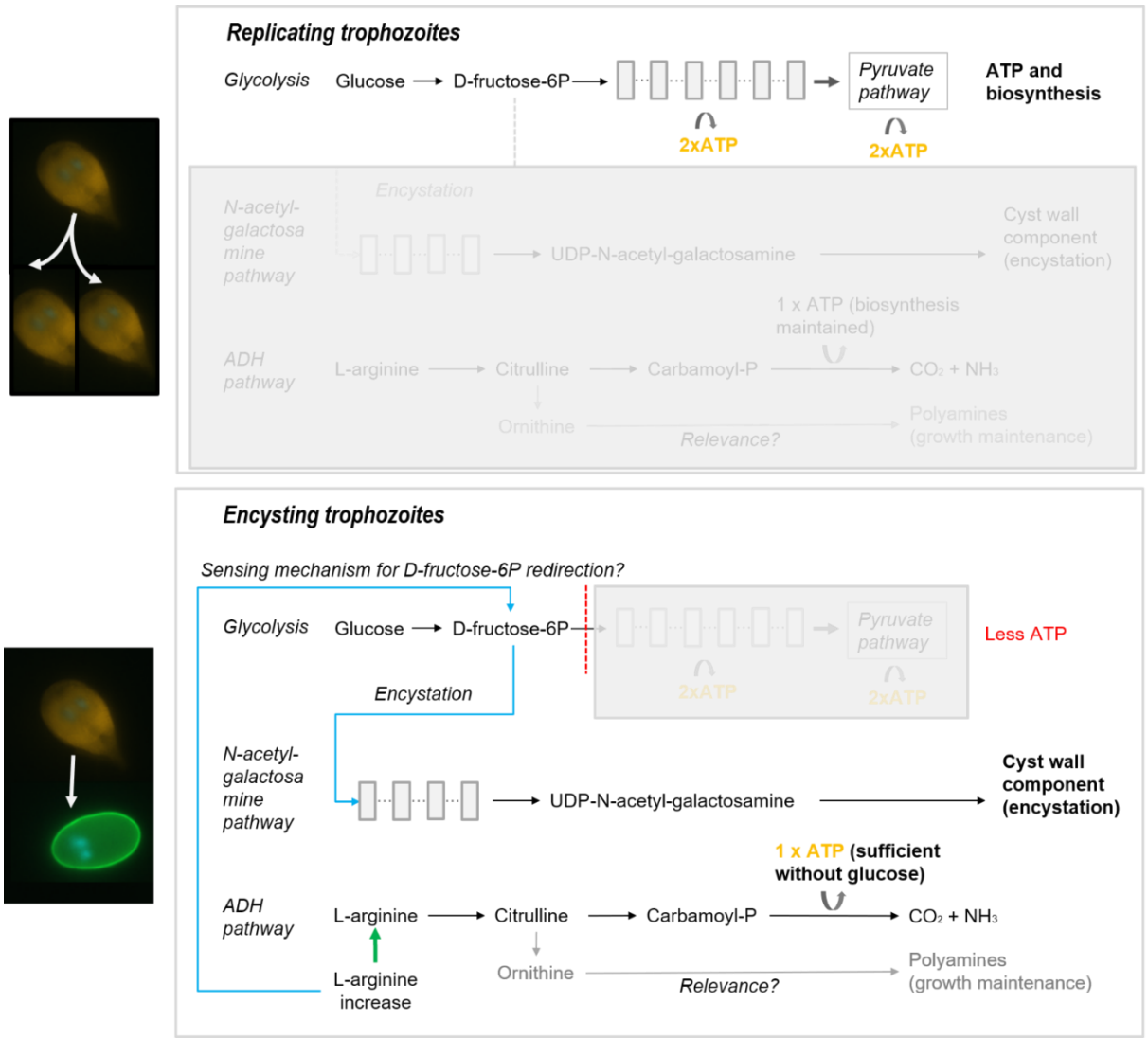

Figure 15. Proposed model for metabolic switch in G. duodenalis *upon changes in the life cycle (inspired by Pham et al. 2017). During trophozoite growth, glycolysis is proposed to dominate*

ATP generation (upper box). Upon a still poorly understood signal (see Figure 1), encystation is initiated (lower box). I propose that arginine levels will vary depending on host food intake (type, amount, and timing) and that sufficient access to arginine is a prerequisite for encystation (blue arrow). Glucose is drawn as being shunted (blue arrow from D-fructose-6P) into cyst wall biosynthesis through the N-acetyl-galactosamine pathway (Jarroll et al. 2001). Upon arginine influx to the infectious site, I propose a resulting major ATP generation from ADH, and glucose utilization mainly for cyst wall biogenesis. Note that some individual parasite cells may have better or worse access to arginine due to, e.g., their position in a colony of parasites which would contribute to variation in the encysting phenotype. Little grey boxes in pathways represent metabolite intermediates. Large shadowed boxes indicate proposed low pathway activity. For more detail, enzymes involved and expression data I refer to Jarroll et al. 2001 and Pham et al. 2017.

Arginine as substrate for nitric oxide is not determining infection

Arginine is certainly utilized by both parasite and host. However, it is not clear whether competition for the amino acid occurs or how relevant its role is during infection. In contrast to the above parasite-focused explanatory model, a role for arginine in host protection as the substrate for NOS has also been proposed for arginine (e.g. Eckmann 2003; Stadelmann et al. 2012). The idea is supported by in vitro observations of NO-induced trophozoite growth inhibition, inhibition of excystation, but parasite inhibition of NO-release by host cells has also been shown. Combined, this has been interpreted as a host-parasite interaction through arginine and NO (Eckmann 2003). This idea focuses on arginine for host defenses and (implicitly) considers the presence of ADH in *Giardia* spp. to indicate a parasite "response" (on the evolutionary time-scale) rather than arginine having a significant value of its own in the parasite (although the paper does recognize the efficient transport and catabolism of arginine in the parasite). The NO-hypothesis predicts (again implicitly) better parasite growth when arginine is absent, since host defenses would not be as strong without the substrate for NO. My data contradicts a model in which arginine *mainly* is important for host defenses. Host data on local gene expression or systemic cytokine profiles from the different experimental groups in the current thesis could provide indications on the impact on the host. However, for the parasite the absence of arginine was harmful for replication in WT and (with my sensitivity) made no difference in $mTOR^{\Delta IEC}$ hosts. This is consistent with arginine mainly being important for the parasite and the host relying on other defenses.

Is polyamine signaling a contributor to encystation commitment?

In addition to the metabolic shift during life cycle change from trophozoite growth to encystation proposed by Jarroll (2011) and Pham et al. (2017) and me, secondary metabolites of arginine metabolism may contribute to life stage switching in *G. duodenalis*. In contrast to assemblage A, the assemblage B genome has an arginase gene (Franzén et al. 2009), suggesting an important function for e.g. ornithine and polyamine metabolism. Both ADH and arginase catabolism generate ornithine, which is a precursor for polyamines in eukaryotes. In several

eukaryotic systems, high growth rates – here corresponding to replicating trophozoites – correlated with high polyamine levels (reviewed in (Goyns 1982)). Goyns further proposes polyamines to mainly be growth supporting (not inducing) since a decrease in polyamine levels only influences (mammalian) cell survival after 2-3 generations. Applying this idea to *G. duodenalis*, utilization of ornithine from catabolized arginine for polyamine generation is a possibility which could support trophozoite growth maintenance. If the ADH flux increases (as proposed during encystation) a shift in ornithine availability and subsequently polyamine levels (and the balance between different types of polyamines) could occur and support commitment to encystation. Supporting a role in the *G. duodenalis* life cycle, addition of a 10mM putrescine analogue (which caused growth inhibition in *Trypanosoma cruzi* and an anaerobic trichomonad) caused morphological abnormalities upon cell division and other growth defects in trophozoites in vitro (Maia et al. 2008). Unfortunately, encystation was not investigated in that study.

In my model (Figure 15), low ADH-activity during trophozoite growth is predicted and ornithine in this stage would, in the model, come mainly from arginase activity (for assemblage B parasites). Available arginine might be utilized by the assemblage B arginase and generate polyamines to support trophozoite growth and continue generating ATP from glucose. If activation of the highly efficient ADH pathway upon encystation leads to intra-parasite competition for arginine, and if ornithine (as polyamine precursor) is more efficiently generated by arginase than by OCT in the ADH pathway, a decrease in polyamine concentrations could result. If the trophozoite growth-maintaining signal is removed or reduced, this could support commitment to encystation in *G. duodenalis*. It has been reported that *G. duodenalis* (assemblage A) commits to encystation in vitro, i.e. does not return to replicative growth once the encystation program is initiated even if the triggers for encystation are removed (Sulemana, Paget, and Jarroll 2014). Investigations into *G. duodenalis* arginase activity (as compared to OCT) and changes in polyamine levels during the transition from trophozoite replicative growth to encystation would provide first clues to test this hypothesis. In such studies, a comparison between assemblage A and B encystation would be informative, since no arginase gene has been found in assemblage A. Analysis of differences in the activity of involved metabolic enzymes and comparisons of (theoretical) metabolic fluxes could help to generate specific, testable hypotheses concerning assemblage-specific mechanisms. If a polyamine-level encystation-commitment mechanism exists in *G. duodenalis*, its contribution to commitment is likely to be slow and supportive rather than constituting the initial and major regulatory mechanism.

Diets have an impact on cyst shedding

The relatively small difference between arginine-containing and arginine-free diets in this study is likely due to the intrinsically low cyst shedding in this model. It turned out that the change from our facility's standard mouse diet to control food for experiments (which should be highly similar to the standard diet) reduced cyst shedding in WT control animals; particularly the number of animals with detectable cysts in feces was reduced from 100% to 32% (compare Figure 7 and Figure 11). Major differences between the standard food used for normal housing

and my experimental diets (control and arginine-free) are shown in Table 7 (see also Appendix 13). When selecting the experimental diets, a crystalline diet was preferred for exact control of amino acid content. This means that each amino acid was added separately, and not as an estimated content of total protein added to the diet. A control diet could be designed to keep the total nitrogen/(artificial) protein content equal by adding more of other amino acids. Another option was to accept a slight shift in concentrations of protein/total amino acid contribution-fat-carbohydrate-contents and keep amino acid percentages the same. Choosing to compensate nitrogen/protein content has the drawback of also manipulating other amino acids, meaning a change in the major class of molecules of interest in this experiment. Especially since experiments of mTOR nutrient sensing were planned, this was not an appealing choice. I instead opted for the latter, as is reflected in the differences in total fat, protein and carbohydrates seen in Table 7. The 1% L-arginine diet was the standard concentration for the crystalline complete diet from the provider.

Table 7. Diet comparison. A selection of nutrient contents is presented here. For full specifications, see Appendix 4.

	Standard facility diet ("Fortified energy rich for mouse")	Experimental control diet	Experimental arginine-free diet
Fat (% in diet)	16	18	18.4
Protein (% in diet)	27	16	14.4
Carbohydrates (% in diet)	57	66	66
L-arginine (% in diet)	1.5	0.98	0

Given that all WT hosts shed cysts in pilot experiments with the standard facility diet, and only 32% in the experimental setting, these dietary differences appear to have had a drastic effect on cyst shedding. Since arginine content also varied, there is an actual tendency in this data indicating that most cysts were detected in hosts with a 1.5% arginine content and the least cysts were detected in WT hosts with 0% arginine diets. However, since other nutrient components also changed between 1.5% and 1% arginine content, and the experiments were not performed in parallel or otherwise designed for direct comparisons, this is speculative. It can also not be excluded that other factors, e.g., microbiota composition of mice, also changed during the ~10 months period during which experiments were carried out. However, given the strictly standardized breeding, housing and animal handling procedures in our facilities I consider that explanation alone unlikely. Diets exerting the observed effect on cysts *through* changes in microbiota composition rather than via a direct nutrient-access effect on the parasite is a more likely explanation and an interesting research question. Major improvements in the experimental setup include 1) optimizing the control diet for high cyst shedding while ensuring the possibility to remove arginine without otherwise changing the diet; and 2) including microbiota analysis of uninfected mice with normal and arginine-free diets.

Intestinal epithelium mTOR deficiency is beneficial for G. duodenalis *cyst shedding*

G. duodenalis generated more cyst shedding in (tissue specific) mTOR-deficient hosts ($mTOR^{\Delta IEC}$) than in WTs. mTOR is important for nutrient sensing and inducing arginine uptake (Visigalli et al. 2007), as well as cell cycle regulation and cell proliferation (Laplante and Sabatini 2012), which are central functions in intestinal epithelium. The epithelium is 1) responsible for whole-organism nutrient uptake, and 2) rapidly renewing (e.g. Blander 2016). Therefore, mTOR-deficient hosts can be considered weakened in the parasite niche with regards to cell proliferation/healing and they are likely to have an altered nutrient absorption phenotype.

I show that *G. duodenalis* benefits from this host phenotype by producing more cysts. Interestingly, dietary arginine deprivation had an effect on parasite reproduction (cysts) in WT but not in mTOR-manipulated hosts (comparing arginine-sufficient and arginine-free diets between $mTOR^{\Delta IEC}$ hosts). Furthermore, the number of trophozoites was not higher in either $mTOR^{\Delta IEC}$ group compared to WT on arginine-sufficient diet, indicating that the favorable effect supported encystation but not replication. In summary, the effect on cyst numbers of arginine-free diets seen in WT was not detected in the mutant hosts.

Healthy, adult humans and mice can synthesize arginine via the intestinal-renal axis (Morris 2006). $mTOR^{\Delta IEC}$ epithelia are morphologically and cellularly different from WT and do not heal as well upon irradiation injury (Sampson et al. 2016). If *G. duodenalis* disturbed the epithelium upon attachment (support for this view is found e.g. in (Allain et al. 2017; Liu et al. 2018)), $mTOR^{\Delta IEC}$ epithelia are likely to heal worse than WT epithelia and become more leaky. Therefore, infection in these hosts could result in increased luminal nutrient levels, including arginine which is synthesized in epithelial cells, presumable also in $mTOR^{\Delta IEC}$ hosts. Such leakiness could generate an infectious niche which is equally rich in arginine in hosts fed arginine-sufficient and arginine-free diets. Such arginine leakiness being a major determinant for infection when comparing $mTOR^{\Delta IEC}$ and WT hosts in these experiments is supported by the fact that cyst numbers but not trophozoite numbers were different between WT and mutants combined with the evidence and arguments presented above for a encystation-specific role for arginine in *G. duodenalis* growth and life cycle progression. Measuring arginine levels in intestinal lumen in WT and $mTOR^{\Delta IEC}$ hosts combined with measurements of parasite regulation of ADH pathway and other encystation-specific genes would be a way to approach this hypothesis.

4.2 Phenotypic plasticity in protozoan parasites

Data on two protozoan parasites are here discussed in the context of phenotypic plasticity, i.e., the parasites' (in this case) capacity and tendency to **produce different phenotypes not due to genetic differences but in response to environmental stimuli**. The two parasite species were exposed to types of stimuli which they are likely to have been exposed to over evolutionary time-scales. In the case of intracellular *E. falciformis*, strong immune responses are well documented and were used as stimulus. For *G. duodenalis*, arginine supports parasite growth in vitro, and arginine-free host diets were used as stressors in those infections. In both studies (in mouse models), reduced oocyst (*E. falciformis*) and cyst (*G. duodenalis*) shedding was observed in more immune competent hosts and under arginine-deplete conditions, respectively.

Reduction in oocyst/cyst generation could be the result of either reduced replication in intestinal stages in the host, or of hampered oocyst/cyst formation, or even host destruction of successfully encysted parasites. For *G. duodenalis*, I excluded a difference in trophozoite replication, therefore concluding that arginine depletion acts specifically on cysts. *G. duodenalis* was able to respond plastically and opportunistically and generate more cysts in weakened hosts (mTOR mutants), which we have reason to think are providing more arginine to the parasites (see above). In contrast, *E. falciformis* was not able to generate more oocysts in hosts with lower-than-WT immune stimuli on the parasite. For *E. falciformis*, the evidence therefore suggests no (undetectable) plastic capacity to differences in immune stimulus. *G. duodenalis* is responding plastically by increasing reproductive success in the weakened mTOR-mutant host, and a leaky epithelium which makes arginine available could be causing this effect (see mTOR discussion section above). More targeted experiments are required though, to elucidate whether mTOR-deficient hosts are indeed providing the lumen and parasites with more arginine than WT hosts on normal arginine-diets. The very limited cyst shedding in WTs on arginine-free diets indicate the lower limit of *G. duodenalis* plastic capacity with regards to arginine and suggest that a minimal arginine availability is necessary for encystation.

Active phenotypic plasticity in response to arginine in G. duodenalis

Plastic capacity in *G. duodenalis* can be an **active response** by the parasite and then a sensor for arginine can be proposed. If the effect is **passively imposed** a direct and mechanistically limited effect of arginine availability would be the case. The latter could be defined as, e.g. an isolated reduced flux through ADH and arginine catabolism of arginine resulting in less products in those pathways but no or marginal effects beyond these reactions. However, since arginine is consumed as substrate for ATP generation, its metabolism through ADH is linked to growth (Edwards et al. 1992), and ADH enzymes are regulated (Stadelmann et al. 2012), it can be assumed that variations in arginine availability are actively *sensed* and cause an active response. Responses beyond the catabolism of arginine by ADH or arginase, i.e. subsequent events are a predictable effect of changes in arginine availability. Above I propose such an active response (Figure 15) since I suggest a change in ADH flux and generation of ATP to

cause the shunting of D-fructose-6P from glycolysis into the N-acetyl-galactosamine (cyst wall component) pathway, and this in turn to induce or stimulate encystation.

Unpredictable stimuli promote phenotypic plasticity

The strategies employed by parasites to cope with external stimuli – for instance active sensing and plasticity or genetically canalized programs which disregard stimuli –will be linked to the nature of the stimulus, e.g. predictability. For immune defenses, on a coarse-grained level they might be considered predictable. They change in the type of molecules involved and intensity but also this is (relatively) predictable for the parasite. Since maintaining plastic capacity can be assumed to be more costly than genetic canalization (Reece, Ramiro, and Nussey 2009) this suggests that *E. falciformis* has adapted a canalized program which *assumes* certain immune stimuli. If one optimal phenotype exists this is the predicted outcome. If instead different distinct phenotypic optima exist because of varying and unpredictable external stimuli, phenotypic plasticity is expected to be selected for. (DeWitt and Scheiner 2004)

Considering *G. duodenalis* infections in the mouse model, mice in the wild have a calory intake comparable with, or slightly higher, than that of laboratory mice fed ad libitum (Austad and Kristan 2003), suggesting no lack of carbohydrates and substrate for glycolysis in wild mice. Together with the model in Figure 15 this predicts no carbohydrate limitation on trophozoite replication in the wild. However, the access to amino acids (such as arginine) from meat, nuts and seeds may indeed be limited and unpredictable (J. M. Rhoads and Wu 2009) and according to the model in Figure 15, cyst shedding would be equally unpredictable. Arginine access would vary between host individuals, meaning that it is unpredictable when efficient arginine-dependent encystation can take place in different infected individuals. This situation should promote phenotypic plasticity (i.e. sensing arginine) for encystation, so that trophozoite differentiation is initiated when arginine access is sufficient. This may not be the only requirement, and commonly a stressor induces life cycle progression in microorganisms (e.g. Reuner et al. 1997; Dean et al. 2009; Loomis 2014). Possibly, stressed trophozoites arrest or slow down growth and undergo differentiation into cysts when arginine becomes available. An optimum could be to generate a certain number of cysts within a short time-frame to increase chances of successful transmission, also similar to the *Trypanosome* spp. synchronization to differentiate into the stumpy parasite stage (Reuner et al. 1997; Dean et al. 2009; Rojas et al. 2019; Sollelis and Marti 2019).

Considering non-clonal G. duodenalis*: Phenotypic plasticity and genetic variation in wild populations*

I use commercially available (assumed) clonal parasites to demonstrate phenotypic plasticity in an experimental setting; the definition for phenotypic plasticity being that phenotypes vary in response to external stimuli and despite genetic homogeneity (see Definitions). In vitro, *G. duodenalis* assemblage A (WB-6) has a high recombination rate (Le Blancq, Korman, and Van der Ploeg 1992) and both assemblages A and B also display allelic heterozygosity, the latter to

a larger extent (Adam 2011). I can therefore assume that the parasite is not clonal in nature. Phenotypic plasticity and polymorphisms will then exist side by side in nature and possibly contribute to the arginine utilizing and encystation phenotypes.

Genetic diversity is a prerequisite for selection of any trait, including capacity for phenotypic plasticity. The unpredictable arginine availability within a host is here proposed to promote plastic capacity.

I further suggest that dietary differences between host individuals promote genetic diversity. If those are clonal, the parasite relies solely on its phenotypic capacity to thrive and encyst efficiently when necessary. If the capacity to sense arginine (plasticity) is combined with an – for the diet of this particular host – optimal arginine utilization, e.g. determined by the ADI enzyme, a more beneficial encystation phenotype is possible. An encystation phenotype would describe e.g. the number of cysts shed per gram feces and the frequency of shedding. In that particular host, one genotype would be dominant among the shed cysts. However, given the diversity in diets between host individuals (with regards to content and timing) I expect the genetic diversity among cysts from many hosts in nature to be high and an analysis of an environmental cyst sample to show polymorphism in genes related to arginine utilization. An additional contributor to expected genetic diversity is recombination during encystation (Ramesh, Malik, and Logsdon 2005; Cooper et al. 2007; Melo et al. 2008). A limited number of cysts will initiate a new infection upon ingestion by a new host and meiosis and homologous recombination during encystation would contribute to ensuring genetic diversity among cysts which are shed together.

I particularly expect genetic diversity in genes which contribute to arginine responsiveness and utilization. Supporting this suggestion, ADI, the first enzyme of the ADH pathway, was indeed polymorphic in eight analyzed isolates of assemblage B parasites (and also in assemblage A parasites), analyzed at the amino acid level (unpublished, Christian Klotz, Robert Koch-Institute). If the proposed mechanism to synchronize encystation is indeed important in nature, I would expect polymorphism also in other ADH pathway genes, and potentially in genes of the N-acetyl-galactosamine pathway. Considering that diet is the external stimulus I suggest to synchronize encystation, there could be genetic similarities between parasite genotypes which infect herbivores (e.g. assemblage E, found in hoofed livestock and subtypes of assemblage A found in livestock) which distinguish them from parasite genotypes which infect e.g. strict carnivores (e.g. assemblage F found in cats and C or D in canids) (U. Ryan and Cacciò 2013). Those predictions are however complicated by the still unclear taxonomy of *G. duodenalis* as described in the introduction of this thesis. Nevertheless, access to genome data from naturally occurring *G. duodenalis* infections can be used to assess polymorphic patterns. Analysis of enrichment in polymorphism has been reported and the method by Tataru et al. is particularly interesting here since it allows beneficial mutations (Tataru et al. 2017). Using this or similar methods could be a way to assess whether parasite genotypes on loci relevant for arginine-metabolism and encystation are enriched in polymorphisms compared to the overall genome. In addition, such sequence data from a wide range of sources can be used to ask whether these

genes cluster in a way which reflects host dietary habits. Combined, such analysis would be an interesting extension of the results and new hypotheses presented here.

Conclusions

Infection biology requires the study and analysis of molecular and cell biology, as well as host immune responses, pathogen life cycles, metabolic requirements, multispecies interactions (ecology), epidemiology, and evolutionary biology. In addition, for human purposes pharmacology as well as drug development and resistance can be added to this (incomplete) list. This thesis aims to contribute some basic insights into the biology of two protozoan parasites with a focus on life cycle progression, and to place the findings in a broader perspective by applying an evolutionary concept to evaluate results. *E. falciformis* showed no signs of phenotypic plasticity at the levels of transcription or reproduction (oocyst shedding) in response to variations in host immune stimulus. *G. duodenalis* instead displays phenotypic plasticity in response to a biologically relevant stimulus, nutrient availability. I argue that the capacity to respond plastically is an adaptation to unpredictable availability of a key metabolite: arginine. Making use of the timing in host food intake, or specifically that of arginine, could be an elegant solution to synchronize encystation to ensure successful transmission. If (selected) phenotypic plasticity is necessary for efficient transmission due to the intrinsic unpredictability of the stimulus (diet), I expect to see this reflected in *G. duodenalis* genomes as polymorphism in arginine metabolizing genes. For one gene, ADI, such unpublished data confirms the hypothesis.

Taken together, this thesis contributes new insights to *E. falciformis* and *G. duodenalis* behavior during in vivo infections. The *E. falciformis* life cycle transcriptome is additionally a resource and reference for future projects. New data on *G. duodenalis* dependence on arginine for encystation in vivo highlight the importance of studying the complete life cycle in order to understand parasite growth requirements and overall biology. The data presented encourage to investigate arginine dependency in encystation protocols, thereby possibly improving them and importantly, determine specific conditions under which *G. duodenalis* replicates, initiates, and commits to encystation.

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Author declaration

I, Totta Ehret Kasemo, hereby declare that I am the sole author of this thesis. To the best of my knowledge this thesis contains no material previously published by any other person except where due acknowledgement has been made. This thesis contains no material which has been accepted as part of the requirements of any other academic degree or non-degree program, in English or in any other language. This is a true copy of the thesis