DISSERTATION

THE PATHOGENESIS AND ENVIRONMENTAL MAINTENANCE OF *MYCOBACTERIUM ULCERANS*

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 2014

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ABSTRACT

THE PATHOGENESIS AND ENVIRONMENTAL MAINTENANCE OF *MYCOBACTERIUM ULCERANS*

Buruli Ulcer Disease (BUD) is a severe, neglected tropical disease of the skin caused by the acid-fast bacillus *Mycobacterium ulcerans*. The disease is characterized by necrosis of subcutaneous adipose tissue, and healing with contracture and/or intense scarring of the skin. Little is known about the host response to *M. ulcerans* from transmission and infection, through the course of disease and resolution. Understanding the host-pathogen interaction is key to development of treatment programs for this neglected disease. In this dissertation, a systems biology approach was used to evaluate a laboratory mouse model of *M. ulcerans* infection and an analysis of the capability of *Anopheles gambiae* mosquitoes to maintain and transmit the bacterium is described following the introduction and literature review (Chapters 1 and 2).

In Chapter 4, the histology and immune responses in a mouse model that mimics human *M. ulcerans* infection is described, providing insight into the host response during active infection with *M. ulcerans*. Specifically, non-toxigenic, virulent *M. ulcerans* was inoculated into the mouse footpad, and the resulting progression of infection and immune response was characterized in both a wild-type C57BL/6 mouse and an immunodeficient *Rag1*^{tm1Mom} (Rag$^{-/-}$) mutant mouse strain. Assessment of the bacterial burden in the mouse as a correlate of the infectious process was documented and demonstrated a persistent or latent feature of infection. Additionally, a mechanism of host immunosuppression was described in

immunocompetent animals, in the absence of the toxin mycolatone, highlighting the need for better understanding of virulence determinants employed by the bacilli during infection.

Chapter 5 reports an expansion of the mouse model to investigate the transmission of *M. ulcerans* by a mosquito vector. After exposure to *M. ulcerans*, larval *A. gambiae* mosquitoes experienced significant developmental delay, resulting in reduced survival and stunted growth. Adult *A. gambiae* demonstrated bacterial contamination of their external mouthparts with live *M. ulcerans* bacteria. The contamination pattern of adult mosquitoes implicates these insects in the mechanical transmission of *M. ulcerans*. The mouse model from chapter 4 was used to evaluate mosquito borne transmission of the bacillus. Infected mosquitoes were allowed to take a blood meal from mice. The subsequent immune response of the mice was measured for sero-reactivity against *M. ulcerans*. In addition, larval mosquitoes were documented to readily consume water-borne *M. ulcerans*, consistent with their feeding mechanism. Thus, larval mosquitoes represent a reservoir or point of environmental maintenance of the pathogen.

Chapter 6 details the initiation of a study to investigate the metabolic effects of the exposure of adult mosquitoes to virulent *M. ulcerans*. The developmental delay and subsequent stunted growth of adult mosquitoes after exposure to *M. ulcerans* was analyzed through the mass spectrometric investigation of small molecules of metabolism in an attempt to elucidate the biological effects of exposure. In summary (Chapter 7), the availability of a well-defined animal model for BUD provides a valuable tool, which can be used to investigate the specialized pathogenic features of this emerging infection and explore novel disease interventions.

ACKNOWLEDGEMENTS

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LITERATURE REVIEW CHAPTER 1: *MYCOBACTERIUM ULCERANS*

History of Buruli Ulcer Disease

Buruli Ulcer Disease (BUD) is a physically disfiguring and socially debilitating ulcerative disease of the integument caused by *Mycobacterium ulcerans* and is classified as a neglected tropical disease. Despite initial characterization of the disease in 1897 by Sir Albert Cook in Uganda, basic questions remain regarding the route of transmission, environmental maintenance, and disease pathogenesis. In 1948, formal discovery and description of *M. ulcerans* infection defining a "new mycobacterial infection in man," was achieved by MacCallum et al. (1948) to include ulceration with atypical histological and bacteriological characteristics in the Bairnsdale region of eastern Victoria, Australia (2). Preceding this description, and the tentative assignment of a mycobacterial etiology, scattered reports of mycobacteria-implicated ulcerations in Africa and Australia could be traced back to the second half of the nineteenth century. Over the following decades, hundreds of cases of BUD were reported in sub-Saharan Africa (3, 4), further refining the description of the disease. While a dozen names have been proposed to describe infection with *M. ulcerans*, including Bairnsdale Ulcer, the designation of Buruli Ulcer was coined by Clancey et al. (1962) corresponding to an endemic riverine region in Uganda and the location of many of the first characterized patients (5). During these early years, continued investigation of *M. ulcerans* was complicated by the inability to culture the bacteria. In a serendipitous breakthrough, the first successful culture was obtained using a

defective incubator maintaining 33°C, the temperature coinciding with *M. ulcerans* tropism for the outer layers of the skin.

Epidemiologic and bacteriologic investigations were continued throughout the 1970's. This time was characterized by the discovery of risk factors associated with infection, namely standing water and insects (6, 7), and attempts to refine the case definition (8, 9). The development and testing of a purified protein derivative skin test reagent, termed Burulin, was in important breakthrough for the diagnosis of Buruli Ulcer disease (10). However, difficulties were arising regarding the specificity and application of the tool in endemic areas (11). Importantly, during this time, the only known toxin to be produced by a *Mycobacterium* was identified to be secreted by *M. ulcerans* and its effects evaluated in a guinea pig model (12). It would not be until 25 years later, with the assistance of Delphi Chatterjee from Colorado State University, that the toxin would be identified as a small, lipid-like polyketide implicated in a variety of potent cytotoxic and immunomodulatory effects associated with infection (13). Research in the 21st century has uncovered a vast amount of information regarding *M. ulcerans* infection. The first successful culture from an environmental location provided great insight into the mechanisms of bacterial persistence outside the host and the environmental niche of the pathogen (14). In 2004, the World Health Assembly passed a resolution calling for advanced research into the disease. Shortly thereafter, multiple research consortia were formed to streamline the efforts of collaboration for the development of prevention and control strategies. The standardization and acceptance of criteria for staging and guidance for healthcare workers greatly improved the long-term prognosis of Buruli Ulcer disease (15). Recent advances in Buruli Ulcer disease research have led to the development of new

diagnostic approaches using mass spectrometry (16), a developing understanding of the host

immune response (17), and continued studies investigating the potential vector capacity of

insects in the transmission of the bacteria (18). The current state of research into this emerging

pathogen is fraught with many challenges, further compounded by its place at the bottom of

the research funding scale compared to other neglected tropical diseases (19).

Taxonomy, Phylogeny, and Genetics

Mycobacteria are a diverse and ubiquitous genus of microorganisms, encompassing

species of pathogenic and non-pathogenic bacteria (Figure 1). The genus *Mycobacteria*

contains the infamous pathogens *M. tuberculosis* and *M. leprae*, in addition to members of the
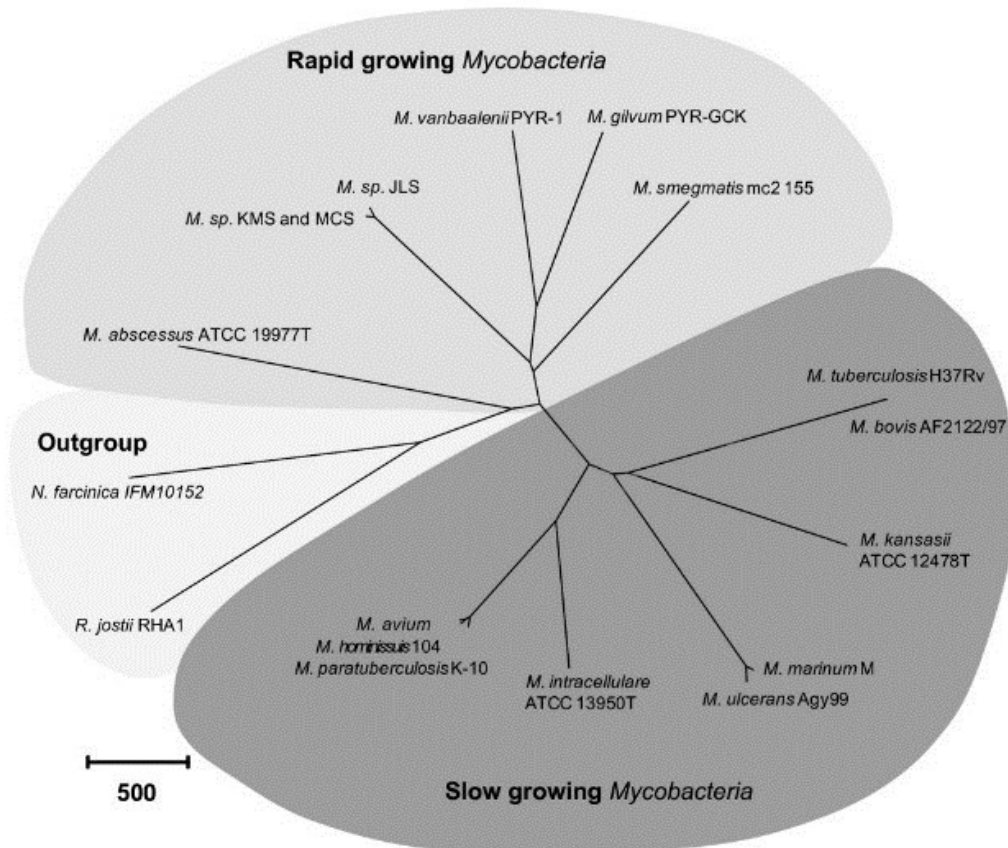
Figure 1: Phylogeny of Mycobacterium genus. Lineages are denoted based on growth rate of organisms. Scale represents amino acid difference. Adapted from Veyrier et al. BMC Evol. Biology. 2009 9:196 (1).

Mycobacterium avium complex (MAC) and a wide array of newly designated non-tuberculous

mycobacteria (NTM) of significant public health concern. The evolution of *M. ulcerans* is

primarily associated with the genetic divergence from a common ancestor shared by

Mycobacterium marinum and the acquisition of a large circular plasmid (pMUM001) encoding

the polyketide toxin, mycolactone (20). This divergence occurred approximately one million

years ago and describes the evolutionary diversion from a human and fish pathogen into a

devastating infection of the skin and underlying tissue in humans (Figure 2). Despite the
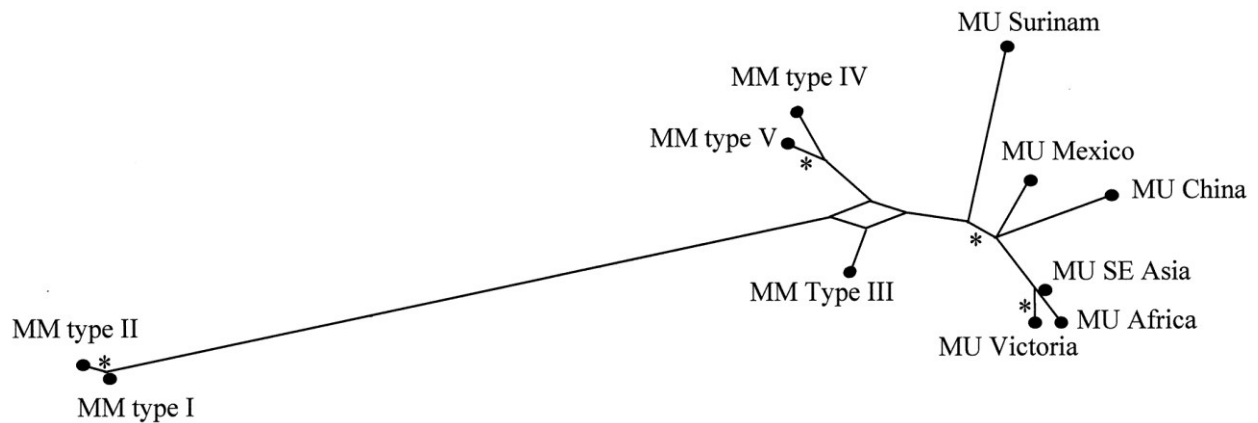
Figure 2: Splits chart of the phylogenetic relationship among M. ulcerans (MU) and M. marinum (MM) genotypes. Edges have greater than 80% bootstrap support. Asterisk denotes edges with greater than 60% bootstrap support. Adapted from Stinear et al. J. Bact. 2000 182:22.

contrasting phenotypes of *M. marinum* and *M. ulcerans*, the pair share nearly identical genome

sequences (98.3% homology), compared to a 78.5% identity to *M. tuberculosis* (21, 22). The

complete genome sequence of *M. ulcerans* consists of a circular chromosome of 5632 Kb and a

virulence plasmid of 174 Kb, interspersed by 771 pseudogenes and 304 insertion sequence

elements. Through lateral gene transfer and reductive evolution, the *M. ulcerans* genome has

lost approximately 1000kb of DNA compared to its *M. marinum* progenitor, split between

twelve regions of between 2 and 53 kb in size (23). Multilocus sequence analysis (MLSA)

revealed clear delineation between *M. marinum* and *M. ulcerans*, also describing clonality

among genotypes from different geographic locations (23, 24). It is suggested this genetic

speciation is in response to environmental changes and mammalian host adaptation, and

perhaps indicates a passage through an evolutionary bottleneck (21). The evolution from the

generalist *M. marinum* to a specialist, niche-adapted pathogen is characterized by gene loss

due to DNA deletions and rearrangements mediated by some of the 213 copies of *IS2404* and

91 copies of *IS2606* insertion sequence elements, disrupting over 110 genes (22). Such

disruptions involving cell wall biosynthesis, carbon and amino acid metabolism, and the

reduced transcription levels thereof help to explain, in part, the slow growth of *M. ulcerans*

compared to its progenitor (22), in addition to the contribution of reduced genetic redundancy.

The insertion sequence elements account for nearly 475 kb of DNA distributed throughout the

genome. Most notable of these are 79 kb of sequence variation specifically among the PE/PPE

genes encoding Gly-Ala rich cell envelope proteins, comprising 45% of the 157 regions of

difference between *M. ulcerans* and *M. marinum*. *M. ulcerans* has low variability in house-

keeping genes and other genetic elements within isolates from the same geographic region.

However, the genomic sequences of geographic isolates can be differentiated by single

nucleotide polymorphisims (SNP) and variable number tandem repeats (VNTR) throughout the

genomes. Ghanaian strains, for example, contain approximately 100 SNPs compared to the

reference strain AGY99, whereas Japanese strains can contain as many as 26,000 SNPs. VNTR

analysis identified 9 VNTR loci as useful molecular targets to study the relationships among

isolates (25). The combined use of multiple sequence differentiation tools (MLSA, VNTR, IS-PCR,

SNP), the location differences of the insertion sequence elements IS2404/IS2606, and

mycolactone structure, reveal 12 distinct geographic clades (Figure 3). Interestingly, all African
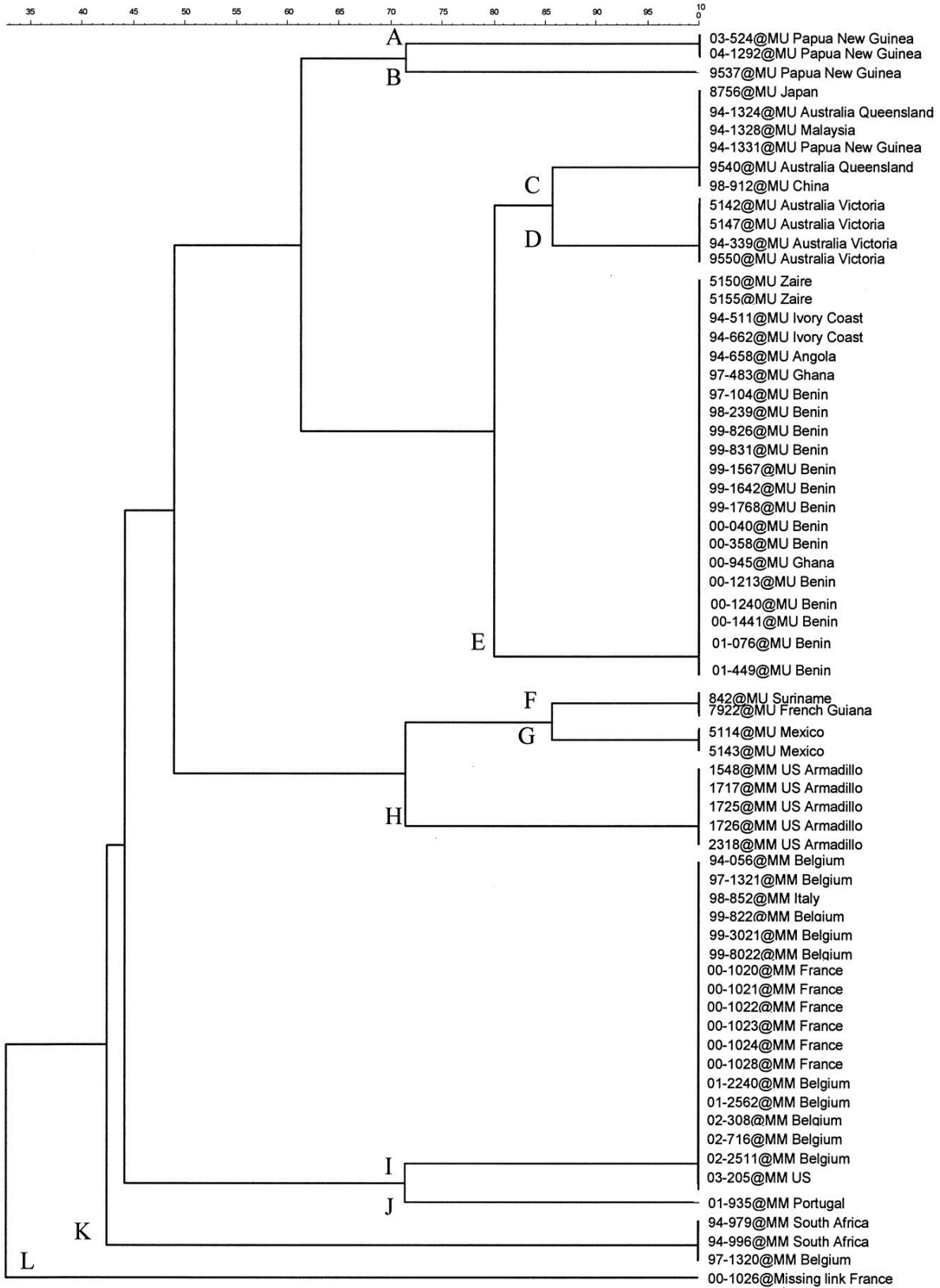
Figure 3: Categorical dendrogram displaying the clustering of genotypes of both M. ulcerans (MU) and M. marinum (MM) isolates from different geographic origins. Adapted from Stragier et al. J. Bact. 2005. 187:5.

isolates from West and Central Africa, including historical isolates, belong to the same genotype (24, 26-28), and display marked spatiotemporal homogeneity (29).

Epidemiology and Transmission

Buruli Ulcer disease is the third most common mycobacterial infection in immuno-competent people following tuberculosis and leprosy (30, 31). In fact, BUD has replaced tuberculosis and leprosy as the most prevalent mycobacterial disease in BUD endemic communities, affecting up to 22% of the population within affected communities (32). Additionally, over the past decade, there has been a noted increase in BUD in several West African countries (33-35). Buruli Ulcer disease has been reported in more than 30 countries, but the most significant burden of disease remains in sub-Saharan Africa, and particularly the Gulf of Guinea region (30, 36-38). Australia has a long history with *M. ulcerans* and manages active foci of infection in the southeastern coastal regions, specifically coastal Victoria, Far North Queensland, Bairnsdale and Point Lonsdale (39-47). Recently, successful cultures from environmental samples have demonstrated the appearance of *M. ulcerans* in aquatic environments, though environmental subsistence and the precise source of infection for humans remain unknown (14, 48). The route of infection is also unknown. The organism is postulated to enter at sites of trauma to the skin (49, 50), following contact with contaminated water or vegetation (51). Insects of the Hemiptera and Diptera orders are implicated in the transmission of Buruli Ulcer in many geographic regions, specifically the aquatic biting insects from the families Belostomatidae and Naucoridae (52, 53), in addition to multiple genera of mosquitoes (54). Indeed, an association between *M. ulcerans* and these insects has been well described, although an understanding of the true dynamics of transmission and interaction via

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insect systems in a natural setting remains undefined. A multitude of studies have investigated the association between these insects and *M. ulcerans (53, 55-62)*. Epidemiological studies have described a higher rate of *M. ulcerans* DNA contamination of captured mosquitoes during an outbreak of disease compared to a non-outbreak period of time (63). Additionally, the biting water bugs were shown to actively transmit the pathogen to mice in the laboratory via contaminated raptorial arms and salivary glands (58). Taken together, these studies describe a close association between the pathogen and insects, and while it is possible that their role in the disease lifecycle may be limited to environmental maintenance of the bacterium and not directly transmission, they represent a ubiquitous reservoir. No confirmatory evidence for human-to-human transmission exists (64), although prolonged contact or via bite wound has been suggested to lead to ulceration (65, 66).

The primary risk factors associated with transmission of Buruli Ulcer disease include a close association with stagnant or standing water, a lack of protective clothing, such as long pants and shoes, and biting aquatic insects. Unfortunately, these risk factors fail to explain the true dynamics associated with the emergence and development of disease. The complex political and socio-economic interactions in endemic areas must be accounted for to develop a clear understanding of the epidemiology of disease. An analysis of the socio-political climate of the endemic region along the Nyong River in Cameroon provided novel insights into mechanisms of disease transmission and susceptibility of humans (67). Deforestation and ecological disruptions leading to a transformation of the human interactivity within an ecosystem are a crucial factor in the emergence of Buruli Ulcer. Indeed, these ecological disruptions have been documented to be associated with disease outbreaks (68, 69). A multi-

disciplinary approach to investigating the epidemiology of disease will lead to a better understanding of the infection dynamics. As an example from a historical perspective, the endemic Akonolinga district in Cameroon has undergone significant socio-economic upheaval in the last century. The transition from sustainable agriculture to large-scale commercial farming operations has resulted in wide-spread deforestation and disruption of the aquatic ecosystem (67). The general health of the population in this region has been negatively impacted by the reduced diversity of food and reduced community interaction. A well-documented and poorly understood feature of Buruli Ulcer disease are the disproportionate rates of infection in young children. Correlating this epidemiological finding to the generalized risk factor of environmental disruption, it may be surmised that the reduced food diversity has a greater impact on the general health and immunological development of children, thereby increasing their susceptibility to infection. This interaction could be used as an alternative model to predict emergence of the disease in other geographic locations (70). While the social sciences are often marginalized by biological researchers, the "One Health" perspective on the surveillance and research of emerging diseases will expand our understanding of the true cause and effect relationships that underlie the emergence and transmission of Buruli Ulcer disease (71).

Disease Manifestations

The pathogenesis of *M. ulcerans* infection is believed to follow an initial inoculation of the dermis, however the mechanisms behind this process are deeply hypothetical. The bacilli then replicate in the subcutaneous tissue resulting in necrosis (72-76).

After exposure and an undefined incubation period, the disease is observed to begin as a nodule, papule, plaque, or localized edema at or near the site of exposure (Figure 4).

Unmanaged cases can progress to large ulcerative lesions which if left untreated, result in debilitating complications from scarring with

Figure 4: Plaque formation (left) and ulceration (right) characteristic of Buruli Ulcer disease (adapted from WHO, Intl.)

contracture or loss of limbs or vital organs (31, 49, 77). The time to development of an ulceration from a nodule or other early disease state is suspected to be weeks to months, although the true incubation period has not been characterized to include diverse geographic locations and strain phenotypes (78). In an Australian cohort of Buruli Ulcer patients, the incubation period for the disease was described to range between 34 and 204 days, with the average incubation period being 135 days post exposure (79). Exposure in this study was described as a documented visit to an endemic region. However, these results may not be generalizable to other endemic regions and infection with other geographic isolates.

Diagnosis and Treatment

Diagnosis of *M. ulcerans* infection is based on clinical appearance using the World Health Organization case definition and common molecular techniques (34, 77, 80). Misclassification is a problem in both pre-ulcerative and ulcerative stages of disease due to the high abundance of both infectious diseases (cutaneous leishmaniasis) and cancer (squamous cell carcinoma) with a similar presentation. Case confirmation requires two positive tests out of

the following: Ziehl-Neelsen Acid Fast Bacilli (ZN-AFB) staining, culture, polymerase chain reaction (PCR), or histopathology (31, 34, 77, 81-83), but if confirmation occurs at all, it is well after initial clinical diagnosis and provides limited information for clinical management decisions. Additionally, the three later methods are not likely to be adaptable to rural, resource poor areas. Treatment options for BUD are limited, but management strategies continue to improve. Based on recent research, randomized trials, and increasing clinical experience, a consensus document was prepared to standardize the approach to case management in 2007 (84). Historically, aggressive surgical excision of nodules and lesions followed by skin graft was the treatment of choice based upon the prevailing view that antibiotics were ineffective. Recent studies using antimycobacterial drug therapy, specifically rifampin-based regimens, have shown efficacy if utilized during early stages of disease (33, 85-88), though disease presentation during infection, geographic limitations, and variable incubation times influence the options for treatment as the disease progresses. Prospective studies in humans and model animals have now shown that treatment with antibiotics alone will lead to healing without recurrence (88-90). The consensus treatment program includes daily rifampin for 8 weeks in conjunction with streptomycin or clarithromycin for a further 4-8 weeks (91). Currently, surgery is infrequently recommended as a primary intervention, although there is still a significant role for surgery in the initial management of severe cases or when antibiotics are contraindicated (92). Debridement of necrotic tissue with the goal of improving the rate of wound closure is indicated by the presence of bacilli during histological examination of lesion margins post antibiotic treatment, or when the lesion has been present for greater than 75 days (93).

Nearly 20% of patients experience a paradoxical reaction to antibiotic therapy, and this phenomenon has also been described in animal models of treatment (94-96). It is believed that the antibiotic therapy causes a reversal of the immunosuppression described during active Buruli Ulcer disease, resulting in an intense immunological reaction to persisting bacteria and the occasional development of new ulceration (97). This immunosuppression is primarily ascribed to action of the toxin Mycolactone. However, the potency of rifampin against *M. ulcerans* infections indicates the contribution of a protein or peptide-based virulence factor involved in immunosuppression or persistence. Interestingly, some anecdotal reports of a naturopathic approach to disease management have had some success. Application of sterile, high mineral-content clay to the lesions resulting in a hypoxic environment and the use of high temperature wraps (as indicated by the bacterium's preferred growth temperature of 32°C) are viable options when antibiotic or surgical intervention would not be well-tolerated (98, 99). The further characterization of animal models of *M. ulcerans* infection in concert with increased awareness and reporting of the disease have significantly reduced the morbidity associated with Buruli Ulcer disease over the past decade.

Physiology and virulence factors of M. ulcerans

Generally, non-tuberculous mycobacteria (NTM) represent a diverse group of opportunistic pathogens capable of producing multifarious infectious phenotypes. Infections of lungs, skin, soft tissue, joints and bones as a consequence of NTM infection results in an annualized prevalence range between 7.2 and 35 cases per 100,000 persons in the North America alone (100, 101). It is assumed that the prevalence and incidence of NTM infection is significantly higher in disadvantaged areas where disease reporting and surveillance are lacking.

Members of the NTM classification cause a wide degree of infections, and while genotypic and phenotypic differences occur between species, many common mycobacterial traits are responsible for the potency of these infections. The physiology of mycobacteria during infection has been thoroughly researched over the last century, describing a wide array of virulence factors implicated in the survival of the bacilli, its resistance to therapeutics, and the persistence associated with many infections.

Mycobacteria, in general, have evolved diverse strategies to survive within and without the human host. Established mycobacterial virulence factors include the prevention of the acidification of phagocytic vesicles (102), prevention of phagosome-lysosome fusion (103), resistance to antimicrobial components of the serum (104), modulation of cytokine secretion (105), the ability to replicate intracellularly (106, 107), and various genetic mutations promoting antibiotic resistance (108). In common with other bacterial pathogens adapting to new or more stable environments such as *M. leprae* and *Yersinia pestis*, genetic perturbations have been documented in the genome of *M. ulcerans*, including the accumulation of insertion sequence elements and pseudogenes, genome downsizing, and the acquisition of foreign genetic material conferring a selective advantage (22). As a result of reductive evolution and pseudogene formation, strains of *M. ulcerans* have lost many genes encoding some common mycobacterial virulence factors, in addition to resultant alterations in physiological processes and metabolism. While the *M. ulcerans* cell envelope and associated metabolites have been less well studied than other mycobacteria, inferences from the genome sequence can provide clues to the metabolic activity in regards to potential virulence determinants of *M. ulcerans*, namely components of carbon and lipid metabolism. However, little is known about metabolic

strategies employed by *M. ulcerans* during survival in any one of the several distinct niches thought to be occupied by the bacilli. Indeed, *M. ulcerans* employs profoundly different survival mechanisms within the host compared to other mycobacterial pathogens. If *M. ulcerans* successfully occupies a distinct insect associated niche, as has been implicated, the synthesis of predicted chitinases/transglycosidases may be of significance (Burulist: *MUL_0371, MUL_2210, MUL_2681*) (109). These enzymes may provide a mechanism of attachment to or degradation of the N-acetyl-glucosamine polymers that comprise the major component of insect exoskeletons, thereby promoting colonization of insect tissues.

A noteworthy deletion in the *M. ulcerans* genome includes reduction of ESX loci from five in *M. marinum* to only three in *M. ulcerans* and the loss of associated effector proteins from 18 to 2, respectively (22). The disruption of genes encoding immunodominant proteins involved in this system, such as ESAT-6, CFP-10, and *hspX,* may represent selection pressure-induced changes resulting in immune evasion mechanisms. In other mycobacterial species, the *esx1* locus encodes protein secretion machinery (Type VII) involved in intracellular spread and immunogenicity (110). Additionally, loss of this system may contribute, in part, to the reduced phagocytic uptake and extra-cellular persistence common to *M. ulcerans* infection.

Lipid-based molecules are significant antigenic components of the mycobacterial cell wall that are also involved in virulence (111). The loss of many of these components in *M. ulcerans* can be explained by the reduction of polyketide synthase (PKS) genes from 27 in *M. marinum* to 12 in *M. ulcerans*. The contraction of lipid synthesis machinery coincides with a reduction of the *MmpL* family of lipid and polyketide transporters from 25 in *M. marinum* to six in *M. ulcerans*. Of the 12 remaining PKS genes, 10 are utilized in the production of important

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cell-wall-associated lipids including mycolic acids, phthiodiolones, phenol phthiodiolones, mannosyl-phospholipids, and mycobactins (112, 113).

As an example, phenolic glycolipids (PGL) are an abundant component of the cell wall of most mycobacteria and, as major virulence factors, are involved in host-immune modulation (114). *M. ulcerans* bacilli are able to synthesize the phenolphthiodolone lipid intermediate, although they lack the necessary machinery to add the rhamnosyl moiety and complete the PGL molecule (115). Additionally, neither *M. ulcerans* nor *M. marinum* contain an analogous PKS locus for the synthesis of sulfolipids (22).

Despite the inability to synthesize many prominent lipid species, *M. ulcerans* retains a full complement of the fatty acid synthase I/II machinery (FAS I/II) (22), and many other lipid-associated enzymes. The conservation of anabolic lipid mechanisms highlights the importance of particular lipids in the physiology of *M. ulcerans*. Conversely, the degradation or utilization of host-derived lipids is also implicated as a virulence factor of *M. ulcerans* (116). The catabolic phospholipase enzymes have already been identified as virulence factors in other species of mycobacteria (117). The phospholipase enzymes hydrolyze phospholipids into distinct subunits depending on the enzyme subtype. Subsequently, products of this enzyme-catalyzed degradation are involved in downstream signaling events within the host (118), and are characterized to play a role in pathogenesis of *M. tuberculosis* infections (119). However, while the phospholipase C and D enzymes are actively synthesized and secreted during growth of *M. ulcerans*, their role during infection with *M. ulcerans* remains uncharacterized. The functional disruption or loss of some of the major immunodominant components important to other

mycobacterial infections may confer a selective advantage to this emerging pathogen. A

summary of these components is included in the table below:

Table 1: Comparison of virulence factors between *M. ulcerans* and *M. marinum* based on genetic sequence are summarized (adapted from Demangel et al. Nat Rev Micro, 2009).

	M. marinum M	M. ulcerans Agy99
Genome size and arrangement	• Circular chromosome; 6,637 kb • Circular mercury resistance plasmid (pMM23; 23 kb)	• Circular chromosome; 5,632 kb • Circular mycolactone-associated plasmid (pMUM001; 174 kb)
Number of genes	5,424	4,160
Number of pseudogenes	65	771
Number of insertion sequences (ISs)	7 ISs; *Myma01* (7 copies), *Myma02* (7 copies), *Myma03* (4 copies), *Myma04* (5 copies) and *Myma05*, *Myma06* and *Myma07* (2 copies of each)	IS2404 (213 copies) and IS2606 (91 copies)
Number of PE and PPE genes	175 PE genes and 106 PPE genes	69 PE genes and 46 PPE genes
Number of ESX secretion systems	5	3
Number of ESX effectors	18 *espA* paralogues and 31 *esx* paralogues	2 *espA* paralogues and 14 *esx* paralogues
Number of phospholipase C genes	Seven phospholipase C paralogues (*plcB*, *plcB1*, *plcB2*, *plcB3*, *plcB4*, *plcB5* and *plcB6*)	One gene that encodes phospholipase C (*plcB*); others have become pseudogenized or lost by DNA deletion
Number of lipoproteins	88 genes that encode lipoproteins	77 genes that encode lipoproteins; *lipY* has been lost by deletion
Phenolic glycolipids	Mycoside M	Phenolphthiodiolone lipid backbone cannot be glycosylated as the glycosyl transferase (MUL_1998) has been pseudogenized
Mycolactone	Not produced	Mycolactone A and B

The secretion of an extra-cellular matrix (ECM) and the formation of a biofilm is another

striking feature of *M. ulcerans* that is likely involved in virulence. The formation of a biofilm

confers an advantage for many pathogenic bacteria, especially those of environmental origin

(120). Biofilms of other human pathogens, such as *Pseudomonas aeruginosa* and *Haemophilus*

influenzae have been well characterized and early evidence from *M. ulcerans* suggests biofilm

formation may also be implicated in pathogenesis and the colonization of environmental and

insect niches (121). The structure of the biofilm generated by *M. ulcerans* differs from classic

biofilms, appearing as a thick covering of the outmost layer of cells (122). This finding contrasts

with biofilms generated in other pathogenic bacterial species, which usually demonstrate a

matrix of ECM and distributed bacterial cells (123). Lipids and lipoglycan components of the

mycobacterial cell envelope such as lipoarabinomannan and PIM have also been described as

components of *M. ulcerans* ECM, in addition to a significant contribution of glucose

polysaccharides and a large variety of chaperone proteins (DnaK, GroEL, GroES) (122).

The prolific biofilm and ECM produced by *M. ulcerans* has a diverse functional role, in

both environmental persistence and pathogenesis. Aquatic plants are known to secrete organic

compounds which can be used as a substrate for bacterial growth (124). A recent *in vitro* study

by Marsollier et al. (2004) described the role aquatic plant extracts have in the promotion of

biofilm formation of *M. ulcerans* (121). This study demonstrated a significant decrease in

doubling-time (from 80 to 40 days in the study) upon exposure to crude organic plant extracts,

suggesting *M. ulcerans* gains a distinct advantage by associating with and utilizing compounds

synthesized by aquatic plants .

During pathogenesis of *M. ulcerans* infections, the ECM is involved in resistance to

antibiotics, immune evasion, and increased cytotoxic effects (122). This finding coincides with

other biofilm forming bacteria (125, 126). However, the impact and abundance of the *M.

ulcerans* exotoxin and other uncharacterized virulence factors must also be accounted for when

assigning the pathological contributions of the ECM.

Toxin synthesis

Long regarded as the primary virulence factor for *M. ulcerans*, the exotoxin mycolactone

has been shown to be responsible for, in part, the dramatic cytotoxic and immunomodulatory

effects described during infection. Acquisition of the large pMUM001 virulence plasmid

encoding the toxin is the primary distinguishing factor associated with divergence from the *M.

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marinum common ancestor. As the only species which actively produces a toxin, *M. ulcerans* is unique within the Mycobacterium genus, and the cytopathologic effects tentatively attributed to the toxin underlie the case definition of Buruli Ulcer disease. The existence of a secreted factor involved in virulence was postulated as early as 1965 (127), although it would not be until 1999 that the toxin would be purified and formally described as the first macrolide synthesized by a bacterial pathogen (13). Structurally similar compounds have previously been characterized to include antibiotics (erythromycin), an immunosuppressant (rapamycin), antifungal agents (amphotericin B), among others, although the host derived target of action of these compounds can differ (128). Macrolides are complex polyketides produced as secondary metabolites of many soil bacteria of the order Actinomycetales and have a wide array of functional effects. Indeed, mycolactone is suggested to contribute to the development of pathologic features of Buruli Ulcer disease, although the specific role of the toxin in the pathogenesis of disease remains under intense investigation.

Structurally, the mycolactone toxin is comprised of a 12-membered ring with two attached unsaturated fatty-acid side chains, encoded by the extrachromosomal pMUM virulence plasmid. Different geographical isolates produce different structural variants of mycolactone, identified as mycolactone A-G (Figure 5). Structural variants are identified by the number and location of hydroxyl groups and double bonds, and the length of the lower polyunsaturated acyl side chain, while the structure and arrangement of the upper side chain and the 12-membered ring appear to be conserved among geographic isolates.
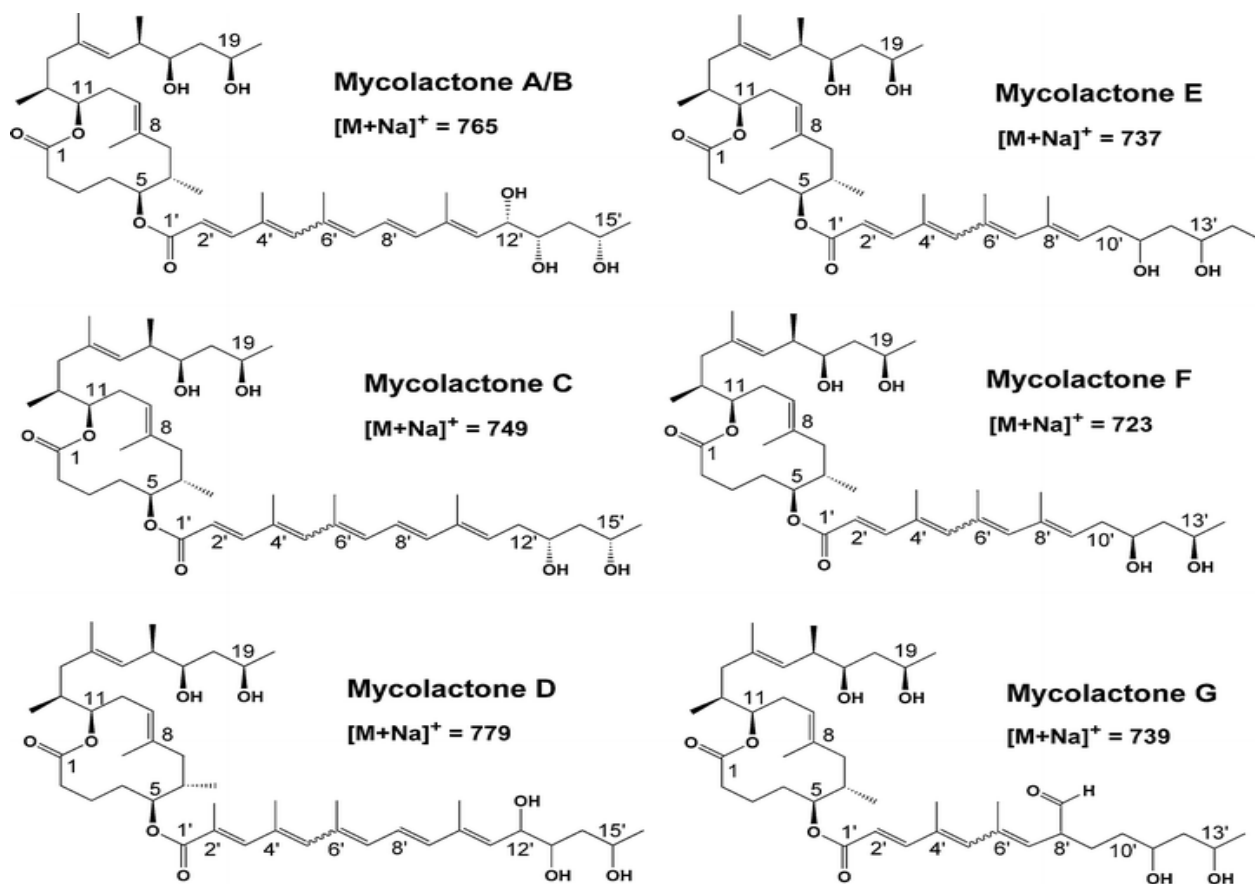
Figure 5: Mycolactone congeners of the five naturally occurring structures (A/B-F) and one unnatural structure (G). Adapted from Hong et al. *Nat. Prod. Rep.* 2008.

A number of *in vitro* and *in vivo* studies in human-drived cell lines and animal models, have documented a wide array of mycolactone-mediated effects. In a model system developed by Hall et al. (Plos Pathogens 2014), a unifying mechanism of the effects exerted by mycolactone was proposed to describe broad spectrum inhibition of protein translocation to the endoplasmic reticulum (129), as opposed to inhibition of translational processes previously proposed (21, 130) . While the specific molecular consequences of this action are still under investigation, a diverse set of immune proteins were found to be sensitive to mycolactone (BCA, MIP-2, G-CSF, GM-CSF, IL-27, C5/5a, IL-1RA), depending on the cell type stimulated and

the route of stimulation. Other in vitro studies have shown numerous cell types to be sensitive to mycolactone (73), resulting in immunomodulation and decreasing the efficiency of the immune response (107). The toxin is also purported to be involved in the painlessness of the lesions (131). Regulation of the toxin in vivo has not been investigated, although mechanisms attributed to toxin regulation have been discovered in vitro (132, 133). Overproduction of the mycobacterial siderophore, mycobactin, is correlated with a down-regulation of mycolactone. In addition, the supplementation or removal of specific carbohydrates in growth media has been shown to affect regulation of mycolactone synthesis. This evidence suggests that environmental signals may influence regulation of the toxin in different niches and pathologic conditions (132).

Host-Pathogen Interactions

Pathogenesis

Infection with *M. ulcerans* is unique among other common mycobacterial infections with regard to the tissue tropism, lifestyle, and phenotype of the host immune response. Unlike other common mycobacterial infections, which tend to persist in an intracellular environment, Buruli Ulcer lesions predominantly contain extracellular bacilli often exclusively in the cutaneous, subcutaneous, and adipose tissues (72). Lesions can occur on any part of the body, though they tend to occur on limbs or extremities, and absent secondary infection, are painless. Histologic assessment demonstrates extensive coagulative necrosis with a proportionally low acute inflammatory response as the hallmark criteria of Buruli Ulcer disease, although there is considerable variation in the severity and presentation of the disease among geographic areas. Some investigators have considered subcategorizing *M. ulcerans* strains into eco-varieties or

ecovars to better explain the presentation diversity that can occur (134). To date, investigation into the mechanisms behind this pathogenesis have focused on the contribution of the toxin mycolactone. However, it is not clear how mycolactone contributes to the cytotoxicity or immunosuppression during clinical infection, as the extent of tissue degeneration and cytotoxicity exceeds that which would be attributable to mycolactone alone (73). It is important to consider that infection with non-toxigenic *M. marinum, M. haemophilum*, and *M. tuberculosis* can result in necrosis and cytotoxicity via other mechanisms (49, 135).

The pathogenesis of *M. ulcerans* infections is categorized based on clinical appearance of the lesions into a preulcerative stage (represented by a nodule, plaque, or local edema), ulcerative stage, and healed stage (80). After initial exposure, the bacilli survive and multiply during a transient, intracellular phase within the host. Initial colonization of neutrophils occurs during the early stages of infection (2-48 hours) and promotes persistence of the bacteria in the tissue (73). Through a cytotoxic mechanism, the bacilli are released from their phagocytic incubators and begin an extracellular lifestyle, resulting in the transition to the preulcerative presentation. Histologically, this stage is characterized by abundant extracellular bacilli and massive inflammatory cell infiltration. Over a period of weeks to months, expanding necrosis of the subcutaneous and adipose tissues results in sloughing of the outer layers of skin leading to ulceration. Histologically, ulcerative disease is characterized by discrete foci representing extended acellular necrotic areas containing "clumps" or microcolonies of extracellular bacilli, elastolysis, and necrosis of recruited immune cells. An important consideration during this time is the abundant collagen degradation, fibrosis and vasculopathy that impairs immune cell migration to the lesion. This pathogenesis also limits the permeation of antimycobacterial

treatments to locations containing susceptible bacilli. As necrosis of the subcutaneous tissues progresses, the ulcer becomes undermined and can spread beneath healthy epidermis. Spontaneous healing has been documented in some cases. The mechanism behind this phenomenon is unknown, and unmanaged healing can result in intense fibrosis and disfiguring scar formation.

Innate Immunity

The skin represents a physical and innate barrier against infection. Within the skin, specialized cells (eg. keratinocytes, Langerhans cells, tissue-resident macrophages) recognize and initiate immune responses to mycobacteria. This response is coordinated via signaling through pattern recognition receptors (PRR). Among the PRRs, the toll-like receptors (TLR) critical for recognition of mycobacterial pathogens are TLR2, TLR4, and TLR9 (136). A recent study by Peduzzi et al (2007) described the presence and activation of both plasmacytoid and myeloid dendritic cells during early lesion formation (137). A finding consistent with other dermatological conditions (138). Initial innate cell recognition of the pathogen leads to a proinflammatory signaling cascade, characterized by high levels of IL-6, IL-8, IFN-γ, and TNF-α.

Adaptive and Humoral Immunity

The immune response to *M. ulcerans* infection can be correlated to the location of the bacteria during infection. Early infection response is characterized by the intracellular localization of infecting bacilli. The initial induction of a cell-mediated immune response is a reaction to this intracellular infection. As infection progresses, and bacilli become predominantly extracellular, the cell mediated response is no longer effective. This correlates with the transition to a response characteristic of an extracellular infection, and the down-

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regulation of a proinflammatory, cell-mediated response. The pathogenic mechanisms of *M. ulcerans* result in an inefficient and often transitory cell-mediated response, even though effector mechanisms of this response are associated with protection.

The initial immune response to Infection with *M. ulcerans* in humans begins with the induction of a local, neutrophilic inflammatory response in conjunction with the signaling of cell-mediated immunity involving cytokines such as TNF-α, IFN-ɣ, IL-1, IL-2, IL-12 and IL-15 (139) that are characteristic of a Th1 immune response. Indeed it has been suggested that a Th1 immune response is protective and may prevent the development of disease in people exposed to *M. ulcerans*, while a Th2 response does not (140). However, the cellular response during ulcerative infection is minimal, due to virulence-mediated immunosuppression, resulting in the down regulation of the protective IFN-ɣ cytokine, in particular. Other studies have reported the discovery of idiopathic T-cell anergy (140, 141) and variable cytokine profiles throughout the lesion and over time (139, 142, 143). This suggests that at some point during infection, an unknown mechanism results in suppression of CMI, facilitating proliferation of bacilli and, inappropriately, a transition to the generation of a non-protective Th2 type response. Concordantly, others have demonstrated that expression of cytokines such as IL-10 and IFN-ɣ and activation of dendritic cells (a key cell for the induction of adaptive immunity) can vary considerably within ulcerative lesions (137), and throughout infection. Subsequently, a transition to a Th2 type response associated with lymphocyte apoptosis, necrosis, and fibrosis at the infectious foci including a significant decrease in protective cytokines. The mechanisms behind the transition to a non-protective response resulting in expansion of the lesion is uncharacterized but a correlation between the gradation of immune responses in leprosy and

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Buruli Ulcer has been suggested (144). This non-protective response is associated with a reoccurrence of disease or the maintenance of persistent bacteria resulting in a chronic state of infection.

The antibody response during *M. ulcerans* infection has been evaluated in the context of surveillance and diagnostics for many years (82, 145-147). *M. ulcerans* specific antigens elicit a steady antibody response during infection (148), and this response has been developed for use as a screen for exposure to the pathogen (149). Unfortunately, cross reactivity among environmental mycobacteria as well as possible previous exposure to *M. ulcerans* limits the application of this assay (148), as many other mycobacteria share immunogenic antigens (150). From an immunological perspective, the production of specific antibodies against *M. ulcerans* and their role in the development of immunity has two important aspects. First, the presence of specific antibody near the time of infection is associated with a beneficial effect (151), as pre-ulcerative nodules frequently contain extracellular bacilli (49). Secondly, how antibody-mediated immunity (AMI) and the downstream signaling of Fc receptors (FcR) (152, 153), polymeric Immunoglobulin G (IgG) receptors (154), and parenteral immunoglobulin (155, 156), interact with CMI to mount an effective immune response. The interaction between antibodies, FcR, and subsequent FcR signaling, may represent a novel aspect of immunogenesis during *M. ulcerans* infection that is involved with the generation of a Th1 response.

History and Distribution

Mosquito-borne pathogens are responsible for significant morbidity and mortality on a global scale. The *Anopheles gambiae* complex *(A. arabiensis, A. bwambae, A. merus, A. melas, A. quadriannulatus, and A. gambiae* sensu stricto) plays a central role in the transmission of many diseases, due to diverse habitats and geographical distribution of the insects. Before 1962, *A. gambiae* mosquitoes were considered a single species. By 1964, six distinct species had been described (identified above), categorized by differences in larval habitat and adult female resting and feeding behaviors. While the various *A. gambiae* species differ in their capacity to transmit pathogens, the anthropophilic nature of *A. gambiae* sensu stricto describes a long history of an intimate association between humans and the mosquito. *A. gambiae* is generally described as an afro-tropical mosquito, with the majority of the population distribution occuring in sub-Saharan Africa (Figure 6). However, 10 species of the *Anopheles gambiae* sensu
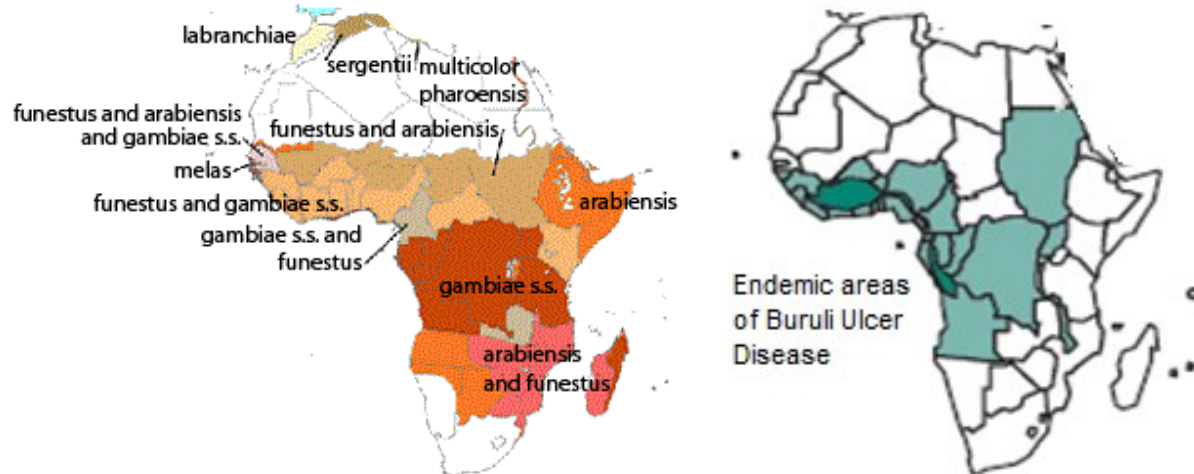
Figure 6: The geographical distribution of Anopheles spp. (left) and endemic areas of Buruli Ulcer disease (right), showing significant overlap. Adapted from CDC.gov and Bulletin of the World Health Organization vol.83 n.10. (2005).

lato complex have been identified in Australia. These species show similar variability in terms of host-seeking behavior and vector competence described in the African complex of mosquitoes. Mosquitoes from multiple genera have been implicated to be involved with the environmental persistence of *M. ulcerans* in multiple geographic locations. While no definitive proof exists describing active transmission of *M. ulcerans* by mosquitoes, there is a growing body of evidence suggesting a role for mosquitoes in the maintenance of the bacilli in the environment.

Best known for the transmission of malaria, lymphatic filariasis, and numerous viral infections, the Anopheline mosquitoes have been implicated as disease vectors for millennia. The effects of ongoing climate change are predicted to result in expansion of suitable environments for Anopheline development (157), which will have a significant impact on the incidence of many mosquito-borne pathogens. The hematophagous nature of the mosquito provides the insects with a protein source for egg development but also results in exposure to pathogens and, subsequently, the potential to transmit those microbes to susceptible organisms.

Development in aquatic environments

Early development of mosquitoes occurs in an aquatic environment and the subsequent lifecycle of the insects is dependent on a close association with water. Larval mosquitoes are commonly found in habitats endemic for Buruli Ulcer disease (158). Development of larval mosquitoes occurs primarily at the air-water interface near plant stems and algal mats, where the larvae feed using labral head fans to filter microbes and organic matter from the water column (159, 160). The idea that larval mosquitoes may serve as bioaccumulators or concentrators of environmental *M. ulcerans*, thereby initiating the passage of *M. ulcerans*

through an aquatic food web has been explored (62, 161). Thus, it is accepted that larval mosquitoes will readily consume *M. ulcerans* and promote the maintenance of the bacilli in the environment.

Mechanical transmission and Vector Capacity

Risk factors for the development of Buruli Ulcer disease, including an association with standing water and the protective effect of bed nets described previously, implicate an insect in the transmission of the disease. Indeed, much of the sympatry existing between *A. gambiae* and *M. ulcerans* is a result of many common themes regarding the geographic and environmental location of the two organisms, which is a requirement for assignment of mosquitoes as a vector. Together with mosquitoes, other insects have been implicated in transmission of *M. ulcerans* and have received considerable attention, specifically members of the Naucoridae and Belostomatidae genra (52, 53, 59, 60). However, these insects are not hematophagous and only incidentally bite humans (54). Many correlative studies have suggested that adult mosquitoes may serve as vectors of the bacilli (30, 61, 63, 162). The complex biology associated with the movement of pathogens from the mosquito gut to the salivary gland is appreciated during interaction with other pathogens. Unfortunately, there is no evidence that mosquitoes are biological vectors of bacterial diseases. However, mechanisms of mechanical transmission require far less biological interaction than salivary transmission. As an example, *Francisella tularensis*, the causative agents of Tularemia, has been shown to be mechanically transmitted by mosquitoes in some settings (163, 164). The overlap in the geographic distribution and the shared association with water between the *A. gambiae* and *M. ulcerans* highlights the potential interaction that may be occuring in nature.

Differences in vector competence among members of the Anopheles complex have been described and are attributed to differing preferences for feeding on humans versus animals, the tendency to enter structures, and an ability to recover after environmental disturbances. It is also suggested that interactions between host and pathogen influence the probability that mosquitoes are able to successfully transmit a pathogen. Interaction between mosquitoes and transmissible pathogens have been investigated in many systems, and the molecular consequences of this interaction are being characterized to discover novel strategies employed by both the mosquito and the pathogen that influence this dynamic. The mosquito attempts to control the internal development of pathogens, often resulting in immune stimulation and imposing a fitness cost. Many pathogens exploit mosquito systems to their advantage, resulting in increased probability of transmission and survival of the pathogen. While there are likely far fewer biological interactions occurring during instances of mechanical transmission compared to biological transmission, a study of the physiology of *A. gambiae* and *M. ulcerans* during varying spatial and temporal exposures will provide significant insight into mechanisms of pathogen control by the mosquito and immune evasion and persistence by the bacilli, leading to transmission or maintenance of the pathogen.

CHAPTER 3: MOUSE MODEL OF *M. ULCERANS* INFECTION

Introduction

The pathogenesis of *M. ulcerans* infection is hypothesized to follow an initial contamination of the dermis, followed by replication of the bacilli in both an intra- and extracellular environment within the host subcutaneous tissue, concurrent with the production of a toxin that may contribute to necrosis of the tissue (72-76). Unfortunately, the contribution and presence of the toxin remains undefined during infection in humans. Therefore, we sought to characterize infection with a mycolactone negative isolate and compare the pathogenesis of disease in our mouse model to the human case definition. The disease originate as a nodule, papule, plaque, or localized edema, then progresses to ulcerative lesions. The lesions, if left untreated, result in debilitating complications from scarring with contracture to loss of limbs or vital organs (31, 49, 77).

Multiple studies have used animal models to investigate infection with *M. ulcerans* (13, 131, 165, 166). These studies have demonstrated the mouse and guinea pig susceptibility to infection with *M. ulcerans*, describing similar pathogenesis of disease to that seen in humans (167, 168). To date, few studies have investigated *M. ulcerans* infection dynamics in immunodeficient mouse models (17). In order to better understand the mechanisms of persistence and immunosuppression, a comparison between wild-type and immunodeficient animals is advantageous. The aim of this present work is to characterize the host immune response and disease pathogenesis of a mycolatone-negative isolate by comparing

immunocompetent and immunocompromised mice after infection. The direct contribution and concentration of mycolactone in human lesions is not well described, and indeed it has been shown that the plasmid encoding this toxin is very unstable (169). A comparative investigation of the infection dynamics in immunodeficient and immunocompetent mice will provide insight into mechanisms employed by the pathogen irrespective of the contribution of mycolactone.

M. ulcerans strains from different geographic regions have variable virulence phenotypes. This variability is commonly correlated with the synthesis (or lack of) of mycolactone isotypes or the ratio of isotypes. However, it is likely that other virulence factors play a significant role in pathogenesis. Our studies suggest cell-mediated immunity plays an important role in the outcome of disease, and that the host becomes immunosuppressed early in the course of infection during the asymptomatic stage of disease, irrespective of the presence of the toxin. Reactivation of disease into a severe, chronic state is characterized by an uncoordinated, cytokine-driven response, concomitant with antigen-specific pathogen recognition. This supports the often conflicting data derived in human studies, and demonstrates that a systematic approach to understanding the immune response and its effect on BUD disease progression is required for further investigation of novel diagnostics and interventions for BUD.

Materials and Methods

Animals

Eighty 8-week-old female wild-type C57BL/6J and immunocompromised B6.129S7-Rag1tm1Mom/J (Rag$^{-/-}$) mice were obtained from The Jackson Laboratory Center for Mouse Models of Heart, Lung, Blood and Sleep Disorders (Bar Harbor, ME). All mice were maintained

in the Animal Biosafety Level 3 facility at CSU, with sterile rodent chow and water ad libitum, with 5 animals per cage. The studies involving animals were approved by the Institutional Animal Care and Use Committee at Colorado State University, which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International.

Bacterial Strain and Culture

M. ulcerans strain 94-511 was selected based on its virulence in humans (originally a clinical isolate from endemic Cote d'Ivoire), and its ease of culture and manipulation in multiple environments (24, 72). *M. ulcerans* 94-511 has been documented to produce mycolactone (72), although the strain used for this study was considered mycolactone negative. Confirmation that the strain was mycolactone-negative was achieved by whole DNA extraction from cells and an absence of a 174kb plasmid encoding the toxin, in addition to the absence of reactivity upon PCR analysis of the ketoreductase B (KR) domain of the polyketide synthase (mls) gene. For the preparation of the *M. ulcerans* inoculum, acid-fast bacteria (AFB) were grown at 32°C in static liquid Middlebrook 7H9 medium for approximately 1 month, or until the formation of a pellicle. The resultant pellicle was harvested and allowed to grow again at 32°C in low-mode shaking liquid Middlebrook 7H9 medium supplemented with oleic acid, albumin, dextrose, and catalase and (OADC) for approximately 2 months. Cells were harvested via filtration from liquid media through 0.22µm membrane, acid fast stained, and counted in a hemocytometer for determination of concentration. A glycerol solution was added to a concentration of 10% and the culture was aliquoted into working infectivity stocks at approximately 10^{10} colony forming units (CFU)/ml.

Experimental Infection

Mice were infected subcutaneously in the left hind footpad using a tuberculin syringe with 20µl containing approximately 6×10^6 AFB diluted in sterile water (using the right hind footpad as a control), or via aerosol with 6×10^6 AFB using a Middlebrook Aerosol Exposure chamber (GlasCol, LLC, Terre Haute, IN). The progression of disease was monitored with time points of 1, 7, 14, 28, 60, and 142 days post infection (DPI). At time points, animals were euthanized using CO_2, their lung, spleen, draining lymph node (DLN) (popliteal), and footpad were removed, individually homogenized, and stored on ice for cytokine assays or immediately plated for CFU.

Determination of Bacterial Burden

M. ulcerans proliferation was assessed in infected mouse lung, spleen, draining lymph node (DLN) (Popliteal), and footpad tissues of infected mice at each of the previously described time points. Tissue specimens were aseptically harvested from euthanized mice into sterile tubes containing 4.5 ml of physiologic saline and briefly homogenized with a PRO250® homogenizer (PRO Scientific, Oxford, CT). Four 10-fold dilutions of each homogenate were spread onto plates containing Middlebrook 7H11 agar (Difco Laboratories, Detroit, MI) enriched with OADC. Plates were incubated at 32°C and CFUs counted after 4 months to ensure growth of all cells present.

Cytokine concentrations in mouse tissues

Identical volumes of tissue were collected from each animal at each time point. Tissue homogenates from the DLN, spleen, and footpad were briefly centrifuged for 10 minutes at 1000xg to remove cellular debris. The supernatant was collected and assayed to determine

protein content with a bicinchoninic acid assay (BCA) (Pierce, Rockford, IL) and diluted to a

working concentration of 1 mg/ml to standardize the protein concentration. The samples were

then assayed in triplicate for the cytokines interleukin-2 (IL-2), IL-4, IL-5, IL-6, IL-10, IL-12, IL-13,

IL-17a, IL-23, Interferon-ɣ (IFN-γ), tumor necrosis factor- α (TNF-α), and transforming growth

factor-beta (TGF-β), and evaluated against tissue from uninfected control mice. After analysis a

selection of cytokines are presented here. The enzyme-linked immunosorbent assay (ELISA)

(Qiagen) was used for detection of soluble cytokines in infected mouse tissues. Per the

manufacturer's instructions, samples were added to wells of an anti-mouse cytokine antibody

coated plate. The plate was developed using and Avidin-HRP reporter and read using

wavelength correction subtracting the reading at 450 nm from the reading at 570 nm.

Histopathological Studies

The DLN and footpads were harvested, fixed in 10% formalin, and embedded in paraffin.

Sections were cut and stained with hematoxylin and eosin (H&E; IHC Tech, Aurora, CO). Digital

microphotographs were taken using a Nikon Eclipse 51E microscope and a Nikon DS-Fi1 camera

with a DS-U2 unit and NIS elements F software. Images are reproduced without manipulations

other than cropping and adjustment of light intensity.

Statistics

Differences between the means of the CFU load of experimental groups and between

the concentrations of individual cytokines, was analyzed at each time point using a two-tailed

Student t-test, and a *P* value of ≤0.05 was considered significant. Pearson's correlation analysis

was performed on the cytokine samples to evaluate the significance of change over time.

Results

Mouse footpad inoculation results in an inflammatory condition of the skin whereas aerosol challenge does not

Although aerosol exposure to *Mycobacterium ulcerans* represents an unlikely initial infection in human cases, many nontuberculous mycobacterial infections (NTM) demonstrate a tropism for the respiratory system (170). Aerosol challenge of wild-type C57BL/6 mice and the immunodeficient Rag$^{-/-}$ mice with virulent *M. ulcerans* resulted in a transient, asymptomatic infection, and perhaps a period of non-replicating persistence followed by death due to lack of nutrients (Figure 7C). Specifically, *M. ulcerans* was cultured from lung homogenates for up to 60
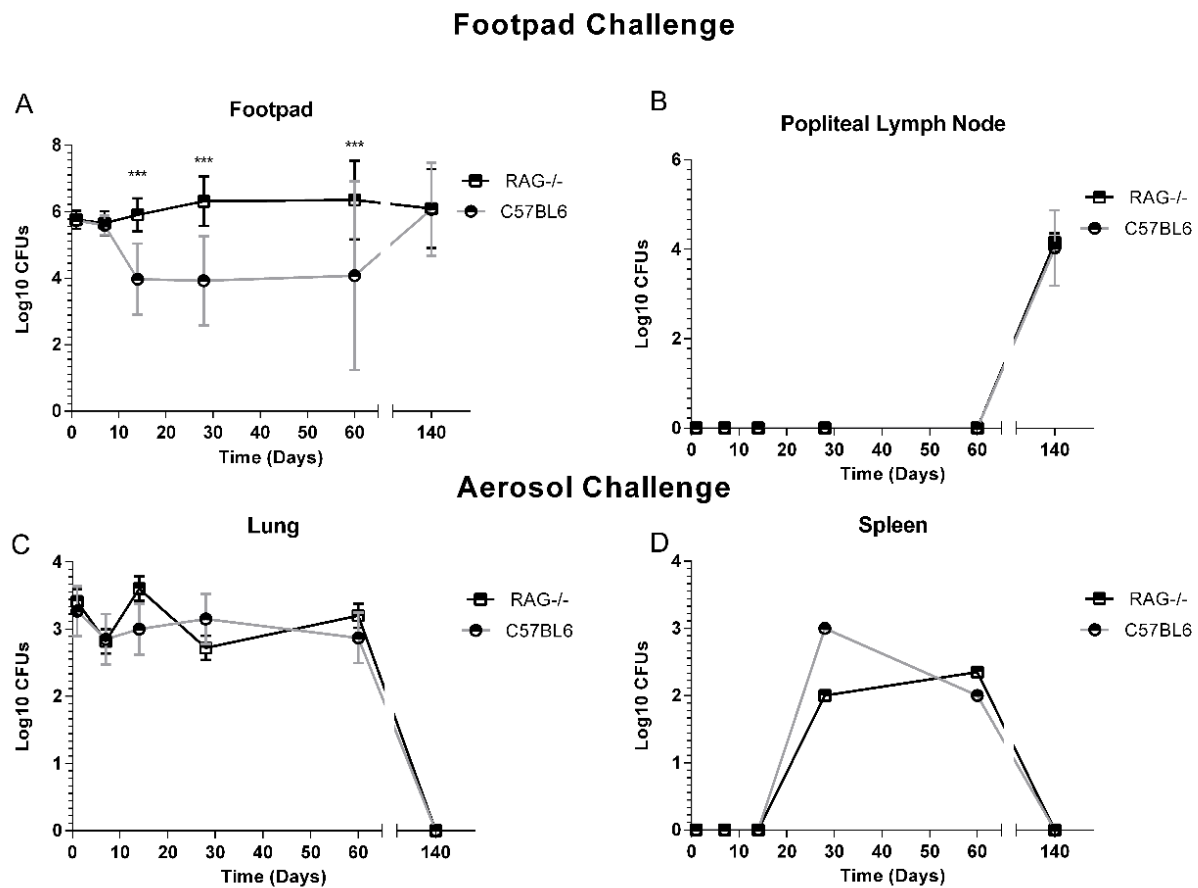
Figure 7: Colony forming units (CFU) in mouse tissues during infection. *** denotes P≤0.001.

days post infection (DPI); no viable bacilli were recovered after this time point. Furthermore, up

to 2 $\log_{10}$ colony forming units (CFUs) were also seen in the spleen and liver during the later

time points (28 and 60 DPI), but not by 142 DPI following aerosol exposure (Figure 7). Our

studies demonstrated no culturable *M. ulcerans* in peripheral blood, nor did the mice show

clinical signs or external pathology representative of a cutaneous *M. ulcerans* infection during

any point of the infection. However, the presence of disseminated M. ulcerans in the spleen of

aerosol infected animals suggests hematogenous trafficking of bacilli. In contrast to aerosol

infection, inoculation of the footpad resulted in measureable CFU throughout the course of

infection monitoring. Organisms were observed in the footpad of Rag$^{-/-}$ mice throughout the

course of infection, while in C57BL/6 mice there was a reduction in CFU at day 14, colonization

persisted (Figure 7). Further, *M. ulcerans* was cultured in the draining lymph nodes in both

immunocompetent and immunocompromised mice during the chronic disease state (Figure

7B). *M. ulcerans* is known to grow preferentially at 32°C, in accordance with the cooler outer

layers of the skin and peripheral tissues (171). In our studies, aerosol exposure and the

subsequent respiratory infection of the mice was shown to result in a limited, transient

infection, even in a severally immunocompromised model, consistent with the known biological

tropism of *M. ulcerans*. We therefore chose to pursue the footpad as the only route of

exposure in our additional experiments.

Gross pathological features

In human patients, the *M. ulcerans* bacilli are hypothesized to gain entry to the

subcutaneous layers of the skin at the site of an existing wound or as a result of a puncture.

Footpad inoculations of mice were performed using 5x10^6 *M. ulcerans* CFUs in a 20μl sterile

water suspension. Injection of the bacterial suspension resulted in a slight wheal formation

within the footpad. At 1 DPI, the footpad presented with mild edema and erythema in all mice,

who also avoided using the mildly swollen foot. By 7 DPI the

swelling had subsided to nearly that of baseline, and usage of

the foot was unrestricted. These visual observations of the

gross pathology continued until 28 DPI, when a reappearance

of the mild edema and a more pronounced erythema was

apparent. Between 28 and 60 DPI, the swelling previously

seen in the footpads was reduced to baseline appearance. By

60 DPI, visible signs of infection were absent in all mice and

mice appeared to use the inoculated foot without restriction.

Neither did the mice appear to suffer from fatigue, malaise,

and isolation as has been described in other mouse models of

infection (172), although these variables were not individually

Figure 8a (top): dorsal lateral view of C57BL/6 hind footpads at 142DPI. Figure 8b (bottom): view of plantar surface of C57BL/6 hind footpads at 142DPI showing proximal spread of inflammation in infected left hind footpad. Right hind footpad as control.

tested. Mice developed a reactivation of disease after an asymptomatic period of

approximately 50 days and by 142 DPI, all remaining mice were sacrificed due to edema and

erythema of the footpad which had expanded proximally (Figure 8) compared to previous

observations documented on 1 and 7 DPI, accompanied by severely restricted usage of the foot

and other signs of illthrift such as huddling, immobile disposition, and ruffled fur. Mice were

sacrificed before the development of complete footpad ulceration for ethical reasons. Few

differences were observed in the gross pathology of either C57BL/6 or Rag^{-/-} mice infected with

M. ulcerans. Specifically, all mice presented with a similar pathological syndrome over the

course of the infection, although there were differences in the type and kinetics of the

pathology observed between the footpads of each strain (Figure 9A).
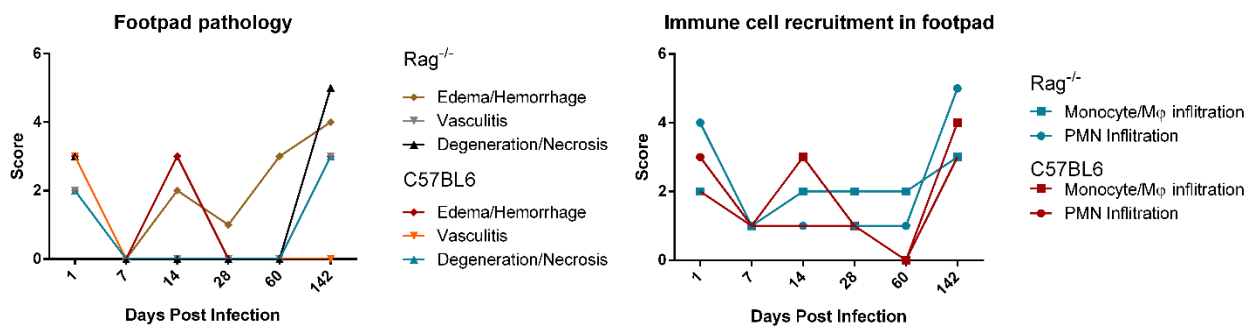
Figure 9a (left): Histology score of footpad tissue for pathology during infection. Figure 3b (right): Histology scores for immune cell infiltration into footpad tissues during infection.

Histopathological features

Histological analysis of the infected footpad and DLN was performed to asses host

immune cell recruitment and the condition of host tissues during infection. Mice were infected

with 5×10^6 *M. ulcerans* CFUs in a 20μl sterile water suspension in their left hind footpad and

sacrificed periodically after infection to determine bacterial numbers in tissues (Figure 7). At 1

DPI, the Rag^{-/-} and C57BL/6 mice had a similar presentation, represented by focally extensive

areas of dermal/subcutaneous edema and moderate to severe infiltration of

polymorphonuclear neutrophils (PMN) and few macrophages. Muscle-fiber and collagen
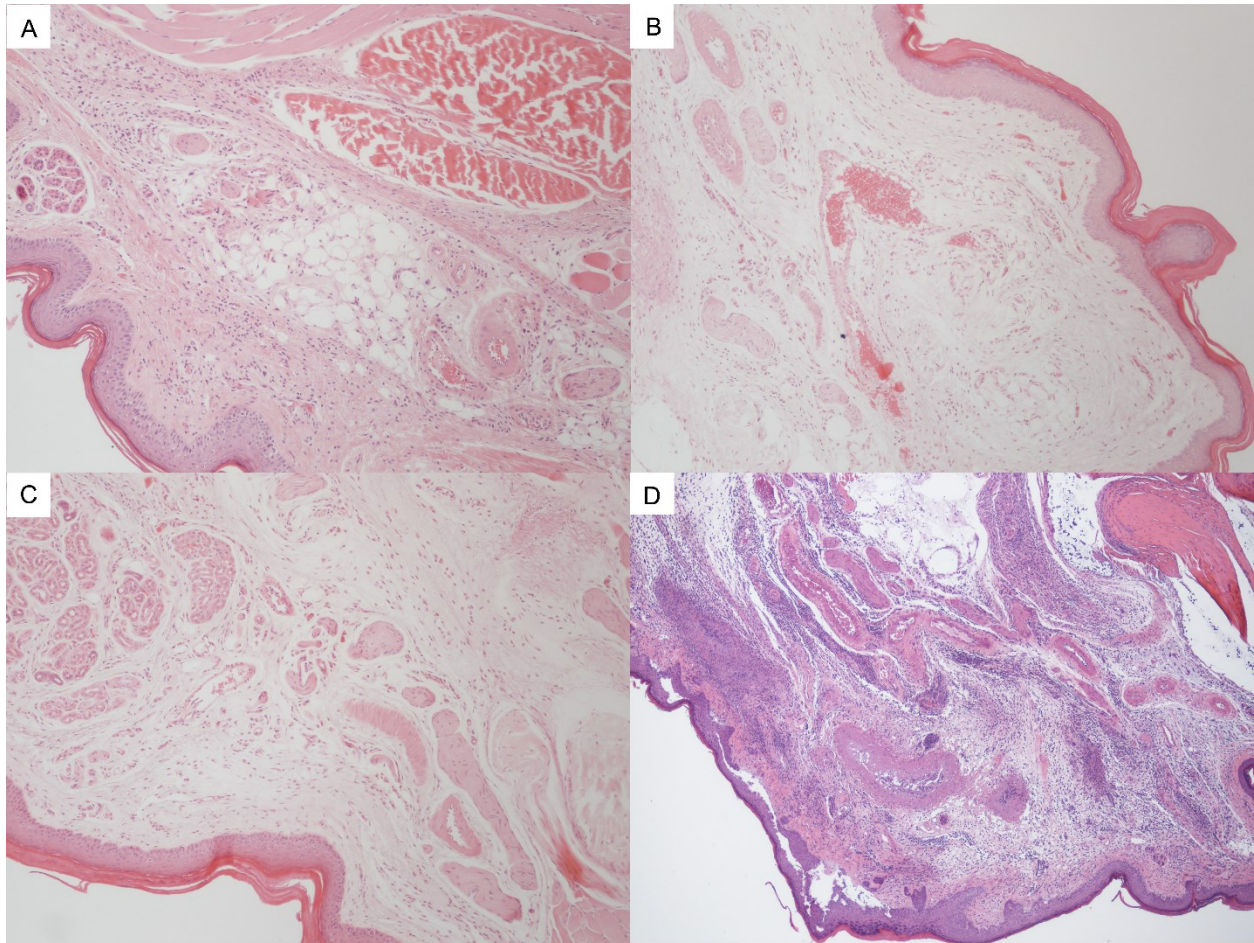
Figure 10: Representative footpad tissue micrographs with hematoxylin and eosin staining (H&E) from C57BL/6 (WT) mice demonstrating initial immune cell recruitment at 1DPI (10x)(A). Immune down-regulation resulting in normal appearance of footpad tissue at 28DPI (10x)(B), and 60DPI (10x)(C). Intense immune cell influx to footpad tissue resulting in edema and tissue necrosis by 142 DPI (4x)(D).

degeneration was observed in both mouse strains, in addition to mild leukolytic necrosis of

infiltrating PMN (Figure 10A). By 7 DPI, there was no statistical difference in CFUs between

groups of mice. In addition, the popliteal lymph node was quiescent and histologically

unremarkable until 7 DPI, when the C57BL/6 mice began to demonstrate reactive hypertrophy

and hyperplasia with formation of secondary follicles and active germinal centers in the cortex

and expansion of the paracortex (Figure 11).
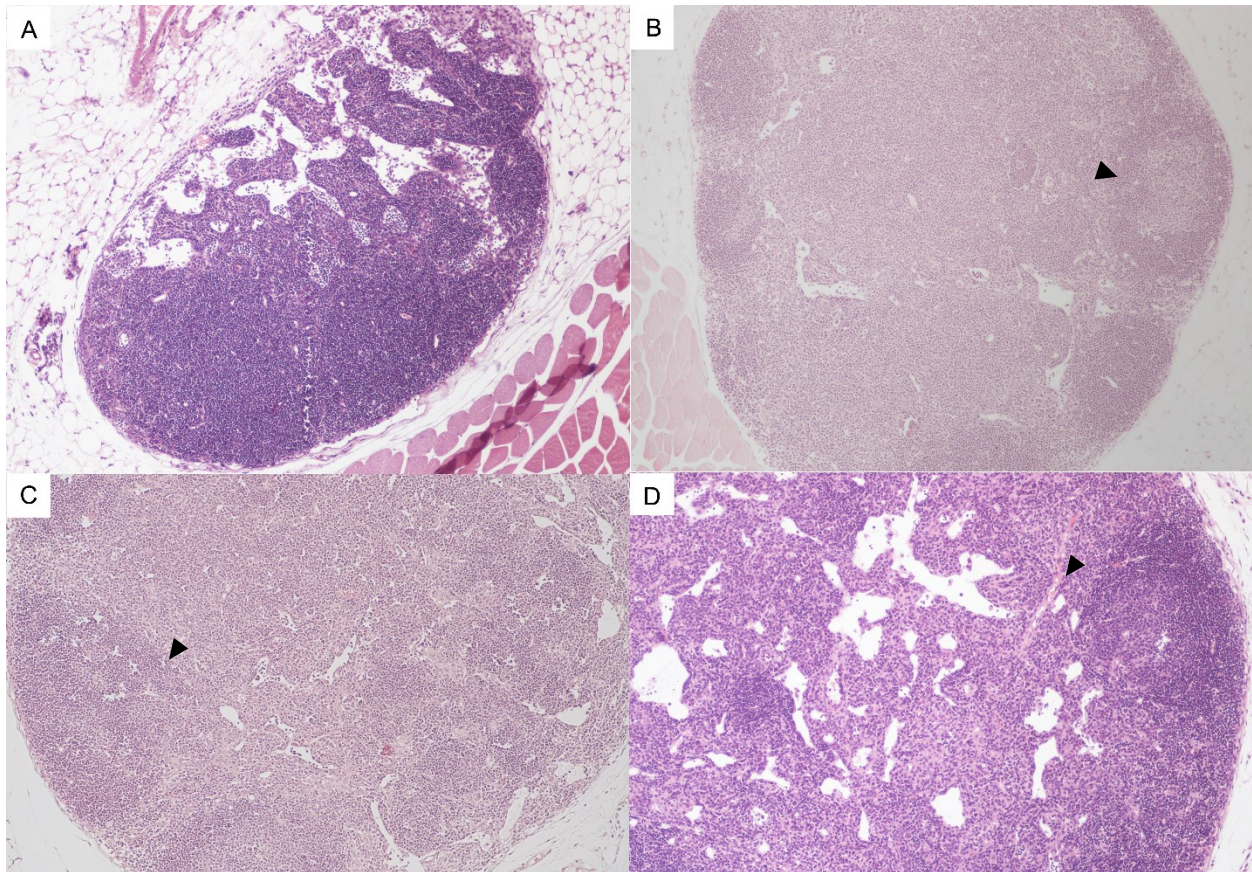
Figure 11: Representative popliteal lymph node micrographs with hematoxylin and eosin staining (H&E) from C57BL/6 (WT) mice demonstrating moderate neutrophil infiltration at 1 DPI (10x) (A). Moderately hypertrophic node containing secondary follicles and active post-capillary venules at 28 DPI (10x) (B). Hypertrophic node with several large secondary follicles and expanded paracortex at 60 DPI (10x) (C). Node with high cell density in all compartments by 142 DPI. Few secondary follicles with active germinal centers and deep paracortex containing high number of mature plasma cells (10x) (D).

Rag$^{-/-}$ mice retained comparably small nodes composed of dense stroma and few discernible post-capillary venules (PCV) accompanied by mild and dispersed infiltration of the stroma by neutrophils (Figure 13).

By 14 DPI, the immunocompetent C57BL/6 mice were able to mount an immune response against infection and reduce the bacterial load in the footpad by approximately 2 log$_{10}$. Immunodeficient Rag$^{-/-}$ mice were unable to mount an adaptive immune response and CFUs in footpad tissues increased by 1 log$_{10}$ over the course of 28 DPI (Figure 7). The histological analysis displayed similar immune cell recruitment pattern between strains of mice.

The immunocompetent mice demonstrated a moderate recruitment of foamy macrophages focally in the dermis/subcutis of the pad, with mild edema and few lymphocytes and PMN diffusely throughout the interstitum. Similarly, immunodeficient mice presented with mild edema and a mild infiltration of macrophages and few PMN in a focally extensive area of the dermis/subcutis (Figure 12D).
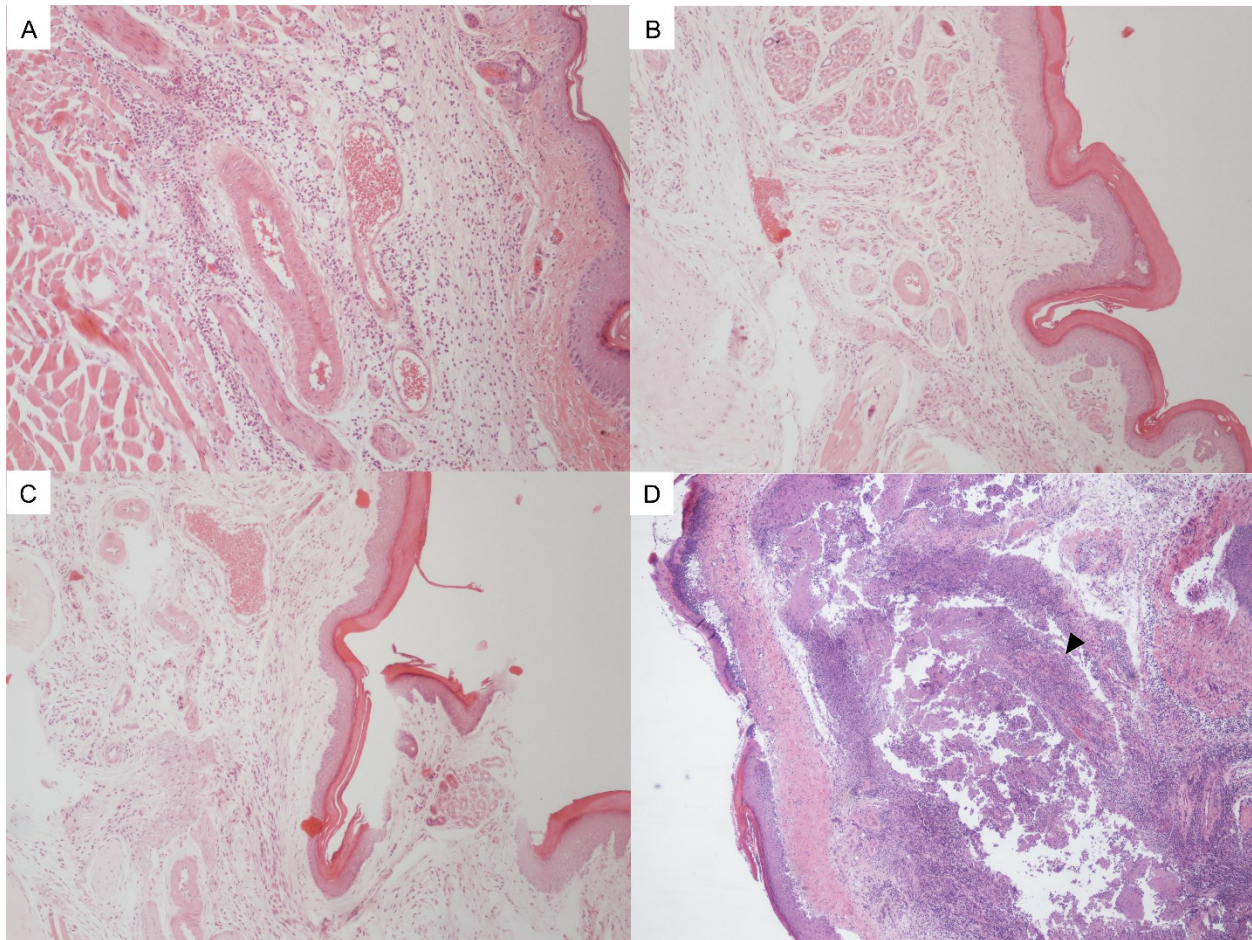
Figure 12: Representative footpad tissue micrographs with H&E staining from Rag-/- (immunodeficient) mice demonstrating initial immune cell recruitment at 1DPI (10x)(A). Immune downregulation resulting in histologically unremarkable appearance of footpad tissue at 28DPI (10x)(B), and 60DPI (10x)(C). Intense neutrophil infiltration to footpad tissue accompanied by sever tissue necrosis and collagen degeneration by 142 DPI (4x)(D).

As expected, Rag$^{-/-}$ mouse lymph nodes remained small and underdeveloped, containing dense reticular stroma and small numbers of scattered PMN, mast cells, and foamy macrophages, with a notable absence of lymphocytes (Figure 13).
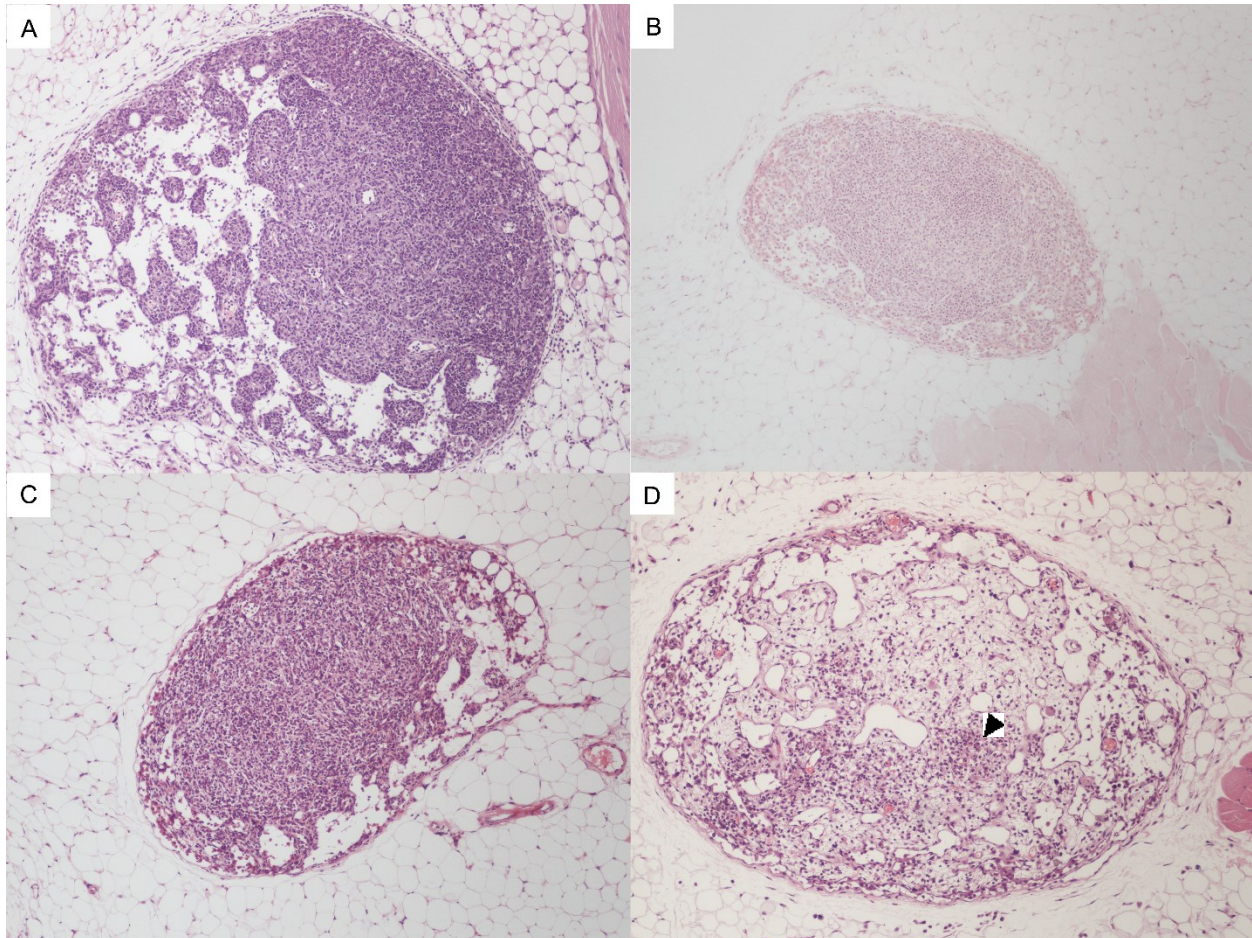
Figure 13: Representative popliteal lymph node micrographs with hematoxylin and eosin staining (H&E) from Rag$^{-/-}$ mice demonstrating dense cortical and medullary stroma, infiltrated by neutrophils and myeloid precursor cells at 1 DPI (10x) (A). Tiny node with foamy macrophages in medullary sinuses, few polymorpho-nuclear cells at 28 DPI (10x) (B). Node with dense reticular stroma and few post-capillary venules. Small numbers of foamy macrophages and no apparent lymphocytes at 60 DPI (10x) (C). Node composed of loose vasculo-reticular stroma with scattered macrophages and few mast cells, and deep paracortex containing high number of mature plasma cells by 142 DPI (10x) (D).

During the period of 28 DPI to 60 DPI, a plateau of the CFU numbers in both strains of mice corresponded to a period of persistence of the bacteria. This plateau phenomenon, or persistence, has been documented in other mouse footpad infection models (173) .

Additionally, during this time period, the infected footpads of both groups of mice progressed

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from minimal, dispersed infiltration of primarily macrophages and few PMN, to a histologically

unremarkable state (Figures 10B-C, 12B-C). During this time course, some bacterial killing was

apparent, although abundant bacilli were cultured from infected footpads, conflicting with the

apparent down-regulation of the host immune response (Figure 9B).

From 60 to 142 DPI, the bacterial load of the C57BL/6 footpad showed a dramatic 2 $\log_{10}$

increase (Figure 7A). Lymph nodes of C57BL/6 mice exhibited the development of an advanced

immune response represented by markedly hypertrophic expansion of both the cortex and

paracortex. The former contained several large secondary follicles and the latter, many active

PCV characterized by plumb endothelial cells and trans-endothelial lymphocyte migration, and

mature plasma cells in the medulla (Data not shown). CFUs in the Rag$^{-/-}$ footpad remained in a

persistent, plateaued state during this time period (Figure 7A).

By 142 DPI, CFU levels in the footpads of both strains of mouse were identical (Figure

1A). In the wild type C57BL/6 mouse, the CFUs increased 2 $\log_{10}$ over the steady state described

from 14 through 60 DPI, to a level statistically indistinguishable from the immunodeficient Rag$^{-/-}$

mouse. This final time point of 142 DPI comprised mice which had failed to control the infection

at a level comparable to immunodeficient mice. All mice presented with severe disease, on

both a histological (Figures 10-13) and gross pathologic level (Figure 8). Within the dermis and

subdermal tissue of C57BL/6 mice, focally and involving musculature, there was moderate to

marked edema, marked infiltration of monocyte-macrophages, moderate PMN infiltration and

multifocal tissue necrosis. Within the C57BL/6 lymph node, high cell density was apparent in all

compartments. There were few secondary follicles, all with active germinal centers, occasional

PCV appeared with hypertrophic endothelium and the medullary cords and deep paracortex

contained large numbers of mature plasma cells. Comparably, Rag$^{-/-}$ mice presented with more severe disease, encompassing most of the soft tissues of the foot (dermis, musculature, nerves, periost). There was severe tissue necrosis with loss of identifiable musculature, infiltration of predominantly PMN with an admixture of fewer macrophages and scattered mast cells. Blood vessels within the affected tissue appeared with leukocytosis, leukocyte margination, transmural infiltration and occasionally fibrinoid necrosis. In a focally extensive area of the footpad of one mouse, the inflammation and tissue necrosis extended to and involved the epidermis with formation of a scab mainly composed of necrotic epithelium and degenerate/effete PMN. The underlying dermal collagen was undergoing degeneration (Figure 10D).

Mouse cytokine production can be correlated to disease state as a marker of disease progress

Infection with *M. ulcerans* in humans and mice induces a local inflammatory response that is characterized by the induction of cytokines such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-1, IL-12 and IL-15 (139) characteristic of a Th1 immune response. Indeed it has been suggested that a Th1 immune response to *M. ulcerans* may prevent the development of BUD in people exposed to *M. ulcerans*, while a Th2 response does not (140). To identify immunological correlates of infection, we performed a broad screen of soluble cytokines from infected mouse tissues (Figure 14). Using a multi-analyte profiling approach, we compared the levels of soluble, inflammatory cytokines in the mouse footpad homogenates between mouse strains during the development of active infection. Additionally, selected cytokine concentrations were measured in the spleen, DLN, and whole blood serum at
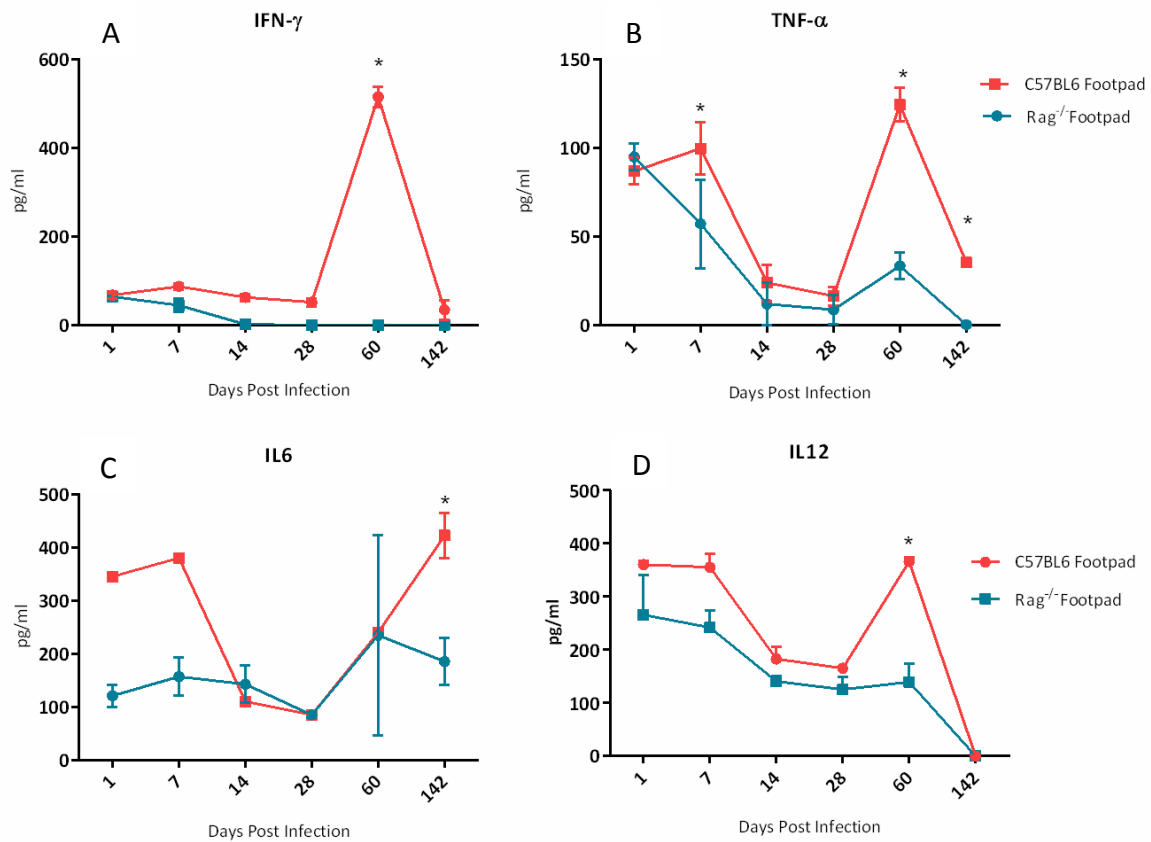
Figure 14: Concentration (pg/ml) of selected cytokines in mouse footpad tissue homogenates over the course of infection. Data point represents experimental mean with standard deviation. * denotes *P*≤0.05

the final time point of 142 DPI using a similar approach. By 7 DPI both strains of mice responded with higher secretion of IL-6 and IL-12 in the footpad homogenates relative to other measured cytokines, and at similar levels (Figure 14C and D). Other cytokines, particularly TNF-α and IFN-γ, demonstrated similar abundance between groups of mice, and occurred at a low level compared to other measured cytokines (Figure 14A and B). During the time course of 14 DPI through 28 DPI, measured cytokines appeared to decrease from initial measured levels and plateaued, which correlated with the pattern observed in the CFU numbers during this time period. By 60 DPI, C57BL/6 mice demonstrated an increased secretion of multiple cytokines, relative to the initial stages of infection. IL-6 and IL-12 rose to concentrations measured in

earlier time points, while IFN-γ demonstrated a nearly 6-fold increase relative to the

measurement at 1, 7, 14, or 28 DPI [e.g. 1DPI-IFN-γ: 90.6pg/ml, 60DPI-IFN-γ: 492.7pg/ml,

experimental mean, n=5]. The increased IFN-γ levels in the C57BL/6 mice correlated with the

reduced bacterial burden documented at this time point.
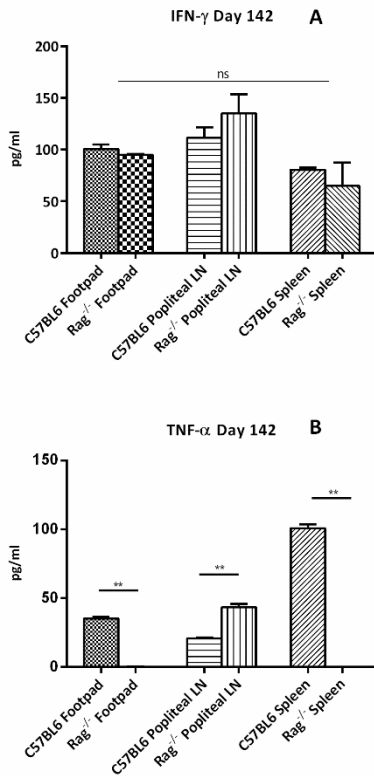
Figure 15: comparison of the concentrations (pg/ml) of IFN-γ (A) and TNF-α (B) in various tissues between strains of mice at the final time point (142DPI)

As described previously, at the terminal time point of 142 DPI similar gross and histo-pathological conditions were apparent in both strains of mice (Figures 10D, 12D). While other measured cytokines remain at a low level, a significant decrease in the production of IFN-γ was documented in C57BL/6 mice relative to that measured at 60 DPI [C57BL/6: 60DPI-IFNγ: 492.7pg/ml, 142DPI-IFNγ: 34.98pg/ml, experimental mean, n=5], although at statistically indistinguishable levels between strains of mice in the various tissues (Figure 15A), and strongly correlates with the rising CFU burden and histological measurements of strong disease activity. The concentration of TNF-α in the measured tissues demonstrates significant differences between strains

of mice by the final time point (Figure 15B). By 142 DPI, CFU levels in immunocompetent mice

rose from a plateau to a level statistically indistinguishable from Rag$^{-/-}$ mice over the same time

period, perhaps due to the down-regulation of protective cytokines.

Discussion

The data from the current study demonstrates the development of active infection after experimental inoculation of the mouse footpad that parallels the suspected human route of exposure and subsequent presentation of disease, without the contribution of the toxin mycolactone. While this mycolactone-negative strain appears attenuated in the wild-type C57BL/6 mice, a finding consistent with other mouse footpad models (174), the mice presented with degrees of pathology over time, eventually resulting in severe disease regardless of the immune state of the host. Mice of both immunological states rapidly developed necrosis of footpad tissues and destruction of the limb, as described previously (165), after a lengthy and variably asymptomatic period of time. Many publications of virulent *M. ulcerans* pathogenesis in experimental infections report extensive inflammatory cellular infiltrates to the lesion (2, 163, 164), consistent with our model. Indeed, the pattern of immune cell recruitment we have described is consistent with the finding of Oliveira et. al. (2005), who described strong initial recruitment of neutrophils followed by monocytes, to be a feature of M. ulcerans infection unrelated to the isolate's virulence (168). Other publications report minimum or absent inflammation in the lesion (30, 134), together with rapid progression into an ulcerative state. Taken together, this opposition provides evidence linking the contribution of other virulence determinants and variable bacterial phenotypes that still result in necrotizing pathogenesis. These studies also highlight a major obstacle in the understanding of M. ulcerans biology. The infectious phenotype of different eco-varieties of the pathogen demonstrate significant variability, even more so when comparing pathogenesis in distinct genotypes of mice (17). In

general, from this study, we were able to obtain valuable insights into the disease progression and the relationship with expression of immunity.

Immunocompetent mice were able to mount an immune response to the bacteria during this time, supported by the recruitment of immune cells into footpad tissue, the production of cytokines, and the initial decrease in bacterial load of the footpad. The pattern of immune cell recruitment during disease in the immunocompetent C57BL/6 mice is consistent with other reports in both mice and humans, in that the initial immune response is characterized by strong neutrophilic infiltration and a transition to a monocytic/macophagic infiltration during later stages (175). The histologic identification of immune cell necrosis and collagen degeneration occurring in the absence of mycolactone production was surprising. This finding supports the conjecture that virulence is not solely determined by the action of the toxin.

At the terminal time point of 142 DPI, the ensuing infection of the mice resulted in a severe inflammatory state characterized by a significant increase in bacterial burden and the disappearance of Th-1 driven, potentially protective IFN-γ and IL-12 production. The recruitment of host inflammatory cells was most dramatic at this final time-point. In addition to the abundance of inflammatory cell infiltrates, extensive tissue necrosis and degeneration of musculature and collagen characterized the severe pathology that precedes ulceration.

All animals eventually succumbed to infection, regardless of immune status, supporting the conjecture that BUD pathogenesis is not entirely dependent on immune status, co-infection, or the presence of the toxin (176). And while the two models end up with the

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same outcome, the path to this outcome differs between strains of mice. The detailed histological analyses described here demonstrate that in the absence of mycolactone, cellular infiltration drives BUD pathology in the later, chronic disease state (Figures 10D, 12D). Analysis of soluble cytokines in the lesions revealed high IL-6 expression in both C57BL/6 and Rag^{-/-} mice (Figure 14), and an inverse relationship between IL-6 and IFN-γ. IL-6 was shown to increase when IFN-γ decreased in the C57BL/6 mouse and appears to be continuously elevated in the Rag^{-/-} mouse in the absence of IFN- γ. An over production of IL-6 has been associated with severe, chronic inflammatory diseases (177), and has been reported as a differentially regulated cytokine in BUD patients compared to community controls (178). Additionally, IL-6 has been documented as a marker of innate immune activation in clinical specimens of BUD patients (137). Indeed, high levels of IL-6 have been reported to enhance other mycobacterial infections such as *Mycobacterium avium*, when occurring both intra- and extracellularly (179, 180). The abundant infiltration of neutrophils and monocytes to lesions in our model is consistent with the role IL-6 has in the augmentation of cell adhesion molecules and neutrophil transmigration (181), suggesting that IL-6 drives leukocyte recruitment to the lesion.

Our results indicated a mechanism of localized immunosuppression in concordance with immune cell infiltration in C57BL/6 mice. While other immune avoidance strategies of *M. ulcerans* have been thoroughly described (73), the specific role of IL-6 in this system requires further study. Defects in antigen processing and presentation of macrophages, specifically suppressing cell-mediated immunity as a result of over-expressed IL-6, have been documented (182). The source of IL-6 induction remains unclear, as TNF-α was not demonstrated to be a significant factor during the course of infection. Previous studies described mycobacterial cell

wall components such as muramyl dipeptide and lipoarabinomannan as responsible for

inducing IL-6 production in other models (183, 184), suggesting that a mycobacterial product

may be involved with IL-6 induction in our model. The up-regulation of IL-6 and IL-12

demonstrated here may represent novel biomarkers of *M. ulcerans* infection which may be

further explored and eventually developed and applied to detect risk of development of BUD.

The pathologic state of the C57BL/6 mice during reactivation of disease (>140 DPI),

represents a failure to adequately control the infection, despite a fully complemented immune

system, in our model of BUD. Additionally, the absence of IL-4 and IL-5 in our model would

suggest that Th-2 immunity did not play a role in the development or reactivation of disease.

Thus, it appears that the Th-1/Th-2 paradigm does not apply here, as has been seen with other

mycobacterial infections (185, 186). The molecular mechanisms involved in

immunosuppression and the activation and subsequent degeneration of T cells is in need of

further characterization, but could represent a crucial piece of understanding of the immune

evasion strategies of *M. ulcerans.*

Finally, this study demonstrates significant immunosuppression and persistence of

the *M. ulcerans* bacilli without the contribution of mycolactone. The characterization of

additional virulence factors affecting disease progression with or without the presence of

mycolactone will significantly enhance our understanding of *M. ulcerans* infection.

CHAPTER 4: ROLE OF *ANOPHELES GAMBIAE* IN THE TRANSMISSION AND MAINTENANCE OF *M. ULCERANS*

Introduction

M. ulcerans is a known environmental pathogen, but its specific niche and the mechanism of maintenance in the environment remains unknown (187). The source of transmission of the pathogen is also unknown, but many aquatic invertebrates have been implicated in this process (161). The primary risk factors in the development of disease are a close association with standing or stagnant water, the use of protective clothing, and inadequate wound management (188). Reports of *M. ulcerans* DNA contamination of wild-caught mosquitoes and the successful culture of live *M. ulcerans* from predatory water bugs (Naucoridae, Belostomatidae) have led the initiation of a plethora of studies investigating the association between *M. ulcerans* and aquatic invertebrates (48, 52, 53, 59, 60, 62, 189).

Anopheles gambiae, the infamous malaria vector, is commonly found in regions endemic to Buruli Ulcer disease, especially in sub-Saharan Africa (190). *A. gambiae* larvae develop in small, diverse bodies of water and filter bacteria from the water column as a source of nutrition. The aquatic lifestyle of the Anopheline mosquito, both as larvae and adults, potentiates the hypothesis that these mosquitoes could also be associating with natural populations of *M. ulcerans*. In this study, we investigate the association between these two organisms and the role *A. gambiae* may play in the transmission and maintenance of *M. ulcerans* through the use of laboratory models of transmission and a thorough investigation of the contamination of the insects. Previous studies have investigated the interaction between

M. ulcerans and other genera of mosquitoes, specifically describing a positive correlation between disease incidence in Victoria, Australia and M. ulcerans-DNA contamination of wild caught mosquitoes. Within the laboratory, a landmark study by Wallace et al. (2010) described the interaction among multiple genera of mosquitoes, wild-type and toxin-negative M. ulcerans, and the implications this interaction has in ecological trophic relationships (54). However, this study lacked an analysis of the interaction between *M. ulcerans* and any of the ubiquitous members of the *Anopheles* genus. The study described here includes a similar analysis of the role of *A. gambiae* to transmit and maintain the *M. ulcerans* pathogen within the laboratory and the implication of such interaction.

Materials and Methods

Mosquito growth

1st-instar larval mosquitoes were acquired from the colony maintained at the Arthropod-borne Infectious Disease Laboratory (AIDL) at Colorado State University. 100 larval mosquitoes were distributed to individual cages containing 250ml of sterile water and supplemented daily with finely ground fish food. All cages were setup in duplicate for the individual treatment groups and each experiment was repeated in triplicate. Mosquitoes were monitored daily and allowed to develop over a period of 10 days in a controlled environment of 28°C and 70% humidity. Mosquito cages were supplemented with sterile water and raisins, ad libitum, upon emergence of adult insects. Upon termination of the study, adult mosquitoes were aspirated from the cages and knocked down in 4°C. 10 mosquitoes each were distributed for the generation of technical replicates and their use in subsequent assays.

Bacterial culture

Mycolactone-negative *Mycobacterium ulcerans* strain 1615-GFP was kindly donated by Dr. Pamela Small from the University of Knoxville, Tennessee. The culture was propagated at 32°C for a period of 3 weeks on Middlebrook 7H11 (Difco Laboratories, Detroit, MI) plates supplemented with Kirschner Selecta-Tabs and 10 µg/ml kanamycin (7H9+), then aliquoted into infectivity stocks at a concentration of 10^{10} cells/ml. Expression of GFP was confirmed in the final culture with a fluorescent microscope. Contaminating bacteria from mosquito samples were acquired by vortexing the tissues of interest in 7H9+ liquid media, performing a 10-fold serial dilution and plating the dilutions on Middlebrook 7H11 plates with supplements as described.

Mosquito treatment groups

100 1st-instar larval mosquitoes were exposed, in duplicate, to live and dead supplemental bacteria, or no supplemental bacteria. 10^2 cells/ml of live *M. ulcerans* 1615-GFP, 10^2 cells/ml of dead, ɣ-irradiated *M. ulcerans* 1615-GFP, and dead, ɣ-irradiated *Mycobacterium tuberculosis* were added to the sterile water upon the addition of larval mosquitoes. All groups received approximately 100 mg of fish food daily, in addition to the single dose of supplemental bacteria.

Mosquito survival and fitness

The mosquito's survival to adulthood was measured by counting the number of emerged adults and generating a Kaplan-Meier survival curve among the treatment groups. The relative fitness of emerged adults was evaluated by measuring the wing size of emerged

mosquitoes as a proxy for body size. Both wings were removed and measured electronically via the publicly available ImageJ program (imagej.nih.gov/ij/) using a line measurement plugin.

DNA extraction of bacilli from mosquito tissues

Mosquitos were collected and dried for 30 minutes under a vacuum. After this time, 500ul of liquid N2 was added and the mosquitoes were incubated for 30 seconds. Dryed insects were homogenized 100 μl sterile DNase/RNase free water with a handheld Eppendorf homogenizer then centrifuged at 10,000xg for 10 minutes to remove debris. The supernatant was retained and 100 μl of 1M HCl was added and incubated for 30 minutes at room temperature for decontamination, then subsequently neutralized by adding an equal volume of 1M HCl. DNA was extracted and purified using diatomaceous earth, as described (191). Briefly 50 μl of the decontaminated sample was added to 50 μl of lysis buffer L6 (120g guanidinium thiocyanate (GuSCN) (Fluka Chemie; Buchs, Switzerland) dissolved in 100 ml 0.1M Tris-HCl at pH 6.4, with 22 ml 0.2M EDTA and 26g Triton X-100 (Sigma Aldrich; St. Louis, MO) and stirred overnight) in a clean Eppendorf tube and briefly mixed. 10 μl of proteinase K (20 mg/ml) was added before an overnight incubation at 60°C in a shaking incubator. To capture DNA, 10 μl of diatomaceous earth stock solution (10g diatomaceous earth (Sigma Aldrich; St. Louis, MO) in 50 ml H20 containing 500ul of 37% (wt/vol) HCl), then placed in a shaking incubator for 2 hours. Mixed samples were then centrifuged at 5000xg for 5 minutes. The resultant pellet was twice washed with 200 μl of L2 buffer (120 g GuSCN in 100 ml 0.1M Tris-HCL pH 6.4), then centrifuged and the pellet washed first with 70% ethanol, then 200 μl acetone. The washed pellet was dried under vacuum then resuspended in 20 μl TE buffer and incubated for 20 minutes at 65°C. After

incubation, samples were centrifuged at 2000xg for 5 minutes and the supernatant transferred to a sterile, PCR grade 1.5ml tube.

PCR analysis of extracted DNA

PCR analysis was performed as described (192). Primers for the amplification were MU1 (5'-GGCAGGCTGCAGATGGCAT-3') and MU2 (5'-GGCAGTTACTTCACTGCACA-3') directed against the IS2404 sequence and producing at 549-bp fragment in the presence of *M. ulcerans* DNA. Briefly, 2 µl samples of purified DNA were amplified in buffer supplied by the manufacturer of *Taq* polymerase (Roche, Indianapolis, IN) in 20 µl reaction mixture containing 1U of Faststart *Taq* polymerase, 1 uM primers, 1.5 mM MgCl2, and 200 uM (each) deoxynucleoside triphosphates. Amplification occurred in an automated thermal cycler (MJ Research) with the following conditions: denaturation at 94°C for 2 minutes, amplification by 35 cycles of 1 minute steps at 94, 66, and 72°C, and final extension at 72°C for 7 minutes. 15 µl of the completed reaction were run on at 1.5% agarose gel in Tris-borate-EDTA buffer stained with SYBR safe DNA stain (Life Technologies, Grand Island, NY), and visualized with UV transillumination on a gel-doc XR+ system (Biorad) using Image Lab software.

Immunofluorescence

10 larval and 10 adult female mosquitoes from each treatment group were subjected to immunofluorescence (IFA) screening of internal and external tissues for contamination by *M. ulcerans*. The head, midgut, and salivary glands of adult female mosquitoes were removed and immediately placed in 4% paraformaldehyde solution for 10 minutes for fixation and washed in phosphate buffered saline (PBS). After fixation, the tissues were permeabilized by the addition of 0.1% Triton X-100 for 10 minutes and washed in PBS. To distinguish between green mosquito

auto-fluorescence and the GFP expressing bacilli, tissues were first probed using a rabbit anti-*M. ulcerans* whole cell antibody developed at Colorado State University. The anti-*M. ulcerans* antibody was then probed with a goat anti-rabbit Cy5 labeled antibody (Life Technologies). NucBlue DAPI nuclear stain (Life Technologies) was applied for a period of 5 minutes as a counter-stain. Fluorescently labeled tissues were viewed by epifluorescence microscopy (Olympus, Center Valley, PA) equipped with a standard epifluorescent attachment filter set. Larval mosquitoes were removed from the development cages and immediately placed in 4% paraformaldehyde for fixation. Larval mosquitoes were viewed with epifluorescent microscope scope as previously described, without additional fluorescent labeling. Images are reproduced without alteration besides cropping and adjustment of light intensity.

Transmission experiments

Adult female mosquitoes from each treatment group were analyzed for their ability to mechanically transmit the contaminating *M. ulcerans* bacilli. Firstly, the heads of adult female mosquitoes were removed and assayed to confirm the presence or absence of live *M. ulcerans* bacilli as described. A second set of 10 adult female mosquitoes were used for saliva collection, a third set of 10 adult female mosquitoes were used for an artificial blood meal, and a fourth set of 20 adult female mosquitoes were used for a blood meal from C57BL/6 mice.

For saliva collection, 10 adult female mosquitoes were collected from each treatment group and anesthetized by a 5 minute exposure to a cotton ball soaked with triethylamine (Sigma Aldrich). The wings and legs were removed from the mosquito bodies and the proboscis was placed into a capillary tube containing 7H9+ media. Mosquitoes were allowed to salivate into capillary tubes for 45 minutes. After this time period, mosquitoes were

removed from the capillary tube and the tube was placed into a cryovial containing 100 μl 7H9+

media and centrifuged at 1,000xg for 10 minutes. The resulting sample was divided in half; with

half subjected to DNA extraction and PCR analysis for the *M. ulcerans* insertion sequence

IS2404. The remaining half was 10-fold serially diluted, plated on 7H11+ plates and incubated as

described.

For the artificial blood meal, 10 adult female mosquitoes were collected and placed

into a clean carton with a mesh lid 24 hours in advance of the experiment, with clean water and

raisins provided ad libitum. Approximately 4 hours before the experiment, the food and water

were removed to encourage feeding. A temperature controlled water flow mechanism (VWR

Radnor, PA) was connected to a glass blood meal apparatus. A hydrated hog gut membrane

was attached to the bottom of the glass apparatus and the device was filled with 100 μl

defibrinated sheep blood. Mosquitoes from all treatment groups were allowed to feed on

individual artificial blood meal devices for approximately 1 hour. After the artificial blood meal

apparatus was placed on the cages, mosquitoes were stimulated to feed by exhaling breath

over the cages. After feeding, the defibrinated blood was collected, divided in half, and assayed

in a similar method as the saliva collection.

Mouse Transmission

For the mouse blood meal, 20 adult female mosquitoes were collected, divided, and

placed into a clean carton with a mesh lid to make duplicate groups of 10 mosquitoes per

treatment group. Eight C57BL/6 mice were gently anesthetized by an intraperitoneal injection

of 100 μl ketamine-xylazine solution (100 μl ketamine, 75 μl xylazine, 825 μl PBS) and allowed

to rest for 10 minutes. The anesthetized mice were placed onto the mesh lid of the mosquito

cage and remained in place for approximately 1 hour. A separate group of mice received an inoculation of 10^6 *M. ulcerans* cells in a 10ul inoculation into the tail. An additional control group of mice received a mock puncture inoculation in the tail using a 22 gauge needle dipped in a suspension of *M. ulcerans*. Every 14 days post inoculation or mosquito blood meal, a 20ul blood sample was collected from the tail vein of all mice. Blood was allowed to clot and the sera was retained for western blot analysis against *M. ulcerans* whole cell lysate.

Statistics

Statistical significance of the survival and fitness data was determined using a two-sided student's t-test between individual treatment groups.

Mouse Care

Twenty 8-week-old female wild-type C57BL/6J mice were obtained from The Jackson Laboratory Center for Mouse Models of Heart, Lung, Blood and Sleep Disorders (Bar Harbor, ME). All mice were maintained in the Animal Biosafety Level 2 facility at CSU, with sterile rodent chow and water ad libitum, with 5 animals per cage. The studies involving animals were approved by the Institutional Animal Care and Use Committee at Colorado State University, which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International.

Results

Within the aquatic environment, larval
mosquitoes are known to filter bacteria from the water
column as a source of nutrition. During mosquito
development in the contaminated water, *A. gambiae*
larvae readily consume the *M. ulcerans* bacilli,
measured by fluorescent microscopy of the larval
alimentary canal (Figure 16). Larval mosquitoes readily
consume supplemental bacteria, but the effect on
survival and development of adult mosquitoes is only
encountered in groups consuming live *M. ulcerans*.
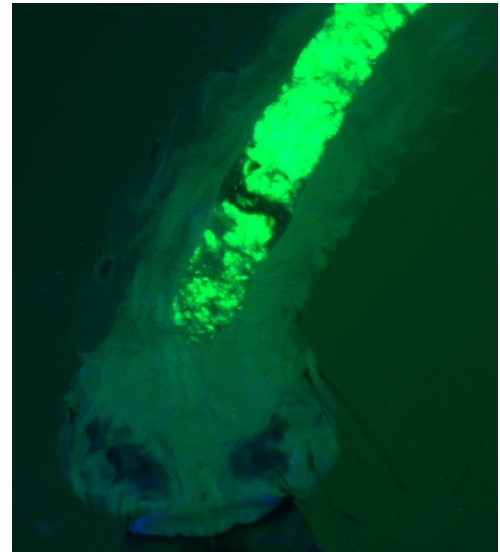
Figure 16: Alimentary canal of larval *A. gambiae* mosquito packed with *M. ulcerans* 1615-GFP, viewed under a fluorescent microscope (10x).

Upon emergence from the aquatic environment, adult mosquitoes remain in contact with the

contaminated water source. To determine the contamination patterns of adult mosquitoes,

emerged adult female mosquitoes were collected, fixed, and fluorescently labeled. Since most

vector-borne pathogens are transmitted by mosquitoes via salivary expression into the

puncture wound, we first analyzed the salivary glands of adult female mosquitoes and

discovered no measurable contamination by *M. ulcerans* using IFA. The salivary glands were

also homogenized and subjected to PCR against the *M. ulcerans* insertion sequence IS2404 and

culture. We found no contamination of the mosquito salivary glands using this approach.

Additionally, analysis of the midgut tissues revealed no contamination by *M. ulcerans*.

A microscopic analysis of the distal end of the female mosquito's proboscis reveals a complex tissue structure composed of chitinous scales and sensory hairs. IFA analysis of this structure demonstrated intense contamination by *M. ulcerans* bacilli (Figure 17). These
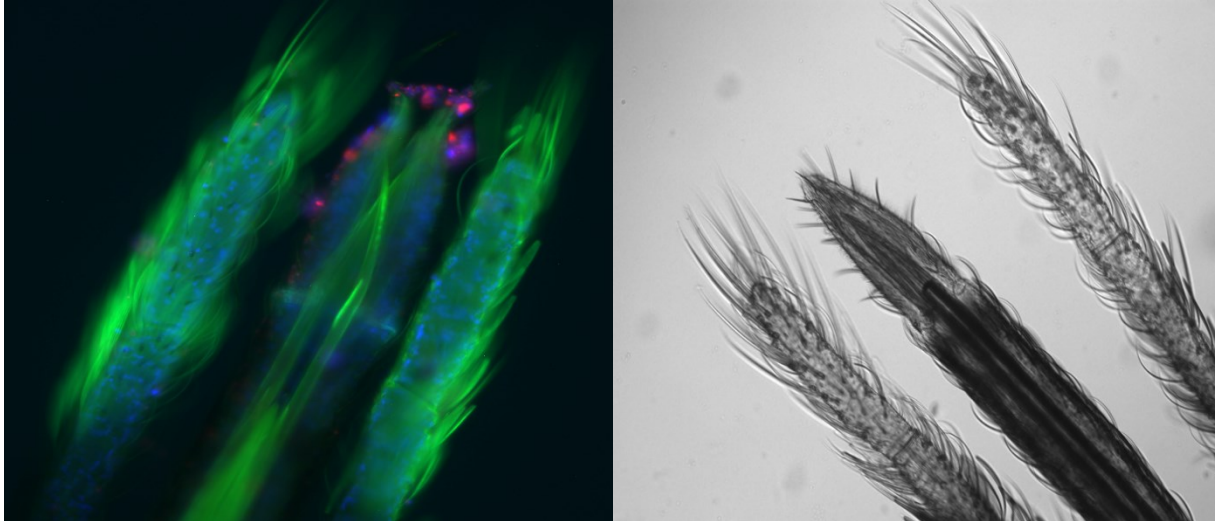
Figure 17: Immunofluorescence image of labral tissues of mosquito proboscis at 40x magnification. *M. ulcerans* bacilli (labelled in red) contaminated these external structures (left). High contrast microscopy of labral tissues of proboscis for reference (right).

contaminating bacilli are active and demonstrate positive growth upon culture (data not shown). The length of time that the contaminating *M. ulcerans* remains alive on the mosquito mouthparts was not measured. From these data, it could be suggested that mosquitoes may transmit the bacilli through a mechanical mechanism, as opposed to the common biological mechanism of other pathogens. All mosquitoes sampled from the live *M. ulcerans* groups demonstrate this external pattern of contamination. The γ-irradiated *M. ulcerans* and *M. tuberculosis* groups do not show a similar pattern of contamination, although few γ-irradiated *M. ulcerans* cells could be detected on the mosquito mouthparts.

An analysis of survival to adulthood among the treatment groups reveals a significant

difference in survival among the treatment groups. As a source of nutrition, the ɣ-irradiated *M.*
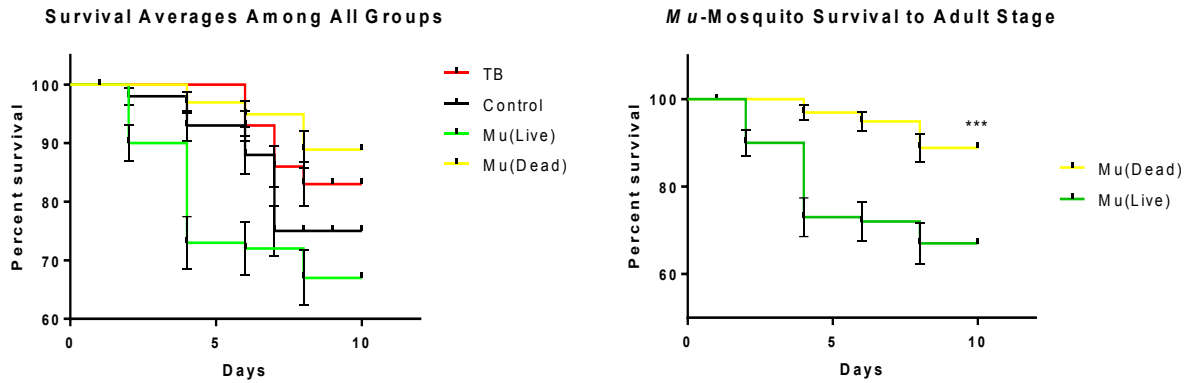
Figure 18: Kaplan-Meier plot of Survival to adulthood for mosquitoes from all treatment groups (left). Individual *M. ulcerans* (Mu) exposed treatment groups separated for comparison revealing a significant difference in survival (right). ***= p.001

ulcerans and *M. tuberculosis* bacilli provide a significant increase in survival compared to the

control groups, which rely solely on fish food. Conversely, mosquitoes which have consumed

live *M. ulcerans* bacilli during development are measured to have a significantly reduced

survival rate compared to the controls (Figure

18). In addition, the emerged adult

mosquitoes are significantly smaller than

their counterparts which have consumed

control fish food and ɣ-irradiated bacilli

(Figure 19). Interestingly, when *M. ulcerans*

bacilli are used as a monotypic food source

(without supplemental fish food), the effect

on the larval mosquitoes is lethal (data not

shown). According to our study, this effect
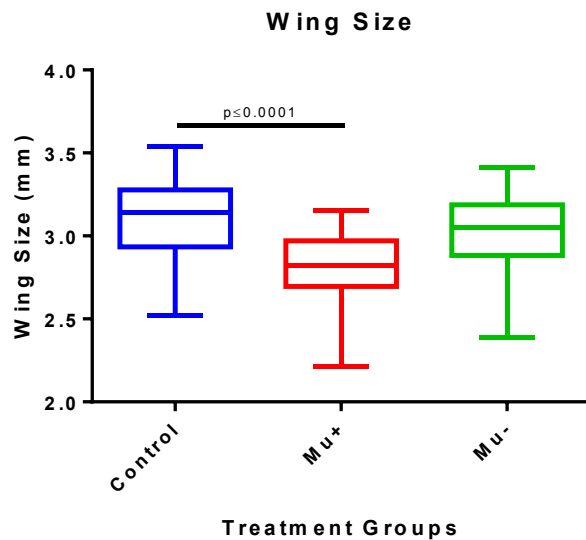
Figure 19: Comparison of the wing size of emerged female mosquitoes as a proxy for body size. Adult mosquitoes are significantly smaller than their control counterparts as a result of exposure to live *M. ulcerans*.

seems to be specific to *A. gambiae* mosquitoes, as Culex sp. mosquitoes can consume live *M. ulcerans* bacilli as a monotypic food source, even in concentrations as high as 10^{10} cells/ml without any measured developmental effects or reduced survival (data not shown).
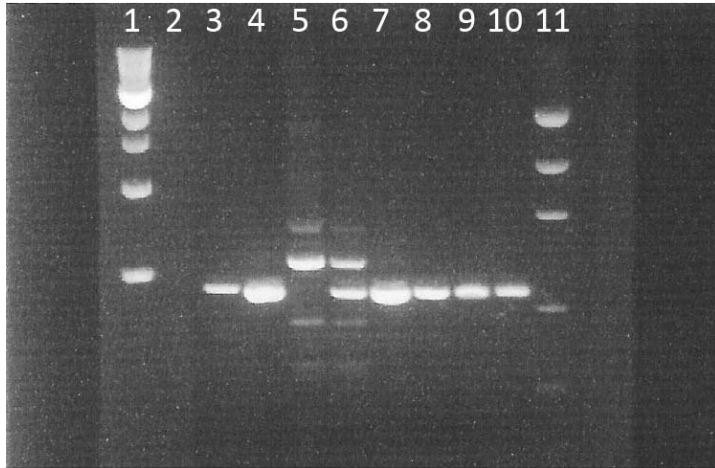
Figure 20: PCR analysis of experimental samples. Lanes:
1: high mass DNA ladder
2: control mosquito homogenate (negative control)
3: *M. ulcerans* gDNA (1ug) (positive control)
4: Mosquito homogenate spiked with *M. ulcerans* gDNA (1ug)
5: homogenate of mosquitoes exposed to M. tuberculosis
6: homogenate of mosquitoes exposed to M. tuberculosis spiked with *M. ulcerans* gDNA (1ug)
7: homogenate of mosquitoes exposed to live *M. ulcerans*
8: homogenate of mosquitoes exposed to dead *M. ulcerans*
9: saliva sample from mosquito exposed to live *M. ulcerans*
10: saliva sample from mosquito exposed to dead *M. ulcerans*
11: low mass DNA ladder

To determine if the contaminated mosquitoes could mechanically transmit the bacilli, contaminated mosquitoes were collected and saliva was extracted. The collected samples were then interrogated for the presence of *M. ulcerans* via PCR against the IS2404 sequence and culture. This procedure did not detect any replicating bacteria in the collection medium via culture (data not shown). The PCR analysis results were positive for the presence of *M. ulcerans* DNA in the saliva sample, as expected (Figure 20; Lanes 9,10), by the presence of a 549-bp band. No contaminating bacilli or *M. ulcerans* DNA was detected in the saliva collected from the control mosquitoes (data not shown).

To further refine the transmission hypothesis, contaminated and control mosquitoes were allowed an artificial blood meal through a hog-gut membrane and by feeding on anesthetized mice. After demonstrating the positive contamination of adult female mosquitoes

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Figure 21: PCR analysis of samples from transmission experiments. Lanes:
1: high mass DNA ladder
2: control mosquito homogenate (negative control)
3: *M. ulcerans* gDNA (positive control)
4: water sample from live *M. ulcerans* mosquito cage
5: defibrinated blood after artificial blood meal of mosquitoes exposed to live *M. ulcerans*
6: homogenate of mosquito exposed to live *M. ulcerans* before artificial blood meal
7: homogenate of mosquito exposed to live *M. ulcerans* after artificial blood meal
8: homogenate of mosquito exposed to live *M. ulcerans* before mouse blood meal
9: homogenate of mosquito exposed to live *M. ulcerans* after mouse blood meal
10: *M. ulcerans* gDNA (positive control)
11: low mass DNA ladder

from the same group (Figure 21: Lanes 6,8), the remaining mosquitoes were placed in a clean cage as described. The mosquitoes and the defibrinated blood were collected and analyzed. Subsequently, mosquito tissues did not demonstrate growth of live *M. ulcerans* upon culture but remained PCR positive (Figure 21: Lane 7). The collected blood did not demonstrate positive culture or PCR signals after the feed (Figure 21: Lane 5). Contaminated and control mosquitoes were also allowed to feed on anesthetized mice for a period of 1 hour. The subsequent analysis of mosquito exposed mouse serum demonstrated no reactivity to whole cell lysate via western blot over a period of 20 weeks (data not shown). Mice inoculated with the sham needle puncture did not develop antibodies against *M. ulcerans* antigens during the study period

Discussion

Due to the significant overlap in the geographic and environmental distribution of both *A. gambiae* and *M. ulcerans*, it is highly likely that these two organisms interact on some level. Other studies have demonstrated an association between mosquitoes and outbreaks of Buruli Ulcer disease (61). Indeed, the use of bed nets has been shown to reduce the risk of infection

with *M. ulcerans*. Unfortunately, the role mosquitoes play in the transmission and maintenance of *M. ulcerans* in the wild remains elusive. In this study, we report two distinct contamination patterns of *A. gambiae* mosquitoes. Larval mosquitoes readily consume introduced *M. ulcerans*, and may serve as a bioaccumulator of the pathogenic bacteria in the wild. A study by Mosi et al (2008), demonstrated the contamination of the raptorial arms of predacious water bugs after the consumption of contaminated mosquito larvae, further encouraging the role of mosquitoes in the passage of *M. ulcerans* through the aquatic food web (52). In this study, we show larval mosquitoes can successfully maintain live, active *M. ulcerans* in their alimentary canal during their development. Larval mosquitoes are a prominent member of the aquatic food chain, and are eaten by a wide variety of higher order insects, amphibians, birds, and fish. The association between larval mosquitoes and *M. ulcerans* bacilli may represent a ubiquitous mechanism of maintenance of the bacteria in the natural setting. However, it is not likely that mosquito larvae would be exposed to the concentration of bacilli used in this study while developing in their natural habitat, although the actual distribution and concentration of *M. ulcerans* in the wild in unknown.

Once emerged from the water column, adult mosquitoes must still associate with the aquatic environment for breeding and hydration. The appearance of contaminated bacilli on the external structures of the adult mosquito and the lack of internal contamination of gut and salivary glands suggests that contamination of the adult mosquitoes occurs post emergence from the aquatic stage of development. These emerged, contaminated mosquitoes are small and may be more likely to be eaten by higher predators. While it may seem disadvantageous for the pathogen to negatively affect the development of the mosquito, it further promotes the

idea of a biological relationship between the two organisms. However, it is unknown whether the fitness cost from the association with the live *M. ulcerans* is due to larval consumption of the bacilli, the external contamination of the adults, or through some other mechanism. Size of the adult mosquito can influence epidemiologically relevant traits, thus the probability that mosquitoes actively transmit the contaminating pathogen is low (193). Although, it could be suggested that contaminated mosquitoes with reduced fitness may also contribute to environmental maintenance of the bacilli.

Our transmission studies further implicate mosquitoes' role in maintenance and not as a vector. In our model, the contaminated mosquitoes were not able to measurably transmit the pathogen (Figure 22). While the true infective dose is unknown, it is highly likely that a potential dose received from

Figure 22: The position of the adult female anopheles mosquito during a blood meal. Arrow indicates the anatomy of the proboscis and labral tissues during the bloodmeal. Image credit: who.org.

a mosquito would contain very few, if any, cells. The mouse model of transmission did not demonstrate any measurable immune recognition of the pathogen potentially vectored by the mosquitoes. Our mouse transmission study models the potential transmission of bacilli from contaminated mosquitoes to fully immunocompetent mice. However, it is suspected that the nutritional status or general health of human populations plays a significant role in their susceptibility to *M. ulcerans* infection, and the inclusion of an immunosuppressed model would be advantageous. Epidemiological studies in the field have not been able to definitively

demonstrate mosquito-borne transmission. However, it is still possible that mosquitoes could play a role in transmission, and the development of a model to accurately predict this phenomenon would require more extensive study.

Finally, analysis of contaminated insects via PCR, while incredibly sensitive and specific, can result in easily misinterpreted data. Our procedure of tandem PCR and culture demonstrated that positive PCR signals do not predict the presence of live, transmissible *M. ulcerans* bacilli upon culture. The source of the captured DNA must be considered when analyzing PCR results, as DNA from lysed cells and as a part of the *M. ulcerans* extra-cellular matrix may be exceedingly abundant.

Current studies suggest a limited role for mosquitoes in the transmission of M. ulcerans, and perhaps a significant role in environmental maintenance. While the findings of Williamson et al (2014) highlight the importance of a puncture-type inoculation for eventual development of classical disease, thus the implication of mosquitoes, a wide variety of blood-feeding insects have yet to be evaluated. The family Ceratopogonidae contains 4,000 species of blood-feeding insects, better known as the biting midge. This family of insects is responsible for the transmission of the filarial worm *Mansonella* and a wide variety of viral pathogens. Additionally, pool-feeding insects such as horse flies (family Tabanidae) and black flies (family Simuliidae) are aggressive and widely distributed blood-feeding insects responsible for the transmission of Trypanosoma, the filarial worm Loa Loa, anthrax, tularemia, and Onchocerciasis (194-198). A member of these insect families, with their global distribution and established vector capacity, could represent the enigmatic source of transmission that many have been searching for.

CHAPTER 5: ANALYSIS OF MOSQUITO METABOLOME AFTER EXPOSURE TO *M. ULCERANS*

Introduction

Infection with *Mycobacterium ulcerans* results in a necrotizing ulceration of the subcutaneous

tissue and is a major cause of morbidity in more than 30 countries (63). West and Central

Africa, Australia, and similar tropical localities have reported an increasing incidence of the

disease over the past decade (199, 200). Exposure to the bacteria is thought to occur from a

yet unknown, but persistent, environmental niche. Serological studies of patients in endemic

areas indicate high sero-prevelence rates compared to disease incidence rates, suggesting that

exposure to the pathogen without the development of disease is common. After exposure, and

over a variable incubation period, infection can progress from a painless nodule, plaque, or

edema to severe ulceration. It is likely that *M. ulcerans* persists within a complex food web,

through the passage and maintenance by various arthropods and mammals within a particular

ecosystem (201). Recently, other non-human mammals have been discovered to be susceptible

to infection by *M. ulcerans*, potentially indicating a diversity of reservoirs used by the bacilli to

promote persistence in the environment (45). However, the mechanism used to bridge the

environmental reservoir and susceptible populations has remained elusive, despite numerous

studies.

Several insect genera have been investigated for their ability to maintain and transmit

the pathogen, including mosquitoes (Culex, Anopheles, and Aedes) and biting water bugs

(Naucoridae, Belostomatidae) (54, 202). Epidemiological studies have reported strong

associations between *M. ulcerans* and mosquitoes in endemic areas, with Buruli Ulcer patients often recalling mosquito bites after visits to endemic areas (61). Thus, a close association with insects has been proposed as a potential source of infection (189). Before vector-borne transmission was suspected by investigators, it was widely believed that the acid-fast bacilli (AFB) could be introduced into a previously existing cut or abrasion and subsequently result in Buruli Ulcer (203). This mode of exposure was deemed unlikely in a current study by Williamson et al (2014), which demonstrated a lack of pathology associated with *M. ulcerans* infection when abraded guinea pig skin was inoculated with a suspension of *M. ulcerans* (204). This study also suggested that the mechanism of exposure to most likely result in classical Buruli Ulcer disease was via injection of the bacteria into the skin, further implicating vector-borne transmission.

Laboratory studies have confirmed *M. ulcerans*' ability to colonize many invertebrate species. The mechanisms employed by the bacilli to colonize a particular environmental or invertebrate niche are not well understood, though it is likely that additional virulence factors, not limited to mycolactone, participate in the survival of the bacilli in these varied environments. The polyketide toxin mycolactone is the primary virulence factor encoded by *M. ulcerans*. Although it is well known that mycobacteria employ a diverse set of virulence determinants promoting their persistence in the environment and the host (205), many of which are encoded by *M. ulcerans* (206). Indeed, expression of mycolactone is not required for colonization in some invertebrate models (52).

Complex host-pathogen interactions have been thoroughly researched in other models of vector-borne diseases, describing intricate host-immune and metabolic disruptions leading

to survival and subsequent transmission of pathogens by insects (207). This interaction is influenced by the physiology of both the pathogen and the vector, resulting in intertwined metabolism. An intriguing and valuable research objective investigating the mechanisms of pathogen survival in the host and the dynamics of this interaction have led to innovative strategies for vector and pathogen control (53).

A. gambiae, the infamous malaria vector, is well-known for its ability to transmit a very large and complex pathogen (*Plasmodium falciparum*), and species of the *A. gambiae* complex are distributed throughout geographic locations endemic for Buruli Ulcer disease (190). Some of this sympatry has been documented in studies describing *M. ulcerans* DNA contamination of wild-caught mosquitoes from endemic regions and not from non-endemic regions (61, 63). Thus, it is highly likely, that due to the abundance and distribution of these two organisms, interaction is occurring at some trophic level.

The objective of this study was to examine the interaction between *M. ulcerans* and the *A. gambiae* mosquito using untargeted ultra-high-performance liquid-chromatography coupled tandem mass spectrometry (UPLC-MS/MS) to identify novel metabolic biomarkers of exposure to the pathogen. An untargeted approach in the capture and analysis of metabolites results in an unbiased, holistic methodology to generate a metabolic fingerprint associated with a specified treatment or exposure. This approach has been used to evaluate metabolic perturbations in other pathogenic and nonpathogenic disease states, including cancer, and represents an extremely sensitive tool (208). UPLC-MS/MS based metabolomic studies offer the most versatility when interrogating a sample set containing molecules of diverse molecular characteristics, with the aim to identify and semi-quantify small molecules involved in

metabolism (209). Subsequently, identified molecules are mapped to known metabolic pathways within an organism to assist in the understanding of biological interactions. To evaluate the interaction between these two organisms, *A. gambiae* larvae were allowed to develop in water containing live *M. ulcerans*, dead (ɣ irradiated) *M. ulcerans*, or without supplemental bacteria. Upon emergence, adult mosquitoes were captured and their metabolic patterns were analyzed to investigate pathogen associated effects on development. An understanding of the mechanisms employed by the pathogen to promote its survival in the mosquito system will ultimately provide significant insight into the persistence of the bacilli in the environment and clues towards understanding additional virulence factors utilized by *M. ulcerans* during pathogenesis.

Materials and Methods

Bacterial strain and culture

Mycolactone-negative *Mycobacterium ulcerans* strain 1615-GFP was kindly provided by Dr. Pamela Small from the University of Knoxville, Tennessee. The culture was propagated at 32°C for a period of 3 weeks on Middlebrook 7H11 (Difco Laboratories, Detroit, MI) plates supplemented with Kirschner Selecta-Tabs and 10 µg/ml kanamycin (7H9+), then aliquoted into infectivity stocks at a concentration of 10^{10} cells/ml. Contaminating bacteria from mosquito samples were acquired by vortexing the tissues of interest in 7H9+ liquid media, performing a 10-fold serial dilution and plating the dilutions on Middlebrook 7H11 plates with similar antibiotic supplements.

Mosquito species and maintenance

1st-instar larval *Anopheles gambiae* mosquitoes were acquired from the colony

maintained at the Arthropod-borne Infectious Disease Laboratory (AIDL) at Colorado State

University. 100 larval mosquitoes were distributed to individual cages containing 250ml of

sterile water and supplemented daily with finely ground fish food. All cages were setup in

duplicate for the individual treatment groups and each experiment was repeated in triplicate.

Mosquitoes were monitored daily and allowed to develop over a period of 10 days in a

controlled environment of 28°C and 70% humidity. Mosquito cages were supplemented with

sterile water and raisins, ad libitum, upon emergence of adult insects. Upon termination of the

study, adult mosquitoes were aspirated from the cages and briefly knocked down in 4°C. 10

mosquitoes each were distributed for the generation of technical replicates and their use in

subsequent assays.

Invertebrate infections

100 1st-instar larval mosquitoes were exposed, in duplicate, to live or dead

supplemental *M. ulcerans* bacteria, or no supplemental bacteria. 10^2 cells/ml of live M.ulcerans

1615-GFP, 10^2 cells/ml of dead, ɣ-irradiated *M. ulcerans* 1615-GFP, were added to the sterile

water upon the addition of larval mosquitoes. All groups received approximately 100mg of fish

food daily, in addition to the single initial dose of supplemental bacteria.

Detection of bacteria on mosquito tissues

A sample of 10 mosquitoes was acquired from each treatment group to determine the

presence of contaminating *M. ulcerans* bacilli. Mosquito whole-bodies were collected and

assayed for the presence of contaminating *M. ulcerans* via PCR analysis and culture as

described (Chapter 4).

Survival analysis

The mosquito's survival to adulthood was measured by counting the number of

emerged adults and generating a Kaplan-Meier survival curve among the treatment groups, as

described (Chapter 4). The relative fitness of emerged adults was evaluated by measuring the

wing size of emerged mosquitoes as a proxy for body size. Both wings were removed and

measured electronically via the publicly available ImageJ program (imagej.nih.gov/ij/) using a

line measurement plugin.

Extraction and purification of small molecules

5 adult female mosquitoes from each treatment group were collected and immediately

placed at in 100% methanol at -80°C to preserve their metabolic profile. Frozen mosquitoes

were then placed in a small Eppendorf tube containing 100ul of cold (-20 degree) 100%

methanol and homogenized with a handheld eppendorf homogenizer. The suspension was

briefly centrifuged for 10 minutes at 10,000xg to remove large debris. The supernatant was

then pushed through a 0.22um filter attached to a 1ml syringe to remove remaining small

debris. The filtered methanol extract was used for LC-MS/MS analysis.

Mass Spectrometry

Acquisition: 1 uL injections of the filtered methanol extract were performed on a Waters

Acquity UPLC system. Separation was performed using a Waters Acquity UPLC T3 column (1.8

μM, 1.0 x 100 mm), using a gradient from solvent A (water, 0.1% formic acid) to solvent B

(Acetonitrile, 0.1% formic acid). Injections were made in 100% A, which was held for 1 min, a

12 minute linear gradient to 95%B was applied, and held at 95 % B for 3 minutes, returned to

starting conditions over 0.05 minutes, and allowed to reequilibrate for 3.95 minutes. Flow rate

was constant at 200 µL/min for the duration of the run. The column was held at 50°C, samples

were held at 5°C. Column eluent was infused into a Waters Xevo G2 Q-Tof MS fitted with an

electrospray source. Data was collected in positive ion mode, scanning from 50-1200 at a rate

of 0.2 seconds per scan, alternating between MS and MSE mode. Collision energy was set to 6

V for MS mode, and ramped from 15-30 V for MSE mode. Calibration was performed prior to

sample analysis via infusion of sodium formate solution, with mass accuracy within 1 ppm. The

capillary voltage was held at 2200V, the source temp at 150°C, and the desolvation

temperature at 350°C at a nitrogen desolvation gas flow rate of 800 L/hr.

Processing: XCMS peak detection was performed on both the low and high collision energy

channels (MS and MSe). The datasets were separated following alignment, and the idMS/MS

workflow described previously was applied for generation of indiscriminant MS/MS spectra for

library searching and compound identification (210).

Statistical analysis

All data collected were subjected to principle component analysis (PCA) for dimensional

reduction, linear transformation, and consolidation of variables before any labelling. All

idMS/MS spectra were analyzed by two-way ANOVA ($P \leq 0.05$ considered significant) to generate

a dataset containing only compounds that demonstrated statistically significant abundance

among all treatment groups. From this dataset, the abundance of individual compounds

between treatments were compared using a two-sided student's t-test ($p \leq 0.05$) to generate a

dataset containing compounds dually significant by both ANOVA and t-test. These dually significant compounds were then subjected to library searching for identification.

Library searching and compound identification

MS/MS spectra from dually significant compounds were compared against in-house small molecule libraries developed and validated by the Proteomics and Metabolomics Core facility (PMF) at Colorado State University, the Metlin Mass Spectral database, and NISTv12 for identification via spectral matching and retention time data. Annotation confidence levels as recommended by the Metabolites Standards Initiative were applied (211). Briefly, samples were initially compared against the PMF validated chemical reference library. A compound was identified with level I confidence upon matching the mass spectra, retention time, and *m/z* of the chemical reference standard validated with identical instrumental conditions. Compounds annotated with level II confidence are based upon similarity of mass spectra, exact mass, and m/z of the putative compound with the commercialized Metlin database and NISTv12. Level III identification was based on similarity of the mass spectra of putative compounds to known compounds in a chemical class. Unknown compounds, which were still differentiated and quantified using the techniques described, contain unique chromatographic features and are reported as "unknown."

Results and Discussion

Time course of mosquito exposure and survival

100-1st instar *A. gambiae* larvae were distributed into cages in duplicate. Upon initiation, groups receiving supplemental bacteria (live *M. ulcerans* or ɣ-irradiated *M. ulcerans*)

were dosed. Treatment groups received a single dose of supplemental bacteria at initiation of

the experiment, then received ground fish food daily. Mosquitoes were allowed to develop

over a period of 10 days until they emerged as adults. During this time course, survival of the

mosquitoes were measured via daily counts of larvae. Upon emergence, the number of

mosquitoes which survived to adulthood were counted to evaluate survival over the duration of

development (Chapter 4). Briefly, mosquitoes exposed to live *M. ulcerans* were found to have

reduced survival and fitness by adulthood, compared to the control groups (Figure 23).
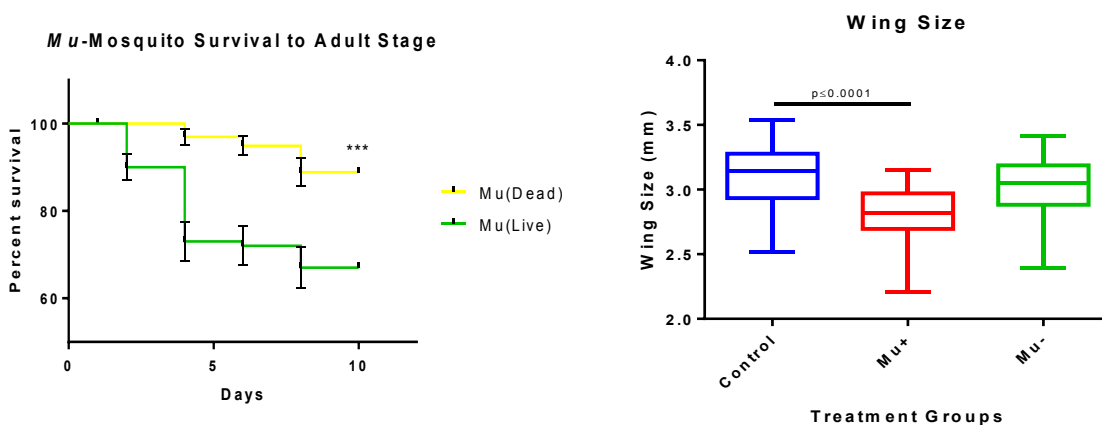
Figure 23: Summary of mosquito survival (left) and size (right) from Chapter 4. Mosquitoes exposed to live *M. ulcerans* are significantly smaller than controls and have reduced survival to adulthood. ***=p<0.001.

Composition and analysis of methanol extracts of mosquito after exposure to *M. ulcerans*

A sample of 15 adult female mosquitoes was collected from each treatment group and

divided into replicates of 5 adult mosquitoes per sample. After homogenization in cold

methanol and filtration, the samples were subjected to UPLC-MS/MS for semi-quantitative

global detection of metabolites. The preservation of the metabolite profile is paramount in any

metabolomics study, hence the use of rapid freezing and methanol quenching. Metabolite

extraction procedures can significantly influence the quality and composition of metabolites in a study, leading to a biased analysis of the biological significance of small molecules (212). Multivariate analysis (PCA) of the metabolite data demonstrates distinct separation of metabolic profiles among treatment groups (Figure 24). Treatment groups are well separated whereas replicates within each

Figure 24: PCA plot of untargeted metabolite analysis demonstrates significant clustering of compounds based on exposure. Control group in black (upper right); dead *M. ulcerans* group in red (bottom left); and live *M. ulcerans* group in green (top left).

treatment are closely clustered. Clustering of compounds based on similar retention times and abundance reduces background and streamlines analysis of significant compounds. Bubble plots of metabolites from this experiment reveal the sheer number of small molecules captured and clusters of significant features (Figure 25). The cluster highlighted in Figure 25 represents a significant cluster of lipids via differential analysis. After data reduction and clustering, a list of 134 compounds were found to have statistically

Figure 25: Bubble plot of total metabolite demonstrates spectral clustering of compounds for all treatment groups. Line separating chart represents *P*<0.05 cutoff for significance. Oval highlighting significant cluster of features as a function of retention time.

significant differences in abundance among treatment groups. From this list, 25 were identified

with level I confidence (211) (Table 2). A significant number of compounds were not

identifiable with Level I confidence (Table 2). This may be due to a lack of coverage of

Anopheles-specific metabolites in spectral libraries. Representative head-to-tail plots of

selected compounds are shown in appendix I and the total ion chromatogram of treatment

groups is shown in figure 26. While metabolomics is quickly becoming a valuable tool for global

assessment of exposure-induced metabolic effects, the confident identification of small

molecules and the assignment of biological significance represents a bottleneck in the analyses

of these studies (212). The expansion of spectral libraries and the availability of open-source

metabolomics data repositories will aid the analysis of future applications of metabolomics.
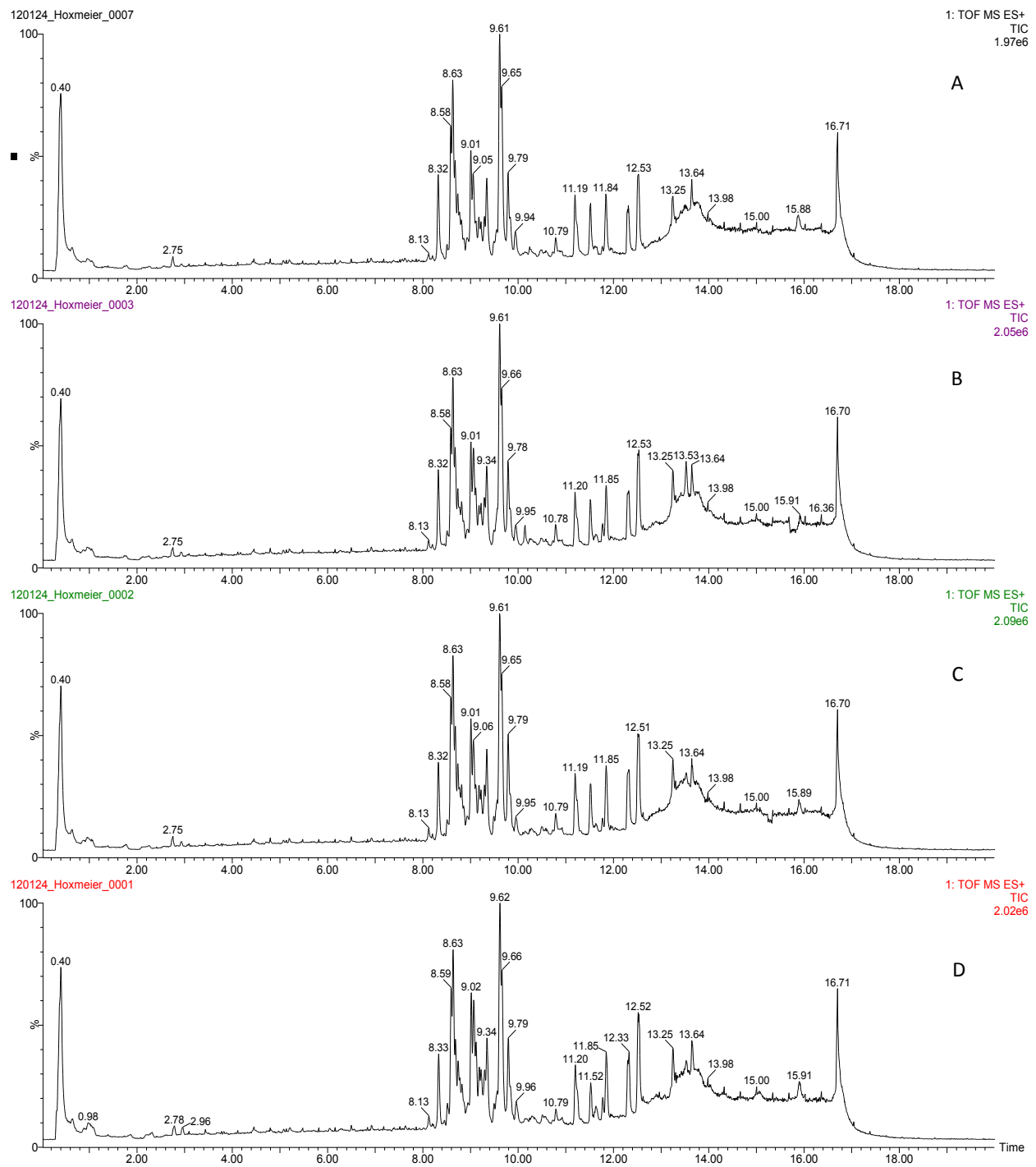
Figure 26: Total ion chromatograms (TIC) by treatment group. A: TIC of control mosquitoes. B: TIC of mosquito exposure to dead *M. tuberculosis*. C: TIC of mosquitoes exposed to live *M. ulcerans*. D: TIC of mosquitoes exposed to dead *M. ulcerans*.

Table 2: Dually significant compounds

ID[a]	Compound Annotation[b]	KEGG ID[c]	Retention time[d]	m/z[e]	P-value[f]	ID confidence[g]	Control vs Mu+ FC[h]	Control vs Mu- FC[i]
C589	unknown	n/a	521.3	440.277	0.003	IV	4.150	2.810
C341	unknown	n/a	484.9	199.133	<0.001	IV	3.450	2.390
C828	Orotic acid-like	C00295	781.3	297.279	0.010	III	3.392	0.536
C724	1-oleoyl-2-palmitoyl-sn-glycero-3-PC 18:1/16:0	C04317	759.8	760.597	0.010	I	2.754	0.896
C814	GPEtn 42:3	C04475	858.6	804.557	0.040	III	2.753	0.483
C355	GPEtn (20:0/20:0)	C04475	737.7	782.579	<0.01	III	2.438	1.067
C753	unknown	n/a	792.7	738.627	0.003	IV	2.346	1.181
C321	unknown	n/a	583.6	510.356	0.010	IV	2.320	1.590
C209	unknown	n/a	807.7	826.541	0.016	IV	2.260	0.755
C426	Lyso-PC 24:0	C04317	575.7	570.355	0.004	III	2.215	1.114
C506	unknown	n/a	792.0	800.528	0.045	IV	2.199	0.765
C591	unknown	n/a	560.4	307.227	0.001	IV	2.110	1.800
C686	GPEtn 42:3	C04475	720.4	804.579	<0.001	III	2.002	1.070
C78	unknown	n/a	650.0	309.243	0.006	IV	1.917	0.851
C295	riboflavin	C00255	229.1	377.146	0.010	I	1.879	1.287
C456	peptide fragment	n/a	556.4	546.284	0.005	III	1.874	0.807
C643	unknown	n/a	772.2	760.593	0.030	IV	1.860	0.985
C465	oxytocin-like	C00746	550.0	955.584	0.012	III	1.851	0.970

C24	Dodecylbenzenesulfonic acid	n/a	752.6	554.177	<0.01	III	1.830	1.050
C493	Sphingomyelin 40:5	C00550	825.5	786.509	0.027	III	1.759	0.806
C737	GPEtn(18:3(6Z,9Z,12Z)/ 18:3(6Z,9Z,12Z))	C04475	829.8	737.539	0.050	I	1.726	1.776
C795	unknown	n/a	589.5	1001.670	0.008	IV	1.688	2.027
C482	unknown	n/a	734.1	331.263	0.005	IV	1.661	1.006
C186	1-hexadecanoyl-sn-glycero-3-PC	C04317	522.7	518.325	0.030	II	1.660	0.740
C443	unknown	n/a	530.8	522.379	0.002	IV	1.600	0.750
C555	dynorphin-like	C01574	646.2	508.341	0.006	III	1.592	1.306
C187	unknown	n/a	517.5	452.277	<0.001	IV	1.570	1.050
C703	unknown	n/a	522.9	544.268	0.009	IV	1.560	1.011
C338	unknown	n/a	549.4	509.345	<0.01	IV	1.560	1.490
C326	GPEtn(7:0/20:4(5E,8E,11E,14E))	C04475	513.6	472.364	0.020	III	1.550	1.415
C52	Lyso-PC 16:0	C05209	520.0	476.279	0.034	III	1.523	0.850
C750	unknown	n/a	763.6	684.581	<0.001	IV	1.449	1.311
C217	Sphingomyelin (d19:1(4E)/26:1(17Z))	C00550	948.0	85.086	0.020	III	1.420	1.220
C240	Lyso-PC 18:0	C04317	618.5	524.372	0.030	III	1.400	0.978
C379	unknown	n/a	774.7	712.611	0.020	IV	1.394	0.850
C71	GPSer (15:0/25:0)	C18125	768.1	761.609	0.004	III	1.380	0.920
C67	1-oleoyl-2-hydroxy-sn-glycero-3-PC 18:1	C04317	546.1	544.340	0.010	I	1.359	0.681
C449	ACTH-like	C02017	508.4	468.309	<0.01	II	1.356	1.410
C442	unknown	n/a	536.3	544.341	0.001	IV	1.353	0.921
C183	Lyso-PE 18:1	C05209	586.4	959.613	0.020	III	1.350	1.570
C390	unknown	n/a	916.8	804.556	0.015	IV	1.326	0.922

C575	ACTH-like	C02017	587.9	587.940	0.033	III	1.309	1.780
C243	2-oleoyl glycerol	n/a	595.1	542.299	0.020	III	1.300	1.080
C344	ACTH-like	C02017	500.0	468.308	0.017	III	1.290	1.285
C461	unknown	n/a	540.6	498.379	0.038	IV	1.259	0.837
C32	GPCho(16:0/16:1(7Z))	C00157	530.5	494.324	<0.01	I	1.241	1.818
C35	1-palmitoyl-2-hydroxy-sn-glycero-3-PC 16:0	C04317	571.6	496.340	0.010	I	1.237	1.192
C83	GPEtn (5:0/24:4(5Z,8Z,11Z,14Z))	C04475	558.3	500.395	0.027	III	1.234	0.986
C335	GPEtn(9:0/18:3(6Z,9Z,12Z))	C04475	545.1	474.379	0.030	III	1.230	0.856
C245	peptide fragment	n/a	528.1	452.277	<0.001	III	1.220	1.930
C799	phenytoin, 1.5,5-Diphenylhydantoin	C07443	528.1	253.100	<0.01	II	1.217	1.958
C444	Lyso-PE 29:5	C05209	530.0	498.379	0.018	III	1.212	0.692
C562	Lyso-PE 20:4	C05209	574.1	361.274	0.014	III	1.202	0.395
C195	unknown	n/a	548.1	466.293	0.020	IV	1.200	2.160
C560	angiotensin-like	C15850	578.8	1031.713	0.010	III	1.197	1.376
C328	unknown	n/a	520.2	516.306	<0.01	IV	1.180	1.600
C107	neurotensin-like	n/a	552.3	502.294	0.040	III	1.150	0.890
C29	1-oleoyl-2-hydroxy-sn-glycero-3-PC 18:1	C04317	589.4	522.356	<0.01	I	1.150	1.332
C512	TG 61:5; [M+Na]+; TG(19:0/20:5/22:0)	C00422	1001.0	703.575	<0.01	I	1.148	0.583
C194	Lyso-PE	C05209	543.5	502.294	0.050	III	1.140	0.840
C794	locustachykinin II	C16098	589.5	1065.693	<0.01	II	1.121	1.712
C440	1-oleoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine	C01233	586.4	480.309	<0.01	I	1.117	1.267
C104	GPCHo (17:1(9Z)/22:5(4Z,7Z,10Z,13Z,16Z))	C05212	520.3	494.324	<0.01	II	1.100	1.400
C581	choline	C00114	526.9	564.307	0.010	I	1.099	1.759

C123	Lyso-PE 16:1	C05209	536.0	311.259	<0.01	III	1.090	1.800
C705	unknown	n/a	520.3	517.311	0.018	IV	1.087	1.410
C80	1-oleoyl-2-hydroxy-sn-glycero-3-PC 18:1	C04317	578.6	522.356	0.020	I	1.074	1.118
C315	iosprene unit	n/a	575.6	975.633	0.014	III	1.070	1.165
C807	unknown	n/a	520.2	1014.612	0.010	IV	1.069	1.773
C711	unknown	n/a	546.1	299.132	0.045	IV	1.065	0.799
C590	Lyso-PC 20:1	C04317	520.3	266.639	0.008	III	1.060	1.360
C790	GPEtn 28:6	C04475	574.1	621.303	0.008	III	1.057	0.512
C712	adrenosterone-like	C05285	546.2	582.300	0.011	III	1.016	0.749
C810	1-heptadecanoyl-2-hydroxy-sn-glycero-3-PC 17:0	C04317	556.7	473.272	0.030	I	1.002	1.208
C422	unknown	n/a	633.1	283.263	<0.001	IV	0.999	2.127
C22	Lyso-PE 16:1	C05209	517.6	474.260	<0.01	III	0.980	1.610
C96	8,11,14-Eicosatrienoic acid	C03242	705.1	305.248	<0.01	I	0.974	0.684
C550	unknown	n/a	708.6	179.143	0.028	IV	0.971	0.819
C401	adenosine	C00212	98.1	141.959	<0.01	I	0.966	0.642
C120	1-octadecanoyl-sn-glycero-3-PE	C04475	623.8	482.324	0.017	III	0.964	0.856
C818	unknown	n/a	728.8	308.265	0.011	IV	0.952	0.692
C223	Lyso-PE 21:0	C05209	264.2	520.338	0.050	III	0.951	1.122
C205	Oleic acid-like	C00712	749.0	283.264	0.044	III	0.943	0.851
C800	1-tetradecanoyl-sn-glycero-3-PC 14:0	C04317	517.6	253.603	0.030	I	0.934	1.375
C709	unknown	n/a	539.7	743.395	0.026	IV	0.923	0.770
C203	GPEtn(17:1(9Z)/17:1(9Z))	C04475	749.1	689.561	0.022	III	0.922	0.589
C95	1-octadecanoyl-sn-glycero-3-PE	C04475	716.0	357.300	0.031	III	0.921	0.642

C577	unknown	n/a	536.3	571.290	0.015	IV	0.910	1.529
C118	unknown	n/a	704.2	235.169	0.010	IV	0.900	0.600
C447	unknown	n/a	525.6	299.628	0.011	IV	0.885	0.744
C234	unknown	C11045	708.9	281.248	<0.01	III	0.880	0.830
C179	20-Hydroxy-(5Z,8Z,11Z,14Z)-eicosatetraenoic acid	C14748	559.8	303.232	0.010	I	0.877	0.904
C180	prostaglandin-like	C00639	697.3	329.248	<0.01	III	0.870	0.670
C408	GPEtn (20:0/18:2)	C04475	670.1	379.283	0.001	III	0.867	0.586
C787	8,11,14-Eicosatrienoic acid	C03242	694.6	305.248	0.030	I	0.856	0.629
C583	unknown	n/a	516.7	298.626	0.010	IV	0.850	0.539
C688	pimelic acid-like	C02656	708.8	245.227	0.001	III	0.850	0.781
C125	Lyso-PC 20:5	C04317	516.8	564.306	<0.01	III	0.840	0.620
C414	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid	n/a	708.6	239.202	0.040	III	0.829	0.809
C178	unknown	n/a	669.9	171.122	0.020	IV	0.828	0.753
C568	unknown	n/a	568.3	318.131	0.037	IV	0.825	0.730
C416	GPEtn(15:0/18:2(2E,4E))	C04475	708.7	264.241	0.010	III	0.820	0.799
C415	3-cyclohexyl-1-propanol	n/a	708.8	83.086	0.019	III	0.818	0.809
C93	Androstane-like	C03772	669.9	267.212	0.004	III	0.806	0.836
C430	unknown	n/a	568.6	279.232	0.017	IV	0.798	0.846
C663	unknown	n/a	946.6	304.299	0.017	IV	0.797	1.120
C322	peptide fragment	n/a	534.3	641.271	0.035	III	0.796	0.907
C366	unknown	n/a	825.0	529.881	<0.001	IV	0.783	0.958
C704	unknown	n/a	523.3	270.120	0.006	IV	0.760	0.851
C808	1-hexadecanoyl-sn-glycero-3-PC 16:0	C04317	559.7	478.327	<0.01	I	0.752	1.125

C227	Deoxyadenosine monophosphate	C00360	360.3	98.512	<0.01	III	0.743	0.770
C38	histamine-like	C00388	990.5	130.159	0.048	III	0.730	0.694
C102	Lyso-PE 16:0	C05209	528.0	245.616	0.020	III	0.730	1.600
C548	unknown	n/a	708.5	139.112	0.001	IV	0.727	0.663
C177	Deoxyadenosine monophosphate	C00360	356.4	98.512	0.030	III	0.710	0.790
C151	Lyso-PC 20:0	C04317	646.5	468.345	0.017	III	0.710	0.790
C336	Lyso-PE 17:1	C05209	549.6	506.323	<0.01	III	0.680	0.391
C593	unknown	n/a	554.9	530.320	0.007	IV	0.670	0.279
C421	GPCho (14:0/17:1(9Z))	C05212	598.9	508.339	<0.001	III	0.654	0.213
C100	GPCho (17:1(9Z)/22:5(4Z,7Z,10Z,16Z))	C05212	586.2	508.340	<0.01	II	0.600	0.170
C641	Dodecylbenzenesulfonic acid	n/a	849.2	703.217	<0.01	II	0.524	0.482
C578	unknown	n/a	534.3	424.324	0.011	IV	0.488	0.275
C766	trans-8, trans-10-Dodecadien-1-ol	C02679	1031.0	141.959	<0.001	I	0.467	0.579
C528	unknown	n/a	24.6	173.021	<0.001	IV	0.463	0.784
C699	unknown	n/a	517.6	398.327	0.033	IV	0.454	0.485
C349	trans-8, trans-10-Dodecadien-1-ol	C02679	1074.8	141.959	0.020	I	0.369	0.398

Table 2 key:
a) In house clustered feature identifier. b) identification of compound based on match of mass spectra, m/z ratio, and/or retention time to available databases. c) compound identifier from Kyoto Encyclopedia of Genes and Genomes (www.genome.jp/kegg/) d) generated via liquid chromatography. e) mass to charge ratio of the largest peak from the mass spectra. f) level of significance of pairwise comparison between treatment and controls groups. g) confidence level of identification based on matching of chromatographic and mass spectral characteristics for each compound to validated compound library or publicly available databases, from Sumner et al. 2007. Metabolomics. h/i) fold change in abundance of compound compared between control and treatment groups. Abbreviations: PC: phosphocholine; GPCho: glycerophosphocholine; GPEtn: glycerophosphoethanolamine; GPSer: glycerophosphoserine; ACTH: adrenocorticotropic hormone; X:N represents number of carbons to double bonds in a given compound.

Phospholipid pathways affected by exposure to *M. ulcerans*

An analysis of the metabolites of contrasting abundance among treatment groups was analyzed in an attempt to correlate the disruption of metabolic pathways with the reduced survival and fitness described in the adult mosquitoes (Chapter 4 and Figure 23). Of the identified compounds with significantly different regulation among treatments, the vast majority belong to the lipid metabolism functional group. The primary metabolites showing the most significant fold change in abundance among treatments belong to the phospholipid and ether lipid class of molecules, comprising the main components of biological membranes (213). In particular, diacylated glycerophosphocholine (PC) molecules were found to be in abundance in live *M. ulcerans* exposure groups (Table 2). While this class of molecules is ubiquitous in membranes, the high levels of these molecules in mosquitoes exposed to live *M. ulcerans* compared to other groups, may indicate an impaired mechanism of PC hydrolysis occurring in live-pathogen exposed groups. Specifically, the accumulation of 1-oleoyl-2-palmitoyl-PC in live *M. ulcerans* groups compared to

Figure 27: Box and whisker plots of the abundance of 1-oleoyl-2-palmitoyl-PC (top)(C724) and 1-oleoyl-2-hydroxy-PC (bottom)(C67) compared to other treatment groups. CTRL:control; Mu-:irradiated *M. ulcerans*; Mu+: live *M. ulcerans*; Trt: treatment

both the control and dead *M. ulcerans* groups suggests a mechanism of live-pathogen induced disruption of utilization or disruption of the mosquito's membrane integrity during exposure (Figure 27).
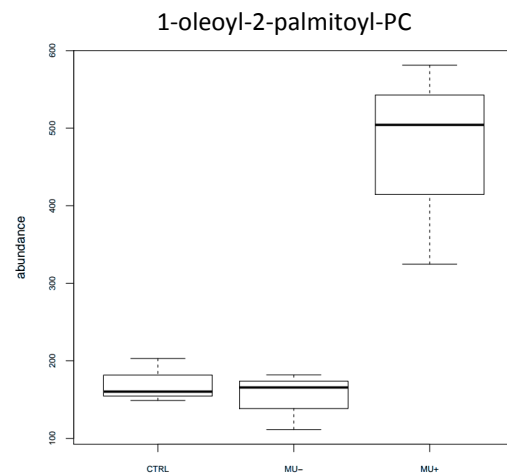
Accumulation of lyso-phosphatidylcholine compounds (LysoPC) (monoacylated glycerophosphocholine) occurs primarily in mosquitoes exposed to dead *M. ulcerans* compared to both the control and live *M. ulcerans* treatment groups. Hydrolysis of the PC molecule resulting in the formation of 2-LysoPC is the result of cleavage of the sn-2 acyl bond potentially by a putative Anopheles encoded phospholipase A2 (UniProt: T1EAP2_ANOAQ) or via enzyme secretion from commensal microbiota assisting in digestion, and represents the most abundant LysoPC in nature (214). This finding suggests that mosquitoes exposed to live pathogen, thus resulting in low abundance of 2-LysoPC compared to controls, may have impaired transcription and regulatory functions as a result (214) (Figure 28).
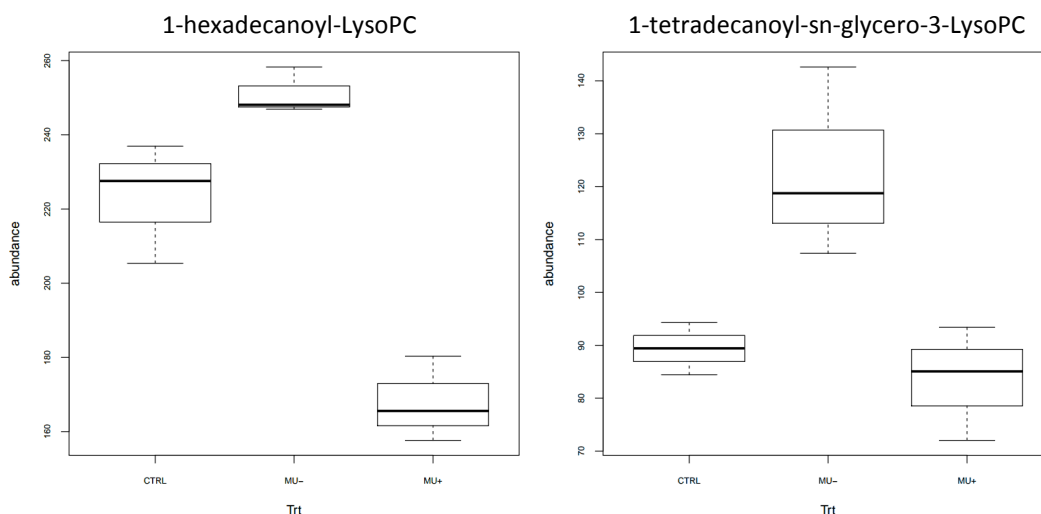
Figure 28: Box and whisker plots of the abundance of 2-LysoPC compounds detected in mosquito tissues. C808 represents the abundance of 1-hexadecanoyl-LysoPC 16:0 (left) and C800 represents the abundance of 1-tetradecanoyl-sn-glycero-3-LysoPC 14:0 (right) in mosquitoes exposed to irradiated *M. ulcerans* (Mu-) in comparison to control (CTRL) and live *M. ulcerans* (Mu+).

Glycerophospholipid metabolism in Anopheles mosquitoes is a critical component of metabolism utilized for the generation of lipid energy sources, components of cell membranes, and signaling pathway modulators (215). Mosquitoes rely on a lipid carrier protein, lipophorin, as a reusable shuttle for the transportation of lipid molecules from sites of storage or synthesis to sites of utilization as an energy source or as precursors to triacylglycerol and phospholipid

synthesis. In contrast with other eukaryotic organisms which store lipids as mixtures of mono-,

di-, and triglycerides, triacylglycerides (TG) are the major component of mosquito lipid storage

and may be cleaved to release fatty acids as an energy source (215). In our model, TG was

found to have significantly higher abundance in mosquitoes exposed to live *M. ulcerans*,

perhaps suggesting some mechanism of pathogen induced disruption of the utilization of this

molecule. In contrast, mosquitoes exposed to dead *M. ulcerans*, had lower abundance of TG

than control mosquitoes (Figure 29), suggesting a reduced requirement for TG accumulation, or

a greater utilization of the molecule

when provided a lipid-rich mycobacterial

diet resulting in increased survival and

size compared to control mosquitoes

(Chapter 4 and Figure 23). An abundance

of unsaturated fatty acids and

glycerophospholipds in mosquitoes is

thought to be an adaptation to an

aquatic environment (216), and the

composition and distribution of these

molecules in insect's tissues has also been

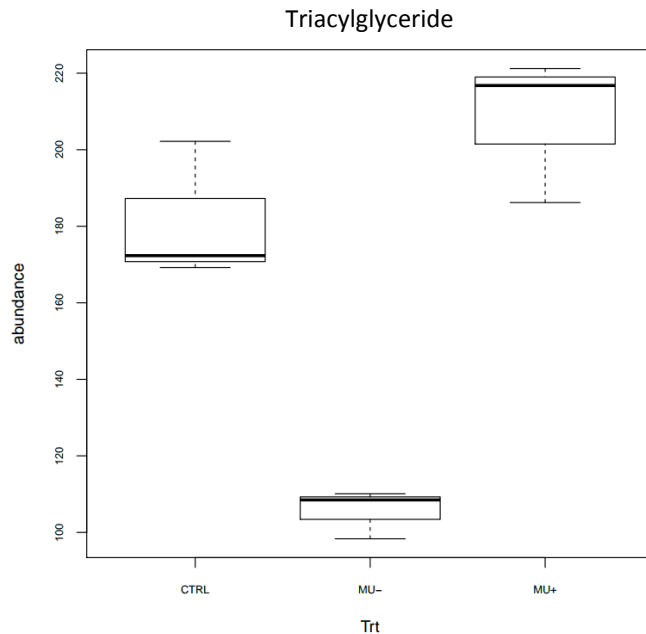shown to differ with diet (217) (Figure 30).

Figure 29: Abundance of Compound 512 (triacylglyceride-TG) in treatment groups. Mosquitoes exposed to dead *M. ulcerans* have significantly lower abundance of TG than control while mosquitoes exposed to live *M. ulcerans* have significantly higher levels than controls.

A

C00157

Diagram of the phosphatidylcholine molecule (PC). R groups represent an assortment of attached fatty acid side chains.

Plc ← → Pld

Pla

B

C04233

D

C00157

PC hydrolysis by *M. ulcerans* encoded phospholipase C (Plc) results primarily in diacylglycerol (DAG). Green dash shows location of enzyme action.

PC hydrolysis by *M. ulcerans* encoded phospholipase D (Pld) results in phosphatidic acid (PA) and soluble choline. Green dash shows location of enzyme action.

PC hydrolysis by phospholipase A (Pla) encoded by Anopheles results in deacylation of the molecule to remove one attached fatty acid resulting in 1– or 2-lysophosphocholine (LysoPC).

D

C00422

DAG from the reaction above can be combined with PC, through the action of an acyltransferase enzyme to form triacylglycerol (TG), a primary energy storage molecule for mosquitoes.

C

C03242

C14748

Fatty acids released from these reactions can undergo elongation resulting in eicosatrienoic acid (top) and 20-hydroxy arachidonic acid (bottom). These polyunsaturated fatty acids articipate as messengers and mediators in a wide variety of signaling events.
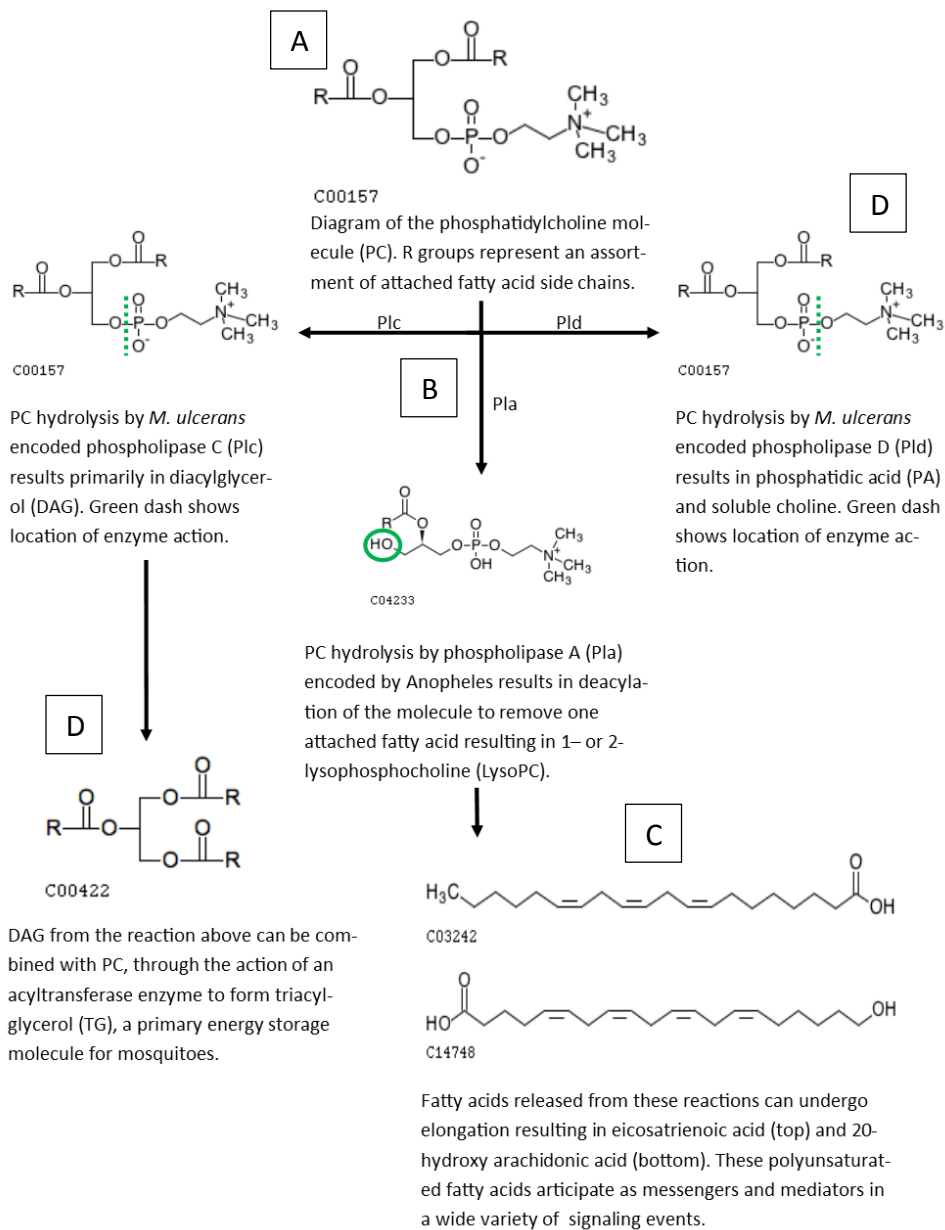
Figure 30: Phospholipids (PL) are a major constituent of biological membranes. PL are characterized by a glycerol backbone attached to a phosphodiester group and a polar head group. Phosphatidylcholine (PC) is a functional class of PL molecule characterized by a choline head group. The fatty acid composition of PC can vary, but is generally composed of one saturated fatty acid and one unsaturated fatty acid, attached in the R position (A). Phospholipase hydrolysis of the PC molecule results in a plethora of subunits involved in many downstream signaling and metabolic processes (B). As PC molecules are cleaved by phospholipase A/B/C/D (Pla/b/c/d), fatty acids are released (C), in addition to other cleavage products such as diacylglycerol (DAG), phosphatidic acid (PA), and choline (D). Fatty acids liberated from PC, DAG, TG, LysoPC can be elongated and/or modified to form ubiquitous signaling molecules. In this study, eicosatrienoic acid and 20-hydroxy eicosatetraenoic acid (hydroxy arachidonic acid) were found to be in lower abundance in groups exposed to *M. ulcerans* than controls. Images obtained from Kyoto Encyclopedia of Genes and Genomes (www.Kegg.jp/kegg/)

Disruption of host-lipid metabolism is a well-characterized feature of many

mycobacterial infections, in addition to an increasing number of intra- and extracellular

pathogens including *Clostridium perfringens, Corynebacterium pseudotuberculosis,*

Pseudomonas aeruginosa, Staphylococcus aureus, Listeria moncytogenes, M. leprae, and *M.*

avium (218). Additionally, histopathological analysis of mouse footpad tissues revealed the

presence of foamy macrophages during late *M. ulcerans* infection (Chapter 3: Figure 10D,

Figure 13). During active and chronic infection with *M. tuberculosis*, the accumulation of TG-rich

lipid bodies in foamy macrophages is used as an energy source for the intracellular pathogen

(219). The development of *P. falciparum* in infected mosquitoes is also associated with

disruption of host lipid metabolic pathways, thus our findings seem consistent with

mechanisms employed by other pathogens for survival in the mosquito (220). The discovery of

the high abundance of TG in mosquitoes contaminated by live *M. ulcerans*, but not dead *M.*

ulcerans, is consistent with these reports and has not yet been described in the mosquito

system.

Hormone-like compounds

Secondary metabolites of the lipid metabolic pathways are involved in immune signaling

in both vertebrates and invertebrates. Hormones are used to signal the generation and

utilization of these lipid mediators. An analog of the ACTH-releasing molecule (corticotropin-

releasing factor, CRF) has been discovered in mosquitoes (221), and ACTH-like molecules are

present in insect cells with phagocytic activity (222). This molecule functions as a stress

response hormone involved in immune cell chemo-attraction, phagocytosis, and capsule

formation, factors of the primary immune response in most insects (223). Counterparts of the

full vertebrate CRF-ACTH endocrine system have not yet been described in Anopheles systems, although analogs of these compounds in insects may represent evolutionary precursors to the vertebrate systems (221). An ACTH-like compound was found to be in high abundance in both mosquito groups exposed to *M. ulcerans* bacteria compared to the control group (Figure 31). A high abundance of this molecule in both groups of mosquito exposed to *M. ulcerans* suggests the initiation of an immunological or
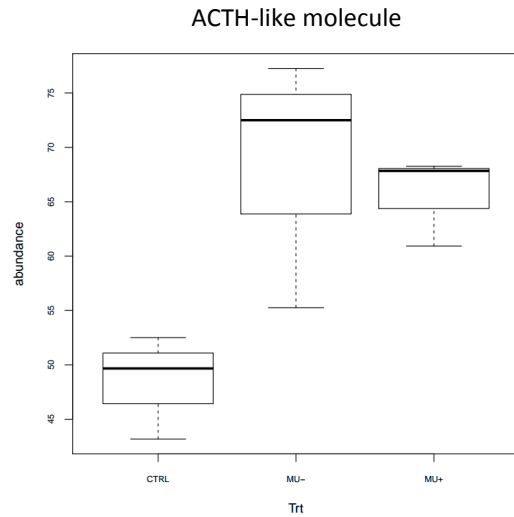

Figure 31: Abundance of C449, an adrenocorticotropin hormone (ACTH)-like molecule found to be in high abundance in mosquitoes exposed to both live and dead *M. ulcerans* compared to controls.

neurological response to the presence of the mycobacteria. The neuroendocrine system is a highly complex network of metabolic pathways and endocrine activity, thus, speculation regarding the effects of differentially regulated compounds within this system must be taken with caution.

In vertebrate systems, hormones such as ACTH stimulate arachidonate release, which can then be converted into eicosanoid compounds such as prostiglandins and lipoxygenase metabolites (224). Eicosanoids are oxygenated metabolites of polyunsaturated fatty acids and are potent signaling molecules involved in inflammation, immunity and the nervous system of vertebrates, invertebrates, and many eukaryotic microbes (225). Interestingly, suppression of eicosanoid synthesis within the host is a major mechanism utilized by entomopathogenic bacteria during infection and during protozoan development in anopheles mosquitoes (226, 227). These findings suggest that modulation of eicosanoid signaling by pathogenic

contaminants of invertebrate systems facilitates survival of these pathogens. In line with this,

mosquitoes in our model of *M. ulcerans* infection were found to have significantly lower

abundance of the eicosanoids 8,11,14-Eicosatrienoic acid and 20-Hydroxy-(5Z,8Z,11Z,14Z)-

eicosatetraenoic acid (20-HETE) (Figure 32), and may represent a rudimentary mechanism of
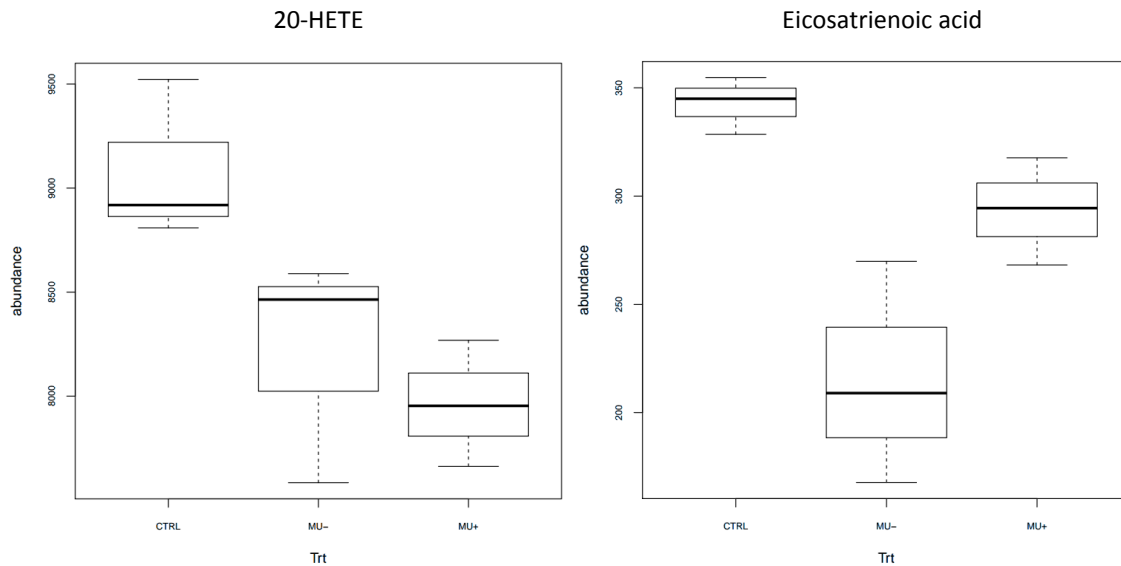
immune suppression.

Figure 32: Abundance of eicosanoid compounds in mosquitoes exposed to live (Mu+) and dead (Mu-) *M. ulcerans* bacteria compared to control mosquitoes. Eicosanoids are mediators of a wide variety of immune signaling processes, and the down regulation of these compounds is suspected to increase the survival of contaminating pathogens. C179 (left) represents the abundance of 20-HETE and C787 (right) represents the abundance of Eicosatrienoic acid.

Fatty acid mediators and signaling

Many effects of eicosanoid modulation are not well known insect systems, although a decreased abundance of these compounds may be due to the active modulation by *M. ulcerans* or due host immune non-recognition of the pathogen, therefore leading to the depressed function of a non-necessary system. For example, the compound 20-HETE, a metabolite of arachidonic acid, is involved in the detoxification response via the cytochrome P450 system (228). Lower abundance of the 20-HETE lipid mediator may result in reduced capacity of the P450 system to generate reactive oxygen species in an effort to combat the contaminating *M. ulcerans* bacilli.

Secondary metabolites

In addition to the various perturbations of lipid metabolic pathways discovered in this study, the abundance of other compounds are deserving of mention. However, the analysis of their role in our model of infection is undertaken with restraint due to the lack of available information regarding the dynamics of these molecules in the anopheles mosquito. Insects use a variety of pheromones during reproduction and host-seeking behavior. The codling moth (*Cydia pomonella*) produces dodecadienol as a precursor in the synthesis of a sex hormone (229). Although the presence of this compound is not yet described in mosquito systems, the significantly reduced abundance of 8-10-dodecadienol in mosquitoes exposed to both live and dead *M. ulcerans* in our model may be a marker of reduced fecundity. However, mosquitoes exposed to dead *M. ulcerans* bacilli have higher survival rates compared to control mosquitoes, in concert with low abundance of the putative pheromone, thus contradicting this finding.

Riboflavin is an essential B vitamin in nearly all pro- and eukaryotic organisms, serving as a precursor for the synthesis of flavin coenzymes and flavin adenine dinucleotide which are essential cofactors for a wide variety of metabolic enzymes and electron transport (230). This molecule was found to be significantly upregulated in mosquitoes exposed to live *M. ulcerans* compared to both control mosquitoes and mosquitoes exposed to dead *M. ulcerans*. In addition to metabolic processes, riboflavin is also involved in areas of yellow pigmentation, such as the eyes and malpighian tubules (231). The accumulation of riboflavin in mosquitoes exposed to live *M. ulcerans*, and the less extreme accumulation in mosquitoes exposed dead *M. ulcerans* is surprising. *Mycobacterium smegmatis* and the
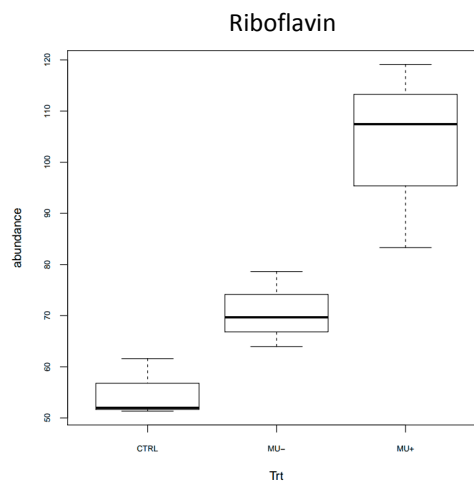

Figure 32: Box and whisker plot showing the abundance of riboflavin in treatment groups. Mosquitoes exposed to live *M. ulcerans* (Mu+) show a significant accumulation of riboflavin compared to the other groups (CTRL: control, and Mu-: dead *M. ulcerans*.

closely related *Corynebacterium diptheriae* is considered to be overproducers of riboflavin (230). The proteins involved in riboflavin synthesis and processing have been documented in the *M. ulcerans* proteome (NCBI accession: WP_011740179 (riboflavin kinase) and WP_011740778 (riboflavin biosynthesis protein)). The dynamics of riboflavin metabolism are not specifically defined in *M. ulcerans*, however a flavin analog (F420-dependent reductase) is synthesized using a riboflavin precursor and is involved in the degradation of aflatoxins (232). The accumulation of riboflavin in our model may be due to an overproduction of the molecule by contaminating bacilli, or a disrupted mechanism of metabolism of the molecule in the mosquito due to *M. ulcerans* exposure (Figure 32). It has also been suggested that an

overproduction or supplementation of riboflavin may help to control the development of P. falciparum in the mosquito (233).

The compounds identified in our model and the putative assignment of biological significance thereof may represent the cumulative effects of virulence mechanisms employed by *M. ulcerans* during contamination of mosquitoes. Many of the features describing disrupted metabolism in the mosquito due to exposure to *M. ulcerans* are novel, although they are based on analogous mechanisms from many similar interactions. While many of the disrupted pathways described here have been documented to be affected by other mycobacterial pathogens in other systems, an metabolic investigation of the host-pathogen dynamics between A. gambiae and *M. ulcerans* how not been done before and will contribute significant evidence towards the understanding of additional virulence mechanisms employed by *M. ulcerans*. This study is the initiation of not only a better understanding of *M. ulcerans* metabolic interaction with A. gambiae, but can be generalized as a complex in vitro model of *M. ulcerans* pathogenesis. Metabolism is inherently temporal and spatial, so continued analysis of this interaction must include additional or repeated measures for a more complete understanding. Representative head-to-tail plots are displayed in appendix I, including some compounds that were not able to be confidently identified with the tools available and are annotated as unknown.

CHAPTER 6: FINAL CONCLUSIONS AND FUTURE DIRECTIONS

Final Conclusions

In comparison to other notable mycobacterial diseases, Buruli Ulcer disease has a

proportionally small impact on global health. Lack of surveillance and health care resources in

the exceedingly rural environment of endemic regions complicates the accurate measure of

disease burden. Some estimates report between 5,000-7,000 new cases per year, with nearly

half of those originating from just Cote d'Iviore (234). *M. ulcerans* infection has a major impact

on the individual however, evidenced by estimates that 66% of people with healed lesions had

some degree of permanent physical disability (235). The median age of patients in the

aforementioned study was only 12 years old. In fact, nearly 50% of patients affected by Buruli

Ulcer disease are under the age of 15 (236). This predilection of infection in children is one of

the most unfortunate aspects of Buruli Ulcer disease. A better understanding of this

phenomenon will likely involve investigation from a variety of different approaches, including

an analysis of the effects of environmental disruption, availability nutritional resources, and

climate change.

Our understanding of the basic mechanisms surrounding the survival and maintenance

of the bacterium in the environment, coupled with a lack of knowledge regarding transmission

of the pathogen and subsequent pathogenic features of infection is evidenced by the increasing

incidence and prevalence of the disease. Recently, the condition has been reported or

suspected in more than 33 countries and the number of reported cases is growing (237). West

and central Africa bears the brunt of *M. ulcerans* infection, similar to other neglected tropical diseases, while other important foci are found in Australia, French Guiana, Peru, and Papua New Guinea (200, 238, 239). Recently, reports of *M. ulcerans* infection have spread to Japan (240). No confirmed infections have been documented in the United States, although evidence of *M. ulcerans* contamination of marsh lands in Louisiana brings the potential for infection close to home (241).

The value and application of animal models of Buruli Ulcer can be appreciated by the recent shift in treatment protocols. Historically, extensive surgery left Buruli Ulcer patients with massive, debilitating scars, not unlike those from unmanaged infection. The extensive use of animal models to assess different treatment modalities has resulted in a better understanding of the host immune response to *M. ulcerans* and the deployment of an effective antibiotic regimen (242). These advances have resulted in reduced severity of cases than what was seen a decade ago (15). The mouse model presented in Chapter 3 is the first to describe a mycolactone-negative infection in immunocompetent and immunodeficient mice resulting in immunosuppression. The strain used in the study is considered attenuated due to the lack of mycolactone expression, although it is clear that many pathogenic features common to infection with *M. ulcerans* are still retained. The analysis of the cytokine concentrations in mouse tissues confirms the extent of immune suppression, resulting in a significant decrease in the concentrations of protective IFN-ɣ in conjunction with an increase in pathology of the footpad. Additionally, the abundance of cytokines IL-6 and IL-12 in both early and late infection may serve as markers for the disease, as the presence of these cytokines has been documented in other studies (142). However, due to lack of experimental data, the variability of cytokine

expression due to host and environmental factors resulting in a specific presentation or prognosis as not been substantiated. Further clinical studies will help to refine the extent that host-factors influence pathogenesis.

Currently, the route of transmission and mechanism of environmental persistence is unknown. As such, a throng of researchers have published dozens of papers regarding putative modes of transmission or environmental niches for the bacteria, including research found in this dissertation. Despite the intensity of research into these areas, many aspects of *M. ulcerans* pathobiology remain elusive. Ulceration most often occurs on the limbs or extremities of patients and, in one documented instance, on a child's ear (63). This feature led many to suspect vector-borne transmission of the disease. Since the early 1999, many groups have investigated the role of aquatic insects in transmission, with varying results. Two reports stand out among many: the first cultivation of *M. ulcerans* from an aquatic insect (14) and the transmission of *M. ulcerans* by a biting water bug (60). In the first study mentioned, *M. ulcerans* was cultured from a water strider (*Gerris sp.*), thus establishing the role of aquatic insects in the maintenance of the bacilli in the wild. The second study was the first to describe transmission via aquatic insect to a mouse through contaminated raptorial arms. Unfortunately, the insect used in this study (*Naucoridae sp.*) is not hematophagous and does not associate with humans. Other studies have described a strong association between *M. ulcerans* and mosquitoes of many genera. As of yet, no study has been able to document mosquito-borne transmission of the bacilli, although many still suspect this route to be likely. Transmission via puncture wound as a result of a mosquito bite containing active *M. ulcerans* in the context of immunomodulatory mosquito saliva remains an attractive hypothesis. Additional studies using

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more thorough microbiological methods for detection of active *M. ulcerans* bacilli in and on mosquito tissues, beyond PCR, is greatly needed. Unfortunately, endemic regions of Buruli Ulcer disease are not conducive to the application of intricate scientific methods. Despite evidence presented here (Chapter 4 and 5), that mosquitoes represent an unlikely vector of *M. ulcerans*, in addition to a recent study with corroborating conclusions (161), the well documented sympatry between the pathogen and mosquitoes highlights the need for continued study into the extent of association between these two organisms.

There exists a major discrepancy between two primary areas of research into the biology of *M. ulcerans*. The discrepancy lies in the significant lack of research into virulence mechanisms employed by *M. ulcerans* beyond the toxin mycolactone, compared to other aspects of the disease. Non-toxigenic members of the *Mycobacterium* genus have caused infections for thousands of years, resulting in significant morbidity and mortality. Many of the virulence mechanisms used by other species of mycobacteria have analogous genetic loci in *M. ulcerans* (22), although a characterization of the presence and role of these mechanisms is severely overshadowed by research into the effects of mycolactone. There is little doubt that mycolactone can cause dramatic cytopathology, measured in both *in vitro* cell culture models and model infections in animals. However, the presence of and specific role of mycolactone during clinical infection with different ecovars of the bacilli is severely lacking.

Since the investigation of mycolactone has monopolized research over many years, isolates commonly used during experimental infections produce a significant amount of the toxin. These isolates may have been selected due to their mycolactone-associated virulence, in an effort to analyze its effects. This has led to many important discoveries regarding the

mechanisms of the toxin. However, an important distinction between clinical isolates and commonly used experimental isolates may exist, in that strains over expressing mycolactone may be supra-virulent compared to isolates of an environmental origin commonly infecting people. More specifically, that the over-expressing strains commonly evaluated in model infections do not accurately represent the phenotype of *M. ulcerans* in endemic areas. Experiments presented in this dissertation, in concert with a few studies investigating infection by mycolactone-negative mutants, have documented severe pathogenesis as a result of infection (Chapter 3). Mycolactone was also not required for colonization of *A. gambiae* mosquitos (Chapter 4) and other species of insects (52), indicating the presence of mechanisms for persistence in environmental and aquatic niches irrespective of the toxin. This dissertation has initiated the study of uncharacterized virulence factors that influence pathogenesis. Until more stringent efforts are made to characterize the presence of the toxin during natural infection, an evaluation of additional virulence determinants of *M. ulcerans* will be critical to the understanding of this pathogen during its lifecycle.

A common feature during infection with *M. tuberculosis, M. marinum*, and *M. leprae* is a disruption of host-lipid metabolism (219, 243, 244). Analogous mechanisms were proposed in Chapter 5 regarding disruption of lipid metabolism of mosquitoes exposed to *M. ulcerans* bacilli. The lack of research into other virulence mechanisms encoded by *M. ulcerans* is severely hampering a thorough understanding of this pathogen. Interestingly, *Corynebacterium ulcerans* and *C. pseudotuberculosis* bacteria, phylogenetic relatives of the *Mycobacterium* genus, secrete phospholipase D as an exotoxin involved with virulence (245). The most recent study describing the synthesis and secretion of phospholipase C and D in *M. ulcerans* (116), was published nearly

15 years ago. This finding is significant for many reasons. The activity of the phospholipase C

(Plc) enzyme results in the cleavage of phosphatidylcholine (PC) into diacylglycerol (DAG) which

can then activate protein kinase C, causing malfunction of phagocytes (116, 117) (Figure 17).

Secretion of Plc by other bacterial pathogens has been shown to disrupt tissue epithelium and

increase sub-epithelial tissue destruction via matrix metalloproteinase production (246).

Disruption of phagocytes and subepithelial cell destruction has initially been attributed to

mycolactone (247), with very little analysis of the activities of *M. ulcerans* encoded Plc working

concurrently. Additionally, the activity of *M. ulcerans* encoded phospholipase D (Pld) results in

the cleavage of PC producing phosphatidic acid (PA). PA has been shown to promote

colonization of tissues and apoptosis during infection with *C. paratuberculosis* and may have

analogous mechanisms during *M. ulcerans* infection (248). No additional studies have

investigated the role of this potent, and relatively common, virulence mechanism during

infection with *M. ulcerans*.

Biochemical analyses of host-pathogen interactions are becoming increasingly common,

as the application of more sensitive and complex tools such as mass spectrometry become

more user-friendly. The group of omics technologies are powerful tools for the analysis of these

interactions. The use of unbiased, global analysis of biological conditions is useful for the

discovery of biomarkers and descriptive studies, although a holistic approach is necessary for

complete understanding. The use of omics tools can provide a significant amount of data, but

reductionist use of these tools limit their application to the understanding of component parts

of an interaction. LC-MS based metabolomic studies are inherently more holistic than the other

omics applications, due to their analysis on a comparatively larger scale, especially in the case

of host-pathogen interactions. Metabolism is an intrinsically dynamic process, complete with temporal and spatial effects. A single analysis of the metabolome of a given interaction will only capture a small snapshot of the metabolites present. To further enhance the biological significance of the data collected, and to get a complete picture of the interaction, repeated measures must be analyzed. The application of metabolomics in Chapter 5 is an initiation of a study to better understand the virulence mechanisms promoting survival of *M. ulcerans* in mosquitoes. From this study we were able to gain insight into potential virulence mechanisms employed by the pathogen which subsequently affect the development of the mosquito. Taken together, the data provided here represent a systems biology approach to understanding the biology of *M. ulcerans* in many environments.

Future Directions

The sheer number of unknowns still associated with infection with *M. ulcerans* leaves much room for expanded studies. Since a shared association of environmental locations between mosquitoes and *M. ulcerans* is well established, continued studies investigating the role of mosquitoes in transmission is critical. Unfortunately, many aspects of this investigation are exceedingly difficult. From an epidemiological viewpoint, many factors influence the probability that mosquitoes will actively transmit an infection. These factors have been mathematically modeled and are used to predict aspects of transmission in other mosquito-borne diseases (249). An analysis of these factors as they related to Buruli Ulcer disease would significantly enhance our understanding of the vectorial capacity of mosquitoes to transmit *M. ulcerans*. The probability that a mosquito becomes infected during its lifetime is unknown, although high rates of *M. ulcerans* DNA contamination of mosquitoes in endemic areas suggests

high rates of exposure or at least contact. The next measure would be the mosquito feeding

rate, or the proportion of mosquitoes that have fed on a human. If the probability that

mosquitoes can become infected is high, the rate they feed on humans will influence the

likelihood that the bacteria can be transmitted. As an example, if mosquitoes truly vector the

pathogen, mosquito bites should account for roughly 99% of the reported 7000 cases per year.

Estimates of the biting rate of *A. gambiae* are subject to seasonal rainfall amounts and

proximity to larval habitats, among other factors, although the biting rate of infected versus

uninfected mosquitoes is another variable to consider. Considering the high density of

Anopheles mosquitoes in endemic regions, and the number of mosquito bites received by a

susceptible individual, the rate of infected mosquitoes biting people and successfully

transmitting the pathogen would be very low. Based on our mosquito studies (Chapters 4,5),

the developmental defects associated with exposure to *M. ulcerans* would further reduce the

likelihood that a contaminated mosquito would transmit the infections. However, in a pilot

study we determined that *Culex sp.* mosquitoes do not suffer any developmental delay or

reduced fitness upon exposure to the bacilli. We did not measure the external contamination

rate of the mosquitoes, although stronger, more aggressive mosquitoes may be more likely to

transmit the bacilli. It may be that mosquitoes other than *Anopheles sp*. (or Anopheles species

other than *A. gambiae*) may be better equipped to handle exposure to the pathogen.

A more thorough characterization of clinical isolates for mycolactone expression and

other virulence factors would dramatically enhance our understanding of *M. ulcerans* biology.

Our studies have revealed mechanisms of disruption of host-lipid metabolism in the absence of

mycolactone. These findings represent a solid foundation for the investigation of other

virulence factors that contribute to pathogenesis. A metabolomic study of model infection or

clinical infection has not been performed and would contribute a vast amount of information

regarding host metabolic disruptions upon infection. This avenue of research is attractive, as

metabolic analyses in mammal systems would be easier to perform compared to insect

systems. Libraries of metabolic molecules are better characterized in mammal systems, thus an

investigation of this nature may provide more biological significance than our investigation of

the mosquito model. Infection by a selection of strains with diverse phenotypes could be

modeled in mice. Subsequently, an analysis of host metabolism over the course of infection

could be compared to gross pathogenesis and histological assessment. It is likely that many *M.*

ulcerans ecovars would utilize a base set of virulence factors, such as phospholipase C/D, and

the presence of mycolactone might affect the temporal and spatial characteristics of lesion

development. Additionally, the metabolomic analysis of mycolactone negative infection in

mammals would provide a sound basis of evidence for the effects of virulence factors without

the contribution of mycolactone.

A major research gap in the analysis of *M. ulcerans* infection is lack of knowledge

regarding the actual infective dose or the contribution of dose in the pathogenesis during

infection. Most infectious disease have a strong relationship between dose and incubation

period. One of the only studies to investigate the incubation period of Buruli Ulcer disease

described an average of 135 days in an Australian cohort of patients (79). This finding may

suggest that due to the exceptionally slow growth of the bacilli coupled with what is likely to be

a very small dose, they bacteria require a long incubation period in order to achieve a

population sufficient to produce a clinically symptomatic infection (48). A dose response study

in a model infection of a rodent (*Thryonomys swinderianus*) found a relationship between dose

and onset of symptoms (250). The authors found that the high inoculum dose resulted in an

earlier onset of lesions with more severe presentation compared to the low dose, and

speculate that the infective dose may account for differences in clinical presentation. However,

another study using different doses to infect armadillos found no difference in pathology

between doses, but a reportable difference in pathology within each dose (251). Unfortunately,

the lowest dose reported used in the armadillo study was $3x10^5$ cells per inoculation, whereas

the low dose used in the *Thryonomys* model was $3x10^8$ cells per inoculation. It is extremely

unlikely that an insect vector would introduce 300 million cells during a bloodmeal. Thus, new

studies investigating the pathogenesis of low-dose infections is required if an insect vector is to

be seriously considered. Along these lines, the sialome (molecular components of saliva) of

mosquitoes contains a large number of bioactive molecules including anti-inflammatory and

immunomodulatory agents, and allergenic compounds (252). These salivary components play a

crucial role in pathogen transmission and the induction or suppression of host-immune and

inflammatory responses (253). The injection of *M. ulcerans* bacilli in the context of

immunomodulatory components of mosquito saliva is an attractive hypotheses investigating

vector-borne transmission. Since little is known about the pathogenesis of infection after

inoculation with fewer than 300,000 cells, a study describing the pathogenesis of infection

when 10-100 cells are inoculated with purified mosquito salivary extracts could provide

definitive data regarding the capacity of mosquitoes to transmit the disease, the contribution of

mosquito saliva in the initiation of infection, and the dose required for development of classical

infection.

The unfortunate position on the bottom of the funding scale compared to other

neglected tropical disease will continue to influence our understanding of *Mycobacterium*

ulcerans. Increased awareness and the dissemination of knowledge are the primary

countermeasures against the continued emergence of these deadly and disfiguring infections.

This practice is supported by passionate and dedicated scientists. A holistic approach to

understanding the mechanisms involved in disease transmission and persistence in the

environment in concert with better knowledge of factors that influence host susceptibility

provides the ideal environment for continued advancement.

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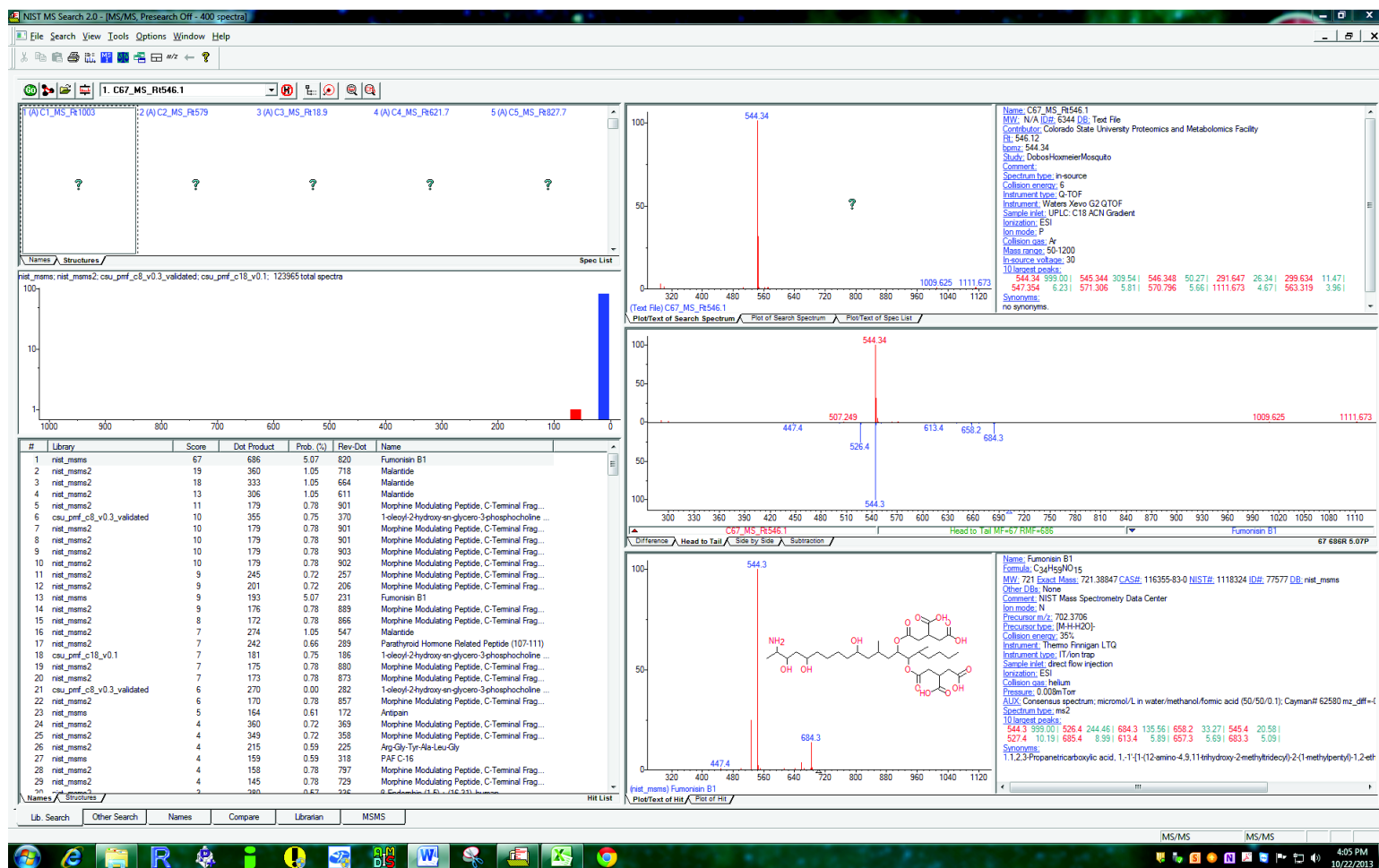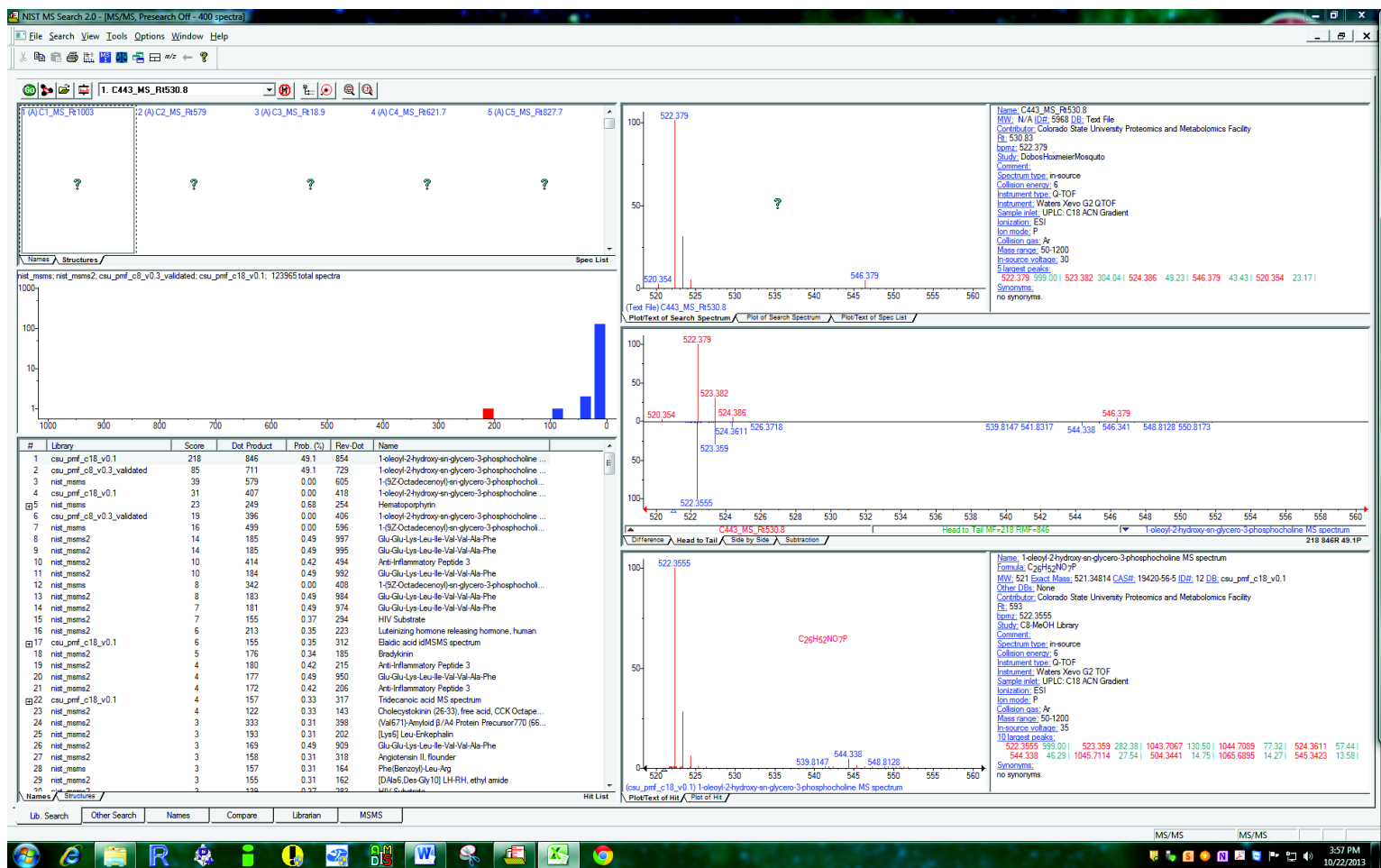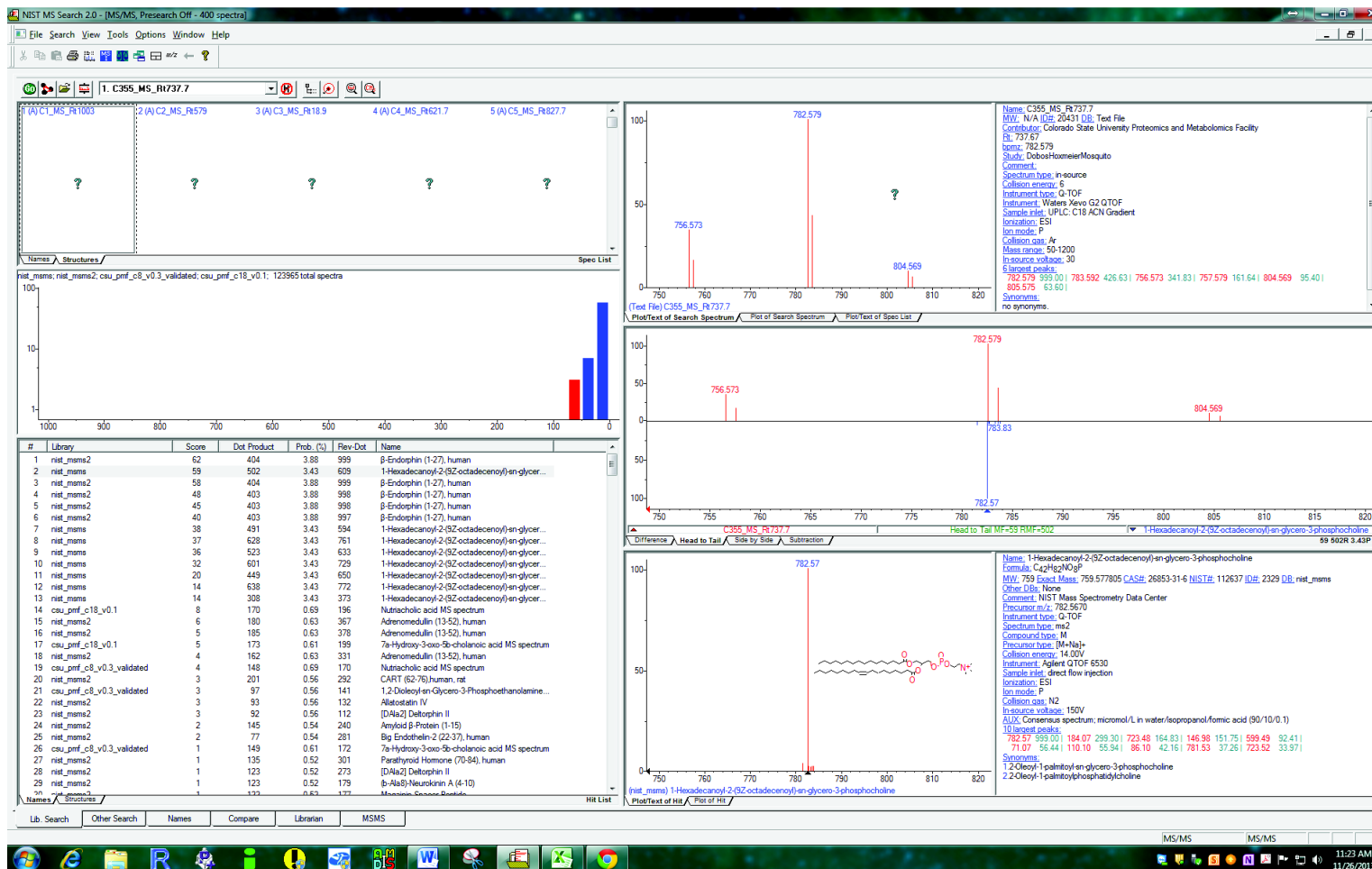
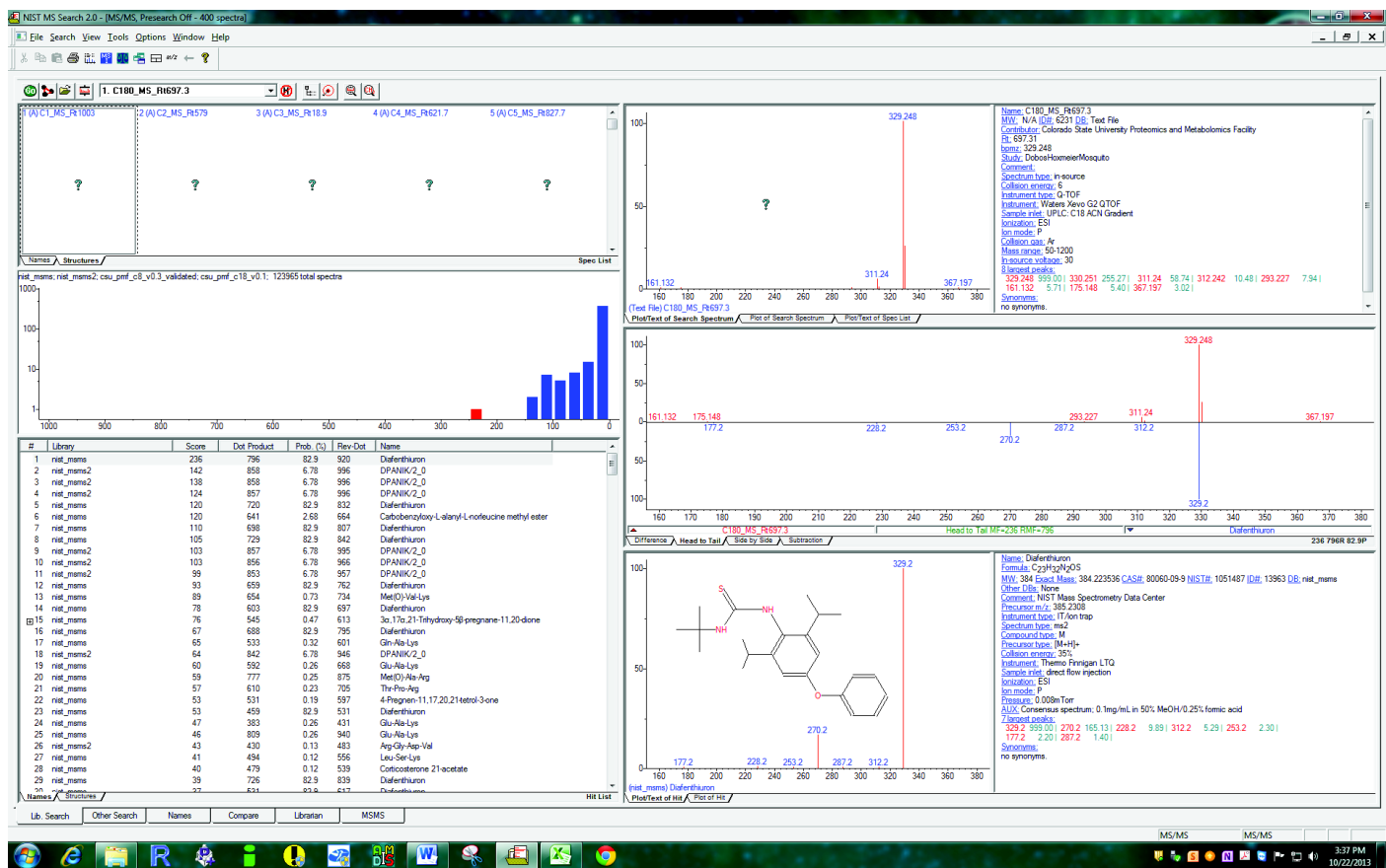Appendix figure 1: NIST spectral match and head-to-tail plot for compound C67.
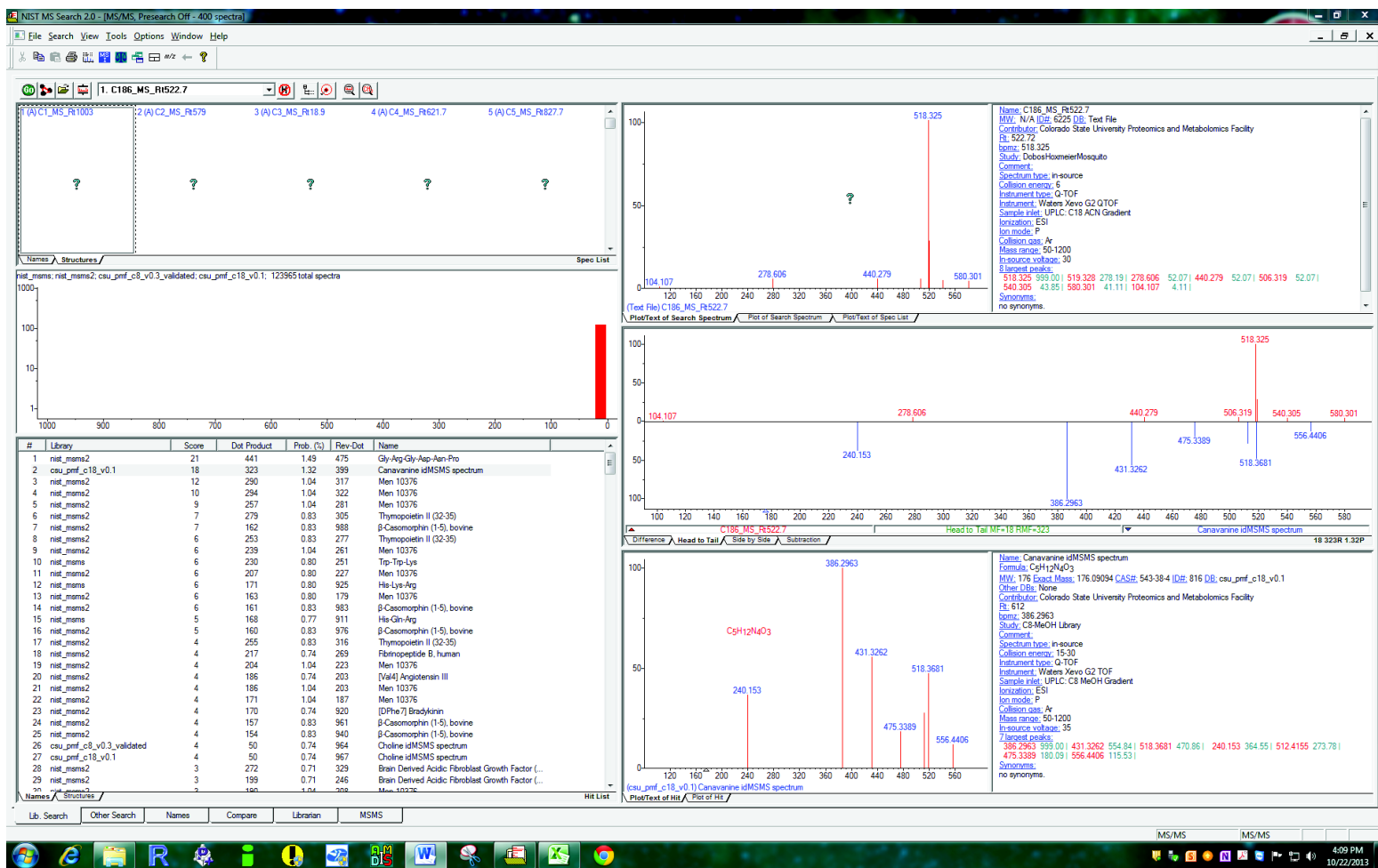
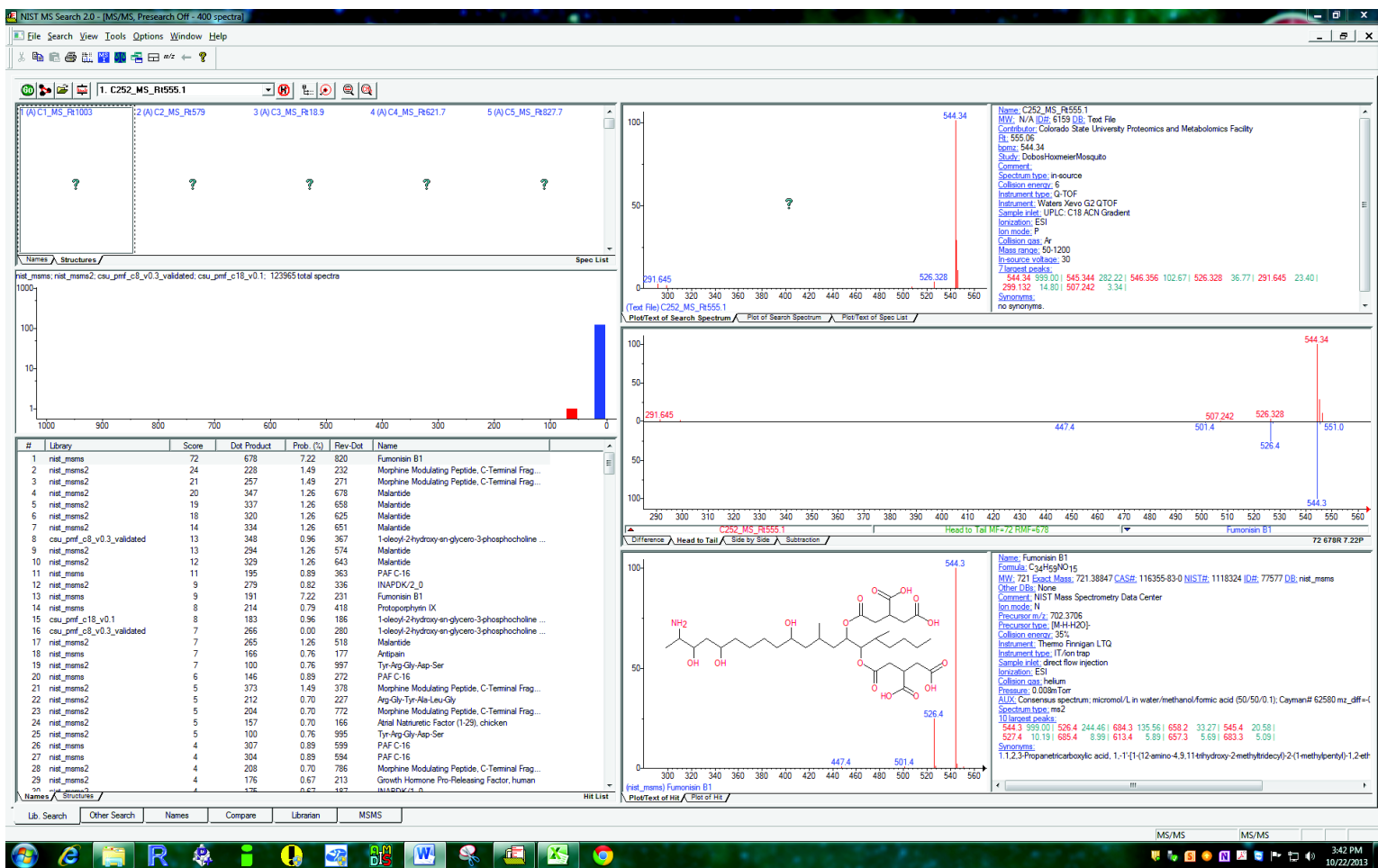Appendix figure 2: NIST spectral match and head-to-tail plot for compound C100.

Appendix figure 3: NIST spectral match and head-to-tail plot for compound C440.
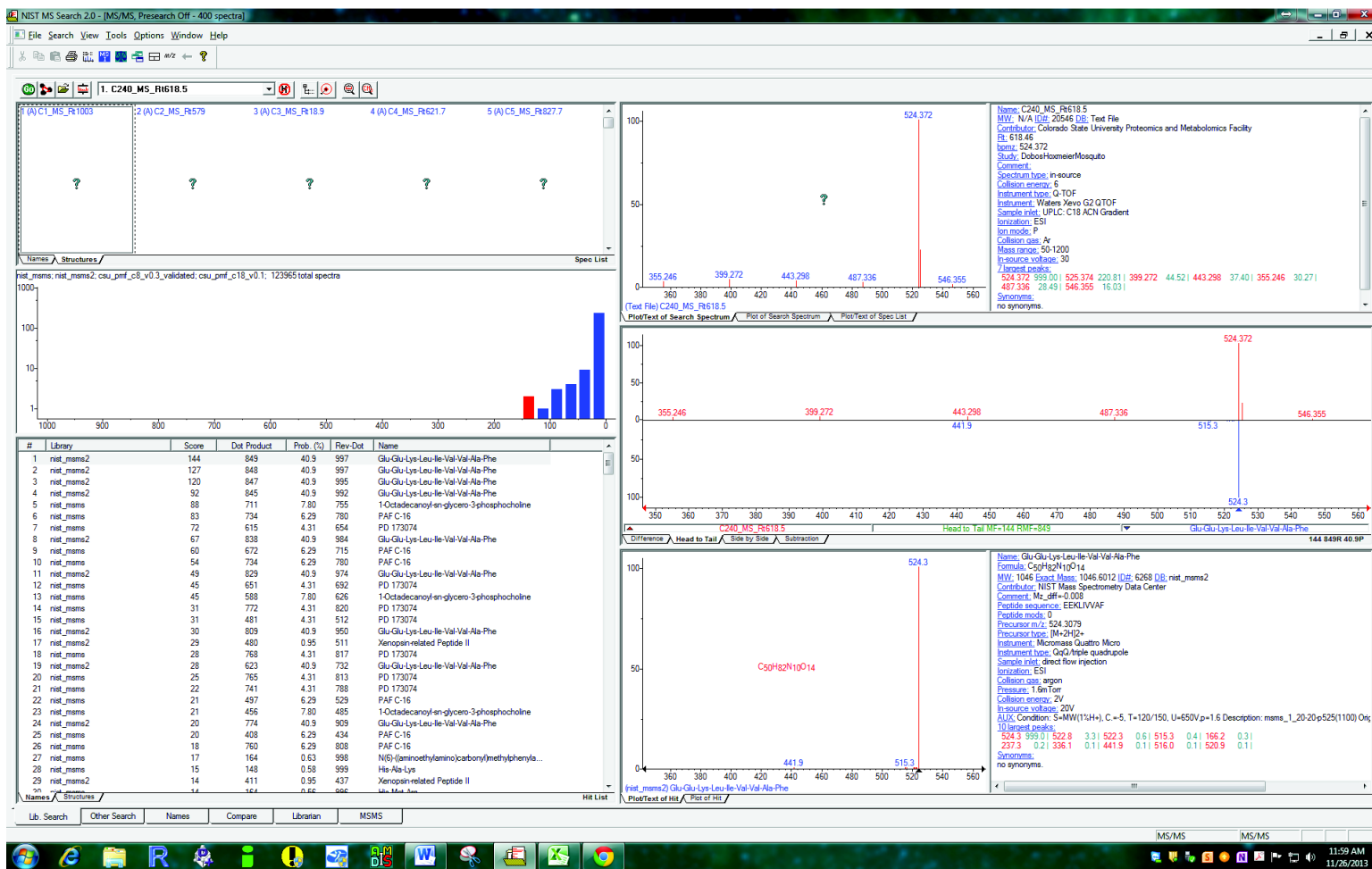
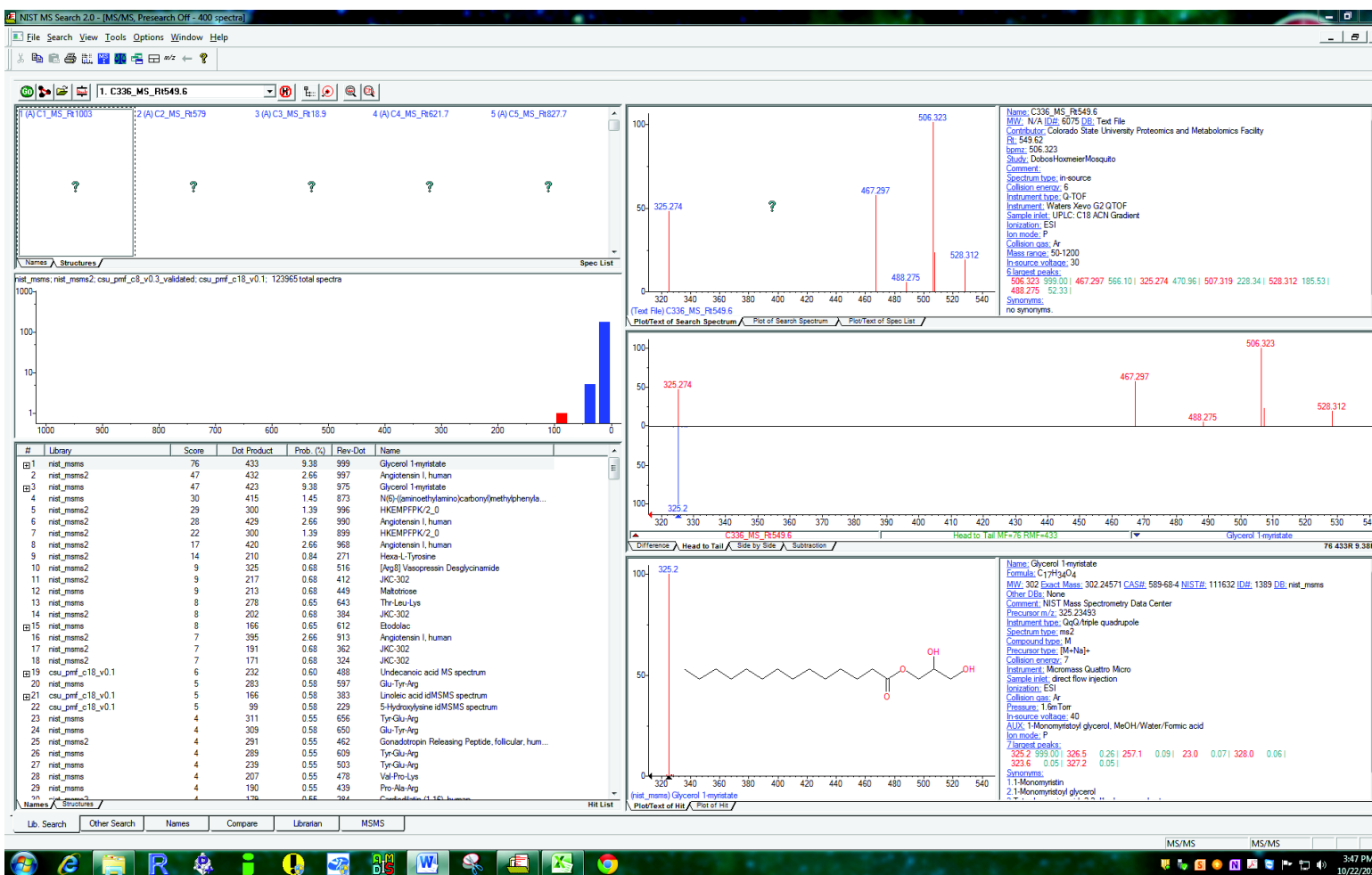Appendix figure 4: NIST spectral match and head-to-tail plot for compound C355.

Appendix figure 5: NIST spectral match and head-to-tail plot for compound C180.

Appendix figure 6: NIST spectral match and head-to-tail plot for compound C252.

Appendix figure 7: NIST spectral match and head-to-tail plot for compound C240.

Appendix figure 8: NIST spectral match and head-to-tail plot for compound C336.