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Emerging Global Diseases
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Program

- 8:30 AM Registration and Poster Set-up
- 9:00 AM Opening Remarks: **Dennis Kyle**, Director of CTEGD
- SESSION 1 — Moderators: Marianna Agudelo, Derek Huck, Victoria Mendiola**
- 9:10 AM **Grace Vick**, Center for Tropical and Emerging Global Diseases, UGA
A SNARE-like *Plasmodium* rhoptry neck protein is required for merozoite invasion
- 9:30 AM **Megna Tiwari-Crowe**, CTEGD, CCRC, Dept. of Biochemistry & Molecular Biology, UGA
O-fucosylation promotes stable expression of a nucleocytoplasmic protein in *Toxoplasma gondii* and can be detected by new antisera in other parasites and protists
- 9:50 AM **Saniya Sabnis**, CTEGD, CVI, and Dept. of Infectious Diseases, UGA
Chronic *Plasmodium* infections cause persistent changes in the host immunological landscape
- 10:10 AM **BREAK — POSTER VIEWING (even posters)**
- SESSION 2 — Moderators: Emily Bremers, Kaelynn Parker, Corey Reynolds**
- 10:50 AM **Steven Maher**, Center for Tropical and Emerging Global Diseases, UGA
Screening and hit validation of novel anticoccidial compounds
- 11:10 AM **INTRODUCTION OF EARLY CAREER SCHOLAR**
- 11:15 AM **Astra Bryant**, Dept. of Physiology & Biophysics, University of Washington, Seattle
The thermosensory specializations of a skin-penetrating human-parasitic nematode
- 12:10 PM **LUNCH — POSTER VIEWING**
- SESSION 3 — Moderators: Saniya Sabnis, Clyde Schmidt, Grace Woods**
- 1:10 PM **Wayne Cheng**, CTEGD and Center for Vaccines & Immunology, UGA
Increased Duffy binding protein 1 expression correlates with *Plasmodium cynomolgi* growth in continuous culture
- 1:30 PM **Lindsay Berardi**, Department of Microbiology, UGA
Wolbachia surface lipoprotein Wbm0152 inhibits ESCRT complexes
- 1:50 PM **Magdalena Argomaniz**, CTEGD and Center for Vaccines & Immunology, UGA
A *Plasmodium vivax* strain that expresses fluorescent proteins throughout the life-cycle
- 2:10 PM **Fiiifi Agyabeng-Dadzie**, CTEGD and Dept. of Genetics, UGA
Single-oocysts and whole genome amplification with long-read sequencing in *Cryptosporidium*
- 2:30 PM **BREAK — POSTER VIEWING (odd posters)**
- SESSION 4 — Moderators: Marianna Agudelo, Victoria Mendiola, Corey Reynolds**
- 3:10 PM **Kaelynn Parker**, CTEGD and Dept. of Cellular Biology, UGA
Communication is key: Investigating interorganellar communication in *Toxoplasma gondii*
- 3:30 PM **Camila Marques-da-Silva**, CTEGD and Dept. of Cellular Biology, UGA
Type I Interferon treatment enhances the efficacy of radiation attenuated sporozoite vaccination against malaria
- 3:50 PM **INTRODUCTION OF THE KEYNOTE SPEAKER**
- 4:00 PM **David Sibley**, Institute for Public Health, Washington University in St. Louis
Cryptosporidiosis: microbial interactions in the gut
- 5:00 PM Concluding Remarks: **Dennis Kyle**

Poster Presentations

- P1 **Emily Bremers**, Center for Tropical and Emerging Global Diseases, UGA
Stereospecific resistance to tetrahydro- β -carboline antimalarial is mediated by a PfMDR1 mutation
- P2 **Sabrina Pizarro**, EPIC and Dept. of Genetics & Biochemistry, Clemson University
Trypanosoma brucei targeted drug discovery
- P3 **Corey Rennolds**, CTEGD and Dept. of Genetics, UGA
Prospective isolation and molecular characterization of stem cells in the rat tapeworm, *Hymenolepis diminuta*
- P4 **Mario Uchimiya**, Complex Carbohydrate Research Center, UGA
Network for advanced NMR and CCRC NMR Facility: Opportunities for metabolomics
- P5 **Madelaine Usey**, CTEGD, and Dept. of Cellular Biology, UGA
To inhibit or not to inhibit: the *Toxoplasma gondii* homolog of ATPase inhibitory factor 1 (TgIF1)
- P6 **Edward D'Antonio**, Dept. of Natural Sciences, University of South Carolina, Beaufort
Exploration of 3-nitro-2-phenyl-2*H*-chromene analogues for potent antitrypanosomal activity
- P7 **Billy Erazo**, Depts. of Medical & Microbial Immunology and Cellular & Molecular Pathology, University of Wisconsin, Madison
Role of essential members of the necroptosis pathway to *Toxoplasma gondii* infection
- P8 **Benjamin Hoffman**, CTEGD and Dept. of Cellular Biology, UGA
Hypothetical protein Tb427.2.5810 is a nuclear effector of CK1.2 signaling in the African trypanosome
- P9 **Nupur Kittur**, Center for Tropical & Emerging Global Diseases, UGA
Integration, exploration & reuse of clinical & epidemiological datasets on the ClinEpiDB platform
- P10 **Susanne Warrenfeltz**, Center for Tropical & Emerging Global Diseases, UGA
Eukaryotic Pathogen, Vector and Host Informatics Resources (VEuPathDB.org)
- P11 **Justin Wiedeman**, Center for Tropical & Emerging Global Diseases, UGA
Conditional knockdown of Aurora and Polo-like kinases in *Trypanosoma cruzi* using a hammerhead ribozyme reveals essential roles for the kinases in parasite cell division
- P12 **Christofer Zepeda Guisa**, Center for Tropical & Emerging Global Diseases, UGA
Looking for a START in *Toxoplasma gondii*: Insights into membrane contact site members
- P13 **Leonor Sicalo Gianechini**, Dept. of Infectious Diseases, UGA
Investigating phenotypic, genotypic and genomic changes in a drug-susceptible isolate of *Haemonchus contortus* under field selection with fenbendazole
- P14 **Elyssa Campbell**, Dept. of Infectious Diseases, UGA
The NIH/NIAID Filariasis Research Reagent Resource Center (FR3)
- P15 **Joseph Dainis**, CTEGD and Dept. of Infectious Diseases, UGA
Feeding frenzy! Exposure to conditioned media from highly virulent *Naegleria fowleri* increase cytopathic effects of lowly virulent isolates

- P16 **Jett Flentje**, Center for Tropical and Emerging Global Diseases, UGA
Malaria parasites do not manipulate pathways controlling vitellogenesis in the mosquito *Anopheles stephensi*
- P17 **Derek Huck**, CTEGD and Dept. of Entomology, UGA
Commensal bacteria enable development of mosquito larvae on detritus diets
- P18 **Anna Husted**, Dept. of Natural Sciences, University of South Carolina, Beaufort
Expression, purification, and characterization of arginase from *Leishmania infantum*
- P19 **Gaurav Kumar**, Dept. of Molecular & Cellular Biology, Kennesaw State University
Poly(A)-Binding Protein 2 and DRBD3 are physiologic targets of CBL0137, a lead compound for Human African Trypanosomiasis drug development
- P20 **Miryam Andrea Hortua-Triana**, Center for Tropical & Emerging Global Diseases, UGA
Characterization of an Endoplasmic Reticulum Calcium-binding protein in *Toxoplasma gondii*
- P21 **Jillian McKeon**, Eukaryotic Pathogens Innovation Center, Clemson University
Exploring enolase inhibitors as therapeutic agents for *Naegleria fowleri* infection
- P22 **Samuel Nyarko**, CTEGD and Dept. of Cellular Biology, UGA
Understanding the significance of Subunit K in the *Toxoplasma* ATP Synthase
- P23 **Destiny O'Neill**, Dept. of Natural Sciences, University of South Carolina, Beaufort
Early-stage therapeutic drug discovery of Chagas' Disease: Investigation of gossypol-based inhibitors of *Trypanosoma cruzi* glucokinase
- P24 **Aubrey Phillips**, CTEGD and Dept. of Infectious Diseases, UGA
A combination of four nuclear targeted effectors protects *Toxoplasma* against interferon gamma driven human host cell death during acute infection
- P25 **Caroline Palmentiero**, Eukaryotic Pathogens Innovation Center, Clemson University
Development of molecular tools for transgene expression in *Naegleria fowleri*
- P26 **Benjamin Phipps**, CTEGD and Dept. of Genetics, UGA
The joy of cooking for mosquitoes: Identifying blood nutrients required for egg formation in mosquitoes
- P27 **Carrie Baumgardner**, Dept. of Physics & Astronomy, Clemson University
Monitoring energy status in living kinetoplastid parasites using a FRET-based AMPK sensor
- P28 **Gonzalo Seminario-Mondejar**, Center for Tropical & Emerging Global Diseases, UGA
Unraveling the enigmatic feeding apparatus of *Trypanosoma cruzi* — Molecular components of the cytostome-cytopharynx complex —
- P29 **Melissa Sleda**, CTEGD and Dept. of Cellular Biology, UGA
New mitochondrial ubiquinone synthesis inhibitors that are effective against the acute and chronic stages of *Toxoplasma gondii*

- P30 **Baihetiya Baierna**, CTEGD, Dept. of Cellular Biology, UGA
The essential enzymes for the synthesis of ubiquinone are arranged in a large protein complex in *Toxoplasma gondii*
- P31 **Shutong Wang**, Dept. of Biology, Georgia State University
Screening of recreational areas of rivers for potentially pathogenic free-living amoebae in Georgia water bodies
- P32 **Juan Camilo Arenas-Garcia**, CTEGD and Dept. of Cellular Biology, UGA
The role of polyphosphates in *Toxoplasma gondii*
- P33 **Antonia Blank**, Eukaryotic Pathogens Innovation Center, Clemson University
Genetic engineering in *Naegleria*
- P34 **Donovan Cantrell**, Dept. of Biochemistry & Molecular Biology, UGA
Glycosylation weakens Skp1 homodimerization in *Toxoplasma gondii* by interrupting a fuzzy interaction
- P35 **Watcharatip Dedkhad**, Center for Tropical & Emerging Global Diseases, UGA
Exoneme exocytosis and membrane rupture during malaria parasite egress from RBCs
- P36 **Reagan Haney**, CTEGD and Dept. of Biochemistry & Molecular Biology, UGA
Identifying the mechanism of action of a novel antimalarial with collateral drug sensitivity associated with *PfKelch13* C580Y mutation
- P37 **Alexander Garrot**, CTEGD and Dept. of Cellular Biology, UGA
Effect of high-fat diet on vaccine mediated protection from malaria
- P38 **Ruby Harrison**, CTEGD and Dept. of Cellular Biology, UGA
Outcomes of natural versus non-natural pairings of *Trypanosoma cruzi* discrete typing units with the triatomine vector *Rhodnius prolixus*
- P39 **Msano Mandalasi**, CTEGD, CCRC, and Dept. of Biochemistry & Molecular Biology, UGA
Oxygen-dependent regulation of F-box proteins in *Toxoplasma gondii*
- P40 **Katherine Moen**, CTEGD and Dept. of Cellular Biology, UGA
The role of protein disulfide isomerase in the endoplasmic reticulum of *Toxoplasma gondii*
- P41 **Victoria Murphey**, EPIC and Dept. of Biological Sciences, Clemson University
Assessing an actin-binding protein, thymosin beta-4, as a novel treatment for *Acanthamoeba* keratitis
- P42 **James Oristian**, CTEGD and Dept. of Infectious Diseases, UGA
Induced in vitro sexual commitment of *Plasmodium cynomolgi*
- P43 **Anthony Ruberto**, Center for Tropical & Emerging Global Diseases, UGA
Understanding 'sleeping beauties': cellular and molecular characterization of *P. falciparum* ring stage parasites in response to artemisinin
- P44 **Gabrielle Russell**, Dept. of Microbiology, University of Tennessee, Knoxville
Development of an interactive GIS database for the study of molecular epidemiology in *Toxoplasma gondii*

- P45 **Colm Roster**, Eukaryotic Pathogens Innovation Center, Clemson University
Enolase inhibitors are potent therapeutic leads against *Trypanosoma brucei*
- P46 **Alexis Stamatikos**, Dept. of Nutrition, Clemson University
Cholesterol efflux attenuates TLR4-target gene expression in cultured macrophages exposed to *T. brucei* ghosts
- P47 **Cristina Samuel**, CTEGD and Division of Biological Sciences, UGA
Improving the efficacy of malaria vaccination through modulation of reactive oxygen species
- P48 **Clyde Schmidt-Silva**, CTEGD and Dept. of Cellular Biology, UGA
CCR2-mediated recruitment of antigen presenting cells to *Plasmodium*-infected liver
- P49 **Mahvish Rajput**, Dept. of Parasitology, University of Agriculture Faisalabad, Pakistan
Morphological and molecular identification of *Paramphistomum* species from sheep of Hyderabad, Pakistan

Oral Presentations

A SNARE-like Plasmodium rhoptry neck protein is required for merozoite invasion

Grace W. Vick¹, Carrie F. Brooks¹, Vasant Muralidharan¹

¹Center for Tropical and Emerging Global Diseases, Dept. of Infectious Diseases, University of Georgia, Athens, GA

Plasmodium falciparum, the causative agent of human malaria, are obligate intracellular parasites during all replicative stages of their complex two-host lifecycle. Critical for their successful growth and expansion is their ability to invade host cells. Merozoite invasion requires the discharge of proteins into the host from specialized club-shaped organelles, known as rhoptries.

Our work is focused on discovering the function of a conserved membrane protein Pf3D7_1117400, that is predicted to be essential for the asexual life cycle. This protein has distant homology to vesicle SNARE proteins, which mediate membrane fusion in eukaryotic cells. We used CRISPR/Cas9 genetic engineering to create a conditional knockdown of Pf3D7_1117400 and determined that it localizes to the rhoptry neck. Hence, we termed this protein, SNARE-like rhoptry neck protein or SLRON.

Knockdown of SLRON led to parasite death after the first asexual cycle, indicating that SLRON is essential for intraerythrocytic development. We observe that SLRON knockdown parasites egress normally, but fail to convert into the ring-stage form. Moreover, we do not observe merozoites attached to erythrocytes suggesting a defect in early invasion steps prior to the tight junction formation. Using live video microscopy, we see that SLRON-deficient parasites induce rapid echinocytosis in host cells, yet fail to internalize. Finally, we see that in the absence of SLRON, components of the parasitophorous vacuole in early rings are not present, indicating a role for SLRON in PV formation and maintenance. Ongoing work will elucidate the role of SLRON in early merozoite invasion and PV integrity.

O-fucosylation promotes stable expression of a nucleocytoplasmic protein in *Toxoplasma gondii* and can be detected by new antisera in other parasites and protists

Megna Tiwari-Crowe^{1,2,3}, Jen Teal^{1,2,3}, Elisabet Gas Pascual^{1,2,3}, Msano Mandalasi^{1,2,3}, Manish Goyal⁴, Marla Popov², Ron Orlando^{2,3}, John Samuelson⁴, and Christopher M. West^{1,2,3}

¹CTEGD, ²CCRC, ³BMB, UGA; ⁴Dept. of Molecular & Cell Biology, Boston University School of Medicine

Toxoplasma alternates between an asexual and sexual life cycle in which it must adapt to various environments. Of interest, is the O-fucosyltransferase (OFT) SPY, which modifies serine and threonine residues of at least 33 nucleocytoplasmic proteins with a single fucose (O-Fuc). OFT-like genes occur in the parasites *Cryptosporidium* and *Acanthamoeba* and social amoeba *Dictyostelium*. This process is related to the O-GlcNAcylation of nucleocytoplasmic proteins in humans, which has been implicated in mediating stress and nutritional responses. SPY is required for optimal growth of *Toxoplasma* and *Dictyostelium* *in vitro*, and the high degree of conservation of OFT with OGT suggests that OFT may also mediate responses to stress. To facilitate investigation of O-Fuc in *T. gondii* and other protists, we developed antibodies specific for fucose-O-Ser or fucose-O-Thr (anti-FOS/T). Unlike the lectin AAL that reacts with all terminal fucose in cells, anti-FOS/T only detects proteins modified by SPY. Anti-FOS/T labeling in *Toxoplasma*, *Cryptosporidium*, *Acanthamoeba*, and *Dictyostelium* is reminiscent of O-GlcNAc in humans. The O-fucose of *Dictyostelium* includes proteins that overlap with the O-fucose of *Toxoplasma* and is highly responsive to starvation-induced development. By focusing on a GPN-GTPase in *Toxoplasma*, we confirmed that it is highly O-fucosylated in its Ser-rich domain, and that O-fuc is required for it to achieve normal expression levels. We speculate that O-Fuc is responsive to external signals such as nutrition, which may control the availability of the GDP-Fuc precursor required by OFT to modulate the stability of key proteins with disordered Ser- or Thr-rich domains.

Chronic *Plasmodium* infections cause persistent changes in the host immunological landscape

Saniya S. Sabnis^{1,2,3}, Celia Saney², Monica Cabrera-Mora⁴, the MaHPIC Consortium⁴, Ignacio Sanz⁵, Frances Lee⁵, Regina Joice-Cordy⁶, Alberto Moreno^{4,5}, Tracey Lamb⁷, Mary Galinski^{4,5}, Chester J. Joyner^{1,2,3}

¹Dept. of Infectious Diseases, UGA, Athens, GA ²CVI, UGA, Athens, GA ³CTEGD, UGA, Athens, GA ⁴Yerkes, Emory, Atlanta, GA ⁵Dept. of Medicine, Emory, Atlanta, GA ⁶Dept. of Biology, Winston-Salem, NC ⁷Dept. of Pathology, UU, Salt Lake City, UT

While minimally symptomatic, chronic *Plasmodium falciparum* (*Pf*) infections, the norm in endemic areas, predispose individuals to secondary bacterial infections and reduce malaria vaccine efficacy. Thus, we need to understand how chronic infections alter immunological landscapes. Using *P. coatneyi* (*Pc*) infected macaques as a *Pf* malaria model, we defined host immunological and transcriptional changes leading to chronic infection. From whole blood RNA sequencing, infections reached chronicity 50-80 days after sporozoite inoculation. Based on host response transcriptomics, chronicity progression is generally parasitemia-independent but is related to the amount of time an infection has persisted. The acute-to-chronic transition was defined by upregulation in B cells and cytokine signaling gene signatures, and downregulation of interferon (IFN) gamma signaling. However, Type I IFN signaling remained elevated. Inflammatory cytokine gene expression was upregulated during acute phase, and some, e.g., TNF α , stayed elevated in chronic phase. Anti-inflammatory cytokines, e.g., IL-10, increased in acute phase and returned to baseline. Flow cytometry analysis showed an increase in multiple B cell subsets, including CD21⁻ CD27⁻ B cells, and effector memory CD8⁺ T cells. Parasitemia control significantly correlated with B and T cell changes, in addition to IgG and IgM against *Pc*, suggesting these responses are key for parasite control in chronic infections. In sum, we defined the progression of acute to chronic *Plasmodium* infection and identified changes in host immunology that may influence malaria vaccine efficacy.

Screening and hit validation of novel anticoccidial compounds

Caitlin A. Cooper¹, Steven P. Maher¹, Khaley Diagne², Lorraine Fuller³, Roman Manetsch², Dennis E. Kyle¹

¹CTEGD, UGA, Athens, GA ²Dept of Chemistry and Chemical Biology, Northeastern University, Boston, MA

³Dept of Poultry Science, UGA, Athens, GA

Coccidiosis is caused by apicomplexan parasites of the genus *Eimeria*, can affect many species of animals including chickens, and results in billions of dollars in losses to the poultry industry annually. Current coccidiostats must be cycled to prevent the rapid generation and spread of resistance and new treatments are needed, however, the drug discovery pipeline for *Eimeria* is not well-established. Having developed a series of quinolones as competitive inhibitors of the Qo site of the bc1 complex in the mitochondrial electron transport chain of the apicomplexan *Plasmodium falciparum*, we aimed to determine if these inhibitors could be repurposed for treating coccidiosis. We first optimized a high-throughput screening platform featuring *E. tenella*-infected-MDBK cells and validated the model using currently-used coccidiostats, including decoquinate. We then used this platform to screen in a dose-response format 76 *Plasmodium*-active quinolones derived from one of three different chemical scaffolds (THA, P4Q, and PEQ). While we did not note a correlation in potency against the two genera, many analogs inhibited *E. tenella* in vitro with EC₅₀'s as low as 1nM. We next selected three hits for scaled synthesis and performed a pen study in which chicks were fed treated feed before infection with *E. tenella*. The three quinolones demonstrated a modest reduction in the primary study endpoint, cecal scaring, compared to untreated controls. Our results indicate further development of quinolones for treating coccidiosis is warranted and our discovery pipeline could be utilized to expedite this and other promising drug series.

Increased Duffy binding protein 1 expression correlates with *Plasmodium cynomolgi* growth in continuous culture

Wayne T. Cheng^{1,2}, Magdalena Argomaniz^{1,2}, Caitlin C. Cooper², Amadis Vivas^{1,2}, Saniya Sabnis^{1,2}, Sarah Gayle Roberson^{1,2}, Celia L Saney^{1,2}, Mary R. Galinski^{1,2}, Steven P. Maher², Dennis E. Kyle^{2,3}, Chester J. Joyner^{1,2,3}

¹CVI, College of Vet Med, UGA; Athens, GA. ²CTEGD, UGA; Athens, GA. ³Dept. of Infectious Diseases, College of Vet Med, UGA, Athens, GA.

A continuous culture system would revolutionize *Plasmodium vivax* (*Pv*) research but remains elusive. *Plasmodium cynomolgi* (*Pcy*) is a closely-related nonhuman primate malaria parasite that shares many biological traits with *Pv* except that *Pcy* preferentially, but not exclusively, invades and develops within reticulocytes. This difference has supported the adaptation of *Pcy* lines that grow in culture, but the mechanisms that enable continuous culture are undefined. Here, we generated a new line of the *Pcy* Berok strain, termed DC line, to grow continuously in culture and performed whole genome sequencing of parasites collected during adaptation to identify the genetic changes that promote growth in culture. Minimal single nucleotide variants emerged during adaptation. Structural variations comprised of insertions and deletions (INDELS) were more common and suggested that a subpopulation of parasites was selected for during adaptation versus de novo mutations that led to improved growth. INDELS were present in many genes associated with the parasite's metabolism, consistent with the nutrient-limited environment of culture. Interestingly, the DC line also had additional copies of the Duffy binding protein 1 gene that was associated with increased gene expression. Duffy antigen receptor for chemokines (DARC) is the ligand for DBP1, and the loss of this receptor has been shown to restrict *P. yoelii* to invading reticulocytes. Thus, we hypothesized that overexpression of DBP1 by the DC line may alter the invasion preference from reticulocytes to normocytes, enabling the parasite to grow effectively in culture. Indeed, invasion assays showed that the WT line preferentially invaded and developed within reticulocytes whereas there was no preference for the DC line. In summary, these data indicate that metabolic changes and alterations in invasion ligand expression through copy number variation support continuous growth of *P. cynomolgi* in culture. This information may help adapt additional *Pcy* strains to culture and inform efforts for culturing *Pv*.

***Wolbachia* surface lipoprotein Wbm0152 inhibits ESCRT complexes**

Lindsay Berardi and Dr. Vincent J. Starai

Department of Microbiology, University of Georgia, Athens GA

Brugia malayi is a mosquito-borne filarial nematode known to cause debilitating and disfiguring lymphatic illness. Affecting millions of individuals worldwide, current anthelmintic treatments are ineffective in completely eradicating late larval stage and adult worms, resulting in long treatment times and potential disease recrudescence. *Wolbachia pipiens*, the essential endosymbiont of several filarial nematodes - including *Burgia malayi* - has become a promising target for drug discovery, as this bacterium's presence is essential for growth and reproduction of these nematodes. My research therefore seeks to understand the molecular underpinnings of the *Wolbachia*:nematode relationship through expression of select wBm proteins in the eukaryotic model cell *Saccharomyces cerevisiae* to identify proteins which alter conserved eukaryotic biology. One such protein, Wbm0152, a predicted outer membrane Peptidoglycan Associated Lipoprotein (PAL) and has been shown to disrupt endosomal cargo trafficking pathways when expressed in yeast. We have now determined that Wbm0152 specifically inhibits the activity of conserved ESCRT complexes in vivo. The Endosomal Sorting Complexes Required for Transport (ESCRT) are highly conserved, multi-protein complex important for endosomal intraluminal vesicle formation, cytokinesis, and viral budding in mammalian cells. When expressed in yeast, Wbm0152 inhibits ESCRT-dependent protein degradation by preventing the delivery of endosomal cargo to the degradative vacuole. Additionally, Wbm0152 colocalizes with ESCRT subcomplexes -0, -I, -II, and -III, but fails to colocalize with the accessory ESCRT protein Bro1p, leading us to hypothesize Wbm0152 prevents ESCRT complex disassembly. Furthermore, we show that Wbm0152 binds the yeast ESCRT-III subunit, Vps2p, as well as the *B. malayi* Vps2 homolog, Bm6583, when expressed in yeast. Therefore, Wbm0152 likely serves a critical role in maintaining the *Wolbachia*:nematode endosymbiosis by altering host endolysosomal membrane dynamics. Further characterization of the activities of Wbm0152 is likely to fill an important knowledge gap regarding the molecular mechanisms by which *Wolbachia* can persist within the nematode host.

A *Plasmodium vivax* strain that expresses fluorescent proteins throughout the life-cycle

Magdalena Argomaniz^{1,2}, Wayne T. Cheng^{1,2}, Amadis Vivas^{1,2}, Grace Hawkins^{1,2}, Henry R. Davie^{1,2}, Sarah Gayle Roberson^{1,2}, Diego Huet², Steven P. Maher², Chester J. Joyner^{1,2,3}

¹Ctr for Vaccines and Immunology, College of Vet Med, UGA; Athens, GA. ²Ctr for Tropical & Emerging Global Disease, UGA; Athens, GA. ³Dept. of Infectious Diseases, College of Vet Med, UGA, Athens, GA

Plasmodium vivax (*Pv*) persists due to its ability to form dormant liver-stages, known as hypnozoites (HZs). Understanding the molecular makeup of HZs is key to developing new treatments to eliminate HZs, but these experiments have been hindered by the inability to isolate *Pv* HZs for molecular characterization. A transgenic *Pv* that expresses fluorescent proteins throughout the life-cycle would overcome this limitation and make molecular characterization possible. To address this need, *Pv* Chesson parasites were harvested from *Saimiri boliviensis* monkeys and transfected with a plasmid containing *gfp*, *mCherry*, and *nanoluc* reporter genes under two different promoters. GFP was placed under the constitutively expressed *hsp70* promoter, whereas mCherry and Nanoluc were placed under the *lisp2* promoter to enable the exclusion of activating forms from dormant HZs in future isolations. Pyrimethamine resistant asexual stage parasites were recovered about 31 days after transfection and inoculation into a naïve animal. Eighty-nine percent of the resistant parasites expressed GFP. Infected blood was then collected and fed to *Anopheles stephensi* mosquitoes, and GFP+ oocysts and sporozoites were detected. Primary human hepatocyte cultures were inoculated with sporozoites, and both small and large forms expressing GFP were detected by live imaging. Large forms also expressed mCherry as expected. There were no effects on the parasite's development in the liver-stages. This study establishes a fluorescent, transgenic *P. vivax* strain that can be used to isolate hypnozoites for molecular characterization and methods for genetically manipulating *P. vivax* to test specific proteins that may be involved in dormancy.

Single-oocysts and whole genome amplification with long-read sequencing in *Cryptosporidium*

Fiifi Agyabeng-Dadzie¹, Megan Beaudry³, Rodrigo P. Baptista⁵, Sebastian Shaw⁶, Boris Striepen⁶, Jessica C. Kissinger^{1,2,3}, Travis C. Glenn^{1,3,4}

¹Department of Genetics, ²Center for Tropical and Emerging Global Diseases, ³Institute of Bioinformatics, ⁴Environmental Health Science, University of Georgia, Athens, GA; ⁵Houston Methodist Research Institute, Houston, TX; ⁶University of Pennsylvania School of Veterinary Medicine, PA

Cryptosporidium genomics has been hindered by many factors, including small clinical sample sizes with few oocysts and an inability to clone individual parasites. As a result, genomic data for most *Cryptosporidium* species is lacking, making studies of outbreak genotypes and whole genome evolution limited. About 50% of the available genome sequences represent *C. hominis* and *C. parvum*. However, these sequences are fragmented due to short-read approaches, and they represent mixed populations of oocysts. Only *C. parvum* has a telomere-to-telomere assembly (CpBGF T2T). To address these limitations, we utilize multiple displacement amplification (MDA) and Oxford Nanopore Technology long-read sequencing on single-oocyst (4 haploid sporozoites) sequencing (SOS) or MDA on existing DNA samples with very little DNA. MDA was observed to be successful with DNA quantities as low as 39 fg (DNA content in an oocyst), with a 78% amplification success rate. Amplification sites were distributed across the genome randomly, resulting in an overall single oocyst average genome coverage of 17% when mapped to the CpBGF T2T reference. Some concatemers were observed in the sequences, indicating the presence of fragmented input sequences before amplification. By performing SOS with a long-read approach, we are freed from population-level analyses that obscure low levels of diversity, and visualization of recombination events becomes possible in *Cryptosporidium* spp. SOS also facilitates the study of lesser-known *Cryptosporidium* species where the existing DNA quantity or number of oocysts is extremely low.

Communication is Key: Investigating interorganellar communication in *Toxoplasma gondii*

Kaelynn Parker^{1,3} ; Diego Huet^{2,3}

¹Department of Cellular Biology, University of Georgia, Athens 30602, GA, USA ²Department of Pharmaceutical and Biomedical Sciences, University of Georgia, Athens 30602, GA, USA ³Center for Tropical and Emerging Global Diseases, University of Georgia, Athens 30602, GA, USA. diego.huet@uga.edu

While organelles are typically thought of as individual structures in eukaryotic cells, communication between them is required to share signals and metabolites to support their functions. These interactions are supported by areas of close proximity between the two organelles, maintained by protein tethers, and referred to as membrane contact sites (MCSs). Currently, the majority of our knowledge on MCSs is limited to yeast and mammals, which are members of the Opisthokont clade. *Toxoplasma gondii* provides the unique opportunity to investigate MCSs in a divergent model eukaryote. To identify MCS protein candidates in *T. gondii*, we have taken an unbiased proximity biotinylation-based approach by generating localization handles to anchor a biotin ligase to the surface of the mitochondrion, the ER, and the apicoplast, a phylum-specific vestigial plastid organelle. Biotinylation experiments followed by mass spectrometry analysis uncovered MCS protein candidates. Our results include several proteins with expected MCS functions, such as lipid-transfer proteins and small GTPases, as well as apicomplexan-specific proteins with no predictable functions or domains that may serve as potential drug targets. Finally, we will also use our data to generate surface proteomes for the ER, mitochondrion, and apicoplast. The results of this work will both expand our knowledge of membrane contact sites across the evolutionary tree, potentially uncover apicomplexan-specific ones, and provide an additional protein localization resource for the community.

Type I Interferon treatment enhances the efficacy of radiation attenuated sporozoite vaccination against malaria

Camila Marques-da-Silva^{1,2}, Cristina Samuel³, Clyde Schmidt-Silva^{1,2} and Samarchith P. Kurup^{1,2}

¹Center for Tropical and Emerging Global Diseases, University of Georgia, Athens, GA, USA. ²Department of Cellular Biology, University of Georgia, Athens, GA, USA. ³Division of biological sciences, University of Georgia, Athens, GA, USA

Malaria is a life-threatening disease, caused by *Plasmodium* parasites inoculated as sporozoites to humans through bites of infected mosquitos. The sporozoites invade and develop in hepatocytes (liver-stage) before infecting the red blood cells and initiating the symptomatic blood-stage of malaria. Vaccines that generate immune responses targeting *Plasmodium* in the liver would prevent their progression to the blood and prevent clinical disease. Therefore, these are considered ideal approaches to combat malaria. Immunization with live radiation attenuated sporozoites (RAS) is one such approach. RAS infected hepatocytes undergo programmed cell-death, allowing *Plasmodium* antigens to be accessed by the antigen processing and presentation machinery of the liver, which primes protective *Plasmodium*-specific CD8 T cell responses. We discovered that type-I interferons (IFN-I) drive this cell-death pathway in the RAS-infected hepatocytes through the induction of reactive oxygen species (ROS) that trigger lysosomal fusion with the parasitophorous vacuole. The administration of ROS scavengers or blockers of lysosomal fusion hindered this pathway and therefore, the efficient elimination of *Plasmodium* in the liver. Upon treating RAS vaccinated mice with IFN-I, we observed the generation of stronger *Plasmodium*-specific CD8 T cell responses, that resulted in better control of subsequent challenge infections. We believe that future studies that uncover the detailed mechanism of IFN-I mediated elimination of *Plasmodium* in hepatocytes will help identify new approaches to enhance the efficacy of RAS vaccination.

Poster Presentations

P1. Stereospecific resistance to tetrahydro- β -carboline antimalarial is mediated by a PfMDR1 mutation

Emily K. Bremers¹, Joshua H. Butler¹, Leticia Do Amaral¹, Emilio F. Merino¹, Hanan Almolhim², Bo Zhou³, Rodrigo P. Baptista⁴, David Anaguano¹, Maxim Totrov⁵, Paul R. Carlier^{2,3}, and Maria B. Cassera¹

¹Center for Tropical and Emerging Global Diseases, University of Georgia; ²Department of Chemistry, Virginia Tech; ³Department of Pharmaceutical Sciences, University of Illinois Chicago; ⁴Department of Medicine, Houston Methodist Research Institute; ⁵MolSoft LLC, San Diego, CA, USA

Drug resistance has been identified for all clinically available antimalarials, highlighting an urgent need to develop new treatments and better understand common mechanisms of resistance (MOR) in the malaria parasite. We have identified a novel tetrahydro- β -carboline compound, PRC1590, which potently kills malaria. To better understand the mechanism of action (MOA) of PRC1590, we selected for and characterized resistance to PRC1590 in *Plasmodium falciparum*. Through *in vitro* selection of resistance to PRC1590, we have identified that a single nucleotide polymorphism on the parasite's multidrug resistance protein mediates resistance to PRC1590. This mutation results in stereospecific resistance and sensitizes parasites to other antimalarials such as mefloquine. Stage specificity assays have revealed that PRC1590 is most potent during the trophozoite stage, when the parasite forms a single digestive vacuole (DV) and actively digests hemoglobin. Additionally, we identified through fluorescence microscopy that PRC1590 localizes to the parasite's DV, suggesting a putative target associated with this organelle. Our findings help elucidate the MOR and the MOA of this emerging class of antimalarials. In addition, our results suggest a potential link between resistance mediated by PfMDR1 and PRC1590's molecular target.

P2. *Trypanosoma brucei* targeted drug discovery

Sabrina Pizarro^{1,2}, Daniel Call³, Erin Jones^{1,2}, Carrie Baumgardner^{1,4}, Jennifer Golden⁵, Ken Christensen³, James Morris^{1,2}

¹Eukaryotic Pathogens Innovation Center, Clemson University. ²Department of Genetics and Biochemistry, Clemson University. ³Department Chemistry and Biochemistry, Brigham Young University. ⁴Department of Physics and Astronomy, Clemson University. ⁵School of Pharmacy, University of Wisconsin-Madison

Trypanosoma brucei, the causative agent of African sleeping sickness, remains a medical and agricultural concern for much of Sub-Saharan Africa. Glycolysis is critical to the infectious blood stream form parasite, and multiple glycolytic enzymes have been validated as potential drug targets. Trypanosome parasites expressing metabolite biosensors allow for the monitoring of fluctuations in metabolites such as glucose, fructose-1,6-bisphosphate, and ATP with relative ease. Through a screen of parasites expressing a glucose sensor, two inhibitors have been identified. Given the importance of glucose metabolism to the parasite, these inhibitors are toxic to parasites. Optimization of their performance through medicinal chemistry approaches would be facilitated by understanding of their cellular target. Here, we describe the characterization and validation of putative targets identified by thermal proteome profiling. Proteins identified include those involved in glycosomal regulation and vesicular trafficking. To begin to assess these as targets, we will compare compound sensitivity of knockdown and overexpression parasite lines with parental trypanosomes.

P3. Prospective isolation and molecular characterization of stem cells in the rat tapeworm, *Hymenolepis diminuta*

Corey W. Rennolds^{1,2}, Tania Rozario^{1,2}

¹Department of Genetics, UGA, Athens, GA; ²CTEGD, UGA, Athens, GA

Tapeworms are successful parasites due in part to their ability to grow quickly, shed many reproductive body segments (proglottids), and regenerate segments rapidly. The cellular and molecular basis of such continuous, large scale tissue turnover remains poorly understood. The rat tapeworm *Hymenolepis diminuta* contains a sole population of proliferative cells with body-wide distribution that are required for growth and regeneration, like planarian neoblasts, indicative of stem cells (SCs). However, unlike planarians, *H. diminuta* regeneration is not body-wide, consisting only of proglottid regeneration from the neck. Understanding this regenerative ability requires isolating and characterizing the SCs, including determining their potency, functional diversity, and developmental relationships. We are conducting parallel approaches to isolate SCs. First, we are using basic stains and fluorescence activated cell sorting to enrich for SCs. We have found three populations of cells differing in nuclear DNA content, likely corresponding to cell cycle phases, and we plan to use single-cell RNAseq (scRNAseq) of the putative 4N cells to distinguish SC subpopulations, including any pluripotent cells and lineage-restricted progenitors that may exist. Second, we are using existing scRNAseq datasets to discover SC markers, including cell surface receptors. We have already identified one candidate, a protocadherin- α ortholog, and are raising monoclonal antibodies. Single-nuclei RNAseq of anterior body fragments is also underway to improve our ability to detect subtle differences in gene expression between SC subpopulations in the neck. We anticipate that these approaches will yield novel insights regarding the composition of tapeworm SCs and facilitate further work to understand SC function, potency, and plasticity.

P4. Network for advanced NMR and CCRC NMR Facility: Opportunities for metabolomics

Mario Uchimiya¹, Alexander Eletsy¹, John Glushka¹, John Grimes¹, Laura Morris¹, Arthur S. Edison¹

¹Complex Carbohydrate Research Center, University of Georgia, Athens GA 30602

The Network for Advanced NMR (NAN) is an NSF-funded partnership between the University of Georgia, the University of Connecticut, and the University of Wisconsin at Madison (UW-Madison). Our goal is to provide access to state-of-the-art NMR resources for the scientific community. This includes a web portal for instrument search, user management, and data archiving and retrieval, and knowledgebases for biological and materials sciences, especially for users with limited NMR experience. This project includes the installation of two 1.1-GHz NMR instruments, a solid-state instrument currently operational at UW-Madison, and a solution-state instrument to be installed at CCRC in the summer of 2024. The CCRC NMR facility also features several instruments in the 600-900 MHz range with unique capabilities, including sample changers for automation, high-sensitivity cryogenic probes with ¹H, ¹³C, ¹⁵N and ¹⁹F detection, and a unique 1.7-mm 800-MHz system for small samples in capillary tubes. The knowledgebases for metabolomics provides protocols and optimized NMR experiment parameter sets for metabolomics. It also includes example data sets, data processing tools, training, and educational materials. Researchers who are interested in applying metabolomics to their specific research projects can make use of these resources at NAN and the CCRC. This work is supported by NAN, the Edison Lab at UGA, the Georgia Research Alliance.

P5. To inhibit or not to inhibit: the *Toxoplasma gondii* homolog of ATPase inhibitory factor 1 (TgIF1)

Madelaine M. Usey^{1,2} and Diego Huet^{1,3}

¹Center for Tropical and Emerging Global Diseases, University of Georgia, Athens, Georgia, USA;

²Department of Cellular Biology, University of Georgia, Athens, Georgia, USA; ³Department of Pharmaceutical and Biomedical Sciences, University of Georgia, Athens, Georgia, USA

As the powerhouse of the cell, the mitochondrion plays a key role in generating energy. While the mitochondrial ATP synthase is critical for this energy production, its activity must be tightly regulated for the cell to adapt to varying conditions. One key regulator is the widely conserved ATPase inhibitory factor 1 (IF1), which inhibits ATP synthase activity and activates cytoprotective gene expression pathways through a process known as mitohormesis. As we know little about how the ATP synthase is regulated in the parasite *Toxoplasma gondii*, we characterized the *T. gondii* homolog of IF1 (TgIF1). We found that TgIF1 knockout and overexpression had no impact on metabolism or growth under baseline conditions. However, TgIF1 overexpression increased ATP synthase oligomerization and knockout reduced cristae density. Additionally, TgIF1 knockout and overexpression both reduced growth under hypoxic conditions. Furthermore, TgIF1 overexpression improved recovery from oxidative stress, suggesting a mitohormetic role of TgIF1. This was corroborated by RNAseq data which showed that the main group of genes impacted by TgIF1 overexpression are involved in gene expression regulation and RT-qPCR data indicating that TgIF1 levels correlate with the expression of several antioxidants. In summary, while TgIF1 does not significantly impact metabolism, it plays a key role in cristae biogenesis and stress response adaptations.

P6. Exploration of 3-nitro-2-phenyl-2H-chromene analogues for potent antitrypanosomal activity

¹Shane M. Carey, ¹Destiny M. O'Neill, ¹Garrett B. Conner, ²Julian Sherman, ²Ana Rodriguez, ¹Edward L. D'Antonio

¹University of South Carolina Beaufort, Bluffton, SC, USA; ²New York University, New York, NY, USA

Chagas' disease and sleeping sickness are both neglected tropical diseases caused by human pathogenic protozoa, such as *Trypanosoma cruzi* and *Trypanosoma brucei*, respectively. There is currently a lack of effective and tolerable therapeutics to treat these life-threatening illnesses. In 2019, my laboratory and the laboratory of Dr. Artur Cordeiro (Campinas, São Paulo, Brazil) were responsible in identifying a cluster of anti-*T. cruzi* compounds called the 3-nitro-2-phenyl-2H-chromene analogues, by performing a high-throughput screen [Mercaldi, G. F. et al. (2019) *Bioorg. Med. Chem. Lett.* 29, 1948-1953]. Compound *GLK2-003* from this study was determined to inhibit *T. cruzi* glucokinase (*TcGlcK*), in which it revealed an IC_{50} of 6.1 μ M, and it was also proposed to be inhibiting the *T. cruzi* hexokinase. *TcGlcK* is a potential drug-target for *T. cruzi* because its product glucose-6-phosphate serves as a key metabolite in various metabolic pathways in the protozoan parasite, such as the pentose phosphate pathway, glycolysis, and gluconeogenesis. In this study we screened a small compound library consisting of 13 compounds having a 3-nitro-2-phenyl-2H-chromene scaffold. Twelve of these compounds had a one-point change from *GLK2-003*, and by performing this hit expansion, the compounds were first run through a target-based screen vs. *TcGlcK*. Subsequently, two *in vitro* phenotypic screening assays were performed against the trypanosomatid parasites *T. cruzi* and *T. brucei* to explore for the possibility of dual antitrypanosomal biological activity. A structure – activity relationship analysis was carried out along with *TcGlcK* K_i determinations, mode of enzyme inhibition, and *in vitro* antiparasitic IC_{50} determinations.

P7. Role of essential members of the necroptosis pathway to *Toxoplasma gondii* infection

Billy Erazo^{1,2} and Laura Knoll¹

¹Department of Medical and Microbial Immunology, University of Wisconsin, Madison, Wisconsin, USA;

²Cellular and Molecular Pathology, University of Wisconsin, Madison, Wisconsin, USA

The parasite *Toxoplasma gondii* has been shown to have infected more than 60% of specific populations worldwide. In some instances where it's not treated, or the host is compromised, it can lead to life-threatening illnesses. Efficient cell death and innate immune response are essential for controlling the parasite burden during the early stages of the infection. These fundamental responses are still poorly understood because of the complexity of the parasite, shortage of tools, and lack of research interest for the host during the infection. This study investigates how members of the necroptosis cascade contribute at different stages of acute and chronic infection. Our laboratory has found that mice models harboring deletion in a central mediator of necroptosis (RIPK3^{-/-}) show improved survival after oral *T. gondii* infection without reducing parasite burden compared to intraperitoneal *T. gondii* infection. Now, we are focusing on the role of the executioner of necroptosis (MLKL) during intraperitoneal infection of *T. gondii*. Our results will help us understand if essential members of the cell death pathway necroptosis engage in a beneficial or detrimental response for the host during the pathogenesis of toxoplasmosis.

P8. Hypothetical protein Tb427.2.5810 is a nuclear effector of CK1.2 signaling in the African trypanosome

Benjamin Hoffman², Kojo Mensa-Wilmot^{1,3}

¹Department of Molecular and Cellular Biology, Kennesaw State University, Kennesaw, GA 30144,

²Department of Cellular Biology, University of Georgia, Athens, GA 30602, ³Center for Tropical and Emerging Global Diseases, UGA, Athens, GA 30602

The parasitic protozoan *Trypanosoma brucei* duplicates several organelles before cytokinesis. Casein kinase CK1.2, a cytoplasmic protein, regulates cytokinesis, mitochondrial DNA (kDNA) division, DNA synthesis and basal body copy number in the trypanosome: How CK1.2 signaling modulates these nuclear and cytoplasmic processes spanning two cell cycle stages is not understood. An unstudied "hypothetical" protein Tb427.2.5810 was identified as a potential effector for TbCK1.2 actions, from a phosphoproteomic study in which polypeptides that were de-phosphorylated after knockdown of CK1.2 were compiled. Localized in the nucleus, knockdown of Tb427.2.5810 arrested proliferation of bloodstream *T. brucei*. In Tb427.2.5810-deficient trypanosomes, cytokinesis is inhibited, kinetoplast division is inhibited, and nuclear DNA synthesis is reduced whereas basal body copy number increases beyond the norm. Thus Tb427.2.5810 is the first nuclear effector for CK1.2 signaling (NECKS1). In future studies we will determine whether the phosphorylation status of NECKS1 directly impacts its biological roles.

P9. Integration, exploration & reuse of clinical & epidemiological datasets on the ClinEpiDB platform

¹Danica Helb, ²Jay Humphrey, ³Sarah Kelly, ²Nupur Kittur, ¹David Roos; on behalf of the ClinEpiDB team
¹University of Pennsylvania, USA; ²University of Georgia; USA; ³Imperial College London, UK

Data is unquestionably the most important output of scientific research, and data that meets FAIR (Findability, Accessibility, Interoperability, and Reuse) guidelines has tremendous potential for being preserved over time and used for secondary data analysis, accelerating discovery and translational impact. Access to study data is increasingly mandated by journals, funders, and scientists themselves. For example, the US National Institutes of Health (NIH) has recently mandated timely sharing of data generated by NIH-funded research. Building on decades of experience hosting diverse eukaryotic pathogen and host genomics datasets on VEuPathDB.org, we have developed ClinEpiDB.org as a free, open-access, web-based resource to facilitate the management, exploration, sharing, and reuse of de-identified data from field surveillance, clinical and epidemiological studies, and trials. ClinEpiDB currently hosts data from 50 global studies in domains including maternal, newborn & child health and infectious diseases such as malaria and schistosomiasis. An example is data integrated from the Malnutrition and the Consequences for Child Health Study (MAL-ED), a multi-center longitudinal cohort study of childhood diarrhea and malnutrition with >1.8 observations and nearly 900 variables, including data on prevalence of cryptosporidium, giardia, and other eukaryotic pathogens in stool samples from children with and without diarrhea. Recent developments in ClinEpiDB include tools for mapping of geospatial data and a feature allowing users to privately upload and explore their own datasets.

P10. Eukaryotic Pathogen, Vector and Host Informatics Resources (VEuPathDB.org)

Susanne Warrenfeltz and Jessica Kissinger for the VEuPathDB Team
CTEGD, Athens, GA

The VEuPathDB project is an NIAID-funded Bioinformatics Resource Center (BRC) that provides free, online access to genomic-scale data mining resources for investigating the biology and biochemical processes of pathogens, disease vectors, and related taxa. VEuPathDB supports > 600 species of protozoan parasites, fungi and oomycetes, arthropod vectors of disease and selected hosts. VEuPathDB resources empower end-users to leverage diverse Omics data to discover meaningful relationships from large volumes of data in support of hypothesis driven research without requiring specialized computational skills. VEuPathDB integrates advanced search capabilities with data visualizations, analysis tools, and genome browsing to facilitate research concerning a single gene or genome-wide topics such as stage-specific gene expression, transcriptional mechanisms and gene model integrity. Although available data differs between organisms, data types can include genome sequence and population-level variation data, manually curated and automatically generated annotation; transcriptomic, proteomic and epigenetic data, pathways, genome-wide phenotypic analyses, host-pathogen interactions and selected clinical data. A phylogenetic framework provides cross-species functional inference via orthology. User support includes an email help desk, social media, video tutorials, webinars, and a worldwide program of workshops. Please email us at help@VEuPathDB.org for more information.

P11. Conditional knockdown of Aurora and Polo-like kinases in *Trypanosoma cruzi* using a hammerhead ribozyme reveals essential roles for the kinases in parasite cell division

Justin Wiedeman², Ruby Harrison^{1,2}, Drew Etheridge^{1,2}

¹Department of Cellular Biology, University of Georgia, Athens, Georgia, USA; ²Center for Tropical & Emerging Global Diseases, University of Georgia, Athens, Georgia, USA

The protozoan parasite *Trypanosoma cruzi* is responsible for Chagas disease, the most severe parasitic illness of the Americas. Despite the medical importance of *T. cruzi*, our knowledge of the identity and function of essential genes is severely limited by the lack of a conditional knockdown system. Unlike its more genetically tractable cousin *Trypanosoma brucei*, *T. cruzi* lacks the necessary enzymatic machinery to facilitate RNAi based functional studies. Additionally, the diploid nature of the *T. cruzi* genome and arrangement of genes into polycistronic “cassettes” which lack clearly defined promoters, precludes the use of standard molecular approaches to analyze essential gene function. Here we describe the development and use of a functional conditional knockdown system based on a tetracycline-responsive hammerhead ribozyme (HHR) inserted into the 3’ un-translated (UTR) region of genes of interest. Using this new tool, we demonstrated the essential nature of multiple protein-coding genes, including the genes for the Aurora and Polo-like kinases. We discovered roles for these kinases in mitosis and cytokinesis, respectively, with these studies mirroring their known roles in *T. brucei*. Importantly, we demonstrated the effectiveness of the HHR knockdown system in intracellular amastigotes, the medically relevant mammalian stage of the parasite. This conditional knockdown system allows, for the first time, the straightforward functional characterization of essential genes in *T. cruzi*.

P12. Looking for a START in *Toxoplasma gondii*: Insights into membrane contact site members

Christofer K. Zepeda Guisa¹ ; Diego Huet^{1,2}

¹Center for Tropical & Emerging Global Diseases, University of Georgia, Athens, Georgia, USA, ²Department of Pharmaceutical and Biomedical Sciences, University of Georgia, Athens, Georgia, USA

Membrane contact sites (MCSs) are regions of close proximity between two organelles that allow dynamic interaction and exchange of various molecules. In eukaryotes, MCSs have been directly implicated in organellar dynamics, signaling pathways, and transfer of lipids, ions and signaling molecules. Steroidogenic acute regulatory protein-related lipid transfer (START) domain-containing proteins are frequently found at MCSs, where they mediate lipid transfer between organelles. In addition to their lipid trafficking activity, START proteins serve diverse roles throughout the cell including but not limited to organellar dynamics and lipid signaling. Although MCSs and the structural motif of the START domain are conserved throughout animals, plants, and fungi, seldom is known in *Toxoplasma gondii*, a parasitic protist from the Apicomplexa phylum. Three out of the seven predicted START proteins in *T. gondii* were studied. Preliminary data for each gene conferred possible localization to either the plasma membrane, endoplasmic reticulum, or the apicoplast. Topology experiments for the apicoplast-associated START protein suggest that the C-terminus is not exposed to the cytosol, but rather localized to one of the interior compartments of the apicoplast. Membrane extractions will be performed to characterize protein topology and determine its membrane association. Additionally, strategies to create conditional knockdown strains will be developed to analyze the function of these proteins throughout the parasite. Through the mechanisms described, this study will provide preliminary understanding into the localization of putative START proteins in *T. gondii* and thereby provide insights into this class of MCS members.

P13. Investigating phenotypic, genotypic and genomic changes in a drug-susceptible isolate of *Haemonchus contortus* under field selection with fenbendazole

Leonor Sicalo Gianechini¹, Eva-Michelle Belikova¹, Sophia E. Parker¹, Cassan Pulaski¹, Jeferson Lourenco², Elizabeth Redman³, John S. Gilleard³, Erik C. Andersen⁴, Ray M. Kaplan^{1,5}

¹Dept. of Infectious Diseases, College of Veterinary Medicine, UGA, Athens, GA, USA ²Dept. of Animal and Dairy Science, UGA, Athens, GA, USA ³Faculty of Veterinary Medicine, Host-Parasite Interactions Program, University of Calgary, Alberta, Canada. ⁴Dept. of Biology, Johns Hopkins University, Baltimore, MD, USA

⁵School of Veterinary Medicine, St. George's University, Grenada, West Indies.

Benzimidazole (BZ) anthelmintics are crucial for treating parasites in pets, livestock, and humans. Benzimidazoles bind to beta-tubulin inhibiting microtubule formation, and missense mutations in this gene at codons 134, 167, 198, and 200 are associated with BZ-resistance in strongylid nematodes. However, evidence suggests that other mechanisms may also be involved. We are investigating the genetic and genome-wide impact of BZ selection to identify additional loci that could account for the full *in vivo* resistance phenotype. A group of goats infected with a drug-susceptible isolate of *Haemonchus contortus*, received subtherapeutic doses of fenbendazole at intervals of 4-8 weeks. Phenotypic tests were paired with deep amplicon sequencing of beta-tubulin genes. Our data shows that the primary beta-tubulin mutation selected was F200Y (frequency:>80%), with F167Y occurring at frequencies below 5%. A quadratic model analyzing the relationship between egg hatch IC₅₀ and F200Y% exhibited a strong fit (R²=64%), yet 36% of the variability in the resistant phenotype remains unexplained by the model. Haplotype analysis of sequenced amplicons is pending along with whole genome sequencing of individual worms, which will offer more insights into genetic diversity and variant effects on phenotypes.

P14. The NIH/NIAID Filariasis Research Reagent Resource Center (FR3)

Elyssa Campbell¹, Christopher Evans¹, Michael Dzimianski¹, Andrew Moorhead¹

¹Department of Infectious Diseases, College of Veterinary Medicine, University of Georgia, Athens, GA

Since its establishment in 1969, the Filariasis Research Reagent Resource Center (FR3) has supported the filariasis research community. By maintaining the life cycles of multiple species, including *Brugia malayi* and *B. pahangi*, offering live parasites alongside molecular reagents, the FR3 provides investigators with the resources needed to advance filariasis research. Moreover, the FR3 offers support through experimental protocols and technical assistance. Funded by the National Institute of Allergy and Infectious Diseases under its Preclinical Models of Infectious Disease program, all resources are provided free of charge, with the investigator only bearing the cost of shipping. These resources have enabled research studies spanning hundreds of publications, furthering our understanding of these parasites and means towards their elimination.

P15. Feeding frenzy! Exposure to conditioned media from highly virulent *Naegleria fowleri* increase cytopathic effects of lowly virulent isolates

Joseph Dainis^{1,2} ; Dennis Kyle^{1,2,3}

¹Center for Tropical & Emerging Global Diseases, UGA, Athens, Georgia, USA; ²Dept. of Infectious Diseases, UGA, Athens, Georgia, USA; ³Dept. of Cellular Biology, UGA, Athens, Georgia USA

Naegleria fowleri (*Nf*), also known as the brain-eating amoeba, is a eukaryotic, free-living species of amoebae that is the causative agent of Primary Amoebic Meningoencephalitis (PAM). Although there are only on average 5 documented cases of PAM in the United States per year, the infection has a 97% mortality rate. This mortality rate is primarily due to the lack of effective drug treatments used to treat *Nf* infections, as none of the currently available drugs specifically target the amoeba. One explanation for the lack of available drug treatments for *Nf* infections is that there are not enough viable amoeba drug targets. Many of the virulence factors of *Nf* and the specifics of the relationship that the amoeba has with its human host remain poorly characterized. Very little is understood about whether *Nf* amoebae are capable of mediating virulence similar to how other parasitic pathogens, such as *Trypanosoma* and *Leishmania* sp, can through their secretome, which includes extracellular vesicles and secreted proteins. Recent work in the lab has discovered that there are two distinct virulent populations of *Nf* clinical isolates as evidenced by differences in vitro measurements of cytopathic effects on cells and in vivo survival curves in mice. Here, we show that conditioned media isolated from highly virulent *Nf* amoebae can drive lowly virulent *Nf* amoebae to feed faster on diverse mammalian cell monolayers, including Vero monkey green kidney cells and B103 neuroblastoma cells. This result indicates that *Naegleria fowleri* is capable of communicating virulent phenotypes through secreted factors, and future directions for this project will look to identify possible secreted proteins or extracellular vesicle contents that could mechanistically drive this increased feeding rate phenotype.

P16. Malaria parasites do not manipulate pathways controlling vitellogenesis in the mosquito *Anopheles stephensi*

Jett P. Flentje¹, Benjamin L. Phipps^{1,2}, Mark R. Brown^{1,3}, Michael R. Strand^{1,3}

¹Center for Tropical and Emerging Global Diseases, ²Department of Genetics, ³Department of Entomology, University of Georgia, Athens, GA

Mosquitoes infect more than 250 million people with malaria and kill more than 400,000 annually despite global efforts to reduce transmission. Malaria-causing *Plasmodium* spp. have coevolved with *Anopheles* mosquitoes such that they can exploit nutrients mobilized for mosquito egg formation without negatively impacting fecundity. We hypothesized that *Plasmodium* infection alters expression of mosquito genes associated with metabolism and egg production to modulate nutrient availability. *A. stephensi* females were infected with the mouse malaria parasite *P. berghei* and expression of genes regulating lipid mobilization and yolk protein synthesis was measured in infected and uninfected individuals using RT-qPCR. We also measured triglycerides and glycogen in the fat body, an insect nutrient storage tissue, over the course of infection. Surprisingly, we found no differences between infected and uninfected females in any of the endpoints described above. We reasoned that *Plasmodium* avoids perturbing egg production because this would impose a fitness cost to the vector and potentially jeopardize transmission.

P17. Commensal bacteria enable development of mosquito larvae on detritus diets

Derek T. Huck^{1,2} and Michael R. Strand^{1,2}

¹Department of Entomology, University of Georgia, Athens, Georgia, USA; ²Center for Tropical and Emerging Global Diseases, University of Georgia, Athens, Georgia, USA

Mosquitoes like *Aedes aegypti* are best known as blood-feeding vectors of diseases including dengue, yellow fever, and filariasis. Pathogen transmission crucially depends on the abundance of adults in a given population that are competent vectors. Adult abundance is determined by the development of larval mosquitoes that are strictly aquatic. One factor that substantially influences larval development is resource acquisition. Field habitats where mosquito larvae develop primarily contain plant detritus that forms the base of the food web and microbial communities which consist of one or more trophic levels. Mosquito larvae are usually the top-level consumers but the roles of detritus and microbes as resources for development into adults are largely unclear. In this study, we used recently developed methods for producing axenic mosquito cultures with no microbes and gnotobiotic cultures with defined microbial communities to investigate the role of detritus and microbes in the development of *A. aegypti*. Bioassays initially indicated that axenic larvae fail to develop on plant detritus from multiple field sites. However, larval development was rescued by adding cultures of undefined microbial communities obtained from the same sites. Furthermore, we identified simplified communities of 4-8 bacterial species that supported development of larvae into adults on detritus diets. Genome sequencing of select bacteria in these simplified communities revealed that specific microbes in larval habitats provision nutrients deficient in detritus that *A. aegypti* larvae require for development.

P18. Expression, purification, and characterization of arginase from *Leishmania infantum*

¹Anna L. Husted, ¹Edward L. D'Antonio

¹University of South Carolina Beaufort, Bluffton, SC, USA

Leishmaniasis is known to be one of the top 10 global neglected tropical diseases and it affects over 12 million people. Visceral leishmaniasis is considered to be the most severe form a patient can acquire that usually leads to spleen and liver enlargements as well as death, if left untreated. The disease is caused by a human pathogenic protozoan parasite of the genus *Leishmania*. There are over 20 different species of this parasite that are harbored in the female phlebotomine sandfly vector. Clinically available medicine for the treatment of leishmaniasis is generally not that effective and has very harsh side effects. Moreover, the development of new and improved therapeutics has been in demand for many years. We were interested in characterizing arginase from *Leishmania infantum*, as the enzyme is a drug-target, and this particular species behaves differently than many of the other species. *L. infantum* has been reported to evade the human immune system by multiplying and hiding within macrophages where the pH of the environment is quite acidic (pH of ~2), but arginase activity is generally understood to function at much higher pH values (e.g., pH range of 8.0 - 9.5). This is in part due to the formation of a binuclear manganese cluster that anchors a bridging hydroxide deep inside the active site. In an effort to better understand the role of arginase and its mechanism for this parasite, we report on the recombinant overexpression (in an *Escherichia coli* host), purification, and initial molecular characterization studies.

P19. Poly(A)-Binding Protein 2 and DRBD3 are physiologic targets of CBL0137, a lead compound for Human African Trypanosomiasis drug development

Gaurav Kumar¹ and Kojo Mensa-Wilmot^{1,2}

¹Department of Molecular and Cellular Biology, Kennesaw State University, Kennesaw, GA. ²Center for Tropical and Emerging Global Diseases, University of Georgia, Athens, GA

Human African Trypanosomiasis (HAT) is caused by the protist *Trypanosoma brucei*. Discovered by phenotypic screening the carbazole derivative CBL0137 cures HAT in a mouse model of disease. The targets of CBL0137 are unknown. Some compounds in clinical trial are effective because of off-target effects, indicating that some studies to identify targets are incomplete. We used a multidisciplinary strategy to identify physiologic targets of CBL0137. In affinity chromatography, fourteen proteins including UMSBP2 associated with CBL0137. Drug-associated proteins are not automatic physiologic targets: Genetic perturbation of a physiologic target produces molecular phenotypes identical to those obtained by treatment of cells with low concentrations of drug. CBL0137 inhibits DNA synthesis, translation of polypeptides, mitosis, and endocytosis of transferrin in *T. brucei*. Polypharmacology of CBL0137 was established after knockdown of four CBL0137-associated polypeptides. A deficiency of poly(A)-binding protein 2 (PABP2) and RNA binding protein DRBD3 inhibits mitosis and DNA replication, revealing novel functions of the two proteins. We infer that PABP2 and DRBD3, like replication protein A1 (RPA1), but unlike UMSBP2, are physiologic targets of CBL0137.

P20. Characterization of an Endoplasmic Reticulum Calcium-binding protein in *Toxoplasma gondii*

Miryam Andrea Hortua-Triana¹, Rodrigo Baptista^{1,2}, Justin C. Berry¹, Leticia DoAmaral^{1,4} and Silvia N. Moreno^{1,3}

¹Center for Tropical and Emerging Global Diseases, ²Department of Computer Science, ³Department of Cellular Biology and ⁴Department of Biochemistry and Molecular Biology, University of Georgia, Athens, Georgia 30602

Toxoplasma gondii is a pathogen that belongs to the phylum Apicomplexa and infects one third of the world human population. The chronic stages of *T. gondii* remain in the intermediate host for its entire life. Most toxoplasmosis complications are due to reactivation of a chronic infection in immune-deficient patients. As an obligate intracellular parasite, active invasion of host cells is essential for its virulence. In *T. gondii*, stimulation of motility and the subsequent invasion and egress events have been shown to be activated by release of intracellular Ca²⁺ stores. The endoplasmic reticulum (ER), likely the main Ca²⁺ store is important for both Ca²⁺ homeostasis and signaling. We identified a member of the CREC family (Cab45, reticulocalbin, ERC-45, calumenin), a calcium-binding protein (Tg229480 or TgERC), with multiple EF-hand domains that localized to the ER. Deletion of the **TgERC** gene resulted in *T. gondii* mutants with reduced capacity to store Ca²⁺ in the ER. Using chemical Ca²⁺ indicators showed that when exposed to high extracellular Ca²⁺ ([Ca²⁺] ~1.8 mM) the ER of the mutant parasites is unable to retain Ca²⁺. Phenotypic analysis of the Δ TgERC mutant expressing GCaMP6f showed a delayed egress compared to wild type, and the typical spike of Ca²⁺ that precedes egress was significantly diminished. Biotin proximity labelling by TurboID tagged TgERC identified two enriched set of proteins; one associated with ER proteins including the Ca²⁺ ATPase SERCA pump, and a second set associated with the Apicomplexa unique secreted organelles such micronemes and rhoptries. These were corroborated by western blot. **TgERC** modulatesd SERCA activity measured in vitro was important for Ca²⁺ storage. Further functional analysis of **TgERC** interacting partners of the secretome is ongoing.

P21. Exploring enolase inhibitors as therapeutic agents for *Naegleria fowleri* infection

Jillian McKeon, Samuel Kwain, Colm Roster, Dan Whitehead, James Morris
Eukaryotic Pathogens Innovation Center, Clemson University, Clemson, SC

Current treatments for *Naegleria fowleri* infections are inefficacious and mortality rates remain over 95%. Recently, human enolase 2 (ENO2) phosphonate inhibitors have been developed as lead agents to treat glioblastoma. Given the importance of glycolysis to the amoebae (Milanes, 2018), these ENO2 inhibitors were explored as possible therapeutic leads. The phosphonate compounds were potent inhibitors of recombinant *Nf*ENO, with the most potent, (1-hydroxy-2-oxopiperidin-3-yl) phosphonic acid (HEX) having an IC_{50} value of $0.14 \pm 0.04 \mu\text{M}$. To gain insight into how promising inhibitors interacted with *Nf*ENO, we performed molecular docking studies with *Nf*ENO (PDB 7UGH). The docking revealed the phosphonate agents bind to the *Nf*ENO active site with varying affinities (-8.6 to -6.2 kcal/mol), mirroring potency. HEX was also a potent amebicide, with an EC_{50} value of $0.21 \pm 0.02 \mu\text{M}$ (~1500-fold lower than the CC_{50}). A pilot experiment in which amoebae-infected rodents were treated with HEX by nasal instillation increased longevity, with eight of 12 HEX-treated rodents remaining alive (resulting in an undefined median survival time) while the vehicle-treated group had a median survival time of 10.9 days. However, brain extraction analysis showed six of the eight survivors were positive for amoebae, indicating that HEX suppressed the infection but did not eliminate it. In summary, the phosphonate based ENO1 inhibitors were potent *Nf*ENO inhibitors, toxic to *Naegleria* in culture, and showed promise in a rodent study suggesting these compounds could be further developed for use in treatment of infections.

P22. Understanding the significance of Subunit K in the *Toxoplasma* ATP Synthase

Samuel Nyarko^{1,2}, Kaelynn Parker^{1,2} and Diego Huet^{1,3}

¹CTEGD, UGA ²Cellular Biology, UGA ³Pharmaceutical & Biomedical Sciences, UGA, Athens, GA.

Apicomplexans cause major parasitic infections and death worldwide with key contributors being *Plasmodium spp.*, *Cryptosporidium* and *Toxoplasma gondii*. The complex life cycles of these parasites are distinguished by their ability to adapt their metabolism to a wide range of hosts and diverse nutritional needs. In yeast and mammals, the mitochondrial ATP synthase, a protein complex composed of 15 different subunits, is at the centre of cellular metabolic adaptations. In apicomplexans, the ATP synthase is larger than its yeast and mammalian counterparts. The apicomplexan complex harbours 32 subunits, with 17 apicomplexan-specific proteins. Moreover, several canonical ATP synthase subunits have unique, apicomplexan-specific extensions. Using *T. gondii* as a model, we are studying the function of the ATP synthase subunit k, a canonical subunit with an ubiquitin-like extension only found in apicomplexans. This extension is likely important in the metabolic and physiological requirements of these parasites. Using the U1-snRNP gene silencing strategy, we demonstrate that subunit k is indispensable for the parasite lytic cycle, and we unexpectedly observe that the absence of subunit k does not lead to mitochondrial fragmentation. Additionally, our blue native PAGE results indicate that subunit k is crucial in the assembly and stability of the *T. gondii* ATP synthase. In our ongoing research, we aim to utilize immunoprecipitation and ubiquitin binding assays to explore protein-protein interactions and elucidate the function of the ubiquitin-like domain. Our findings offer a basis for the study of the function of Subunit k on mitochondrial function and structure including cristae density in *T. gondii*. Functionally characterizing the role of this subunit will provide insights into the apicomplexan biology, evolutionary adaptations, and potential therapeutic targets.

P23. Early-stage therapeutic drug discovery of Chagas' Disease: Investigation of gossypol-based inhibitors of *Trypanosoma cruzi* glucokinase

^{1,2}Carson S. Frey, ^{1,2}Destiny M. O'Neill, and ¹Prof. Edward L. D'Antonio

¹Department of Natural Sciences, University of South Carolina Beaufort, Bluffton, SC, USA; ²Authors contributed equally to this work.

Chagas' disease is a neglected tropical disease predominantly affecting people in Latin America and is caused by the tropical protozoan parasite *Trypanosoma cruzi*. In a previous study by our laboratory, three derivatives of gossypol were examined in a high-throughput screening, and were identified to exhibit moderate potency against both the potential drug-target *T. cruzi* glucokinase (*TcGlcK*) and *T. cruzi* parasites. To expand on this previous research, a commercially available library of synthetic gossypol analogues was used to screen *TcGlcK* in a hit-expansion study. Through biochemical evaluation, we have found several gossypol analogues exhibiting strong inhibition of *TcGlcK*. To test the enzyme inhibition, we subjected each compound to an enzyme inhibition kinetics reaction. The collected data for each compound was plotted for both $1/v$ and $[S]/v$ against the inhibitor concentration. The results of this compound screening include the inhibitory constant (K_i) determinations. The K_i values from the compound screening were then compared to the K_i of naturally occurring gossypol, which was observed to have a value of 7.7 ± 3.2 micromolar.

P24. A combination of four nuclear targeted effectors protects *Toxoplasma* against interferon gamma driven human host cell death during acute infection

Aubrey J Phillips^{1,2}, Brittany Henry^{1,2}, L. David Sibley³, Alex Rosenberg^{1,2}

¹CTEGD, UGA, Athens, GA ²Dept. of Infectious Diseases, CVM, UGA, Athens, GA. ³Dept. of Molecular Microbiology, Washington University School of Medicine, St Louis, MO, USA

In both mice and humans, Type II interferon-gamma (IFN γ) is crucial for regulation of *Toxoplasma gondii* (*T. gondii*) infection, during acute or chronic phases. To thwart this defense, *T. gondii* secretes protein effectors hindering the host's immune response. For example, *T. gondii* relies on the MYR translocon complex to deploy soluble dense granule effectors (GRAs) into the host cell cytosol or nucleus. Recent genome-wide loss-of-function screens in IFN γ -primed primary human fibroblasts identified MYR translocon components as crucial for parasite resistance against IFN γ driven vacuole clearance. However, these screens did not pinpoint specific MYR-dependent GRA proteins responsible for IFN γ signaling blockade, suggesting potential functional redundancy.

Our study reveals that *T. gondii* depends on the MYR translocon complex to prevent host cell death and parasite premature egress in human cells stimulated with IFN γ post-infection, a unique phenotype observed in various human cell lines but not in murine cells. Intriguingly, inhibiting parasite egress did not prevent host cell death, indicating this mechanism is distinct from those described previously. Genome-wide loss-of-function screens uncovered TgIST, GRA16, GRA24, and GRA28 as effectors necessary for a complete block of IFN γ response. GRA24 and GRA28 directly influenced IFN γ driven transcription, GRA24's action depended on its interaction with p38 MAPK, while GRA28 disrupted histone acetyltransferase activity of CBP/p300. Given the intricate nature of the immune response to *T. gondii*, it appears that the parasite has evolved equally elaborate mechanisms to subvert IFN γ signaling, extending beyond direct interference with the JAK/STAT1 pathway, to encompass other signaling pathways as well.

P25. Development of molecular tools for transgene expression in *Naegleria fowleri*

Caroline Palmentiero¹, James Morris¹

¹Eukaryotic Pathogens Innovation Center, Clemson University, Clemson, SC

The pathogenic free-living amoeba, *Naegleria fowleri*, is the causative agent of primary amoebic meningoencephalitis (PAM). This infection has a fatality rate of 97%, in part due to the lack of readily available, effective drugs against this eukaryote. The development of molecular tools to enable trans-gene expression in this organism will provide vital insight into gene function while allowing genetic validation of potential drug targets. We have designed a transfection vector containing constitutive promoters to express both antimicrobial resistance and reporter genes. This vector, termed pCJ2, contains a puromycin resistance gene downstream of 1090 base pairs of the 5'UTR and 350 base pairs upstream of the 3'UTR of a putative actin gene. The construct also includes a green fluorescence reporter gene, eGFP, downstream of 1090 base pairs of a predicted ubiquitin promoter. We explored various transfection approaches for our organism, including using the Viafect reagent, Amaxa Nucleofector technologies, and electroporation of flagellated cells (100V, 500 μ F, 400 Ω). Future studies will optimize our expression vector, exploring the utility of the UTRs of other genes to drive expression. In addition, we will work to incorporate additional molecular techniques, such as CRISPR/Cas9 editing, to enhance transgene expression. For example, by integrating a landing pad for integration of our vectors for stable maintenance. Development of these techniques will ultimately allow us to bring the power of genetics to bear on understanding this important human pathogen.

P26. The joy of cooking for mosquitoes: Identifying blood nutrients required for egg formation in mosquitoes

Benjamin L. Phipps^{1,2}, Jett P. Flentje¹, Mark R. Brown^{1,3}, Michael R. Strand^{1,3}

¹Center for Tropical and Emerging Global Diseases, ²Department of Genetics, ³Department of Entomology, University of Georgia, Athens, GA

The blood-feeding behavior of female mosquitoes underlies their effectiveness as disease vectors. Mosquito lifestyles range from autogenous, meaning they can produce eggs without consuming vertebrate blood, to anautogenous, meaning they rely on nutrients in blood for egg formation. Highly anautogenous mosquitoes, such as *Anopheles* spp. that spread malaria, must take at least one blood meal to support each gonotrophic cycle, and thus can transmit pathogens from one vertebrate host to another. Protein is thought to be the primary blood component needed to synthesize egg yolk, and our previous work shows that *ad libitum* access to sucrose solutions containing bovine serum albumin (BSA) partially recapitulates egg production relative to a single blood meal in the model mosquito *Aedes aegypti*. However, this failed to fully rescue fecundity in anopheline mosquitoes, suggesting these species require additional blood components for egg production. Here we tested the ability of artificial blood meals with different nutritional profiles to rescue egg formation in non-blood fed *Anopheles* malaria vectors. BSA alone or supplemented with physiologically relevant concentrations of free amino acids (AAs) failed to support egg formation in most females, suggesting AAs are not the only nutrients required from blood. Supplementation of BSA meals with iron and lipids, however, increased yolk deposition in oocytes. Since mosquito-borne pathogens are thought to exploit blood nutrients to mature within the vector, we next plan to feed *An. gambiae* artificial meals containing the deadly human malaria parasite *Plasmodium falciparum* to identify blood components required for development of malaria parasites and probe the link between mosquito fecundity and vector competence.

P27. Monitoring energy status in living kinetoplastid parasites using a FRET-based AMPK sensor

Carrie L. Baumgardner¹ and James C. Morris²

¹Department of Physics and Astronomy, Clemson University, Clemson, SC. ²Department of Genetics and Biochemistry, Clemson University, Clemson, SC

The central carbon metabolism pathways of the kinetoplastid parasites, including *Trypanosoma brucei* and *Leishmania spp*, have unusual features that make them attractive targets for therapeutic intervention. While most studies on these pathways have been performed using isolated enzymes or cell lysates, new approaches using biosensors in live cells have been developed that will enable near real-time assessment of pathway function and regulation. One master metabolic regulator, AMP-activated protein kinase (AMPK) is activated in response to depletion of energy stores in the cell. The sensor responds to increases in ratios of AMP to ATP and ADP to ATP. Here, we have developed a *T. brucei* parasite cell line that heritably expresses the FRET-based heritable AMPK sensor, ExRai-AMPKar, to measure energy status changes in living parasites. To score sensor activity, parasites were monitored by flow cytometry. Pilot experiments include testing the sensor with a known AMPK activator, AICA riboside (AICAR). Additional studies, including those assessing sensor response in cells that have been nutrient challenged, will be discussed. Transfection studies using the sensor in *L. donovani* and *L. amazonensis* are also ongoing, as we anticipate leishmania may respond differently to nutrient challenges.

P28. Unraveling the enigmatic feeding apparatus of *Trypanosoma cruzi* — Molecular components of the cytostome-cytopharynx complex —

Gonzalo Seminario-Mondejar¹, Nathan Chasen¹, Ronald Etheridge¹

¹Center for Tropical & Emerging Global Diseases, University of Georgia, Athens

Among the pathogenic trypanosomatids, *Trypanosoma cruzi* possesses a unique feeding apparatus called the cytostome-cytopharynx complex (SPC), similar to its free-living kinetoplastid relatives. The SPC functions as the primary mode of endocytosis, allowing *T. cruzi* to capture and internalize extracellular material from its host. Despite its role in host nutrient acquisition, our understanding of the SPC's construction and operational mechanics remains remarkably scant and what we do know has been derived primarily from structural studies. However, we still lack a detailed understanding of the true complexity of the molecular components which potentially make up this organelle.

In order to expand our understanding of the proteomic composition of each of the distinct subregions of the SPC organelle, we have implemented a proximity labeling technique based on the promiscuous biotin ligase known as TurboID. To begin, we fused TurboID to the previously identified myosin associated protein (MyAP) which is both essential for endocytosis and targets to the SPC microtubule rootlet fibers. Our now complete proteomic survey of the MyAP interactome has allowed us to confidently identify over 100 unique proteins. Importantly, our analysis identified a number of previously verified SPC rootlet targeted proteins including the MyAP associated myosin motor MyoF, CP1, CP2, etc. Encouragingly, the majority of the novel identified proteins also have no orthologs in the SPC-deficient *T. brucei* while still being present in free-living kinetoplastids which retain the SPC.

Targets identified in these surveys will be validated using endogenous epitope-tagging with our in-house conditional knockdown system in order to assess their localization and functional contribution to SPC mediated endocytosis.

P29. New mitochondrial ubiquinone synthesis inhibitors that are effective against the acute and chronic stages of *Toxoplasma gondii*

Melissa A. Sleda^{1,2}, Victoria M. Clifton^{1,2}, Satish R. Malwal³, Khaly Diagne⁴, Eric Oldfield³, Roman Manetsch⁴, and Silvia N.J. Moreno^{1,2}

¹Center for Tropical and Emerging Global Diseases, UGA, Athens, GA, USA. ²Dept. of Cellular Biology, UGA, Athens, GA, USA. ³Dept. of Chemistry, University of Illinois at Urbana Champaign, Urbana, IL, USA. ⁴Dept. of Chemistry and Chemical Biology, Northeastern University, Boston, MA, USA

The current treatments against toxoplasmosis are only effective against acute stages with little effect against bradyzoites found in tissue cysts. The mitochondrion of *T. gondii* is a validated target and one of the major antitoxoplasma drugs, atovaquone, inhibit the mitochondrial electron transport chain (ETC) through inhibition of the coenzyme Q:cytochrome c oxidoreductase. The ubiquinone (UQ) molecule consists of a water soluble quinone head and a lipophilic isoprenoid tail that anchors UQ to membranes. Previously we showed that inhibition of the synthesis of the UQ isoprenoid tail by lipophilic bisphosphonates was an effective way to control the acute infection with *T. gondii*. Here we test inhibitors of the isoprenoid and ubiquinone pathways against the acute and chronic stages of *Toxoplasma gondii*. We found tested three bisphosphonate derivatives (BPH-1218, BPH-1236, and BPH-1238) that inhibited the replication of ME49 (a type II cystogenic strain), altered morphology and reduced the viability of *in vitro* and *ex vivo* derived bradyzoites. Most interestingly, BPH-1218 and BPH-1236 reduced the number and size of tissue cysts in the brains of chronically infected mice. In addition, we tested inhibitors of the mitochondrial electron transport chain and found several quinolone derivatives that were effective at decreasing bradyzoite viability and one that was able to protect mice against a lethal acute infection. Altogether we showed that inhibition of the UQ pathway and electron transport chain are viable targets for acute and chronic stages of Toxoplasmosis.

P30. The essential enzymes for the synthesis of ubiquinone are arranged in a large protein complex in *Toxoplasma gondii*

Baihetiya Baierna^{1,2}, Silvia NJ Moreno^{1,2,#}

¹Center for Tropical and Emerging Global Diseases, University of Georgia, Athens, Georgia, USA ²Dept. of Cellular Biology, University of Georgia, Athens, Georgia, USA

The mitochondrion of *Toxoplasma gondii* and of other apicomplexan parasites contains several divergent mitochondrial enzymes. Evidence for this is that the clinically used drug atovaquone specifically inhibit the *T. gondii* mitochondrial electron transport chain (ETC) at the coenzyme Q: cytochrome c reductase. Coenzyme Q (ubiquinone, UQ) is an essential electron carrier in the ETC and a liposoluble antioxidant. UQ is synthesized in the mitochondria by a group of at least 12 enzymes, and many of these enzymes form a multiprotein complex. Most of the information available on the UQ synthesis enzymes were obtained with yeast. However, some enzymes' roles remain unclear. With the aim to characterize the enzymes involved in the synthesis of the UQ molecule and to investigate the potential presence of a multi-protein complex, we first mined the *T. gondii* genome for homologues genes. However, we were not able to find homologues for all the essential enzymes in this pathway. We first characterized the *T. gondii* homologues TgCoq3 and TgCoq5 and determined their essentiality for parasite growth. We used a subcellular fractionation strategy for enrichment of mitochondrial membranes and TurboID proximity biotinylation to identify enzymes involved in this pathway. The results revealed multiple Coq enzyme homologs. We then tagged these proteins to demonstrate their mitochondrial localizations and confirmed that all of them are part of a high molecular weight complex. Overall, our work was able to demonstrate for the first time that *T. gondii* contains a mitochondrially localized UQ synthesis complex.

P31. Screening of recreational areas of rivers for potentially pathogenic free-living amoebae in Georgia water bodies

Shutong Wang¹; Farideh Shahbazi¹

¹Department of Biology, Georgia State University, Atlanta, Georgia, USA

Acanthamoeba, Naegleria fowleri, Balamuthia mandrillaris, and Sappinia pedata are free-living amoebae causing severe human infections. Acanthamoeba can lead to Acanthamoeba keratitis and granulomatous amoebic encephalitis (GAE), while Naegleria fowleri causes primary amoebic meningoencephalitis (PAM). Balamuthia mandrillaris can cause granulomatous amoebic encephalitis (GAE) with high mortality. Sappinia pedata infections are rare but linked to amoebic encephalitis. Several cases of Free-Living Amoeba (FLA) infections have occurred in Georgia, highlighting the need for water body monitoring to prevent human infections.

In this study, water samples were collected from various water bodies in Georgia and subjected to differential centrifugation to isolate fractions potentially containing amoebae. Subsequently, the water samples were cultured on non-nutrient agar with heat-killed E. coli as a growth medium. Microscopic examination was conducted to identify microorganisms with morphological similarities to amoebae. DNA extraction was performed from the water samples, followed by PCR amplification using primers specific to Acanthamoeba, Naegleria fowleri, Balamuthia mandrillaris, and Sappinia pedata. Gel electrophoresis was then employed to analyze the presence of target gene fragments. This approach aimed to detect the presence of amoebae in the water samples and assess their genetic diversity.

P32. The role of polyphosphates in *Toxoplasma gondii*

Juan Camilo Arenas-Garcia^{1,2}; Myriam Andrea Hortua-Triana²; Catherine Li²; Mayara Bertolini^{1,2}; Emily Brooke Schuman³; Henning Jessen⁴; Silvia N J Moreno^{1,2}

¹Center for Tropical & Emerging Global Diseases, UGA, Athens, GA, USA; ²Dept. of Cellular Biology, UGA, Athens, GA, USA; ³Dept. of Microbiology, UGA, Athens, GA, USA; ⁴The Albert Ludwig University of Freiburg, Freiburg, Germany

Polyphosphate (PolyP) is a linear polymer of tens to hundreds of phosphate residues that can reach millimolar levels in protozoan parasites while the concentration in host cells is at the micromolar level. In bacteria, PolyP functions as phosphate reservoir, energy source, chelator of metal ions, regulator of metabolism, stress response, differentiation, and gene expression. In eukaryotes, PolyP plays an equally large number of diverse roles. This catalog of apparently unrelated functions may be the result of PolyP's ability to function as a protein-stabilizing scaffold. *T. gondii* stores PolyP at molar levels in acidocalcisomes which are hydrolyzed upon alkalization followed by release of calcium into the cytosol. Nothing is known about the function of acidocalcisome PolyP in the *T. gondii* infection cycle, virulence, or the establishment of the chronic infection. In *Saccharomyces cerevisiae*, a vacuolar transporter chaperone complex (VTC complex) synthesizes PolyP, in expense of Pi and ATP, and translocates PolyP to the yeast vacuole and acidocalcisomes. The VTC complex is composed of 5 subunits (VTC1-5), in which VTC4 acts as the catalytic subunit. ToxoDB supports the presence of two homologous genes: TGGT1_298630 (TgVTC2) and TGGT1_299080 (TgVTC4). We created mutants for both genes and found that TgVTC2 and TgVTC4 are essential for the *T. gondii* lytic cycle, and that TgVTC2 and TgVTC4 co-localize with the plant-like vacuolar compartment (PLVAC) markers VP1 and CPL, and with the Zinc transporter previously localized to the PLVAC and acidocalcisomes. Both mutants showed a significant decrease in PolyP content. An interactome analysis of TgVTC4 revealed a new potential component of the VTC complex (TGGT1_213910) that is being characterized. In addition, we are studying the role of PolyP as a protein-like chaperone and its role in acidic calcium storage.

P33. Genetic engineering in *Naegleria*

Antonia Blank¹ and James Morris¹

¹ Clemson University, Eukaryotic Pathogens Innovation Center

Naegleria fowleri, commonly referred to as the ‘brain-eating amoeba’, is the causal agent of the rare yet fatal condition known as primary amoebic encephalitis (PAM). Exposure to the pathogen is usually connected to activities that involve instillation of contaminated waters into the nose, allowing the amoebae entry into the brain via the nasal passages. In contrast, its non-pathogenic relative *Naegleria gruberi* provides a safe model organism and can serve in understanding mechanisms without the risk of exposure and connected health risks.

Manipulating gene expression in *Naegleria* has long posed challenges, hindering essential genetic validation crucial for drug development. This study aims to establish CRISPR/Cas9 system ribonucleic acid protein (RNP) complex and adequate delivery methods for gene editing in the brain-eating amoeba and its non-pathogenic counterpart. Our methodology involved the expression and partial purification of SaCas9, a protein predicted to have a molecule mass of 128 kDa. Additionally, we designed and expressed gRNAs targeting the enolase gene for gene ablation. To achieve this, we employed a homology repair template incorporating a stop codon in each reading frame, along with an M13 recognition sequence that facilitates PCR validation of gene editing success. This construct, along with RNP, were subsequently used in transfection experiments with varying approaches. Transfection success was monitored via PCR analysis. To date, we continue to optimize transfection, with upcoming efforts applying our findings in targeting other potential drug targets.

P34. Glycosylation weakens Skp1 homodimerization in *Toxoplasma gondii* by interrupting a fuzzy interaction

D.A. Cantrell¹, R.J. Bieber Urbauer^{1,3}, H.W. Kim^{1,2}, R.J. Woods^{1,2}, J.L. Urbauer^{1,3}, Z.A. Wood¹, C.M. West^{1,2,4}

¹BMB, ²CCRC, ⁴CTEGD, UGA, Athens, GA; ³Department of Chemistry, University of Georgia, Athens, GA

The Skp1; Cullin1; F-Box Protein (SCF) complex is one of several E3 ubiquitin ligases responsible for proteomic control throughout eukaryotes. Target specificity of the SCF complex is mediated by a wide range of F-box proteins (FBPs) which associate with the SCF complex through the Skp1 adaptor protein. Within the intracellular parasite *Toxoplasma gondii*, Skp1 is regulated through glycosylation which alters Skp1’s FBP binding repertoire, and homodimerization which competes with FBP binding. Despite identifying these modes of regulation, we still lack a mechanistic understanding of how they regulate the function of Skp1. Here, we propose that glycosylation weakens the Skp1 homodimer by disrupting a fuzzy self-association of its 34-amino acid C-terminal region (CTR). We found that TgSkp1 forms a homodimer with an apparent affinity comparable to a previously measured FBP-Skp1 interaction. Glycosylation of Skp1’s disordered CTR significantly weakens Skp1’s homodimer, an effect reproduced by CTR deletion. Scrambling the CTR maintains the high affinity homodimer ruling out an extension of the canonical dimer interface. Replacing the CTR with an equal length poly-serine stretch weakens the homodimer to an equal degree as deletion of the CTR, indicating a composition dependent and length independent effect. For a variant with an internal disordered 12-amino acid stretch deleted (Δ loop), a CTR dependent weakening of homodimerization by high salt was observed, implicating electrostatics in CTR mediated dimerization. All Atom Molecular Dynamics simulations of the TgSkp1 dimer indicate a self-association of its CTR mediated by charge block associations. Taken together, our data indicate that glycosylation weakens homodimerization by disrupting a fuzzy self-association mediated by Skp1’s CTR, freeing Skp1 for FBP binding.

P35. Exoneme exocytosis and membrane rupture during malaria parasite egress from RBCs

Watcharatip Dedkhad¹, Manuel A. Fierro¹, Vasant Muralidharan¹

¹Center for Tropical and Emerging Global Diseases, Dept. of Infectious Diseases, University of Georgia, Athens, GA

The malaria parasites reside and proliferate via schizogony within the parasitophorous vacuole (PV) in the host red blood cells (RBCs) before they break through two membranes. The egress of merozoites requires a poorly understood signaling pathway that results in the exocytosis of specialized secretory vesicles known as exonemes, which is thought to lead to membrane breakdown. These exocytic processes have not yet been observed in live parasites. We established a mutant where we targeted mRuby3 to the PV membrane and a pH-sensitive GFP or super ecliptic pHlourin (SEP) to egress specific secretory vesicles or exonemes. SEP is non-fluorescent in the acidic environment of exonemes before exocytosis. SEP fluorescence can be observed only upon exocytosis when exonemes are exposed to a neutral pH such as plasma membranes and PV. Surprisingly, our data showed exocytosis occurred 3 hours before natural parasite egress. Time-lapse imaging showed the PVM breaking down rapidly after the rounding up of the PVM took place. Contrary to the study in gametocytes, we did not see more than one opening in the PVM rupture during merozoite egress from the RBCs. We have previously shown that an ER-resident calcium-binding protein (Pferc) is essential for egress. Using SEP/Pferc-glmS and SEP/Pferc-M9 double mutants, we show that PFERC is required for exoneme exocytosis.

P36. Identifying the mechanism of action of a novel antimalarial with collateral drug sensitivity associated with *PfKelch13* C580Y mutation

Reagan S. Haney^{1,3}, Jopaul Mathew^{4,5}, Emilio F. Merino^{1,3}, David Anaguano^{2,3}, Vasant Muralidharan^{2,3}, Maxim Totrov⁸, Paul R. Carlier^{6,7}, Maria B. Cassera^{1,3}

¹Dept. of Biochemistry & Molecular Biology, ²Dept. of Cellular Biology, ³Center for Tropical and Emerging Global Diseases, UGA, Athens; ⁴Dept. of Chemistry ⁵Virginia Tech Center for Drug Discovery, Virginia Tech, Blacksburg, VA; ⁶Dept. of Pharmaceutical Sciences ⁷Dept. of Chemistry, University of Illinois at Chicago, Chicago, IL; ⁸Molsoft LLC, San Diego, CA

Malaria is a devastating disease that caused approximately 608,000 deaths in 2022 worldwide. Cases of malaria have increased from previous years in part by the quick development of resistance to current antimalarials. Resistance to antimalarial drugs, such as artemisinin and its derivatives, creates an urgent need to discover and develop new chemotherapeutic agents that engage new targets in the malaria parasite. This research focuses on a novel antimalarial (PRC1584) discovered by our research team. During our investigation to identify the molecular target(s) and the mechanism of action (MOA) of PRC1584, we discovered that it has collateral drug sensitivity with one of the known mechanisms of resistance to dihydroartemisinin (DHA). DHA resistance can be conferred by a single nucleotide polymorphism (SNP) in the *PfKelch13* gene known as Kelch13 (K13) and a few SNPs have been reported, with C580Y and R539T being the most relevant mutations. Preliminary data indicate that parasites carrying a K13 C580Y mutation are more susceptible to PRC1584 treatment. Therefore, we hypothesize that K13 or its interactors may be potential molecular targets of PRC1584. We selected a set of *Plasmodium falciparum* DHA-resistant strains with K13 C580Y mutations and assessed PRC1584 EC₅₀ values, measured the amount of K13 protein, and performed localization experiments to evaluate if PRC1584 colocalizes with K13. Altogether, these experiments and ongoing chemoproteomics studies will reveal if K13 or its interactors are the molecular target(s) or are involved in the MOA of PRC1584. Identifying the MOA of PRC1584 will guide its pre-clinical development to prevent late-stage failure.

P37. Effect of high-fat diet on vaccine mediated protection from malaria

Alexander C. Garrot^{1,2}, Carson Bowers^{1,2}, Samarchith P. Kurup^{1,2}

¹Department of Cellular Biology, University of Georgia, Athens, GA. ²Center for Tropical and Emerging Global Diseases, University of Georgia, Athens, GA

High-fat diet (HFD) is typically associated with obesity and increased health risks such as cardiovascular and liver diseases. HFD changes liver composition and helps recruit myeloid cells and other antigen-presenting cells (APCs) to the liver as a response to higher levels of inflammatory metabolic activity. We have shown that cells of the myeloid lineage recruited to the liver following natural infections or sporozoite-based vaccinations acquire *Plasmodium* antigens from the infected hepatocytes and present them, priming CD8 T cell responses against malaria. HFD is also known to induce reactive oxygen species (ROS) in hepatocytes, driving the elimination of *Plasmodium* parasites from the liver, allowing the APCs access to Plasmodium antigens. We therefore hypothesized that a high fat diet in mice prior to RAS vaccination may induce higher levels of T cell activation and help generate long-standing memory responses. We show that mice fed HFD for 4 weeks prior to *Plasmodium berghei* radiation-attenuated sporozoite (RAS) vaccination exhibit better control of a *P. berghei* challenge infection. However, the total frequencies and numbers of activated circulating or memory liver resident CD8 T cells remained unaffected by HFD. These preliminary experiments suggests that dietary changes prior to RAS vaccination can enhance protection from malaria. Through future studies, we look to uncover the immune mechanisms that underlie this process.

P38. Outcomes of natural versus non-natural pairings of *Trypanosoma cruzi* discrete typing units with the triatomine vector *Rhodnius prolixus*

Ruby Harrison^{1,2}, Kevin Vogel³, R. Drew Etheridge^{1,2}

¹Center for Tropical and Emerging Global Diseases, University of Georgia, Athens, GA; ²Department of Cellular Biology, University of Georgia, Athens, GA; ³Department of Entomology, University of Georgia, Athens, GA

Triatomine insects, colloquially termed kissing bugs, are fecal-oral vectors for the bloodborne parasite *Trypanosoma cruzi* which causes Chagas disease in humans. *T. cruzi* is geographically widespread in the Americas and is genetically diverse, with seven discrete typing units (DTUs) currently recognized: TcI-VI and TcBat. The triatomine *Rhodnius prolixus* is a major vector for *T. cruzi* in Central America and northern South America and is overwhelmingly associated with TcI strains in natural settings. In dipteran (fly) vector systems, non-natural vector/pathogen pairings are reported to result in significantly decreased vector fitness and parasite success compared to natural pairings. We therefore hypothesized that this phenomenon would be recapitulated in the triatomine-trypanosome system. Adult-stage *R. prolixus* orally infected with the Brazil strain (TcI; natural pairing) versus the Y strain (TcII; non-natural pairing) of *T. cruzi* however displayed no measurable differences in survival. Similarly, overall kinetics of parasite expansion and long-term persistence within the insect digestive tract did not differ between treatments. In contrast, preliminary data suggest *R. prolixus* colonized by Brazil strain *T. cruzi* show higher transmission of parasites in fecal droplets compared to Y strain colonized insects. Taken together, our results indicate that non-natural vector-pathogen pairing in the case of *R. prolixus* does not strongly negatively affect either organism but may attenuate *T. cruzi* transmission potential.

P39. Oxygen-dependent regulation of F-box proteins in *Toxoplasma gondii*

Msano N. Mandalasi¹, Elisabet Gas-Pascual¹, Carlos Gustavo Baptista², Bowen Deng¹, Hanke van der Wel¹, Ira J. Blader², and Christopher M. West¹

¹Dept. of Biochemistry & Molecular Biology, CTEGD, CCRC, UGA, Athens, GA, ²Dept. of Microbiology & Immunology, UB School of Medicine, Buffalo, NY

A dynamic proteome is required for cellular adaptation to changing environments, and the SKP1/CULLIN-1/F-box protein/RBX1 (SCF) family of E3 ubiquitin ligases contributes importantly to proteasome-mediated degradation. We examine, in *Toxoplasma gondii*, the influence on the interactome of SKP1 by its novel glycan attached to a hydroxyproline generated by PHYa, the ortholog of the human oxygen-sensor, HIF α PHD2. Strikingly, the representation of several putative F-box proteins (FBPs) is substantially reduced in PHYa Δ parasites. One, FBXO13, is a predicted lysyl hydroxylase related to the human JmjD6 oncogene except for the presence of an F-box domain. The abundance of tagged FBXO13, was reduced in PHYa Δ parasites thus explaining its diminished presence in the Skp1 interactome. A similar effect was observed for FBXO14, a cytoplasmic protein of unknown function that may have co-evolved with PHYa. Similar findings in glycosylation-mutant cells, rescue by proteasomal inhibitors, and constant transcript levels, suggested the involvement of the SCF in their degradation. The effect was selective, because FBXO1 was not affected by PHYa. These findings are physiologically significant because similar effects on FBXO13 and FBXO14 were observed in parasites reared under 0.5% O₂. Minimal impact on steady-state SKP1 modification levels suggests that effects are mediated during a lag phase in hydroxylation of nascent SKP1. The dependence of FBP abundance on O₂-dependent SKP1 modification likely contributes to the reduced virulence of PHYa Δ parasites owing to impaired ability to sense O₂ as a locational signal.

P40. The role of protein disulfide isomerase in the endoplasmic reticulum of *Toxoplasma gondii*

Katherine Moen^{1,2} and Silvia Moreno^{1,2}

¹CTEGD, UGA, Athens, GA. ²Dept. of Cellular Biology, UGA, Athens, GA

Toxoplasma gondii is an obligate intracellular parasite which belongs to the phylum Apicomplexa. It infects most warm-blooded animals including humans as intermediate hosts. During the acute phase of toxoplasmosis, the fast-growing tachyzoite actively invades host cells, undergoes asexual replication, and egresses by rupturing host cell membranes causing damage to host tissues. The endoplasmic reticulum (ER) is a dynamic organelle in eukaryotic cells. It is the site for post-translational processing of proteins and serves as the largest calcium store in the cell. Protein disulfide isomerases (PDIs) are resident ER enzymes and molecular chaperones that catalyze the breakage, formation, and rearrangement of disulfide bonds between cysteine residues in their protein substrates in order to ensure correct protein folding and to regulate ER calcium homeostasis. In mammals, the PDI family, which is a subgroup of the thioredoxin superfamily of proteins, is composed of 21 different proteins, which are not thoroughly characterized in *T. gondii*. Members of the PDI family are functionally diverse but all contain at least 1 thioredoxin-like domain. Those proteins, which carry out disulfide bond manipulation, have canonical CXXC motifs that function as the active site for oxidative protein folding. We characterize TGGT1_211680 (TgPDI1) which is essential for parasite growth and ablation of TgPDI1 caused a defect in parasite replication and host cell invasion. We show that TgPDI1 was important for the proper maturation of microneme and rhoptry proteins in *T. gondii*, and was involved in calcium induced microneme secretion. Interestingly, TgPDI1 was secreted in a calcium induced manner. Through various pulldown methods we were able to identify TgPDI1 substrate candidates including several *T. gondii* secretory proteins.

P41. Assessing an actin-binding protein, thymosin beta-4, as a novel treatment for *Acanthamoeba* keratitis

Victoria A. Murphey^{1,2}; Luis Sanchez Ferrer¹; Anissa Waller Del Valle^{1,2}; Calvin Paulsen^{1,2}; Abigail Goff^{1,2};
Timothy Brinson^{1,2}; Lesly A. Temesvari^{1,2}

¹Eukaryotic Pathogen Innovation Center (EPIC); ²Department of Biological Sciences, Clemson University, Clemson, SC

Acanthamoeba castellanii is an amoeba that can cause *Acanthamoeba* keratitis (AK), a serious eye infection characterized by severe pain, corneal damage, impaired vision, and blindness. In rare cases, the retina, brain, spinal cord, and skin can be infected. The parasite exhibits two life cycle forms: amoebae and cysts. Both forms are found in the eye during infection. Current treatment for AK consists of hourly administration of broad-spectrum antimicrobial drops. No single drug can eradicate both forms of the pathogen while also being non-toxic to eye tissue. Therefore, novel treatments for AK are desperately needed. Thymosin beta-4 (Tβ4) is a cytoplasmic G-actin sequestering protein that promotes wound healing and tissue regeneration. A topical ophthalmic formulation of Tβ4 has been shown, in clinical trials, to promote rapid healing in patients with dry eye syndrome and neurotrophic keratopathy. Thus, we hypothesized that Tβ4 is a viable treatment option for AK. We developed an *in vitro* host cell destruction assay and found that Tβ4 inhibited the destruction of human retinal pigment epithelial-1 (RPE1) cells by the parasite. We determined that Tβ4 hindered encystation, but not excystation, in *Acanthamoeba*. Tβ4 did not reduce parasite viability or increase the proliferation of RPE1 cells. Therefore, protection of the host monolayer was not the result of parasite death or over-proliferation of host cells. Overall, our data suggest that Tβ4 may represent a novel treatment for AK as it may simultaneously disrupt parasite virulence and protect host cells. We are in the process of repeating these studies with corneal cells and determining if Tβ4 can also sensitize the parasite to host innate immunity (e.g., complement lysis).

P42. Induced *in vitro* sexual commitment of *Plasmodium cynomolgi*

James Oristian^{1,2}, Dennis E. Kyle^{1,2}

¹Center for Tropical and Emerging Global Diseases, University of Georgia, Athens, GA, 30601; ²Dept of Infectious Diseases, University of Georgia, Athens, GA, 30601

Plasmodium vivax is the most geographically widespread malaria species, yet our understanding of its unique biology has been hindered by a lack of *in-vitro* culture systems and access to human clinical samples. Phylogenetically related species such as *P. cynomolgi* have been historically utilized in non-human primate models to further our understanding of *P. vivax* biology. Recently, *P. cynomolgi* was successfully adapted to long-term *in vitro* culture, expanding the utility of this model species. Gametocytes, sexually committed parasites capable of mosquito infection, are relatively uncharacterized in *P. cynomolgi* and are essential for mosquito infection, making them an ideal target for *in vitro* study. Previous reports indicate overexpression of gametocyte associated genes Api-AP2G (AP2-G) and Gametocyte Development protein 1 (GDV1) in *P. falciparum* and *P. berghei* leads to massive *in vitro* sexual commitment, yet this has not been accomplished in *P. cynomolgi*. To gain greater insight into the mechanism of sexual commitment in *P. vivax*-like parasites, we are currently generating molecular tools to overexpress *P. cynomolgi*-specific homologues to AP2-G and GDV1 within *in vitro* cultured *P. cynomolgi*. We aim to utilize both the centromere-containing overexpression plasmid, pCyCEN, as well as CRISPR/Cas9 to achieve our goals. *P. cynomolgi* mutants capable of *in vitro* sexual commitment will be tested for mosquito infectivity via standard membrane feeding assay. This work will increase our understanding of sexual commitment in *P. vivax*-like parasites and create a valuable tool for future studies.

P43. Understanding ‘sleeping beauties’: cellular and molecular characterization of *P. falciparum* ring stage parasites in response to artemisinin

Zhe Cheng¹, Anthony A. Ruberto¹, Matthew Tucker², Victoria Mendiola¹, James Oristian¹, Rodrigo Baptista³, Carrie Brooks¹, Vasant Muralidharan¹, Dennis E. Kyle¹

¹Center for Tropical and Emerging Global Diseases, UGA, Athens, GA; ²USDA Agricultural Research Service, Beltsville, MD; ³Houston Methodist Research Institute, Houston, TX

Artemisinin and its derivatives are critical components in drug therapies to treat malaria, but the reduced susceptibility to these compounds in some *Plasmodium* parasites complicates disease control efforts. Prior studies have shown that survival is conferred during the early stages of parasites’ life cycle in the blood. It has been theorized that early forms escape the otherwise deadly effect of artemisinin compounds by entering a state of suspended animation and resuming growth once drug levels decrease, but the molecular mechanisms that support these “sleeping beauties” dormancy-like state remain unclear. We generated a culture-adapted *P. falciparum* strain with reduced susceptibility to artemisinin and whose genetic signature of resistance is not dependent on mutations in regions of the *kelch13* gene typically associated with artemisinin resistance. A distinguishing feature is that they have a copy number amplification of 22 genes spanning a ~75.8 kilobase region on Chromosome 10. To better understand the effects of these structural changes and their potential role in artemisinin resistance, we profiled the transcriptomes of artemisinin resistant- and sensitive forms at single-cell resolution. We reveal numerous differences at the RNA level, including an increased expression of genes in the Chromosome 10-amplified region, as well as altered temporal coordination of gene programs in artemisinin resistant parasites. To identify markers associated with a dormancy-like state, we assessed the transcriptomes of parasites after treatment with the artemisinin derivative, dihydroartemisinin. We identify a small population of ring stage parasites with a distinct transcriptional profile. We speculate that the gene expression signature of these parasites is representative of forms capable of entering a dormancy-like state to mitigate artemisinin-induced stress. These results shed light on the molecular basis of increased tolerance to artemisinin and supports an enhanced dormancy phenotype in artemisinin-resistant forms.

P44. Development of an interactive GIS database for the study of molecular epidemiology in *Toxoplasma gondii*

Gabrielle Russell and Dr. Chunlei Su
UTK, Knoxville, TN Dept. of Microbiology,

Toxoplasma gondii infects warm-blooded animals and humans. It is globally distributed and genetically diverse. In the past two decades, genetic tools have been used to identify thousands of *T. gondii* samples from a variety of hosts, providing a grand view of its population structure. However, the detailed genotyping data are scattered in literature, making it difficult to grasp. There are many static maps presenting *T. gondii* distribution worldwide, but there is lack of an interactive database to facilitate epidemiology research. We will address this problem with an application with mapping as its base that allows for basic user analysis. We are building a comprehensive database of ToxoDB genotyped samples that enables an at-a-glance perspective of genotype distribution and other relevant metadata. The tool should be useful in tracking *T. gondii* transmission and facilitate future epidemiological studies.

P45. Enolase inhibitors are potent therapeutic leads against *Trypanosoma brucei*

Colm P. Roster¹, Danielle LaVigne¹, Jillian E. Milanes¹, Emily Knight¹, Heidi D. Anderson¹, Sabrina Pizarro¹, Elijah M. Harding¹, Meredith T. Morris¹, Florian Muller³, Samuel Kwain¹, Kerrick C. Rees¹, Brian Dominy¹, Daniel C. Whitehead¹, Steven W. Millward², James C. Morris¹

¹Eukaryotic Pathogens Innovation Center, Clemson, SC, USA. ²Department of Cancer Systems Imaging, UT MD Anderson Cancer Center, Houston, TX, USA. ³Sporos Bioventures, Houston, TX, USA.

Glycolysis is critical to the bloodstream form (BSF) of the African trypanosome, *Trypanosoma brucei*. Knockdown of glycolytic enzymes results in poor parasite viability, indicating that glycolytic inhibitors may serve as potent and specific therapies against infection. Phosphonate inhibitors against human enolase (*Hs*ENO2), an enzyme responsible for the interconversion of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate (PEP) in glucose metabolism, have shown potency against ENO1 passenger deleted glioblastoma multiforme. We have investigated whether these compounds inhibit *T. brucei* ENO (*Tb*ENO) and impact parasite viability. An antibiotic known to inhibit ENO, (1-hydroxy-2-oxopyrrolidin-3-yl) phosphonic acid (deoxy-SF2312), was a potent inhibitor of *Tb*ENO (IC₅₀ value of 0.60 ± 0.23 μM). Structurally related synthetic phosphonates, including (1-hydroxy-2-oxopiperidin-3-yl) phosphonic acid (HEX), also inhibited *Tb*ENO (IC₅₀ value of 2.1 ± 1.1 μM). Molecular docking simulations showed that deoxy-SF2312 binds in a similar conformation to the substrate with a binding affinity of -6.8 kcal/mol. In contrast, HEX binds in a unique conformation with a binding affinity of -7.5 kcal/mol. Although these compounds were not trypanocides, modification by adding pivaloyloxymethyl (POM) groups improved toxicity toward *T. brucei*. POMSF and POMHEX had potent activity against parasites with EC₅₀ values of 0.45 ± 0.10 and 0.61 ± 0.08 μM, respectively. These findings suggest that HEX and glycolytic inhibitors are promising therapeutic leads and continued development of the group is warranted.

P46. Cholesterol efflux attenuates TLR4-target gene expression in cultured macrophages exposed to *T. brucei* ghosts

Lawrence Fernando¹, Jing Echesabal-Chen¹, Murphy Miller², Rhonda Reigers Powell³, Terri Bruce³, Joshua Saliutama⁴, Kristina Parman⁴, Kimberly Paul⁴, [Alexis Stamatikos](#)¹

¹Department of Nutrition, Clemson University; ²School of Medicine Greenville, University of South Carolina; ³Clemson Light Imaging Facility; ⁴Department of Genetics & Biochemistry, Clemson University

T. brucei is a eukaryotic pathogen that causes sleeping sickness in humans and nagana in livestock. Infection with *T. brucei* induces a potent pro-inflammatory immune response within infected hosts and this host response is thought to at least be partially due to toll-like receptor (TLR) activation. In response to stimulation by pathogen antigens, TLR4 translocates to lipid rafts, triggering the expression of pro-inflammatory genes. However, cholesterol efflux is considered anti-inflammatory due to promoting lipid raft disruption. In this study, we wanted to assess the impact of *T. brucei* ghosts in facilitating macrophage TLR4 translocation to lipid rafts, and whether promoting cholesterol efflux in macrophages exposed to *T. brucei* ghosts decreases TLR4-target gene expression. When cultured macrophages were incubated with *T. brucei* ghosts, we observed an increase in lipid raft TLR4 protein content, suggesting surface components of *T. brucei* can serve as TLR4 ligands. However, pre-treatment of macrophages with cholesterol acceptors (apoAI or HDL) before *T. brucei* ghost exposure reduced lipid raft TLR4 protein content and the expression of pro-inflammatory TLR4-target genes. Taken together, our results indicate that increasing cholesterol efflux in macrophages may diminish the pro-inflammatory immune response that occurs from *T. brucei* infection by promoting macrophage lipid raft disruption. Future studies in our laboratory will examine whether manipulating cholesterol efflux pathways in vivo alters host pro-inflammatory immune responses that emerge during *T. brucei* infection.

P47. Improving the efficacy of malaria vaccination through modulation of reactive oxygen species

Cristina L. Samuel^{1,3}, Camila Marques-da-Silva^{2,3}, Clyde Schmidt-Silva^{2,3}, and Samarchith P. Kurup^{2,3}

¹Division of Biological Sciences, University of Georgia, Athens, GA, USA; ²Department of Cellular Biology, University of Georgia, Athens, GA, USA; ³Center for Tropical and Emerging Global Diseases, University of Georgia, Athens, GA, USA

Malaria, caused by *Plasmodium* parasites, is a global health challenge. The parasite is inoculated as sporozoites that travel to the liver and invade the hepatocytes, initiating the liver stage infection. Immunization with live radiation-attenuated sporozoite (RAS) stage of the parasite is considered the most effective approach to protect against malaria, with the ability to prevent the onset of blood-stage malaria and therefore the clinical manifestations of the disease. It is known that reactive oxygen species (ROS) produced by hepatocytes can rapidly eliminate *Plasmodium* within the hepatocytes. Considering that elimination of *Plasmodium* RAS in hepatocytes is a prerequisite for priming protective immune responses, we hypothesize that inducing ROS production when delivering RAS would enhance vaccine-mediated protection against malaria. To test this, we treated mice with type-I interferons (IFN-I), an inducer of ROS, or GLX, an inhibitor of ROS, following *Plasmodium berghei* (*Pb*) RAS vaccination. Subsequently, these mice were challenged with virulent *P. berghei* sporozoites. Our study revealed that IFN-I-treated RAS-vaccinated mice generated stronger *Plasmodium*-specific CD8 T cell responses and conferred better protection from the challenge infection, potentially owing to increased accessibility of *Plasmodium* RAS antigens to antigen-presenting machinery. We anticipate future work to lead to new approaches that can exploit the IFN-1 signaling pathway to generate sterilizing immunity against malaria.

P48. CCR2-mediated recruitment of antigen presenting cells to *Plasmodium*-infected liver

Clyde Schmidt-Silva^{1,2}, Samarchith Kurup DVM, PhD^{1,2}

¹University of Georgia, Department of Cellular Biology ²Center of Tropical and Emerging Global Diseases

Malaria, caused by *Plasmodium*, remains a global health crisis that affects nearly half of the world's population. The host is inoculated with *Plasmodium* sporozoites by the bite of infected *Anopheles* mosquito, then the sporozoites infect the hepatocytes where they undergo clinically silent development and replication prior to infecting erythrocytes. This results in the symptomatic, transmissible, and potentially lethal blood-stage of malaria. Therefore, designing vaccines which target the liver-stage provides an ideal opportunity to prevent clinical malaria. The current 'gold-standard' vaccination uses radiation-attenuated sporozoites (RAS) that generate abortive infections in the liver that drive the generation of protective CD8 T cell responses which target the liver stage of *Plasmodium*. Such CD8 T-cell responses are generated by CD11c⁺ CSF1R⁺ antigen-presenting cells (APCs) recruited to the liver from circulation, which subsequently acquire *Plasmodium* antigens from the infected hepatocytes undergoing pyroptotic cell-death. The objective of my project is to determine how CD11c⁺ CSF1R⁺ APCs are recruited to the site of *Plasmodium* infection within the liver. This is an important knowledge gap because enhancing the efficiency of this process would improve the immune responses generated against liver-stage malaria. We show that, following *Plasmodium* infection, infected hepatocytes undergo pyroptosis and release caspase-1 inflammasome complexes which are acquired by Kupffer cells (KCs), which then produce mature IL-1b. IL-1b would induce the expression of the CCR2 ligands, CCL2 and CCL7 in hepatic stellate cells (HSCs), liver-sinusoidal endothelial cells (LSECs), and hepatocytes, enabling the recruitment of CD11c⁺ CSF1R⁺ APCs to the site of *Plasmodium* infection. We expect our findings to open new avenues to enhance immunity to liver-stage malaria in the context of sporozoite-based vaccination.

P49. Morphological and molecular identification of *Paramphistomum* species from sheep of Hyderabad, Pakistan

Mahvish Rajput¹, Muhammad Sohail Sajid¹ and Olivier Andre Sparagano^{2,3}

¹Department of Parasitology, University of Agriculture Faisalabad, Pakistan, ²Department of Infectious Diseases and Public health, Jockey Club College of Veterinary Medicine and Life Sciences, City University of Hong Kong, Kowloon, Hong Kong, China, ³Agricultural Sciences and Practice, Royal Agricultural University (RAU), Cirencester, GL7 6JS, UK

The aim of this study was to assess the prevalence of rumen fluke (*Paramphistome* spp.) infection in sheep slaughtered at the abattoir in Hyderabad, Pakistan, and to conduct a molecular characterization to analyze their evolutionary relationships. Among the 200 rumens examined, 75 (37.5%) tested positive for rumen fluke infection. These results suggest that sheep were infected with a single species of rumen fluke, as they exhibited morphological uniformity. The prevalence of infection was consistent across all months sampled, with the highest rates observed in November (56%), followed by October (38%) and September (34%), and the lowest in August (22%). Although the infection rates varied between sexes, there was no statistically significant difference, with females showing a prevalence of 45% compared to 29% in males. Molecular characterization was performed on six isolated rumen flukes by amplifying the second internal transcribed spacer sequence flanking the 5.8S and 28S ribosomal gene sequences (ITS-2+), yielding a 428 bp fragment that was sequenced for further analysis. Analysis of nucleotide sequences from these isolates revealed high sequence similarities and clustering together with *Paramphistomum epiclitum* in the constructed phylogenetic tree, indicating that all isolates belonged to the same species. This study provides valuable baseline data on the prevalence of rumen fluke infection in southeastern Pakistan and contributes to the molecular characterization of these parasites using the rDNA ITS-2 region, which serves as a useful genetic marker for species identification.