DISSERTATION

PROBING THE METABOLIC SECRETS OF ANOPHELES STEPHENSI MOSQUITOES TO ENHANCE CRYOPRESERVATION TECHNIQUES

Submitted by

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ABSTRACT

PROBING THE METABOLIC SECRETS OF ANOPHELES STEPHENSI MOSQUITOES TO ENHANCE CRYOPRESERVATION TECHNIQUES

The lipid profile and cryopreservation methods of *Anopheles stephensi* mosquitoes have significant implications for mosquito management and research. The intricate life cycle of mosquitoes is governed by lipid metabolism, involving lipogenesis, lipolysis, and fatty acid metabolism, which are critical for transitioning between life stages. Despite the importance of these processes, mosquito cryopreservation has faced challenges, mainly due to the impenetrable nature of mosquito eggs to traditional cryoprotective agents. While other insects like honeybees and fruit flies have seen some success in cryopreservation, mosquitoes have posed unique difficulties. Initial attempts to cryopreserve mosquito eggs were unsuccessful, and there remains a need for effective cryopreservation protocols that maintain the viability and normal development of mosquitoes post-thaw. This study investigates lipid metabolism across mosquito life stages and advances cryopreservation techniques. The lipid profile analysis focused on major lipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelins (SM), and triacylglycerides (TGs), as well as non-bilayer lipids like diacylglycerol (DG) and lysophospholipids (LysoPC, LysoPE). For cryopreservation, a new protocol using methylformamide (MF) with trehalose was developed for first-instar larvae (L1s) older than 1.5 hours post-hatching. The hypotheses were that 1) Lipid metabolism plays a crucial role in the successful development and reproduction of mosquitoes, and 2) Inducing diapause and using suitable cryoprotectants can improve

the recovery rates of cryopreserved mosquitoes. The lipid profile analysis revealed that PE is vital for protein anchoring required for embryogenesis and immune responses and that steroids like 20-hydroxyecdysone (20E) are crucial for molting and development. The cryopreservation study showed that supercooling larvae for up to 60 minutes resulted in normal development to adulthood, although longer durations inhibited adult emergence. Supercooled larvae and their offspring exhibited typical sex ratios and developmental patterns, indicating genetic and phenotypic stability. This research underscores the well-orchestrated metabolic strategies in mosquitoes. It suggests that understanding these biochemical processes is essential for effective cryopreservation, potentially paralleling natural cold survival strategies seen in other insects.

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"Give me a place to stand, and with a lever, I will move the whole world." – Archimedes

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CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

Mosquitoes represent a large group of insects from the Culicidae family and have an essential role in the food chain (Gates, n.d.). Over 3,500 mosquito species are categorized into two subfamilies: Anophelinae and Culicinae. Although only approximately 2.5% are known to be vectors for 78 human disease-causing pathogens (Yee et al., 2022), they are considered the deadliest animals in the world (Franklinos et al., 2019; Gates, n.d.). Known pathogens transmitted by mosquitoes belong to the families of arboviruses such as *Bunyaviridae*, *Flaviviridae*, *Togoviridae*, *Reoviridae*, and *Picornaviridae*. In addition to viruses, there are also Francisellaceae (bacteria), Filaridae and Onchocercidae (nematodes), and *Plasmodium* genus (malaria parasite) (Yee et al., 2022). Mosquito-borne diseases are predicted to intensify as climate change occurs due to increased vector survival, biting rates, longer transmission seasons, increased replication of pathogens within vectors, and shorter reproduction rates (Colón-González et al., 2021).

The intimate mosquito-pathogen interaction is being investigated, including the understanding of virulence, predicting disease epidemiology, and elucidating long-lasting and dynamic interactions. Notwithstanding the mosquito-pathogen interactions, metabolism plays a crucial role in the mosquito life cycle and specially after the pathogen infects the mosquito. Metabolomics is a rapidly growing field that has potential to explore fundamental biological and clinical questions remains unrealized. Metabolomic-based approaches have contributed to our understanding of the dynamic cellular landscape

upon which blood meal uptake and other mosquito developmental processes occur (Chotiwan et al., 2018, 2022; Koh et al., 2020; Mayra et al., n.d.; Melo et al., 2016; Souvannaseng et al., 2018).

Herein, we will discuss the significance of various developmental processes within the *Anopeheles stephensi* mosquito and their association with metabolism. Lipids are essential during reproduction, starvation, diapause, and prolonged flight periods (Arrese & Soulages, 2009). Thus, we will discuss the knowns and unknowns regarding fluctuations of the lipid landscape throughout development. We will also discuss the known roles of present lipids. Additionally, we will discuss the advancements towards developing a cryopreservation method.

1.2 Mosquitopia

1.2.1 Mosquitoes are vectors of medically relevant pathogens.

Mosquito-borne diseases pose a significant global health burden and are most prevalent in tropical and subtropical regions. These diseases are caused by pathogens transmitted through the bites of infected female mosquitoes, primarily belonging to the genera *Aedes*, *Anopheles*, and *Culex*, playing a pivotal role in transmitting pathogens to humans and animals. The most notable mosquito-borne pathogens include malaria, dengue virus (DENV), Zika virus (ZIKV), chikungunya (CHIKV), yellow fever, and West Nile virus (Gianchecchi et al., 2022; Gubler, 2011).

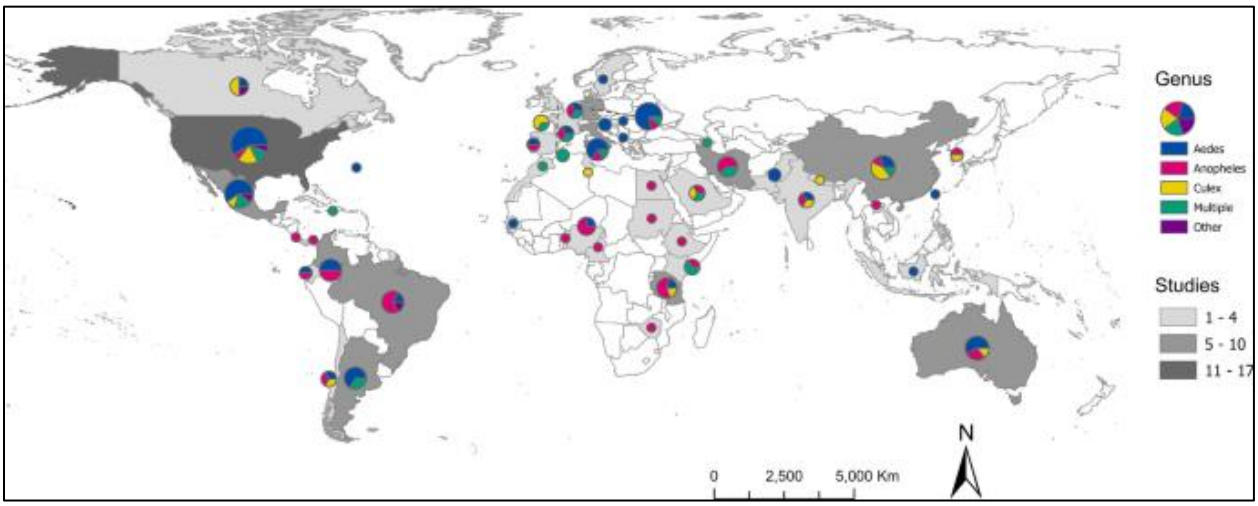

Figure 1.1. Map illustrating the distribution of *Aedes*, *Anopheles*, *Culex*, multiple, or other mosquitoes. Using species distribution modeling (SDM) as a methodological approach to estimate the distribution of vector-borne diseases. The number of studies represent SMD studies at the sub-national or national level. (From Lippi et al. 2023, Copyright © The Author(s) 2023)

The interaction between mosquitoes, pathogens, and vertebrate hosts are characteristics of epizootic, enzootic, and urban cycles and horizontal and vertical transmission. However, as the ecosystem changes, it influences the epidemic cycling and distribution, leading to evolutionary adaptations and unstable transmission limited by their geography (Fouque & Reeder, 2019). In enzootic cycles, also known as the sylvatic or jungle cycle, vertebrates such as birds, small mammals, and nonhuman primates produce viremias, thus considered amplifying hosts (Coffey et al., 2013). Arboviral human diseases usually result from a spillover infection from enzootic cycles, making humans dead-end hosts. Nonetheless, a few arboviruses (e.g., DENV and CHIKV) undergo urban transmission, and at that point, humans act as amplifying hosts (Coffey et al., 2013). In epizootic cycles and horizontal transmission, the pathogen transmission between mosquitoes and vertebrates often leads to outbreaks or epidemics. Vertical transmission occurs when the adult mosquito passes the pathogen to its progeny, which plays a crucial

role in maintaining and amplifying the pathogen within mosquito populations during unfavorable environmental conditions.

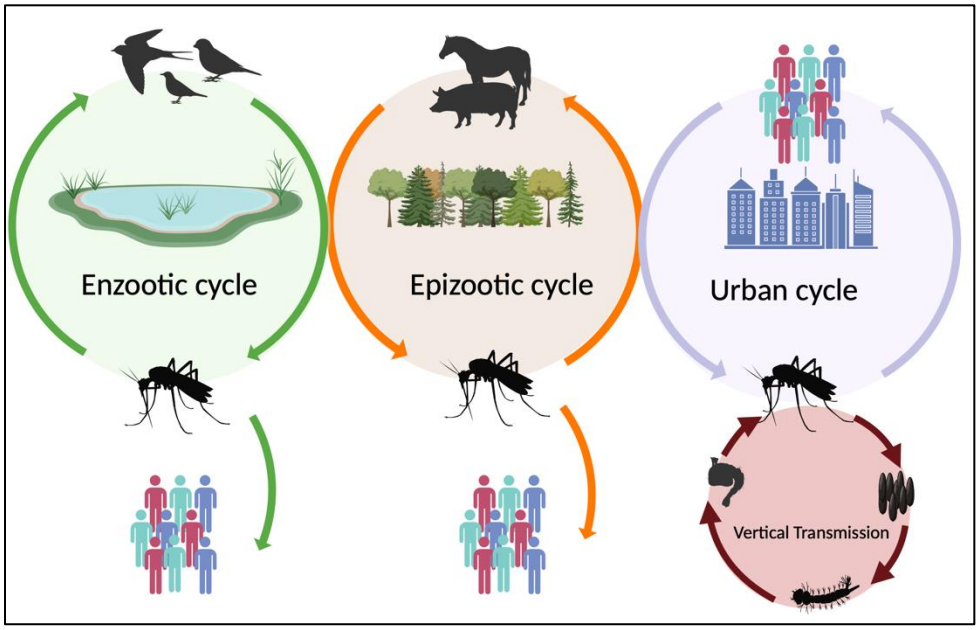

Figure 1.2. Pathogen emergence mechanisms. Mosquitoes transmit medically relevant pathogens through enzootic, epizootic, urban and/or urban cycles. (Figure adapted from Weaver et al, 20118 using Biorender.com)

1.2.2 Vector Control Strategies

Efforts to control mosquito-borne diseases primarily focus on vector management strategies such as reducing mosquito populations and interrupting transmission cycles. These strategies include insecticide-treated bed nets and indoor residual spraying for malaria control, community-based vector surveillance and source reduction through habitat modification, and mosquito larval control measures targeting breeding sites in water bodies (WHO, 2020; PAHO, 2017). Nonetheless, insecticide resistant mosquitoes have sharply increased, developing genetic, physiological, and structural mechanisms to overcome insecticide toxicity (Juache-Villagrana et al., 2022). Thus, genetically modified mosquitoes (GMMs) have emerged as promising tools for controlling the spread of

mosquito-borne diseases. The sterile technique consists of using ionizing radiation and then releasing the adult mosquitoes into the wild with the purpose of interfering with mating activities and thereby reducing the mosquito population (Balatsos et al., 2024). Gene drive technology enables the preferential inheritance of a particular gene to increase its frequency within a population rapidly. This approach can spread genetic modifications that prevent mosquitoes from transmitting pathogens or reducing their ability to reproduce (Hammond et al., 2016). *Wolbachia* is an endosymbiotic bacterium introduced into *Aedes aegypti* and *Aedes albopictus* mosquitoes (Mains et al., 2016, 2019) to interfere with their ability to transmit pathogens. This can be achieved by inducing cytoplasmic incompatibility between pathogen-*Wolbachia*, where incompatible crosses reduce offspring viability (Walker et al., 2011). Molecular techniques, including next-generation sequencing and genomic epidemiology, have enhanced our understanding of vector-pathogen interactions, transmission dynamics, and the evolution of drug and insecticide resistance (Faria et al., 2018; Neafsey et al., 2015). Furthermore, advances in vaccine development, such as the RTS, S/AS01 malaria vaccine, and the Dengvaxia dengue vaccine, offer promising tools for disease prevention, although challenges remain in achieving widespread vaccine coverage and effectiveness (RTS, S Clinical Trials Partnership, 2015; Capeding et al., 2014).

1.2.3 Anopheles spp. Mosquitoes

The genus *Anopheles* comprises over 460 species exhibiting diverse behaviors, ecologies, and roles in disease transmission. However, approximately 41 of these mosquito species are known for their high vectorial capacity to transmit malaria, a protozoan parasite belonging to the *Plasmodium* genus. Malaria is a leading cause of

morbidity and mortality, especially among young children under five years old in the sub-Saharan Africa region (World Malaria Report 2020, n.d.). Approximately 100 *Plasmodium* species have been described, but only five, *P. malaria*, *P. falciparum*, *P. vivax*, *P. ovale*, and, *P. knowlesi* commonly infect human beings (Talapko et al., 2019). Plasmodia infect and multiply within red blood cells, leading to symptoms such as fever, chills, headache, and anemia (Cowman et al., 2016). *P.ovale* and *P.vivax* usually stay dormant in the liver for extended periods, entering the bloodstream up to years later. The liver stage is required for the disease. *Anopheles* mosquitoes also possess the vectorial capacity to transmit the agent of filariasis, the nematode *Wuchereria bancrofti* (Yokoly et al., 2020). Additionally, *Anopheles gambiae* and *Anopheles funestus* are the primary vectors of the O'nyong-nyong virus (ONNV), whose main symptoms are fever and polyarthritis (Alonso & Noor, 2017; Gianchecchi et al., 2022; Gubler, 2011). Moreover, some studies suggest that *Anopheles* mosquitoes may be incriminated in the transmission of arboviruses such as the Mayaro virus (MAYV), Rift Valley virus (RVFV), and the Japanese encephalitis virus (JEV). *An. stephensi* mosquitoes are the primary vector for *P. falciparum* (Llergo et al., 2024).

These mosquitoes originated in Southeast Asia and the Arabian Peninsula. Still, recently, it has extended its geographical range to the Horn of Africa, Sri Lanka, Lakshadweep islands, and the Republic of Sudan (de Santi et al., 2021). They can adapt to different larval habitats, ranging from the margins of freshwater pools to human-made water containers. Thus, this allows for expansion to central urban (Sinka et al., 2020) and rural locations. The expansion of *An. stephensi* mosquitoes represent a critical threat to

global public health. In 2019, the World Health Organization issued a notice to alert public health authorities of its recent expansion, proposing its control and elimination.

1.2.4 Mosquito Life Cycle and Association with Metabolism

Uniqueness and universality are the duality of life itself, resulting in evolution through natural selection. Characteristics of ecology, anatomy, physiology, morphology, and behavior reflect the unique phylogenetic history and ecological niche of a species. The fundamental currency of biological fitness is energy devoted to survival, growth, and reproduction, all governed by mass balance and physical energy laws (*Universal Rules of Life*, n.d.). Heterotrophic organisms derive their energy from consuming plants and then converting this biomass energy into vital fuel through the process of respiration. This crucial biochemical process involves metabolites to drive a wide range of essential biological functions. Although the exact molecular mechanisms of actions are distinct among species, the bottom line lies in their presence for survival and reproduction. Metabolic fluxes are tightly regulated through allosteric regulation, enzyme kinetics, and signaling pathways to maintain metabolic homeostasis and adapt to changing nutrient availability and energy demands. Metabolites serve as the biochemical fingerprints of cellular activity, reflecting the interactions between genes, proteins, and environmental factors. Metabolomics techniques encompass diverse analytical methods, including nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS), and chromatography, enabling the comprehensive profiling of metabolites in biological samples. This allows for mechanistic inquiries and innovative tools to analyze metabolic pathways, which evaluate the interconnectedness of these metabolites that can offer

valuable biological insights (Johnson et al., 2016). The integration of omics approaches, including genomics, transcriptomics, proteomics, and metabolomics, has provided a comprehensive view of mosquito lipid metabolism, facilitating the identification of metabolic pathways, regulatory networks, and metabolic adaptations in response to environmental and physiological changes. Recent publications have demonstrated the utility of omics technologies in deciphering the complexities of lipid metabolism in mosquitoes, revealing insights into lipid biosynthesis, storage, mobilization, and catabolism (Toprak et al., 2020).

The exploration of lipid metabolism in mosquitoes dates back to the early 20th century when researchers began investigating these insects' nutritional requirements and metabolic pathways. Early studies focused on characterizing the lipid composition of mosquito tissues, identifying lipid storage sites, and elucidating the role of lipids in oogenesis and egg development. Notable discoveries during this period include the identification of lipid droplets in mosquito fat body tissues and the demonstrating of lipid synthesis in the ovaries of blood-fed females (Ferreira Barletta et al., 2015; Ziegler & Ibrahim, 2001). The advent of lipidomics and molecular biology techniques in the late 20th and early 21st centuries revolutionized the study of lipid metabolism in mosquitoes, enabling researchers to begin exploring the depths and complexity of lipid pathways at the molecular level. Lipidomic analyses revealed the diversity of lipid classes and species present in mosquito tissues, shedding light on the roles of specific lipids in physiological processes such as energy metabolism, immune response, and pathogen transmission (O'Neal et al., 2020; Reboldi & Dang, 2018).

Mosquitoes undergo a complex life cycle comprised of terrestrial and aquatic environments. The life cycle has four major stages: eggs, larvae, pupae, and adults. Each has a different morphology, habitat, and behavior. The lifecycle begins with female mosquitoes depositing eggs in or near water bodies ranging from stagnant ponds and marshes to artificial containers such as discarded tires and rain gutters. Upon hatching, mosquito eggs give rise to larvae, which inhabit aquatic environments and feed on organic matter and microorganisms. The larval stage is characterized by rapid growth and molting as larvae progress through four instars before pupation. Conditions such as overcrowding can affect the rate of going through these processes and have been shown to reduce parasite transmission significantly (Moller-Jacobs et al., 2014; Murdock et al., 2017).

Furthermore, larval to larval molts are controlled mainly by 20-hydroxyecdysone (20E) and the juvenile hormone (JH), which has previously been studied in *Manduca sexta*, *Drosophila melanogaster* and *Aedes aegypti* (Jindra et al., 2013). Additionally, the ecological interactions between mosquito larvae and their aquatic habitats, including the impact of environmental factors such as temperature, nutrient availability, and predation, cause pressure on larval development and survival (Murdock et al., 2019). Larvae acquire most of their lipids from their mother and these lipids are transferred to the adult stages (Ziegler, 1997; Ziegler and Ibrahim, 2001; Atella and Shahabuddin, 2002), such as eicosapentaenoic acid (EPA) and arachidonic acids from larvae to the functional phospholipids (PL) in adults. Soon after the fourth molt, larvae become inactive during this metamorphosis to become pupae. Pupae are relatively immobile and do not feed, relying on stored energy reserves acquired during the larval stages (Lucas & Romoser, 2001). Upon emerging from the pupal stage, mosquitoes enter the adult stage, where

they assume their characteristic winged form and become capable of flight, mating, and blood-feeding. Adult mosquitoes exhibit sexual dimorphism behaviors, with females typically requiring a blood meal for egg development, while males primarily feed on plant nectar and other sugary sources.

Male mosquitoes primarily feed on plant nectar, which provides them with carbohydrates necessary for energy production, supporting flight, mating activities, and overall metabolic needs. They can transfer nutrients to females through copulation, enhancing female fecundity and reproductive success. This nutrient transfer, known as "nuptial gifts," may include carbohydrates, amino acids, and other essential nutrients. Pathogens exploit the hematophagous lifestyle of female mosquitoes for their transmission. Nonetheless, the physiological processes and environmental factors that influence a dynamic lipid metabolic profile are orchestrated by organs serving specific functions. The midgut is the primary site for digestion and nutrient absorption in mosquitoes. It metabolizes ingested blood, sugar, and other nutrients. The fat body is a multifunctional tissue responsible for lipid storage, metabolism, and immune responses. Energy storage is mainly carried out by adipocytes or tropocytes containing amino acids (AAs) and glycogen as carbohydrates. Lipids are stored in cytoplasmic droplets as triacylglycerides (TAGs). Therefore, the fat body synthesizes, stores, and mobilizes lipids for egg development and overwintering. Malpighian tubules are involved in excretion and osmoregulation. They help regulate the balance of ions and water by removing waste products and maintaining proper physiological homeostasis. The ovary is critical for egg development and maturation in female mosquitoes. It synthesizes yolk proteins and steroids necessary for oogenesis and vitellogenesis, ensuring the production of viable

eggs. The blood meal provides females with amino acids and lipids crucial for oogenesis, process that takes approximately 72 hours to complete. After the blood ingestion, amino acids are extensively increased, which triggers the activation of the rapamycin (TOR) pathway, producing the hormone 20-hydroxyecdysone (20E). Blood meal-derived AAs are fed into the tricarboxylic acid (TCA) cycle for energy production to mobilize nutrients and incorporate them into lipophorin, vitellogenin, and major yolk proteins (Zhou, Flowers, et al., 2004; Zhou, Pennington, et al., 2004).

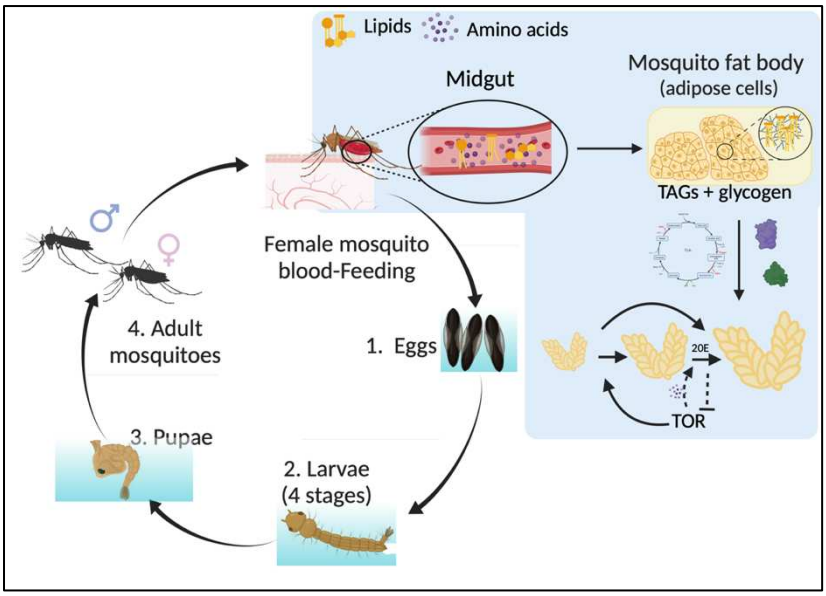

Figure 1.3. Mosquito lifecycle. *Anopheles* mosquitoes undergo complete metamorphosis, progressing through an egg stage (1.), four aquatic larval stages (1st to 4th instars) (2.), an aquatic pupal stage (3.), and finally to the non-aquatic adult stage (4.). To obtain eggs, a female mosquito acquires a blood meal, containing lipids and amino acids that pass through the midgut then to the fat body to undergo metabolic processes and undergo oogenesis. Under moist, the eggs hatch into 1st instar larvae, following development the 2nd, 3rd, and 4th instar stages over a period of ~seven days. The pupal stage lasts for ~two days before transitioning into adults. Figure made with BioRender.com.

1.3 Lipidomics – brief overview

Eukaryotic cells dedicate a substantial portion of their genetic resources— ~5% of their genes—to synthesizing thousands of distinct lipids. The continuous efforts to

investigate lipids' chemical structure and biological significance, a field known as lipidomics, have revealed the complexity and diversity of lipids within eukaryotic cells. Generally, lipids fulfill three functions: energy storage, matrix of cellular membranes, and as second messengers in signal transduction and molecular recognition processes (Van Meer et al., 2008).

Lipids comprise a wide variety of chemical structures containing hundreds or thousands of different molecular species. Lipids have historically been broken down into polar lipids (e.g., glycerolipids) and non-polar lipids (e.g., cholesterol and triacylglyceride (TG)). Lipid Metabolites and Pathways Strategy (LIPID MAPS) in 2005, spearheaded a classification for lipids categorizing them into eight general categories: fatty acyls (FA), glycerolipids (GL), glycerophospholipids (GPL), sphingolipids (SP), sterol lipids (ST), prenol lipids (PR), polyketides (PK), and saccharolipids (SL) (E. Fahy et al., 2009). Each category is divided into lipid classes, which correspond to the polar head group. Furthermore, lipid classes are further divided into subclasses due to different chemical bonds of the aliphatic chain to the *sn-1* glycerol hydroxy group in GPLs. The plethora of lipids due to their physical and chemical properties drive essential cellular processes (Han & Gross, 2022), including but not limited to a) establishing membrane potential and providing hydrophobic barriers for organelle separation; b) providing matrices for transmembrane protein activity regulation via dynamic regulation of cellular membrane; c) serving as primary-, and secondary-messengers; d) serving as energy storage depots; and e) serving as anchors for protein binding to the cellular membrane. Due to their crucial roles, disruption in homeostasis may lead to disease. Recently, the field of lipidomics has bloomed extensively, and studies have evolved to explore special lipid categories such

as mediator lipidomics, sphingolipidomics, phospholipidomics, etc., each with the intention to define the function of lipids in a cell, organ, or organisms.

Early studies on lipid metabolism mainly focused on one molecular species, class, or enzyme-mediated pathway due to the limited viability of biochemical assays for detection. Mass spectrometry's development primarily measured atoms' masses, leading to the discovery of isotopes (Griffiths, 2008). Chemists in the petroleum industry measured the abundances of small hydrocarbons using mass spectrometers in the 1940s. However, it was not until the 1960s that scientists began understanding the complexity of molecules fragmented inside the instruments and unraveling possible applications. Throughout history, liquid chromatography (LC), gas chromatography (GC), TLC, HPLC, MS, and different ionization sources have been used alone or coupled for lipid analysis. Lipids are commonly extracted using organic solvents such as chloroform liquid-liquid extraction methods. The Catalan biochemist and leader in the field of neurochemistry, Jordi Folch-Pi (1911 – 1979), contributed to the biochemistry of lipids by developing the new "gold standard" for lipid extraction known as the Folch's method in 1957 (2001 THE NATIONAL ACADEMY PRESS WASHINGTON, DC). Following Folch's method, EG Bling and W.J. Dyer introduced a similar method in 1959 using a monophasic chloroform/methanol/water mixture (Bligh & Dyer, 1959). In these methods, biological samples are processed and then subjected to MS (Figure 1.4). This is known as 'shotgun lipidomics' or direct infusion (DI). Alternatively, lipids can undergo LC before MS to separate lipids based on the hydrophilicity of the headgroups or the hydrophobicity of fatty acid chains (Swinnen & Dehairs, 2022). Before MS analysis, lipids must acquire a charge through electrospray ionization, producing tiny droplets placed under a high-voltage

capillary. As lipid ions enter the vacuum chamber containing mass analyzers, ions are separated based on their mass relative to change (m/z). However, ions are further fragmented and separated by a second mass analyzer to identify the lipid species better. This is known as MS/MS and the data generated can result in several thousands of features (Cajka & Fiehn, 2014).

Although Preparing samples for metabolomics is generally simpler compared to the processes involved in proteomics or transcriptomics (Perez de Souza et al., 2021), the sheer volume and complexity of data generated by LC-MS/MS necessitate sophisticated tools for data acquisition, processing, and analysis. While a wide array of data processing tools exists, both freely accessible and commercially available, a standardized workflow is crucial for maximizing data utility across a broader spectrum of scientific disciplines(Wanichthanarak et al., 2019). A data processing workflow used at Colorado State University for GC-MS and LC-M-based untargeted global metabolite profiling studies utilizes freely available tools such as XCMS (Smith et al., 2006), RAMClustR (Broeckling et al., 2014), and RAMSearch (Yao et al., 2019), to establish feature detection and alignment, data reduction, and annotation, respectively.

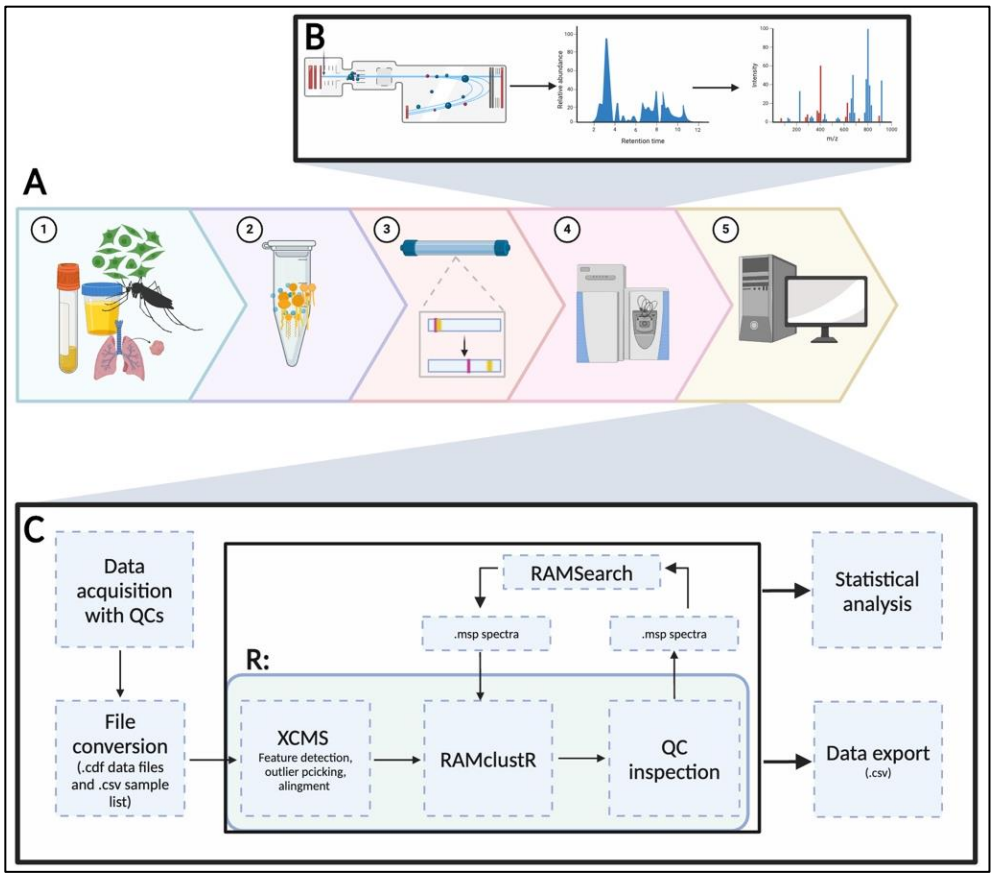

Figure 1.4. General overview of metabolomics workflow. A) Brief overview of sample processing: (1) Tissues, urine, plasma, cells, or whole organisms (mosquitoes) are homogenized. (2) Lipids are extracted using the chloroform/methanol classical method. (3) Liquid chromatography is carried out, followed by (4) mass spectrometry, and subsequently, data acquisition, processing, and analysis are carried out. **B)** Molecules acquire a charge through electrospray ionization, which produces tiny droplets placed under a high-voltage capillary. As lipid ions enter the vacuum chamber containing mass analyzers, ions are separated based on their mass relative to change (m/z). **C)** Standard computational workflow.

As mass spectrometry (MS) has become the state-of-the-art analytical technique to define the lipidome as an expansion of metabolomics studies, it is welcomed to the realm of genomics and proteomics to study a biological system (Dunn, 2011). Over recent years, substantial advancements in this arena have occurred: instrument precision, experimental methodologies, sample processing, and the surge of chemoinformatics and bioinformatic tools for data acquisition, analysis, and integration (Chang et al., 2021).

Therefore, our capacity to detect metabolites across diverse samples has markedly improved, enabling us to establish correlations between these metabolites and specific phenotypes (Lai et al., 2018; Xia et al., 2009).

1.4 Goals and hypotheses of this dissertation

The overarching research goals of this dissertation were to (1) establish the lipid profile of *An. stephensi* mosquitoes at different life stages and (2) develop a cryopreservation method for *An. stephensi* mosquitos. To address the first goal, in Chapter 2, we hypothesized that the lipid profile of *An. stephensi* mosquitoes exhibits fluctuations as they mature from eggs to adults. We, therefore, collected samples at each life stage and carried out mass spectrometry studies to acquire a comprehensive lipidome of *An. stephensi* mosquitoes across life stages. We obtained information about two important transitions such as L1 to L2 and differences between blood-fed versus non-blood-fed female adult mosquitoes. To approach the second goals, in Chapter 3, we dove into the world of cryobiology, and we reported that the first larval stage (L1) at a late stage is best for cryopreservation. We optimized a protocol in which L1s were exposed to subzero temperatures for 10 minutes and 60 minutes with ~40% recovery and successfully generating a second generation (F1).

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CHAPTER 2: INVESTIGATING THE LIPID PROFILE OF ANOPHELES STEPHENSI MOSQUITOES ACROSS LIFE STAGES[1]

2.1 Introduction

The mosquito life cycle is a complex sequence of developmental stages, each demanding precise energy resource orchestration as developing mosquitoes experience aquatic and terrestrial factors. Fatty acid oxidation is essential to generate energy for flight and reproduction (Van Meer et al., 2008). Indeed, the maternal investment of lipids sets the stage for embryonic development. As new larvae emerge, they acquire lipids from the mother via egg lipid deposition (Atella & Shahabuddin, 2002). As they develop through the four instar stages, the lipid repertoire plays a pivotal role in sustaining energy demands during growth, molting, and metamorphosis (Arrese et al., 2001; Wrońska et al., 2023). Insect egg cuticles consist of a protective layer of chitin covered in a waxed single-layered epithelium, fatty acids, and sterols (Wrońska et al., 2023). As larvae progress through the four developmental stages in an aquatic environment, the ecdysteroid hormone is an essential signal (Ekoka et al., 2021).

Lipid metabolism, encompassing lipogenesis, lipolysis, and fatty acid metabolism, is the foundation for providing the necessary energy substrates and structural components for transitioning between aquatic and terrestrial life stages. A detailed comparative analysis of the lipid metabolome of mosquitoes through development has not been conducted despite its likely importance in each stage. Here, we extracted lipids

[1] Adapted from Ramirez *et al*. "Investigating the Lipid profile of *Anopheles stephensi* mosquitoes across developmental life stages". Submitted to Comparative Biochemistry and Physiology – Part D

and other relatively non-polar metabolites from *An. stephensi* at each life stage to describe and compare lipid metabolites between each life stage. We found that several major classes of lipids distinguish *An. stephensi* life stages. Our data supported previous studies implying the role of triacylglycerides (TG) in mosquito maturation, demonstrating their depletion as larvae initially develop and synthesize as pupae emerge into adults. Of particular interest was our finding that the lipidome between larval stage 1 (L1) and 2 (L2) developing mosquitoes was dynamic, including a decreased abundance of several TG and an increased abundance of lysophosphotidylcholines (LysoPC) despite relatively minor phenotypic changes. The adult stage is most commonly studied due to its direct role in disease transmission. While this is beneficial for understanding mosquito-pathogen interactions, a basic understanding of basic mosquito biology is needed. Therefore, we found similarities and differences in adult mosquitoes, even when divided into three samples: males and females, who were blood-fed and sugar-fed. Lipid characteristics in males and blood-fed females clustered much closer than sugar-fed females, suggesting fewer differences between males and blood-fed females.

2.2 Results

2.2.1 Lipids comprise the primary class of metabolites analyzed in our study.

In this study, we sought to preferentially extract lipid and other apolar metabolites for downstream LC-MS analyses. We chose this due to the inherent importance of lipids in the biogenesis of mosquitoes (Wang et al., 2017). Our results demonstrate that lipids represented 62.15% of our analyzed metabolome (Figure 2.1A). Within the lipid-like molecules, superclasses such as endocannabinoids, fatty acyls, glycerolipids,

glycerophosphonolipids, prenol lipids, saccharolipids, sphingolipids, sterols, and steroid derivates were detected (Figure 2.1B). The other primary class of metabolites were unassigned. This is not surprising due to the present state of knowledge in arthropod metabolites. Finally, some classes, such as flavonoids, may be present due to ingesting fish food, commonly used in mosquito husbandry, or otherwise due to missed annotations.

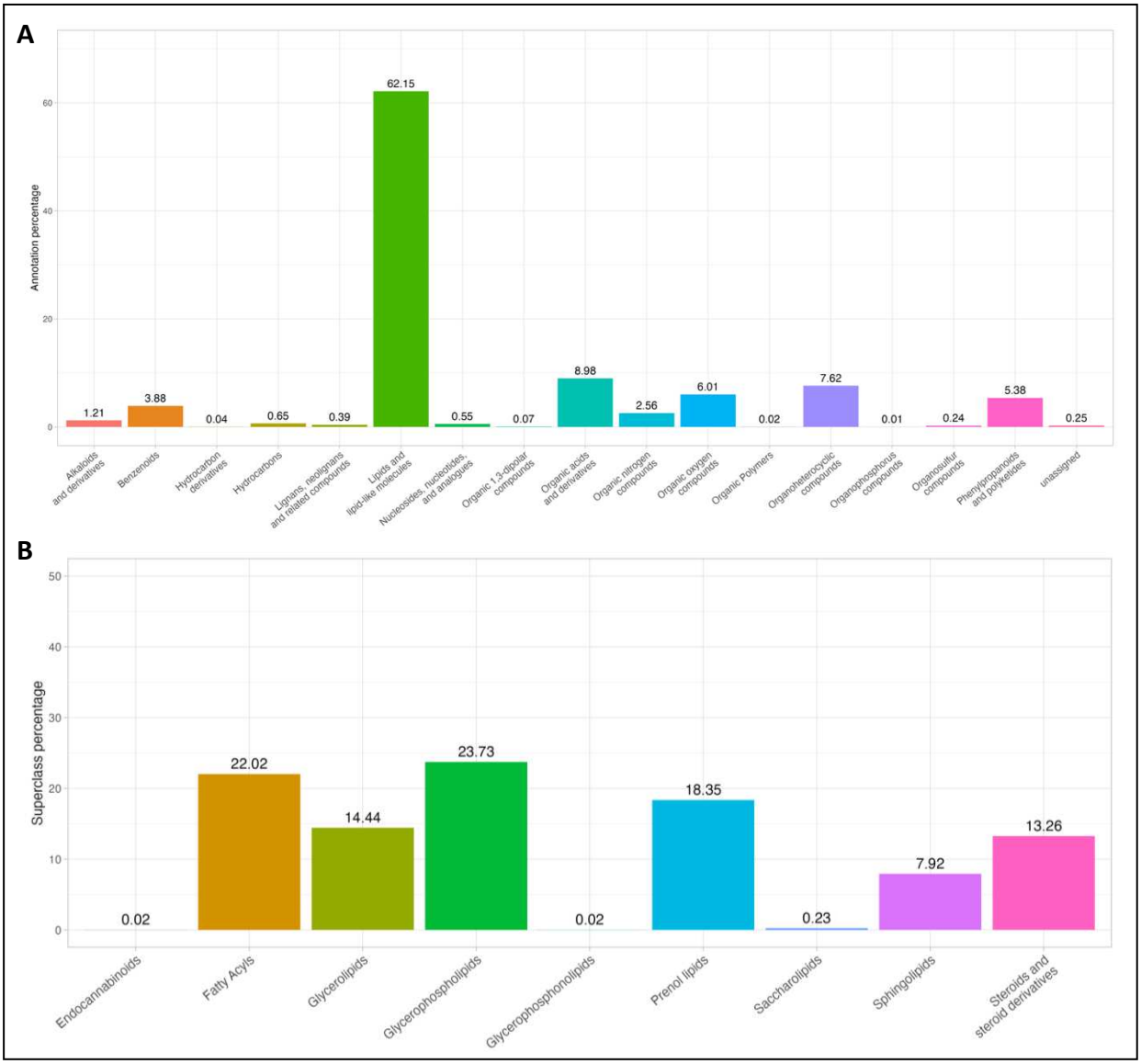


Figure 2. 1. Overall annotations and lipid-like molecules. A) The bar plot illustrated all the compiled compounds from the untargeted mass spectrometry study, including all

life stages, with 62.15% as lipids. **B)** Superclass lipid classification from all the lipid-like molecules.

2.2.2 The lipid metabolome defines each unique life stage of An. stephensi.

Ten thousand four hundred seventy-one unique metabolite features were confidently identified from our dataset. Seven-thousand six-hundred forty-six of these were used to measure variance between principal components across all mosquito life stages. The resulting principal component analysis demonstrated growth stage separation, with 41.8% of the variation explained by the first two principal component axes (Figure 2.2). Interestingly, the clustering of L1-L2 showed less variance, whereas L3, L4, and pupae exhibited a more substantial metabolic shift. The differentiation between L1 and L2 was particularly intriguing, as they are two phenotypically similar stages. This suggests that the underlying lipidomic profiles distinguish these early larval stages despite apparent similarities. Additionally, an analysis of the specific and crucial developmental lipid crusecdysone was found negligible in eggs and adult male mosquitoes and abundant in all other life stages (Figure 2.3).

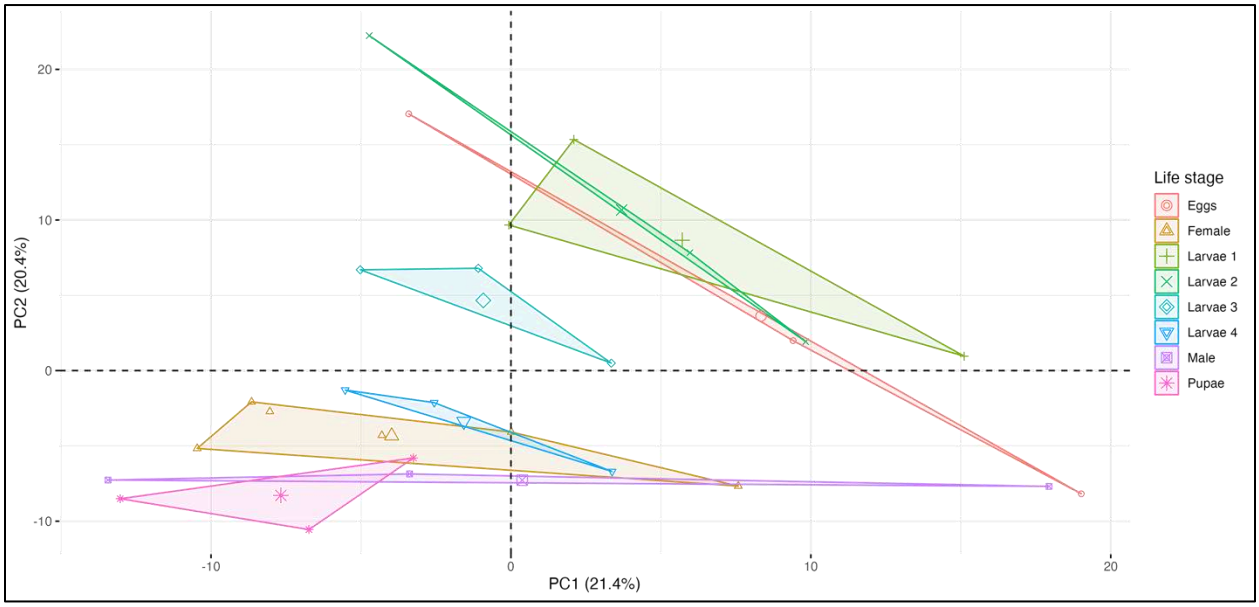


Figure 2. 2. PCA plot illustrating the comprehensive lipid metabolome of An. stephensi mosquitoes at each life stage. Principal component analysis (PCA). The X-

axis has the first principal component, and the Y-axis has the second. Each data point represents a biological replicate for each population, and ten individual samples of each growth phase of mosquito were used as a pool to represent one biological replicate.

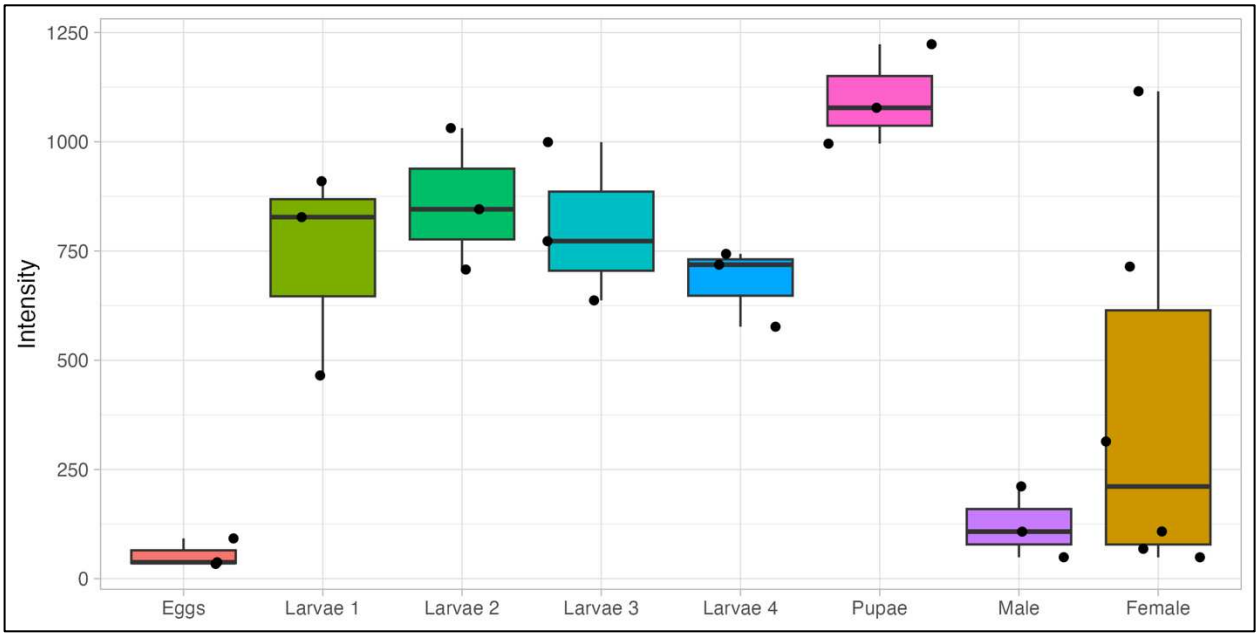
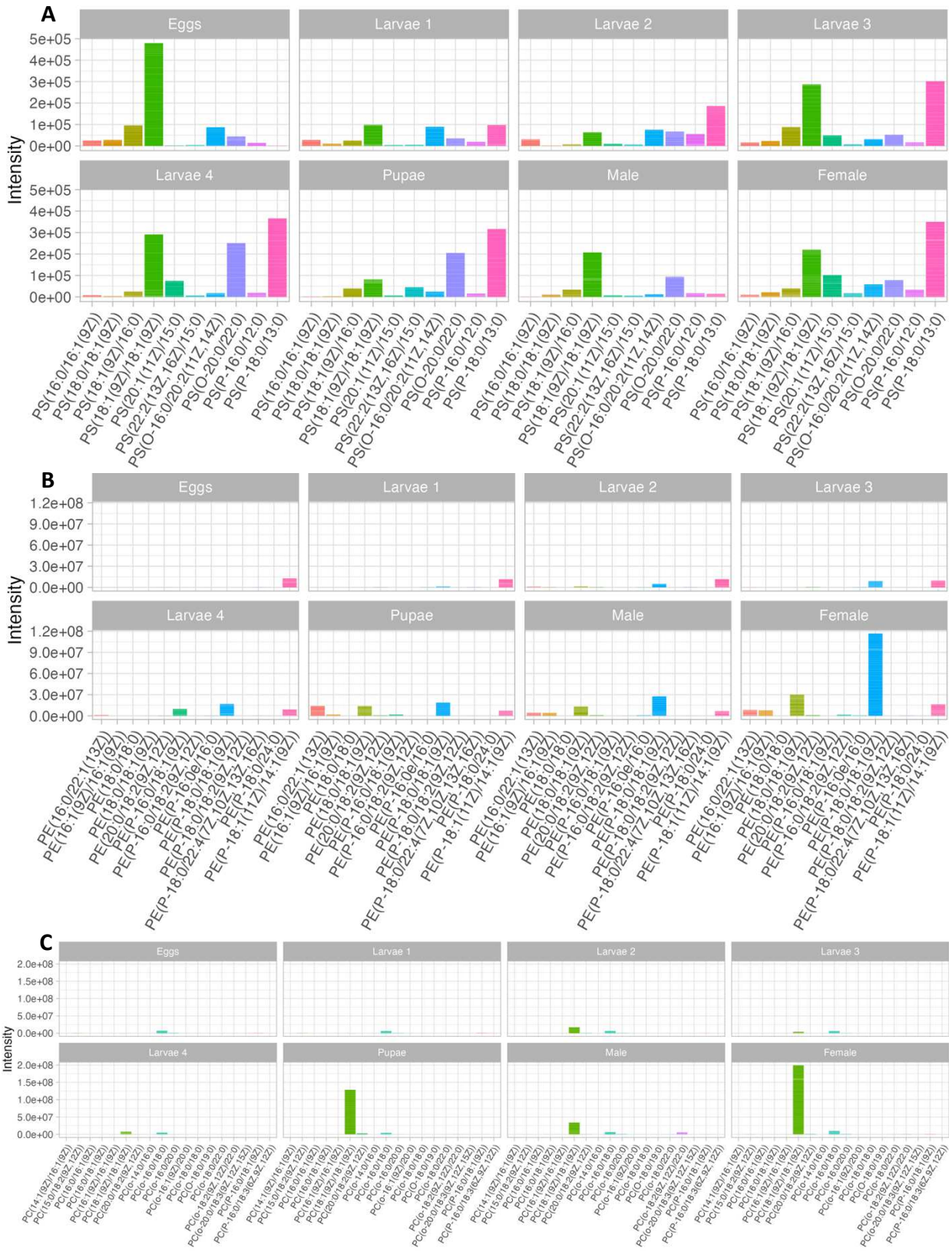
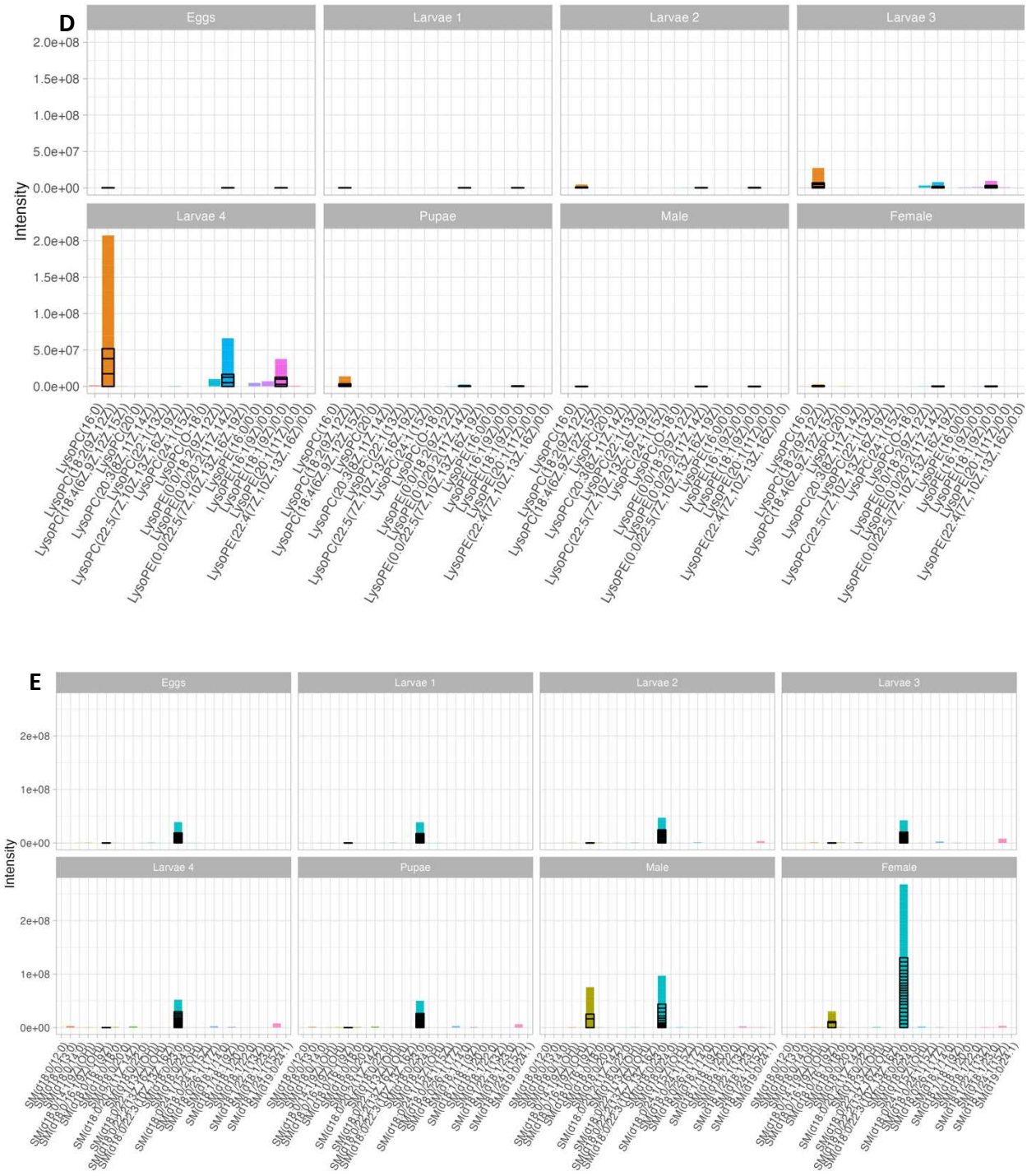


Figure 2.3. **Box plot illustrating peak intensities of 20E at different life stages.** The X-axis has the different life stages, and the Y-axis is the peak intensity of 20E.

An unbiased analysis across all life stages for lipid metabolites identified a predominance of phospholipids (PL), diacylglycerides (DG), and triacylglycerides (TG) as significant contributors to the lipid metabolome of *An. stephensi* across life stages (Figure 2.4A-G). Within these broad classes, phosphotidylserines were present in all life stages, albeit at a lower abundance than either PE or PC and to a lesser extent in L1 and L2 (Figure 2.4A). In contrast, the other two major classes of phospholipids, PE and PC, emerged as significant lipid contributors from pupation through adulthood, with PE most abundant in adult females (Figure 2.4B and 2.4C). Lysophosphotidylcholines (LysoPC) and lysophosphotidylethanolamines (LysoPE) classes were almost exclusively present in the L3 and L4 larval stages versus all other life stages. Lysophosphatidylcholine (LysoPC)

(16:0) was specifically abundant in L4 (Figure 2.4D). DGs and TGs were consistent across all life stages (Figure 2.4F and 2.4G), though TGs emerged as a dominant class of lipids in L4 larvae and remained so in adult males and females (Figure 2.4G).


A
Eggs
Larvae 1
Larvae 2
Larvae 3
Larvae 4
Pupae
Male
Female
Intensity
5e+05
4e+05
3e+05
2e+05
1e+05
0e+00
PS(16:0/16:1(9Z))
PS(18:0/18:1(9Z))
PS(18:1(9Z)/16:0)
PS(18:1(9Z)/18:1(9Z))
PS(20:1(11Z)/15:0)
PS(22:2(13Z,16Z)/15:0)
PS(O-16:0/20:2(11Z,14Z))
PS(O-20:0/22:0)
PS(P-16:0/12:0)
PS(P-18:0/13:0)
B
Eggs
Larvae 1
Larvae 2
Larvae 3
Larvae 4
Pupae
Male
Female
Intensity
1.2e+08
9.0e+07
6.0e+07
3.0e+07
0.0e+00
PE(16:0/22:1(13Z))
PE(16:1(9Z)/16:1(9Z))
PE(18:0/18:0)
PE(18:0/18:1(9Z))
PE(20:0/18:2(9Z,12Z))
PE(P-16:0/18:1(9Z))
PE(P-16:0/18:2(9Z,12Z))
PE(P-18:0/16:0)
PE(P-18:0/18:1(9Z))
PE(P-18:0/18:2(9Z,12Z))
PE(P-18:0/22:4(7Z,10Z,13Z,16Z))
PE(P-18:0/24:0)
PE(P-18:1(11Z)/14:1(9Z))
C
Eggs
Larvae 1
Larvae 2
Larvae 3
Larvae 4
Pupae
Male
Female
Intensity
2.0e+08
1.5e+08
1.0e+08
5.0e+07
0.0e+00


D
Eggs
Larvae 1
Larvae 2
Larvae 3
Larvae 4
Pupae
Male
Female
Intensity
2.0e+08
1.5e+08
1.0e+08
5.0e+07
0.0e+00
E
Eggs
Larvae 1
Larvae 2
Larvae 3
Larvae 4
Pupae
Male
Female
Intensity
2e+08
1e+08
0e+00

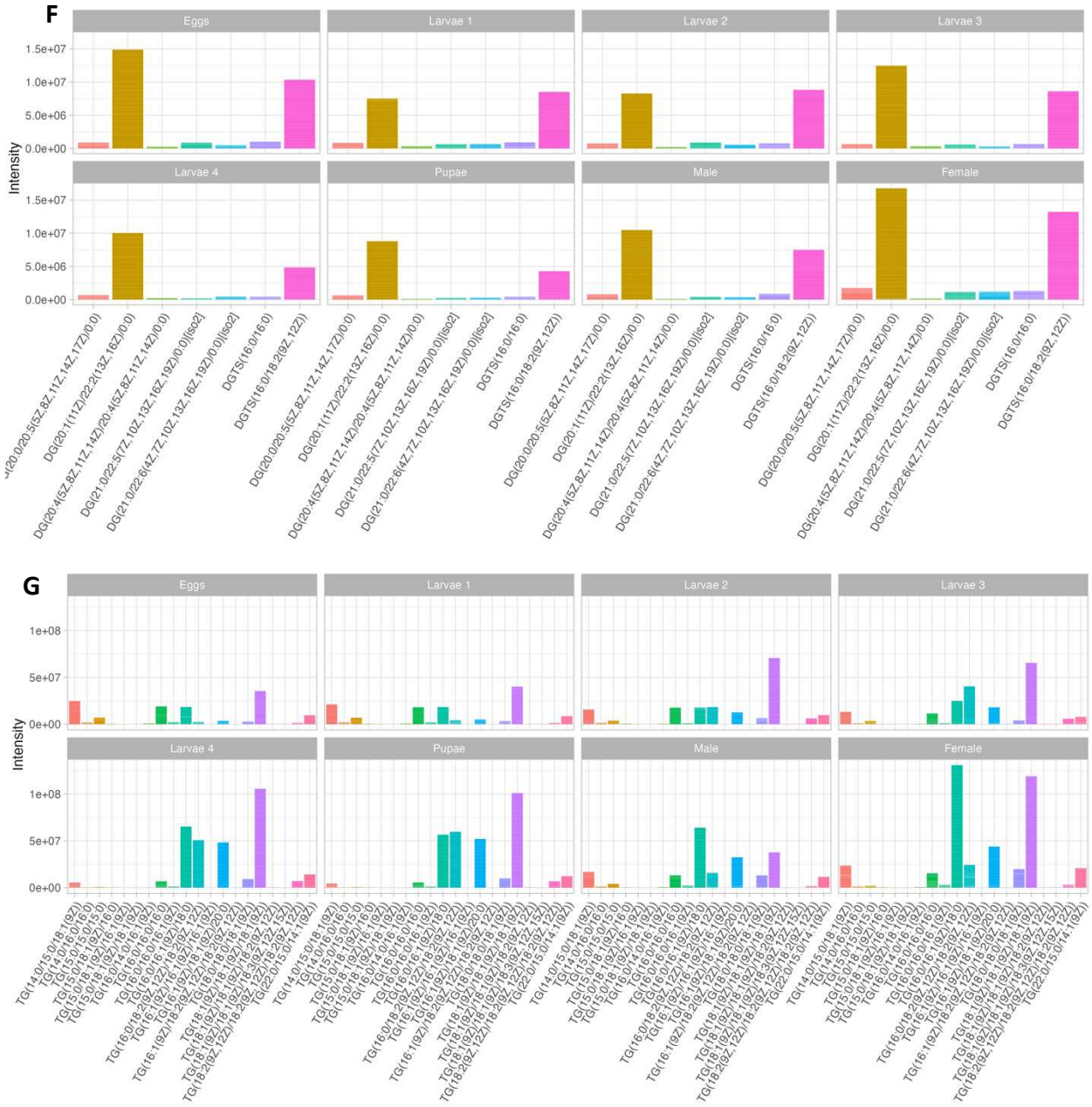


Figure 2.4. Abundance of PE, PC, SM, Lyso-, TG, PS DG, and cholesterol across life stages. Bar plots illustrating the different abundance of biologically essential lipids at each life stage: A) PE, B) PC, C) SM, D) LysoPC and LysoPE, E) TG, F) PS, G) DG, H) Cholesterol.

2.2.3 The lipid metabolome can distinguish L1 from L2 stage larvae.

The transition of 1st to 2nd instar larvae presents few phenotypic changes, including a minor increase in body size. PCA analysis of the metabolome (Figure 2.1) suggested

that differences may occur between these two early larval life stages. An analysis of significant differences demonstrated several differentially

abundant features between L1 and L2 stage larvae (Figure 2.5). Among these, TGs presented as a dominant class of lipids that were significantly lower in 1st instar larvae relative to 2nd instar larvae. Lipid metabolites with increased abundance in 1st instar larvae relative to 2nd instar larvae were heterogenous, with only phophotidylcholines (PC) presenting as a potential dominant class (Table 2.1).

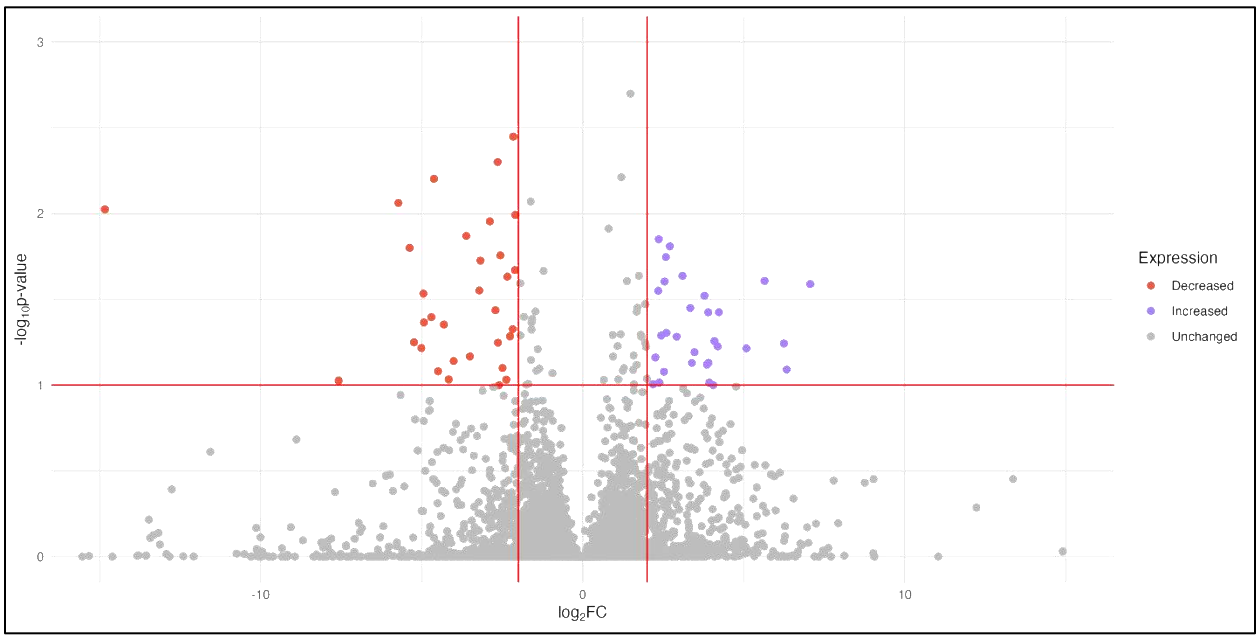


Figure 2.5. Volcano plot of lipids as Larvae 1 transition into Larvae 2. The volcano plot illustrates the unchanged lipids in grey increased in purple and decreased in red. The log_2Fold-Change was set up to 0.1, and -log_{10}P-value was set to -2 and 2.

Table 2.1 List of lipid classes from volcano plot

cmpd	Lipid class	log2fold.change	-log10.pvalue	Abundance (1st vs 2nd instar)	formula	Direct parent class
C00102	LysoPC (18:2)	-3.503	1.167	Decreased	C26H50NO7P	1-acyl-sn-glycero-3-phosphocholines
C00300	UNPD215090	-2.600	1.000	Decreased	C50H72N8O13	Cyclic depsipeptides
C00408	Triricinolein	-2.713	1.437	Decreased	C57H104O9	Triacylglycerols
C00950	1-alkyl-2-acylglycerol-1	-4.930	1.366	Decreased	C41H74O4	1-alkyl,2-acylglycerols
C04280	TG (18:1/20:1/20:1)	-4.493	1.081	Decreased	C61H112O6	Triacylglycerols
C04290	TG (20:0/20:0/20:4)	-5.009	1.216	Decreased	C63H114O6	Triacylglycerols
C04342	TG (18:2/20:1/20:4)	-2.342	1.632	Decreased	C61H104O6	Triacylglycerols
C04350	TG (18:0/20:1/22:6)	-7.580	1.026	Decreased	C63H108O6	Triacylglycerols
C04520	TG (22:1/22:0/18:2)	-4.311	1.353	Decreased	C65H120O6	Triacylglycerols
C05854	1-O-6-O-hexadecanoyl-a-D-glucopyranosyl-2-hexadecanoyloxy-eicosan-1-ol	-2.259	1.285	Decreased	C58H112O9	glycolipids
C05902	TG (20:1/20:1/20:4)	-5.241	1.250	Decreased	C63H110O6	Triacylglycerols
C06878	Nicandrose E	-2.174	1.327	Decreased	C31H54O14	glycolipids
C08738	TG (16:1/20:5/22:2)	-2.640	2.301	Decreased	C61H102O6	Triacylglycerols
C10148	Lanyuamide I	-2.109	1.670	Decreased	C18H31NO2	N-acyl amines
C00009	PC (16:0/18:3)	3.854	1.120	Increased	C42H78NO7P	1-(1Z-alkenyl),2-acyl-glycerophosphocholines
C00471	N-1H-1-3-benzodiazol-6-yl-2-0-acetamido-3-4-5-trimethoxy-13-oxotricyclo-9-5-0-0-hexadeca-1-16-2-7-3-5-11-14-hexaen-14-yl-amino-4-methylsulfanyl-butanamide	3.470	1.192	Increased	C33H37N5O6S	Methionine and derivatives
C00487	1-8-5-ladderane-octanoyl-2-8-3-ladderane-octanyl-sn-glycerophosphocholine	6.246	1.243	Increased	C48H80NO7P	1-acyl,2-alkylglycero-3-phosphocholines

C00679	Spirapril	5.084	1.214	Increased	C22H30N2O5S2	Dipeptides
C00697	7-Ethyl-10-(4-N-aminopentanoic acid)-1-piperidino)carbonyloxycamptothecin	6.334	1.091	Increased	C33H38N4O8	Camptothecins
C01345	N-isobutyl-N-4-methoxyphenylsulfonyl-glycyl-hydroxamic acid	4.085	1.257	Increased	C13H20N2O5S	Benzenesulfonamides
C02913	Dihydrostreptomycin-3-6-bisphosphate	7.061	1.589	Increased	C21H43N7O18P2	Aminocyclitol glycosides
C03119	ethyl-3-22-27-30-32-tetrahydroxy-21-28-bis-hydroxymethyl-2-9-12-31-tetraoxa-18-19-dithia-15-azahexacyclo-19-9-1-1-0-0-0-dotriaconta-3-5-7-10-25-pentaen-4-yl-propanoate	3.101	1.637	Increased	C32H43NO12S2	Benzofurans
C03755	N-acetyl-6-O-sulfo-D-glucosamine	2.437	1.290	Increased	C7H13NO9S	Monosaccharides
C03781	UNPD216962	2.377	1.015	Increased	C35H36O19	Complex tannins
C04983	UNPD15089	2.257	1.162	Increased	C26H34N6O14	Peptides
C05399	FAHFA-O (16:1/18:0)	2.183	1.005	Increased	C34H64O4	Long-chain fatty acids
C06971	Chartreusin	2.580	1.746	Increased	C32H32O14	Naphthopyranone glycosides
C07345	Oxohernandaline	4.227	1.425	Increased	C28H23NO8	Aporphines
C08425	CNP0431759	3.781	1.521	Increased	C45H80O8	Eunicellane and asbestinane diterpenoids
C09903	UNPD226076	2.706	1.810	Increased	C25H37NO3S	Prenylquinones

2.2.4 Lipid metabolism of adult mosquitoes

Adult mosquitoes exhibit a dynamic lipid metabolic profile due to various physiological processes and environmental factors influencing their energy demands, reproduction, and survival. The principal component analysis demonstrated separation amongst the three adult mosquito groups, with 57.8% of the variation explained by the first two principal component analyses (Figure 2.6). Although the clustering of the three groups seems intertwined, explaining the similarities of a life stage, there is a clustering describing the sugar-fed only female mosquitoes. Interestingly, the clustering of male and blood-fed female mosquitoes showed less variance.

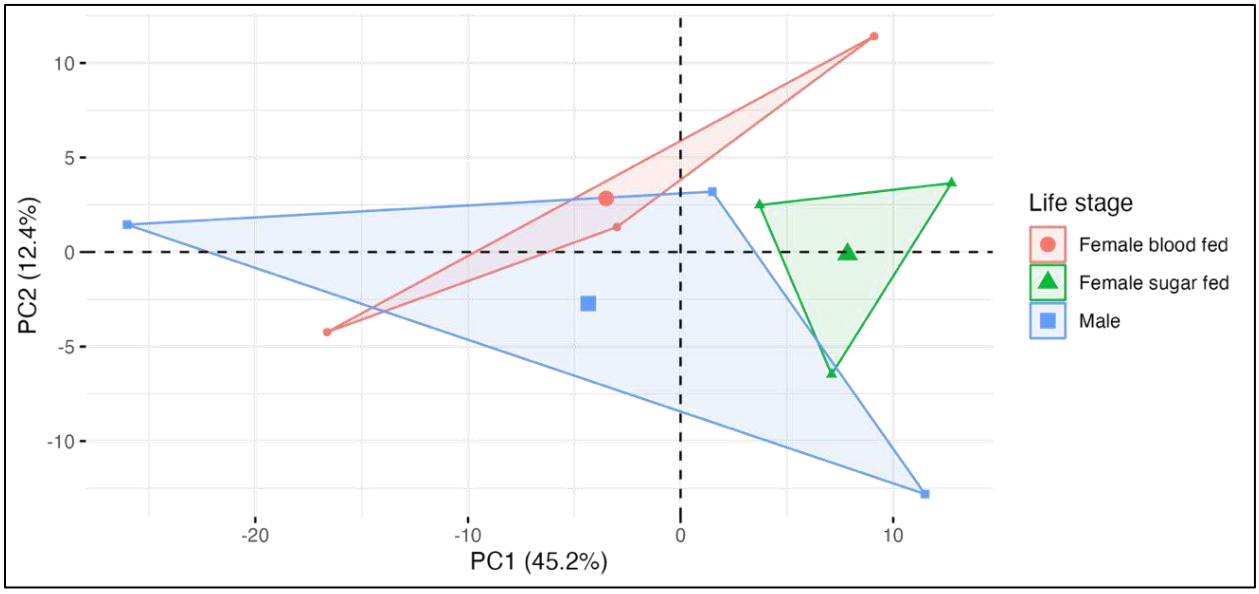


Figure 2.6. PCA illustrates the comprehensive lipid metabolome of adult *An. stephensi* mosquitoes. The X-axis has the first principal component, and the Y-axis has the second. Each data point represents a biological replicate for each population, and ten individual samples of each growth phase of mosquito were used as a pool to represent one biological replicate. Female blood-fed mosquitoes were collected 24 hours after blood meal.

Following the PCA, we carried out an analysis to illustrate what superclasses were found in these samples and what are the most prevalent. In adult mosquitoes, we found

that fatty acyls, prenol lipids, steroids, and steroid derivatives demonstrated 30.64%, 29.77%, and 36.71%, respectively. Other superclasses such as glycerolipids, glycerophospholipids, saccharolipids, and sphingolipids were found in much less percentage presence with 0.29%, 0.87%, 0.58%, and 1.16% respectively (Figure 2.7A). Hydroxystereoids found within the steroids and steroid derivates superclass typically exhibit higher biological activity than less polar steroids. Interestingly, among the three adult mosquito samples (female blood-fed, female sugar-fed, and males), all different classes were detected, with male and female blood-fed mosquitoes showing a more dynamic fluctuation (Figure 2.7B).

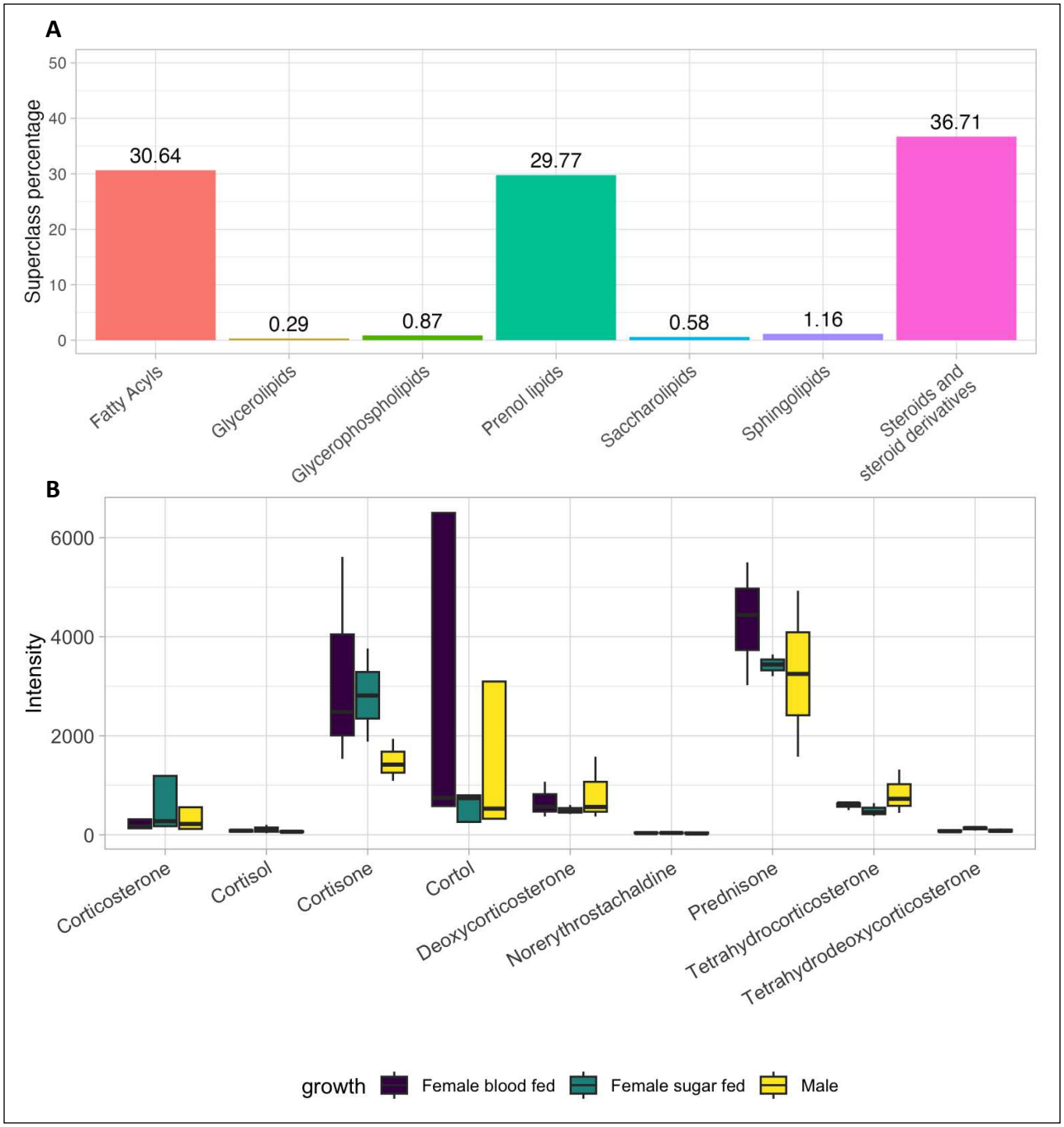


Figure 2.7. Analysis of *An. stephensi* mosquito lipid and lipid-like metabolites. A) Illustrates the percentage of superclass among the three adult mosquito samples: males, females who are blood-fed, and females who are sugar-fed. **B)** Further analysis from the steroids and steroid derivates superclass, hydroxysteroid, were extracted from the data set.

2.3 Discussion

The intricate life cycle of mosquitoes demands a finely tuned orchestration of energy resources, with lipid metabolism emerging as the architectural foundation governing this complex sequence of developmental stages. Our study explores the role of lipid metabolism, encompassing lipogenesis, lipolysis, and fatty acid metabolism, in shaping the transition between the mosquito life cycles. In holometabolous insects, the development transition is tightly correlated to feeding (larva and some adults) and non-feeding patterns (pupae and some adults). Major phospholipids in mosquito cells are PC (~30%) with neutral, cylindrical forms facilitating planar bilayers, and PE (~40%) with an inverted cone-shape with a small, polar head group which promotes a negative membrane curvature (Ratnayake et al., 2023, Trenti et al., 2022). Sphingomyelins (SM) are biosynthesized with the central molecule, ceramide linking to PC and glycosylated by glucosyl or galactosyl ceramide syntheses or phosphorylated by ceramide kinases (YA & LM, 2008). In our studies, PC, PE, and SM were dominant lipids in pupae, adult males, and, notably, adult female mosquitoes. Phosphatidylethanolamine is essential for the formation of glycosylphosphotidyl inositol (GPI) anchors. Proteins requiring GPI anchoring are requisite for embryogenesis, fertility, and immune responses (Kinoshita, 2016), thus supporting their presence as distinguishing features in adult and, notably, adult female mosquitoes. Non-bilayer lipids, such as DG, LysoPC, and LysoPE, do not readily conform to the conventional bilayer arrangements and can induce membrane curvature and participate in vesicle budding, membrane fusion, and protein trafficking, among other functions. These roles may explain the consistent abundance of DG in all life stages and the specific increased presence of LysoPC and LysoPE in the late larval stages of development. Triacylglycerides are the primary source of stored lipids pivotal

for energy metabolism, oogenesis, and diapause in adult mosquitoes (Hun et al., 2021; Tose et al., 2021). They account for the most abundant lipid found in the fat body. TGs are the predominant lipid transferred from the female mosquito to embryos (Tose et al., 2021). It is presumed that more polar lipids are then metabolized through the feeding larval stages, and thus, TGs may show a steady decrease in early larval stages (Sushchik et al., 2013). In our studies, TGs were identified as the dominant lipid class with reduced abundance in 1st instar larvae relative to 2nd instar larvae, which is somewhat confounding. One explanation may be that TGs are rapidly hydrolyzed as larvae hatch from eggs, with subsequent repletion as larvae feed. The rapid hydrolysis of TG to more polar and/or accessible lipids as requisite for hatching is further supported by the recent discovery of an adipose triacylglycerol lipase (ATGL), identified initially as brummer (Bmm) in *Drosophila* (Grönke et al. 2005)(Arrese & Soulages, 2009)). A homolog of Bmm was recently found with a similar activity in *Aedes aegypti*, with homologs also identified in *Anopheles sinensis* and *Anopheles darlingi* (dos Santos et al., 2024). Brummer expression was the most abundant in Drosophila embryos and adults, with negligible expression in 1st and 2nd instar larvae (Grönke et al. 2005). Studies by dos Santos corroborated those findings when Bmm expression was compared across larvae through adult mosquitoes; increased expression of Bmm correlated with decreased amounts of TG in these mosquito life stages (dos Santos et al., 2024). It is reasonable that TGs sequestered in *An.* The Bmm homolog rapidly hydrolyzes *An. stephensi* eggs to fuel hatching. Low Bmm expression in 1st and 2nd instar larvae (as shown in both *Drosophila* and *Ae. aegypti*) would permit rapid replenishment of TGs as larvae feed. Thus, the coordinated use, rebalancing, and accumulation of TGs as mosquitoes develop may be

a universal metabolic mechanism shared across mosquito families and other insect species with similar life cycles. Two LysoPCs were identified as increased lipids in 1st instar versus 2nd instar larvae, which may indicate increased endogenous cholesterol biogenesis upon hatching (Clayton et al., 1964; Clifton & Noriega, 2012).

Steroids are a class of organic compounds characterized by a core structure of four fused carbon rings, biosynthesized from sterols by steroidogenic enzymes found in specialized steroidogenic tissues (Miller & Auchus, 2011). They are vital for various biological functions across all eukaryotic organisms. In mosquitoes, essential steroid derivatives include ecdysteroids and juvenile hormones, pivotal in regulating development and reproduction. We found that crustecdysone (20-hydroxyecdysone, 20E), a prominent ecdysteroid, is present in a greater abundance in all four instar larvae stages and the pupation stage versus other stages. Crustecdysone is an essential mosquito hormone regulating several processes throughout the mosquito life cycle, including molting, insecticide resistance, and fecundity (reviewed in Ekoka et al., 2021). Previously, 20E agonists were shown to be effective insecticides in *An. gambiae*, *Aedes aegypti*, and other mosquito larvae (Beckage et al., 2004). Levels of 20E were also shown to increase as *Ae. aegypti* L4 larvae ecdysis to pupae (Margam et al., 2006).

Interestingly, these previous studies exploring 20E's importance in molting (et al., 2006) and as a target for novel insecticides (Beckage et al., 2004) were conducted on 4th and 3rd instar larvae, respectively. Our studies suggest that 20E may play additional mosquito maturation roles, and its agonists may be more effective on newly hatched eggs, as this hormone is abundant in all larval stages. Hydroxysteroids are more polar than non-hydroxylated steroids due to their hydroxyl groups, which influence their

biological activity and solubility (Szaleniec et al., 2018). Corticosteroids, in vertebrates, are produced in the adrenal cortex and involve two main classes: aldosterone and glucocorticoids. Within the glucocorticoids and aldosterone classes, steroid hormones such as cortisol, cortisone, corticosterone, and prednisone (Timmermans et al., 2019). Interestingly, our data shows the intensity of cortisone and prednisone as higher in blood-fed females. In *Drosophila* and *Culex quinquefascuiatus*, exposure to cortisone increased the sensitivity to fungal challenge (Bartolo et al., 2020). Prednisone is derived from cortisone, a synthetic anti-inflammatory targeting the immune system to relieve redness, itching, allergic reactions, and swelling (Buchwald & Bodor, 2004). When females acquire a blood meal, they face considerable physiological stress due to rapid changes in temperature and pH, gut distension, heme toxicity from hemoglobin digestion, and redox stress in the midgut (Benoit et al., 2011; Champion & Xu, 2017; Lahondère & Lazzari, 2012; Oliveira et al., 1999; Peterson & Luckhart, 2006; Wang et al., 2021). Neurosteroids such as tetrahydrodeoxycorticosterone (THDOC), dihydrodeoxycorticosterone (DHDOC), and tetrahydrocortocosterone (THB) focus on their roles in mammalian physiology, particularly in the central nervous system (Sze & Brunton, 2022). However, investigating their presence and effects in other organisms, such as mosquitoes, might not be as extensively studied. Nonetheless, we found their presence in all three different adult mosquito samples.

In this study, we deployed a mass spectrometry approach to describe the lipidome of *An. stephensi* across all life stages. Our studies supported and built upon data from other metabolomic, proteomic, and genomic analyses and identified potential pathways serving unique roles during mosquito development. Further investigation of these

pathways can now be explored to understand further environmental adaptation parasite resistance and help ensure high-fidelity laboratory management of colonies.

2.3 Materials and methods

2.4.1 Mosquitoes

Anopheles stephensi mosquito eggs were obtained through BEI Resources as part of the Biological Reagents Repository (NIAID, NIH: *Anopheles stephensi*, Strain STE2, MRA-128, contributed by Mark Q. Benedict). Colonies were maintained at 28°C (± 0.5°C) with a 12:12 light: dark photoperiod and 75% (± 2%) relative humidity, with water and sugar provided *ad libitum.* Adult mosquitoes were fed warmed defibrinated calf blood in artificial glass blood feeders. Eggs were collected on coffee filters partially submerged in water cups. They were hatched two days post-oviposition in small bins with deionized water. Larvae were moved to a more significant bin 48-72 hours later to continue development. Larvae were raised on powdered fish food added as a supplement to tap water hatch bins. For experiments, eggs were collected before hatching, L1s (18 hours post-egg-hatching), L2s (36 hours post-egg-hatching), L3s (4 days post-egg hatching), L4s (5 days post-egg-hatching), pupae (7 days post-egg-hatching), nonblood fed adult females and males were collected 3 – 5 days post-emergence, and finally, blood-fed adult females were collected 24 hours after blood feed.

2.4.2 Extraction of small molecules

Ten individual samples of each growth phase of a mosquito were used as a pool representing one biological replicate. Three biological replicates were used for each

population. Samples were immediately submerged in methanol and stored at -80°C until processed. Before sample processing, methanol was thoroughly dried under nitrogen. Metabolites were extracted into 400µL chloroform:methanol:water (2:1:1) by homogenization using a manual pestle homogenizer, followed by incubation at 4 °C for 30 minutes with rocking. Samples were then centrifuged for 10 minutes at 805 x g. The chloroform layer was collected, and samples were thoroughly dried under nitrogen and stored at -80°C until samples were processed for mass spectrometry as described below.

2.4.3 Sample Preparation for mass spectrometry

Samples were received, dried, and stored at -80°C until prepared. Samples were resuspended 200 µL of 80% isopropanol, then shaken at 4°C for an hour, and 100µL of this was added to an HPLC vial insert; 50µL of each remaining sample was pooled to generate a quality control sample. Following an initial LC/MS run to estimate the relative concentration of samples, each sample was recovered from vial inserts by briefly inverting and centrifuging them. The total XCMS extracted signal was calculated and used to measure concentration. Concentrations were then normalized by diluting the recovered samples with 80% isopropanol with brief mixing using a vortex.

2.4.4 UPLC-MS Analysis

The sample order was fully randomized, with a pooled QC sample injected approximately every 8 injections. 3µL were injected per sample for final analysis after normalizing concentration. Separation was achieved using a Waters Acquity UPLC CSH-PhenylHexyl column (1.7µM, 2.0 x 100mm), using a gradient from solvent A (Water, 0.1% formic acid, 2mM Ammonium hydroxide) to solvent B (Acetonitrile, 0.1% formic acid).

Injections were made in 50% A, held at 99% A for 1 min, ramped to 98% B over 7 minutes, held at 98% B for 2 minutes, and then returned to starting conditions over 0.05 minutes and allowed to re-equilibrate for 2.95 minutes, with a 600µL/min constant flow rate. The column and samples were held at 65 °C and 6 °C, respectively. The column eluent was infused into a Waters Xevo G2-XS Q-TOF-MS with an electrospray source in positive mode, scanning 50-1200 m/z at 0.1 seconds per scan, alternating between MS (6 V collision energy) and MSE mode (15-30 V ramp). Calibration was performed using sodium formate with 1 ppm mass accuracy. The capillary voltage was held at 700 V in positive ionization mode. The source temperature was set to 150°C and nitrogen desolvation at 450°C with a flow rate of 1000L/hr. Lockspray infusion of Leucine Enkaphalin (LeuEnk) was used for mass correction, with corrections applied every 40 seconds from 3 scans of 0.1 s each, theoretical mass +556.2771 m/z.

2.4.5 Data Analysis and Statistics

Data were converted from.RAW format to .mzML using Proteowizard MSConvert. XCMS (Smith et al., 2006, Tautenhahn et al., 2008), v3.16.1] version 3.20.0 was used to process raw data using R v4.2.2. The following processing steps were used: (1) Peak detection (CentWave) : ppm = 30 peakwidth = c(2.2, 15), snthresh = 10, prefilter = c(3, 10), mzCenterFun = wMean, integrate = 1, mzdiff = 0.01, fitgauss = TRUE, noise = 2, verboseColumns = TRUE, roiList = list(), firstBaselineCheck = TRUE, roiScales = numeric(0), extendLengthMSW = TRUE. (2) Peak grouping (PeakDensity) : bw = 4, minFraction = 0.5, minSamples = 1, binSize = 0.015, maxFeatures = 50. (3) Retention time correction (PeakGroups) : minFraction = 0.5 extraPeaks = 1, smooth = loess, span = 0.2, family = gaussian, subset = integer(0), subsetAdjust = average. (4) Peak grouping

(PeakDensity) : bw = 1.75, minFraction = 0.45, minSamples = 1, binSize = 0.015, maxFeatures = 50. (5) Missing peak filling (FillChromPeaks) : expandMz = 0 expandRt = 0, ppm = 0, fixedMz = 0, fixedRt = 0. RAMClustR version 1.2.4 in R version 4.2.2 (2022-10-31)) was used to normalize, filter, and group features into spectra. XCMS (Smith et al., 2006, Tautenhahn et al., 2008) output data was transferred to a ramclustR object using the rc.get.xcms.data function, which utilizes the xcms featureValues function. Features with missing values were replaced with small values simulating noise: for each feature, the minimum detected value was multiplied by 0.5. Noise was then added using a factor of 0.5. The absolute value of this value was used as the filled value to ensure that only non-negative values carried forward. Variance in quality control samples was described using the rc.qc function within ramclustR. Summary statistics are provided including the relative standard deviation of QC samples to all samples in PCA space, as well as the relative standard deviation of each feature/compound in QC samples, plotted as a histogram. Features were normalized by linearly regressing run order versus qc feature intensities to account for instrument signal intensity drift. Only features with a regression p-value less than 0.05 and an r-squared greater than 0.1 were corrected. Of 105326 features, 5862 were corrected for run order effects. Features were filtered based on their qc sample CV values. Only features with CV values less than or equal to 0.5 in MS or MSMS data sets were retained. 46087 of 105326 features were removed. Features were clustered using the ramclustR algorithm (Broeckling et al., 2014). Parameter settings were as follows: st = 2.05, sr = 0.5, maxt = 205, deepSplit = FALSE, hmax = 0.3, minModuleSize = 2, and cor.method = pearson. Molecular weight was inferred from in-source MS and MS/MS spectra (Broeckling et al., 2016) using the do.findmain function,

which calls the interpretMSSpectrum package (Jaeger et al., 2017). Parameters for do.findmain were set to: mode = positive, mzabs.error = 0.005, ppm.error = 10, ads = [M+H]+ [M+Na]+ [M+K]+ [M+NH4]+ [2M+H]+ [2M+Na]+ [2M+K]+ [2M+NH4]+ [3M+H]+ [3M+Na]+ [3M+K]+ [3M+NH4]+, nls = [M+H-COCH2]+ [M+H-C2H3NO]+ [M+H-H2O]+ [M+H-NH3]+ [M+H-HCOOH]- [M+H-C6H12O6]+ [M+H-C5H10O5]+ [M+H-C12H22O11]+. MSFinder (Tsugawa et al., 2016) was used for spectral matching, formula inference, and tentative structure assignment. Results were imported into the RAMClustR object. A total score was calculated based on the product scores from the find main function and the MSfinder formula and structure scores. A total of 35499 annotation hypotheses were tested for 10471 compounds. Spectra matches took precedence over computational inference-based annotations. The following database(s) were assigned as 'priority': hmdb, lipidmaps. The database priority.factor was set to 0.95 to decrease scores for compounds that failed to match priority database(s). Each compound's highest total score was selected, considering all adduct hypotheses.

2.4.6 Annotation Summary

Annotations have been computationally assigned and are summarized in csv file, which you can open in excel. InterpretMSspectrum annotates features to assign probably molecular weight. MSFinder is then used to perform automated LC-TOF spectral annotation to assign molecular formulas and structure. MSFinder output is a zipped directory called 'mat' and can be viewed using the MSFinder program. Instructions for viewing these files can be found in a supplemental file. The annotation csv file has two forms: 'all.annotation.csv' and 'assigned.annotations.csv'. The compound is assigned the highest scoring match, and only that highest scoring match is reported in

‘assigned.annotations.csv’, while, as the name suggests all annotations are reported in ‘all.annocations.csv’.

2.4.7 Data visualization

Figures 2.1-2.7 were generated using R (version 4.3.1(2023-06-16)) with the following packages: ggplot2, ggpubr, tidyverse, broom, stringr, tidyr, viridis, plyr, dbplyr, forcats, grepel, RCurl, utils, scales, factoextra, and FactoMineR. For figure 2.1A, the percentage calculation was set so that the total from each super class was divided by all total annotations. For figure 2.1B, only the lipids and lipid-like molecules were plotted across life stages. For Figure 2.2, data set was filtered to only contain the lipid-lipid like metabolites. For figure 2.3, the data was filtered by lipid name 'Crustecdysone'. For figures 2.4A-G, percentages filtering were carried out: PE > 10%, PC > 1.5%, SM no percentage filtering, LPL, PS, DG > 2% , and TG > 1%. For figure 2.5, the mean of the fold change for each metabolite, followed by joining the dataset containing the P-values from larval stage 1 transitioning to larval stage 2 (L1-L2). The increased expression was decided by having a $\log_2$fold change > 2 and -$\log_{10}$P-value < 0.1, decreased expression by having a $\log_2$ fold change < -2 and -$\log_{10}$P-value < 0.1, everything else outside of those parameters were labelled as unchanged. For Figure 2.6, the dataset set was filtered to only contain the adult mosquito stages and lipid-lipid like metabolites. For Figure 2.7A, the percentage calculation was set so that the total from each super class was divided by all total annotations, based on the filtering in Figure 2.6. For Figure 2.7B, the filtered dataset from Figure 2.7A was used, in addition to filtering by “Hydroxysteroids”.

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CHAPTER 3: CRYOPRESERVATION METHODS FOR *ANOPHELES STEPHENSI* MOSQUITOES

3.1 Introduction

The concept of cryopreservation has long fascinated scientists and science fiction enthusiasts alike. This technique, reminiscent of suspended animation in futuristic narratives, promises to indefinitely preserve life or biological materials until they can be revived or utilized. The groundbreaking work of researchers like Polge, Parkes, and Smith in the late 1940s, who discovered the cryoprotective properties of glycerol while attempting to preserve avian spermatozoa, laid the foundation for advancements in cryopreservation and the exploration of suspended animation (Polge et al., 1949). Building upon this foundation, subsequent studies by Fahy and Wowk in the 1980s introduced the concept of vitrification. In this process, biological samples are solidified without forming ice crystals, minimizing cellular damage during freezing (G. M. Fahy et al., 1984). This breakthrough revolutionized cryopreservation methods, enabling the successful preservation of complex biological tissues and organs for transplantation (Wowk et al., 2000). Through innovative research and interdisciplinary collaboration, the field of cryobiology continues to evolve, with each discovery paving the way for future breakthroughs in medicine, biotechnology, and beyond.

At temperatures below -130 °C, the kinetic energy and molecular activity within biological systems decrease. This slowdown leads to a reduction in chemical and biological reactions, including metabolism, enzymatic processes, and diffusion. As a result, the material enters a suspended state until the temperature rises. While ultralow temperatures themselves do not cause cryoinjury, the freezing and thawing procedures

can damage the material through various mechanisms. Cryopreservation employs a multifaceted approach to safeguard cellular structures and functions during freezing and thawing processes. One of the primary challenges of cryopreservation is the formation of ice crystals within cells during freezing, which can cause mechanical damage and disrupt cellular structures. Approximately 70% of the total cell mass is composed of water that, if subjected to freezing temperatures, could form intracellular ice formation (IIF) can be fatal (Mazur, 1960). Cryoprotectants (CPAs) are crucial in controlling salt concentration and mitigating the damaging effects of freezing temperatures on biological materials. These are chemical compounds that can be broadly classified into non-penetrating and penetrating cryoprotectants. Non-penetrating CPAs cannot cross the cell membrane, but this is important for water loss during freezing and thawing to counterbalance the water that will move into the cells with the penetrating CPA, and generally exhibit low cytotoxicity (e.g., sucrose and trehalose). Penetrating CPAs support cell survival by reducing the freezing point of water and inhibiting intracellular ice formation, though these CPAs can exhibit cytotoxicity effects at prolonged exposure timeframes or high concentrations (e.g., DMSO, ethylene glycol) (Figure 3.1).

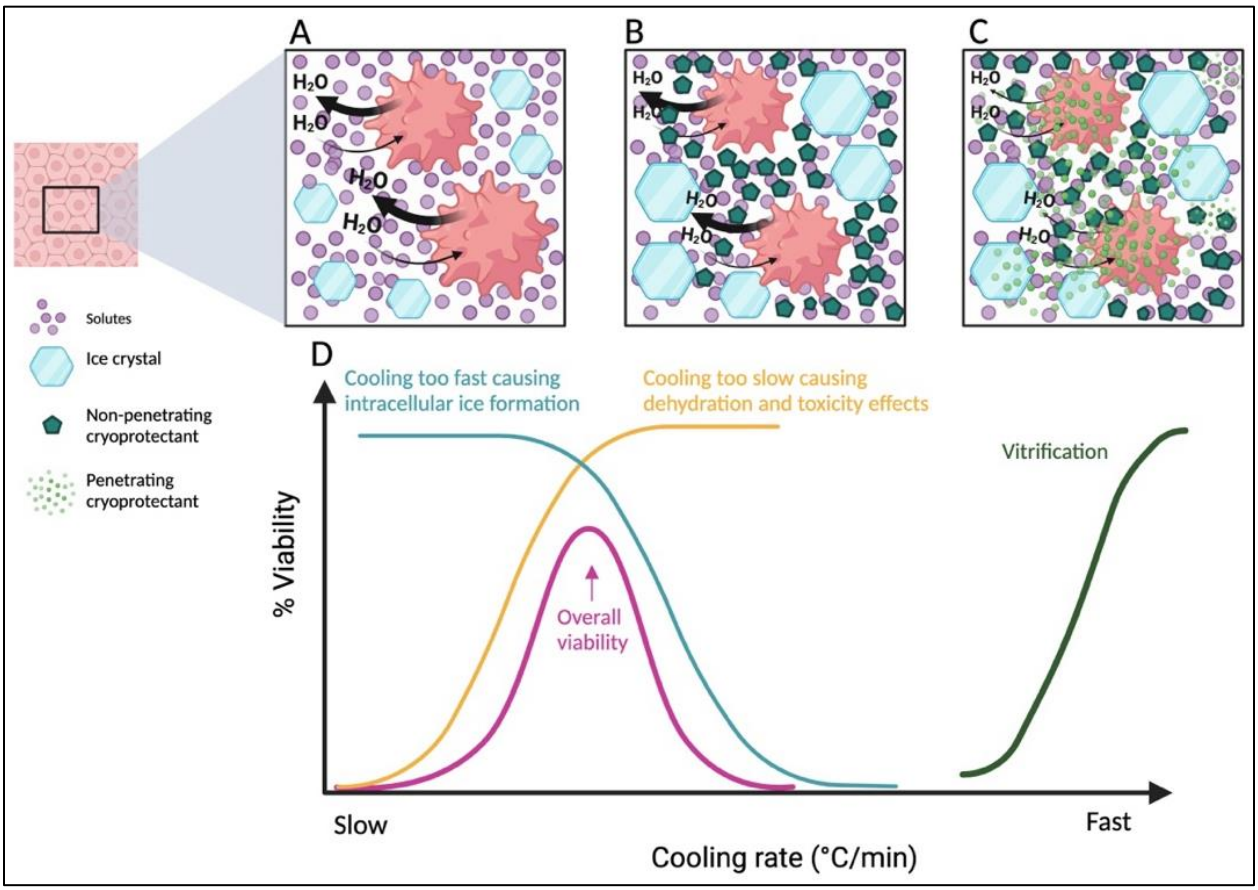

Figure 3.1. Fundamentals of cryopreservation. A) Illustrates cells exposed to freezing temperatures, causing extracellular ice formation (light blue) and solute (purple dots) homeostasis to be lost and causing a hypertonic solution. **B)** Illustrates the role of non-penetrating CPAs (dark green) in inducing a hypertonic solution, but it can still form extracellular and intracellular ice formation. **C)** Illustrates an ideal isotonic solution where both penetrating (light green) and non-penetrating (dark green) CPAs effectively reduce the damaging effects of ice formation/growth on cells. **D)** The graph describes the percent viability on the y-axis and the cooling rate on the x-axis from slow to fast. When applying a fast-cooling rate (turquoise line), IIF is caused; conversely, a slow-cooling rate (yellow line) causes dehydration and toxicity. An overall viability (pink line) exhibits the ideal cooling rate. However, vitrification (dark green) utilizes an ultra-fast cooling rate that can mitigate cryoinjury.

Researchers are exploring alternative cryoprotectants with lower cytotoxicity profiles, such as polyols, sugars, and synthetic polymers, to address cryoprotectant toxicity. Strategies such as gradual exposure to cryoprotectants, pre-conditioning treatments, and post-thaw washing procedures are also being investigated to mitigate cytotoxic effects

while maintaining cryopreservation efficacy. The three main cryopreservation techniques include slow freezing, which involves the liquid phase changing to a solid crystalline phase; vitrification (also known as ultra-rapid cooling), which consists of the solidification to a glass-like state without ice formation; and desiccation (Best, 2015; Seki & Mazur, 2012).

Scaling up cryopreservation techniques for large-scale industrial and conservation applications presents unique cost, efficiency, and logistical challenges. Traditional cryopreservation methods, such as slow freezing and vitrification, may be impractical for large-scale preservation due to high cryoprotectant volumes, lengthy processing times, and specialized equipment requirements. To address these challenges, researchers are exploring alternative approaches such as freeze-drying (lyophilization), encapsulation, and cryopreservation in microfluidic devices. These methods offer potential advantages in reduced cryoprotectant volumes, increased throughput, and compatibility with automation, making them suitable for large-scale preservation of cells, tissues, and even whole organisms. Additionally, advances in cryo-storage facilities, cold chain logistics, and quality control protocols are essential for ensuring the long-term viability and accessibility of cryopreserved materials on a large scale (Lovelock & Bishop, 1959).

The cryobiology field has a rich history of applications across biological systems, with much of its founding principles on single-cell types. Still, it has also expanded to tissues and whole organisms. Initial successes in cryopreserving sperm cells and embryos of various animal species were reported in the mid-20th century, paving the way for broader applications in reproductive biology (Lovelock & Bishop, 1959; Willadsen, 1979). Cryopreservation of tissues, such as corneas, blood vessels, and pancreatic islets,

has been developed for transplantation purposes, extending the shelf life of donor tissues and organs (Karlsson & Toner, 1996; Pegg, 2002). Cryopreservation of tiny organisms, such as nematodes and insects has been completed for research and conservation purposes, enabling the long-term storage of genetic diversity (Barranco & Risco, 2022; Zhan et al., 2021).

Significant advancements in insect cryopreservation have been made in the last two decades, offering promising solutions for conservation efforts and genetic resource management. One notable example is the cryopreservation of honeybee (*Apis mellifera*) semen, a crucial step in preserving genetic diversity and breeding valuable traits (Niño & Cameron Jasper, 2015). Researchers have developed optimized protocols for the cryopreservation of honeybee semen using a combination of cryoprotectants and controlled freezing techniques. Furthermore, researchers have explored vitrification techniques for the cryopreservation of *Drosophila* embryos, achieving high post-thaw survival rates (~68%) and developmental competence (Schreuders & Mazur, 1994; Steponkus et al., 1990; Zhan et al., 2021). Additionally, cryopreservation techniques have been applied to economically essential insect species, such as the silkworm (*Bombyx mori*) enabling the long-term storage of genetic resources and facilitating breeding programs (Jingade et al., 2015).

Efforts to cryopreserve mosquitoes have gained traction in recent years due to their significance in various fields, such as genetic research, vector biology, and disease control. Currently, mosquito lines must be continuously maintained, requiring arduous work that inevitably risks genetic drift, contamination, and the establishment of genetically modified strains. *An. stephensi* mosquitoes transmit the parasite *Plasmodium* and are

known to be distributed throughout Asia. However, recent studies have shown that they are expanding to Africa (Balkew et al., 2020), increasing the number of people at risk of contracting malaria. Although several mosquito control and surveillance strategies have been applied, there still needs to be a gap in fully understanding the vector, *An. stephensi*. With the abovementioned risks, the burden of continuously and laboriously keeping this mosquito line in the laboratory makes it challenging to study the *plasmodium* parasite or other pathogens transmitted by this model. To alleviate this challenge, it is essential to devise a process for preserving *An. stephensi* mosquitoes at one life stage, ideally targeting the least complex stage.

3.2 Results

3.2.1 Establishing methylformamide as cryoprotectant.

Cryoprotectants are not universally effective; different cell types may necessitate distinct cryoprotectant solutions (Bojic et al., 2021). Therefore, our group explored the toxicity of five commonly used cryoprotective agents ethylene glycol (EG), methanol (MeOH), dimethyl sulfoxide (DMSO), N-methyl acetamide (MA), and N-methyl formamide (MF) at a concentration of 1.5 M. MF was the most effective cryoprotectant even while incubating first-larval stage larvae (L1s) in it for 60 minutes at room temperature (Figure 3.2.1).

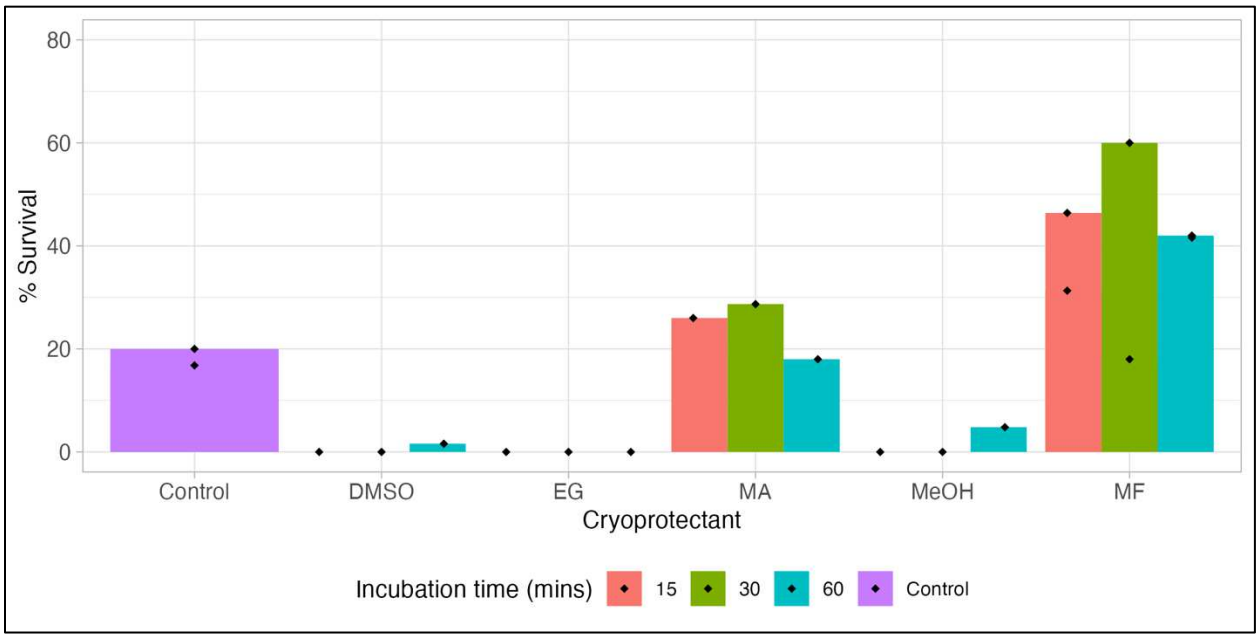

Figure 3.2.1. Assessing cryoprotectants and impacts on survival. For each CPA, 125-150 L1 larvae were incubated in the CPA for 15, 30, or 60 minutes at room temperature, then rinsed in water three times before being returned to normal growth conditions (water, food source, temperature, etc.), for the control group, they were only rinsed and placed back to normal growth conditions. The control group exhibited 16.8% and 20% survival, due to the pupae counted for each group. The group exposed to DMSO exhibited 0% survival at 15 and 30 minutes and 1.6% at 60 minutes. The group exposed to EG and MeOH exhibited 0% survival at the three incubation times. Exposure to MF was replicated twice. This showed 44% and 31.3% survival at 15 minutes, 60% and 18% survival at 30 minutes, 41.6% and 42% survival at 60 minutes. The group exposed to MA exhibited 26% survival at 15 minutes, 28.7% survival at 30 minutes, and 18% survival at 60 minutes.

To determine the permeability of MF across the L1 membrane, we used nuclear magnetic resonance (NMR). L1s were incubated in 1.5 M MF for 0.5-60 minutes, then rinsed larvae in deuterated water (D_2O) three times. NMR was used to analyzed a sample of D_2O from each rinse to determine the amount of labeled MF that diffused out of CPA-treated larvae from each incubation time (Figure 3.2.2). MF rapidly intercalated in the L1 within 1 minute of incubation in the CPA, and with gentle washing, the MF readily diffused out of the L1 larvae in a time-dependent manner. These results confirmed that MF rapidly permeabilizes and efficiently diffuses out of L1 membranes.

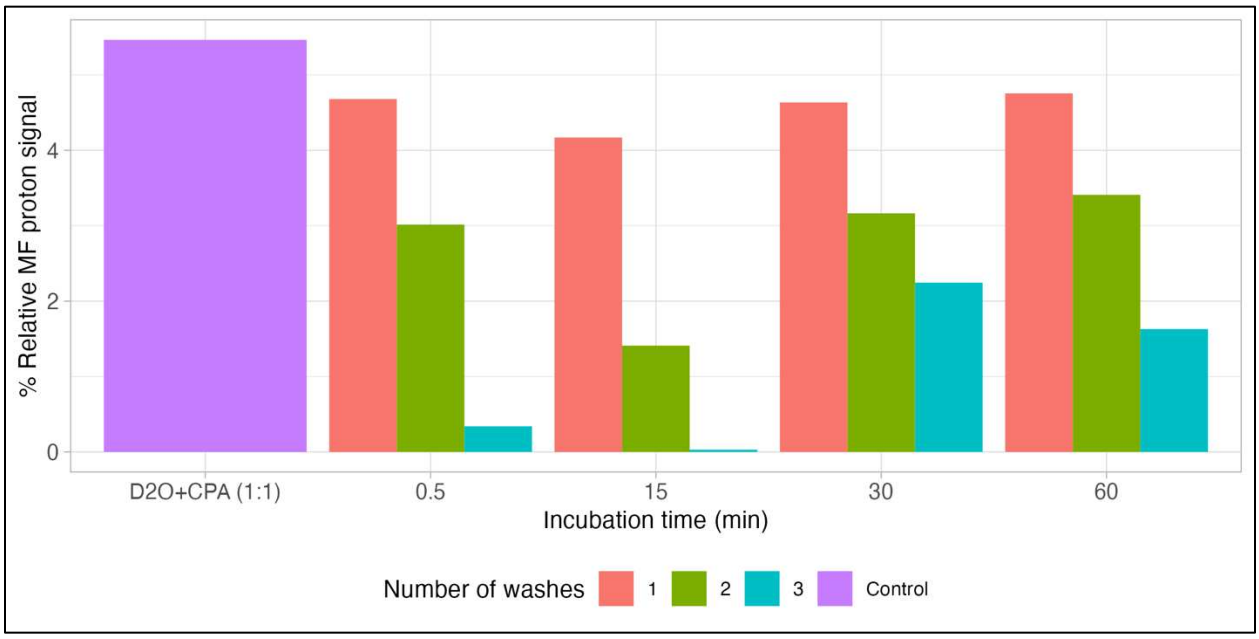

Figure 3.2.2. Methylformamide efficiently permeabilizes L1 membranes. L1s were incubated in 1.5 M MF for 0.5 - 60 minutes. Control is MF + D_2O at a 1:1 ratio. Values are normalized to the NMR signal of D_2O alone.

3.2.2 Impact of temperature and early versus late stage L1 when treated with methylformamide.

To determine the pupation rates of larvae exposed to MF immediately after emergence at (<1.5 hours post-emergence; early L1) or later (>1.5 hours post-emergence; late L1), L1s were exposed to 7 M MF for 1-10 minutes at both room temperature or 0 °C. We found consistently higher survival rates of late-stage larvae (91-99%) compared to early-stage larvae (70-84%) after 1-10 minutes of 7 M MF exposure at 0 °C (Figure 3.2.3).

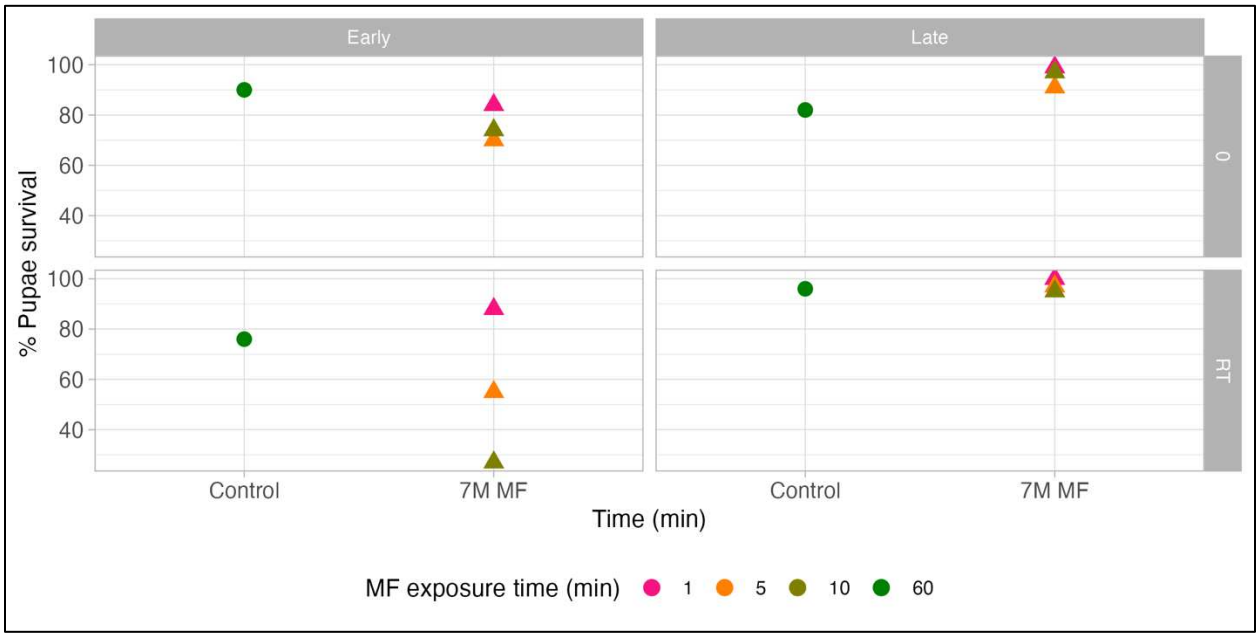

Figure 3.2.3. Impact of age and temperature on L1 development treated with 7 M MF. L1 survival was determined by calculating the number of L1s successfully transitioning into pupae. Columns show the early and late stages; rows show 0 °C (0) and room temperature (RT). This graph illustrates the percent of pupae survival when L1s were exposed to 7 M MF.

Although L1s tolerated 7 M MF was well and successfully transitioned into pupae, we further investigated how many days L1s would take to make this transition and if adding a group that was only exposed to 1.5 M MF would make a difference. Our findings suggest that late-stage L1s tolerated 7 M MF well at room temperature and 0 °C (>90% survival). Interestingly, L1s treated with 1.5 M MF (a common concentrate ion used in slow freeze cryopreservation studies) had lower survival rates compared to those treated with a higher concentration (7 M MF; Figure 3.4A). The pattern of pupation varied based on the incubation temperature and early- versus late stage. Control from late-stage L1 pupation days ranged from 6 - 8 days and 6 - 7 days when incubated at room temperature and 0 °C, respectively. The majority (~80%) of late-stage L1s kept at room temperature were pupated on day 7, and lower fractions (<15%) pupated on days 6 and 8. Late-stage

L1s treated with 7 M MF at 0 °C began pupation on day 6, peaking on day 7, and continued until day 11. Conversely, late-stage L1s treated with 1.5 M MF finished pupating within days 6 and 7. The majority (>65%) of late-stage L1s treated with 7 M MF kept at room temperature pupated on day 7 with lower pupation rates on days 6, 8, and 9, similar to late-stage L1s treated with 1.5 M MF. Control from early-stage L1s pupation days ranged from 7 - 12 days and 6 - 9 days when incubated at 0 °C and room temperature, respectively. Early-stage L1s treated with 7 M MF and incubated at 0 °C had the majority pupated on day 7; lower pupation rates occurred on days 6 and 8 - 12. Conversely, L1s treated with 1.5 M MF pupated at a higher rate on day 6 and dramatically lowered pupation on days 7 through 12. Early-stage L1s treated with 7 M MF and kept at room temperature had the majority pupation at days 6 and 7, lower pupation rates on days 6 and 8 - 10. Surprisingly, L1s treated with 1.5 M MF did not successfully transition to the pupae stage. These results reveal that late-stage L1s are least negatively impacted by 7 M MF treatment, showing their cryoprotectant properties to be feasible and appear to progress through the fours instar larval stages, pupae, and adulthood (Figure 3.2.4B).

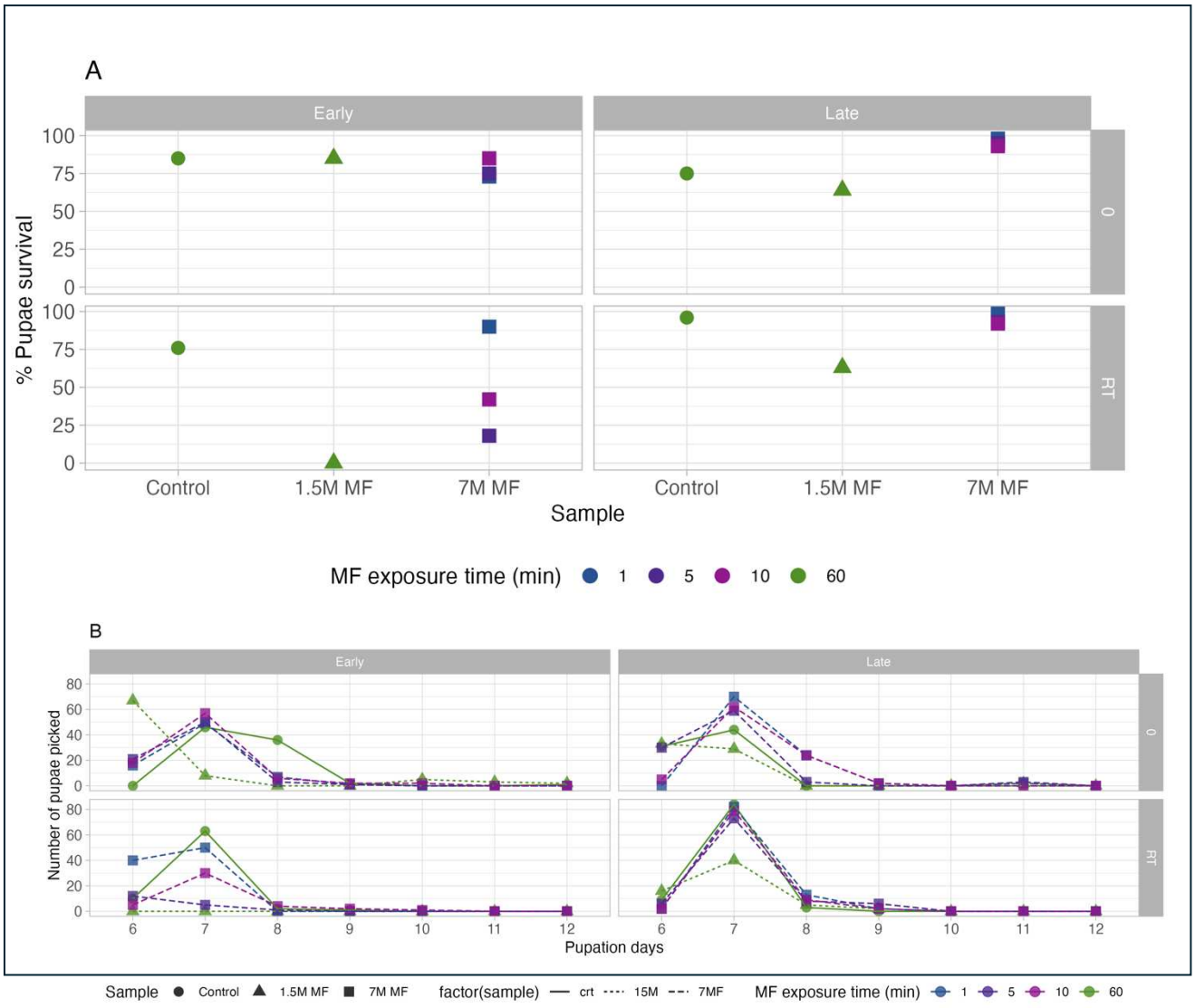

Figure 3.2.4. Impact of age and temperature on L1 pupation days when with 1.5 M or 7 M methylformamide. A) Graphs illustrate the percent pupae survival after L1 was treated with .5M or 7M methylformamide and incubated for 1, 5, 10, or 60 minutes at either 0 °C or room temperature. B) Graphs illustrate the number of pupae picked post-hatching as L1s were exposed with either 1.5 M MF or 7 M MF, with different exposure times, and either kept at 0 °C or room temperature. Control, 7 M MF, and 1.5 M MF treated L1s were monitored daily for the timing of pupation (represented as days post-hatch) from 6 through 12 days. Controls (not MF treated, shown with solids dots and lines) were kept for 60 minutes at either 0 °C or room temperature. Early- and late-stage L1s were exposed to 7 M MF for 1, 5, or minutes. Early- and late-stage L1s were exposed to 1.5 M MF for 60 minutes.

3.2.3 Supercooling protocol optimization and mosquito survival.

After observing good viability and typical pupation rates using 7 M MF at 0 °C with late-stage L1s, we sought to vitrify them and assess viability. This was accomplished by

incubating late-stage L1s in 7 M MF at 0 °C for 5 minutes, then immediately plunging larvae to -40 °C, -85 °C, -120 °C or -155 °C for 5 minutes. L1s were returned to room temperature and evaluated one hour later for survival. We found no larvae survived rapid cooling at temperatures lower than -40 °C. Next, we repeated that experiment using a range of temperatures from -2.5 °C to -28 °C to determine better the exact temperature at which larvae do not survive rapid cooling. All L1s survived at temperatures as low as -16 °C for 1 hour **(Table 3.1)**.

Table 3.2.1. Percent survival of L1s exposed to freezing temperatures for 1 hour.

Temperature	Survival 1 hour after cooling	
	percent	alive / total
Experiment #1		
control	100%	10 / 10
18°C	90%	9 / 10
-40°C	100%	10 / 10
-85°C	0%	0 / 10
-120°C	0%	0 / 10
-155°C	0%	0 / 10
Experiment #2		
control	100%	10 / 10
20°C	100%	10 / 10
8.5°C	100%	10 / 10
-2.5°C	100%	10 / 10
-16°C	100%	10 / 10
-28°C	0%	0 / 10

These results revealed that we would likely need to utilize a pre-cooling step before transferring L1s to longer-term liquid nitrogen storage temperatures (-196 °C). Therefore, our optimized protocol included cooling L1s in water to 0 °C on ice, then transferring to 0 °C 7 M MF for 5 minutes before being transferred to dry ice vapor (-12 °C to -16 °C) for 5

minutes. Larvae were then transferred to 0 °C water, warmed to room temperature, then returned to normal growth conditions. A detailed protocol of the dry ice vapor procedure is provided in appendix B. Over 65% of L1s that were supercooled for 5 minutes successfully transitioned to the pupae stage, comparable to control, non-cooled larvae (Table 3.2).

Table 3.2.2 Survival after cooling

treatment	survival to pupae	
	percent	pupae / intial number of larvae
control	67.6%	186 / 275
cooled	65.6%	180 / 275

Pupae from treated (cooled) and untreated (control) larvae were allowed to progress into adults, which were counted and sexed by cold-anesthetizing adult mosquitoes at 0 °C. A subset of the adult mosquitoes from both the treatment and control groups (F0) were maintained for one additional generation (F1) using standard rearing procedures described above. The number of adult male and female mosquitoes was counted to calculate sex ratios. We found that the percentage of females in the initial colony (F0) was comparable at 48.6% and 49.7% for treated and control colonies. To determine any possible longer-term impacts of cooling on sex ratios, the F0 colonies were blood-fed and allowed to lay eggs, which then progressed into adults and were again counted for numbers of males and females. Again, we found comparable percentages of females in the treatment and control groups (69.0% and 76.7%, respectively). Interestingly, while the rate of females differed between the F0 and F1 generations, within a generation, they were not meaningfully different between treatments and control groups (Figure 3.2.5).

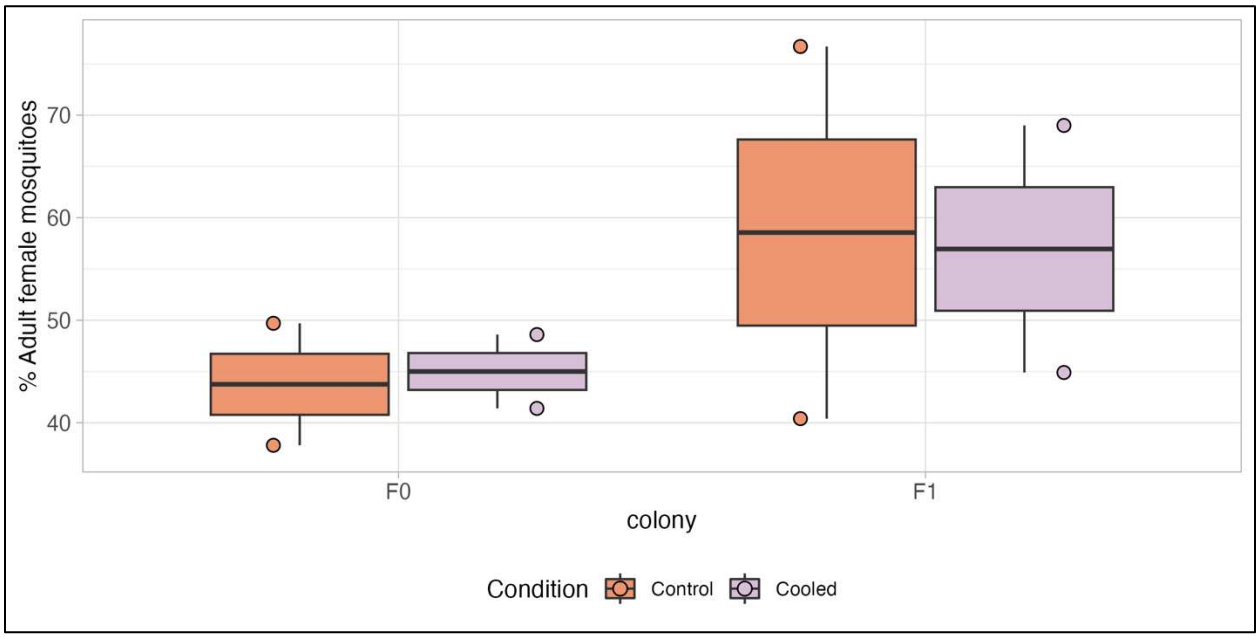

Figure 3.2.5. Evaluation of female to male ratio after cooling L1s with pre-cooling step. Boxplot indicated the percent adult female population after pre-cooling and cooling method (F0) and after one generation (F1). Control groups were not exposed to the pre-cooling step (orange), while the cooled groups were (light pink).

After establishing that late-stage L1s survived after supercooling to -16 °C for 5 minutes, time tolerance experiments were performed to assess viability tolerance. L1s were pre-treated and cooled as above, then rapidly cooled to -15 °C using a thermoelectric cooling plate and held at this temperature for 10 minutes, 60 minutes, or 120 minutes. Larvae were then transferred to 0 °C water, warmed to room temperature, then returned to normal growth conditions as above. Over 35% of larvae cooled for 10 minutes matured to pupae, and 14% and 2% matured to pupae when incubated for 60 and 120 minutes, respectively. Interestingly, L1s supercooled for 120 minutes did not survive past pupation; all others survived through emergence to adulthood (data not shown). Multiple experiments were carried out after, with no viability after 2 hours.

3.3 Discussion

Cryopreservation of insects has been explored from different angles, from preserving the semen of honeybees and butterflies to sperm and embryos of silkworms and drosophila fruit flies and using techniques such as slow freezing and vitrification. However, efforts to cryopreserve mosquitoes have made little progress due to the complex structures of the mosquitoes. Early attempts focused on mosquito eggs. However, due to their vitelline membrane, eggs were impermeable to traditional cryoprotective agents and methods (M. D. Valencia et al., 1996; M. D. P. Valencia et al., 1996). Recent work by James ER *et al.* overcame this barrier by using *Anopheles* species eggs collected 30 minutes after oviposition before the eggs formed the impenetrable chorion. Using deuterated methanol and a two-step pre-cooling process before rapid freezing, they demonstrated hatch rates of ~25%, with normal development compared to non-cryopreserved counterparts (James et al., 2022). Others have recently evaluated cryoprotective toxicity, permeation, and CPA loading using L1s (Campbell et al., 2021; Nesbitt et al., 2021). Our work builds on these recent advances, focusing on L1s greater than 1.5 hours after hatching, allowing more flexibility than requiring freshly laid eggs or newly hatched (<1.5 hours) L1s.

Our protocol utilizes MF, which other mosquito cryoprotection studies did not evaluate. Methylformamide is a commonly used permeating cryoprotectant for cryopreservation of many different species and samples (Best, 2015). Larvae were cooled on ice until acclimated to 0 °C, transferred to 0 °C methyl formamide with trehalose, and then moved to subzero temperatures (-12 °C to -16 °C) for up to 120 minutes. In a complete cryopreservation protocol, frozen larvae would be placed in liquid nitrogen for

long-term storage. Supercooled larvae developed generally compared to their non-frozen counterparts, progressing to pupae and adults with similar timing of emergence when supercooled for up to 60 minutes. Supercooled larvae held at 120 minutes survived through pupation but did not emerge as adults. Adult sex ratios of the treated larvae (F 0) and the following generation (F1) were equivalent to the controls, suggesting normal development.

Cryopreserved mosquitoes must be comparable to their non-preserved controls. Changes to mosquito genetics, epigenetics, fecundity, metabolism, and many other phenotypes can have unintended consequences for colony health and their utility in experiments. Notably, many of these phenotypes are due to alterations in metabolites. An inference of metabolic shifts was shown when comparing timing to pupation for 1.5 M versus 7 M MF-treated larvae and, more importantly, when comparing timing to pupation for early-stage versus late-stage L1s.

Although cryopreservation may seem like an artificial phenomenon, it occurs in nature as part of adaptation, temperature being an abiotic factor. Nowadays, insects have four recognized strategies to survive cold temperatures: vitrification, cryoprotective dehydration, freeze avoidance, and freeze tolerance (). These trigger physiological adaptations and production of cryoprotectants, removal of ice nucleators, extreme dehydration, and antifreeze proteins (). Understanding the molecular and biochemical processes involved in metabolic adjustments is essential for comprehending their survival mechanisms.

3.4 Materials and Methods

3.4.1 Mosquitoes

Anopheles stephensi mosquito eggs were obtained through BEI Resources as part of the Biological Reagents Repository (NIAID, NIH: *Anopheles stephensi*, Strain STE2, MRA-128, contributed by Mark Q. Benedict). Colonies were maintained at 28 °C (± 0.5 °C) with a 12:12 light: dark photoperiod and 75% (± 2%) relative humidity, with water and sugar provided *ad libitum.* Adult mosquitoes were fed warmed defibrinated calf blood in artificial glass blood feeders. Eggs were collected on coffee filters partially submerged in water cups. They were hatched two days post-oviposition in small bins with deionized water. Larvae were moved to a larger bin 48-72 hours later to continue development. Larvae were raised on powdered fish food added as a supplement to tap water hatch bins.

3.3.2 Freezing larvae and Recovery of L1 Larvae (Protocol was published in protocols iO)

3.3.2.1 Cryoprotectant Selection

Larvae at larval stage 1 (L1s) (n = 125/150) were transferred into a mesh basket immersed in water at room temperature. The mesh basket was removed from the water, blotted dry and transferred to 1.5 M EG, 1.5 M MeOH, 1.5 M DMSO, or 1.5 M MA, 1.5 M MF, or water (control) for 15, 30 or 60 minutes at room temperature (22°C -25 °C). At the end of the incubation period, mesh baskets were removed, blotted dry, and L1sw were rinsed by immersion of basket into fresh tap water three times. L1s were returned to regular conditions (28 °C (± 0.5 °C) with a 12:12 light: dark photoperiod and 75% (± 2%) relative humidity and fish food), and survival was assessed by maturation into pupae.

Survival to pupation = ((# of pupae /initial # of L1 larvae) * 100) was calculated for each incubation time for a given CPA solution. In a separate experiment, L1s were incubated in 7 M MF at 0 °C, then immediately transferred to -15 °C for 5 minutes. They were assigned to a mesh basket to a petri dish containing ice water. The dish was immediately removed from the ice and placed on the bench until the water reached room temperature. L1s were then returned to the chamber at 28 °C (± 0.5 °C) with a 12:12 light: dark photoperiod and 75% (± 2%) relative humidity.

3.3.2.2 Determination of the Toxicity of Cryoprotectants

50 or more first larval stage (L1) were collected using a transfer pipette with a blunt end and transferred onto a mesh basket immersed in tap water. L1s were then immersed in the 1.5 M EG, 1.5 M ML, 1.5 M DMSO, or 1.5 M MA/MF, or water alone (control) while in the filter dish for 1, 2, 3, 4, 5, 6, or 8 hours at room temperature (22 °C - 25 °C). After the appropriate exposure time, CPA was drained from each dish, and L1s were rinsed by immersion in tap water while remaining in the mesh basket. Additional two washes were carried out. L1 was returned to tap water with fish food and assessed for survival-based morphological changes from L1-L4 larvae. Percent survival was determined by (Percent survival = # of pupae collected/initial egg count) for each time point for a given CPA solution.

3.3.2.3 Freezing larvae

7 M MF + 0.5 M trehalose in deionized water was the CPA solution made one day before an experiment. On the day of the experiment, CPA was placed in a square culture dish and set on a cooling table at 28 °C. 200 L1s 14 - 20 hours post-emergence were counted, placed in a nylon mesh basket submerged in clean water and put on a cooling

table set to 28 °C and cooled to 0 °C over 30 minutes. After, the basket was removed from the water, blotted dry, and transferred to a dish containing the CPA solution cooled at 0 °C. They were held for 5 minutes at 0 °C followed by rapid cooling to -15 °C. L1s were left on the cooling table at -15 °C (10 minutes – 2 hours).

3.3.2.4 Warming larvae

Larvae were removed from the cooling table and placed at 4 °C for 10 min. The mesh basket was then blotted dry, transferred to a dish containing water, and held at 4 °C for 5 minutes. Then, larvae were placed onto a warming table held at 28 °C for 1-2 hours, and initial viability assessment was performed by watching for movement (including mid-gut movement) using a dissecting microscope. Larvae were returned to husbandry conditions for recovery and development through life stages.

3.3.2.5 Viability studies

The percent survival was calculated by counting pupae that emerged from each freezing incubation and divided by the number of original larvae.

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CHAPTER 4: SUMMARY AND FUTURE DIRECTIONS

4.1 Summary of major findings

The projects described herein explored the lipid profile and advances in the cryopreservation methods of *An. stephensi* mosquitoes. The study investigated mosquitoes' intricate life cycle, focusing on lipid metabolism's role in their development. Key metabolic processes include lipogenesis, lipolysis, and fatty acid metabolism, which are critical for transitioning between life stages. Mosquitoes, as holometabolous insects, exhibit different feeding behaviors across stages, with larval and some adult stages feeding, while pupal stages do not. Major lipids identified include phosphatidylcholine (PC), phosphatidylethanolamine (PE), and sphingomyelins (SM), essential for cellular structures and functions. Triacylglycerides (TGs) are crucial for energy storage and oogenesis, and dynamic changes in TG levels were observed throughout development. The study highlighted the importance of phospholipids like PE for protein anchoring, necessary for embryogenesis and immune responses.

Additionally, non-bilayer lipids like diacylglycerol (DG) and lysophospholipids (LysoPC, LysoPE) play roles in membrane dynamics and protein trafficking. The presence and variations of these lipids were noted across different life stages, supporting various physiological processes. The study also examined steroid biosynthesis, identifying 20-hydroxyecdysone (20E) as a critical hormone in larvae and pupae stages and regulating molting. Other steroids, such as cortisone and prednisone, were more abundant in blood-fed females, indicating their role in stress response. Overall, the study underscored mosquitoes' well-orchestrated metabolic strategies, involving lipid and steroid

metabolism, to navigate their complex life cycle stages, ensuring successful development and reproduction.

The cryopreservation of insects, including honeybees, butterflies, silkworms, and fruit flies, has seen varied success, using techniques like slow freezing and vitrification. However, mosquitoes have posed significant challenges. Initial attempts at cryopreserving mosquito eggs failed due to their impermeable vitelline membrane. Recent breakthroughs by James ER et al. involved collecting *An. stephensi* eggs shortly after oviposition and using deuterated methanol with a two-step pre-cooling process, achieving ~25% hatch rates post-thawing. Further studies have assessed cryoprotective agent toxicity, permeability, and loading in first-instar larvae (L1s). Building on these advances, we developed a new protocol using methylformamide (MF) with trehalose for cryopreserving larvae older than 1.5 hours post-hatching. Larvae supercooled for up to 60 minutes developed typically, though those held for 120 minutes did not emerge as adults. The treated larvae and their offspring exhibited typical sex ratios and development. Cryopreserved mosquitoes must retain genetic, epigenetic, and phenotypic stability to be useful for research. Metabolic shifts inferred from varying MF concentrations and larval stages highlight the need to understand these changes. Cryopreservation parallels natural cold survival strategies in insects, such as vitrification, cryoprotective dehydration, freeze avoidance, and freeze tolerance, which involve producing cryoprotectants and antifreeze proteins. Understanding these biochemical and molecular processes is crucial for effective cryopreservation and insect adaptation.

4.2 Ongoing and future work: investigating the relationship between lipid metabolism and methods to improve cryopreservation methods.

4.2.1 Inducing diapause in Anopheles stephensi mosquitoes

Many factors of an organism's physiology, metabolism, and behavior are governed by internal clocks, which dictate daily or seasonal strategies such as sleep or hibernation. These clocks rely on measuring changes in photoperiod at different time scales. Circadian rhythms synchronized with the 24-hour light-dark cycle corresponding to Earth's rotation, from plant flowering to mammalian hibernation, have been influenced by environmental shifts throughout the year. As winter establishes its presence, dry seasons in the tropics and temperature zones, the lack of food and optimal temperature arise (Denlinger, 2001). Low temperatures affect biochemical processes by decreasing enzyme activity (Sanfelice & Temussi, 2016), membrane fluidity (Rozsypal et al., 2014), and elevating hemolymph viscosity, which may compromise physiological transport processes (Kenny et al., 2018).

Mosquitoes, ubiquitous disease vectors, face considerable challenges during winter, characterized by reduced temperatures and adverse environmental conditions. Despite these obstacles, mosquitoes have developed strategies to endure the harsh winter months, such as diapause and overwintering sites. Diapause, a state of suspended development, is a common strategy employed and generally triggered by photoperiodic cues. It involves metabolic rewiring, facilitated by biochemical adjustments, enabling extended periods of inactivity while conserving energy reserves (Denlinger, 2002). Selecting suitable overwintering sites, they seek sheltered locations such as underground burrows, leaf litter, or human-made structures to evade extreme temperatures and desiccation (Leisnham et al., 2014). Characteristics of entering diapause are decreased

morphogenesis, increased stress tolerance, and reduced metabolism (Karp, 2021). More specifically, these shifts involve energy production, fatty acid metabolism, and cytoskeleton reorganization; in the case of females, lipid droplets accumulate (Zhang et al., n.d.). Diapause does not mean complete cessation of development but significantly decreases the growth rate because it is environmentally programmed (Felipe Araujo Diniz et al., n.d.); diapause can occur at any developmental stage (Denlinger & Armbruster, 2014). In the case of *Cx. pipiens*, diapause is triggered by the photoperiod; they can harbor the West Nile virus and reintroduce it the following summer (Karp, 2021). However, the ability of *Anopheles stephensi* mosquitoes to undergo diapause remains elusive. Furthermore, studying the spatial and temporal organization of essential lipids is pivotal, and it will pave the way towards deciphering molecular mechanisms of action as mosquitoes enter diapause.

Delving into the metabolic intricacies of diapause and cryopreservation methodologies presents promising avenues for deepening our comprehension of mosquito biology, refining vector control methodologies, and safeguarding mosquito biodiversity. While there are similarities between mosquito diapause and cryopreservation in preserving biological material in a dormant state, they serve different purposes and operate at various temperature ranges and time scales. However, understanding diapause mechanisms can provide insights into cryopreservation techniques and vice versa, particularly regarding metabolic adaptations and cellular responses to low temperatures—a promising approach involves inducing diapause as a precursor to cryopreservation at the first larval stage.

Diapause has been described as the elevation of stress tolerance, nutrient storage, and reduced metabolic activity (Hahn & Denlinger, 2011). The association of gene expression with cell proliferation, stress resistance, circadian rhythms, hormonal signaling, metabolic activity, and nutrient storage has been described through transcriptomics (Hahn & Denlinger, 2011). In insects, manipulation of lipids are a characteristic of diapause and serve as the principal energy storage molecule (Hahn & Denlinger, 2011). The accumulation of lipids, especially triacylglycerides (TGs), is the primary fuel consumed during embryogenesis and plays an essential role in egg desiccation resistance. Photoperiod-induced maternal effects regulate the energy reserves stored in eggs, facilitating the accumulation of substantial metabolic resources necessary for extended non-feeding intervals, such as embryogenesis and the dormant pharate larval stage. Variances in lipid content stem from maternal provisioning and intrinsic regulation by embryos and pharate larvae. Notably, significant transcriptional alterations occur in genes associated with lipid metabolism, fostering lipid preservation throughout embryogenesis and the pharate larval stage by diminishing lipid breakdown and increasing biosynthesis of unsaturated fatty acids (Reynolds et al., 2012). A few mosquito species have been reported to undergo diapause, and the life stage at which this occurs has also been captured (Mensch et al., 2021). In the case of adult female *Cx. pipiens* mosquitoes, extreme lipid storage causes fat body hypertrophy (Pinch et al., 2021). In previous study found that fatty acid synthase (FAS) was upregulated in diapause-blood-fed female *Ae. Albopictus* mosquitoes compared to non-diapause-blood-fed female mosquitoes (Batz & Armbruster, 2018). Some Anopheles species have the ability to undergo diapause. However, the specific mechanisms by which An. stephensi

undergoes this phenomenon remain to be fully understood. Therefore, it is crucial to assess the lipid metabolomic plasticity of this vector at each life stage. This project aimed to investigate the expression levels of two fatty acid lipid-producing enzymes: fatty acid synthase (FAS) and ceramide synthase. We performed an RT-qPCR assay to detect expression levels of these genes during the adult life stage in a diapause-induced colony compared to a typical colony (Figure 4.2.1.1). We aimed to understand the roles of these lipid-producing enzymes and how fluctuation in their expression level can alter diapause success.

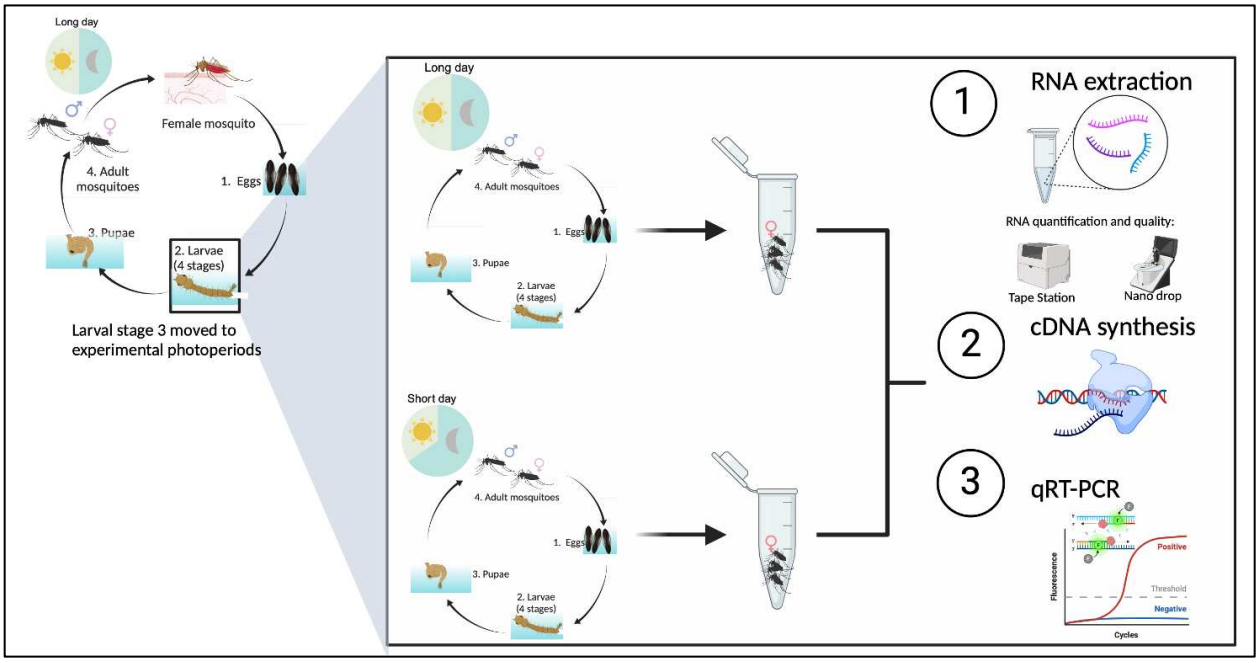

Figure 4.2.1.1. Experimental design to induce diapause in *An. stephensi* mosquitoes. Typical *An. stephensi* mosquito rearing protocol involved a long day (12:12, light:dark photoperiod), once the larvae reached the 4th larval stage, they were either kept in the same room (control- Long day) or moved to another chamber with a short day (8:16, light:dark photoperiod) setting. As the first generation (F0) underwent through all mosquito stages in their respective photoperiod setting, the second generation (F1) female adult mosquitoes were collected. RNA was extracted (1), cDNA synthesis (2), and qRT-PCR (3) were carried out. Image created with BioRender.

While this project is still in progress, we have already taken important steps, including generating various mosquito pools, optimizing RNA extraction methods, and fine-tuning primer concentrations. Additionally, we have successfully collected several group samples. (Figure 4.2.1.2A). After RNA extraction, we assessed the RNA quality and concluded that a pool of ten mosquitoes without wings would be used (Figure 4.2.1.2A). Therefore, we continued to carry out the qRT-PCR assay to test the most effective concentration of actin, the control. Here, it was shown that 10µM was the most effective since the mean C_q value was 28 cycles (Figure 4.2.1.2B). List of the *actin*, *fas*, and *schlank* are shown in Table 4.2.1.1.

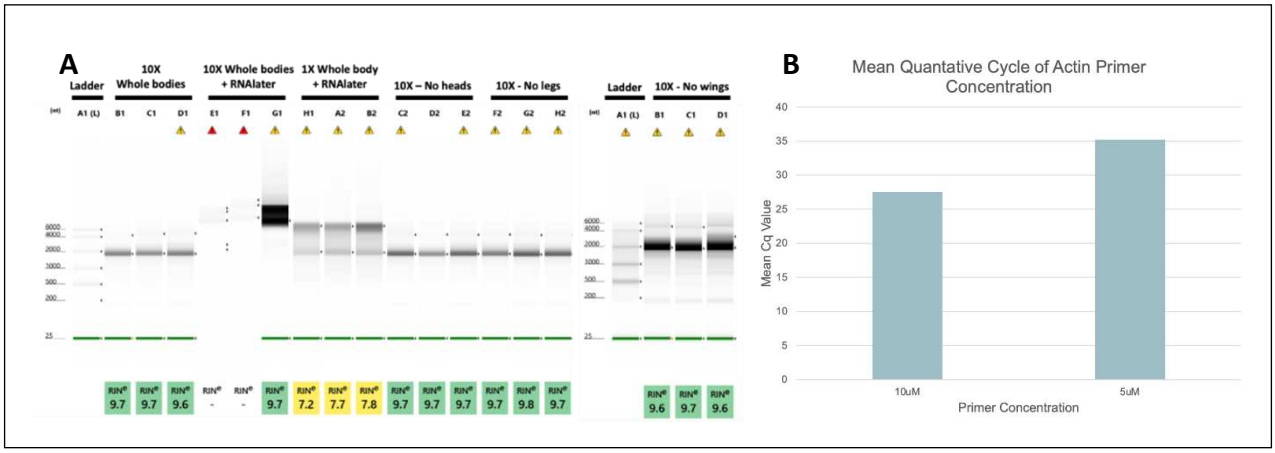

Figure 4.2.1.2. RNA quality and qRT-PCR results. A) Pilot investigating the different mosquito pool with and without heads, legs, wings. Additionally, assessing the effectiveness of RNAlater. **B)** qRT-PCR results illustrating primers against actin at two concentrations, 5- and 10 µM. The y axis shows the primer concentration, and the x axis shows the mean C_q value.

Table 4.2.1.2 PCR primers

Name	Sequence
*actin*_forward	CAGTCAGTCAAGACGTGTCCA
*actin*_reverse	TCGCACATCTTGGATTTGGTT
*fas*_forward	GCATTTTCCTTACGCTCCTGC
*fas*_reverse	TCCCTGGTCTGGCTGTTTTG
*schlank*_forward	CGTCCCGAGTTCAGTTCCGAG
*schlank*_reverse	ATCGGTGCAATCCAAAACCGTTC

While the project was not carried out to full termination, the findings obtained thus far have yielded insightful information. These preliminary results offer a solid foundation for designing and conducting further experiments to explore and address the remaining questions.

4.2.2. Cryopreserved larvae previously diapause-induced

Inducing diapause in mosquitoes before cryopreservation offers a synergistic approach to enhancing recovery rates and maintaining viable populations for research and control efforts. For instance, research on the Mediterranean corn borer (*Sesamia nonagrioides*) where inducing diapause in larvae were more resilient to cryoprotective agents and freezing processes compared to non-diapausing larvae, leading to higher post-thaw recovery rates and normal development (Eizaguirre et al., 2002). Another example involves the codling moth (*Cydia pomonella*). Diapause was induced by manipulating environmental conditions such as photoperiod and temperature. The diapausing larvae showed enhanced cryotolerance, resulting in better survival and development post-cryopreservation compared to non-diapausing larvae (Hasan et al., 2018). These studies suggest that diapause can act as a preparatory phase that equips organisms with the necessary physiological adaptations to withstand the stresses of cryopreservation. These adaptations include changes in metabolic activity, accumulation of natural cryoprotectants, and alterations in membrane composition, which collectively improve the organism's ability to survive the freezing and thawing processes. We therefore explored this approach in *An. stephensi* mosquitoes, exposing adult mosquitoes (F0) to either a long- or short-day condition, two days later females acquired a blood meal. Offspring were carried out in the respective photoperiod and as the second generation

(F1) of female adults acquired a blood meal, their larvae at the first instar stage (L1) were used for the experiments. The four groups included two groups from the long day (regular) colony (10 minutes and 35 days) and two groups from the short day (diapause) colony (10 minutes and 35 days). The regular and diapause colonies exposed to subzero temperatures for 10 minutes showed a 40% and 2.5% recovery, respectively. Additionally, the diapause group went through development as regular colony since they seem to be synchronized and pupated within the same timeline (Figure 4.2.2.1).

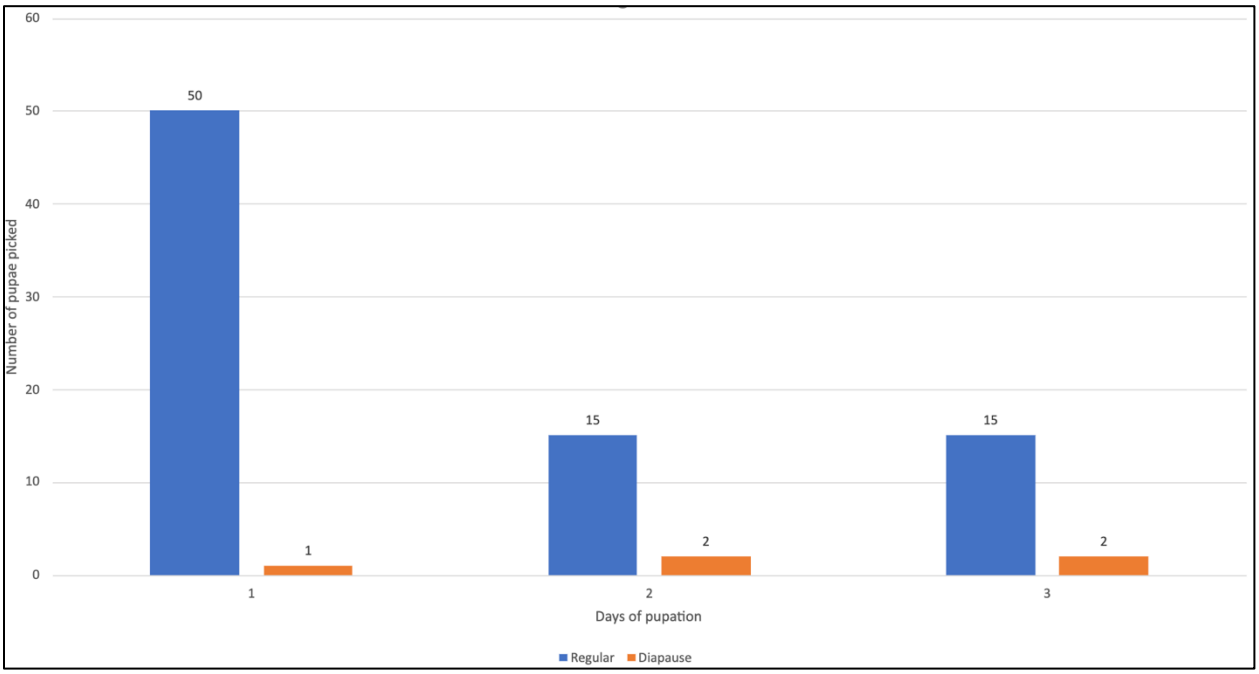

Figure 4.2.2.1. L1s exposed to subzero temperatures. The x axis shows the number of days taken to collect all the pupae from the regular (blue bar) or diapause (orange bar) colonies and the y axis shows the number of pupae picked.

Interestingly, once the L1s were thawed after 35 days exposed at -20 °C, their morphology was intriguing; L1s from the diapause colony maintained a straight morphology versus the regular colony showing a 'shrimp-like' morphology with a few head detached form the thorax (Figure 4 2.2.2A-B).

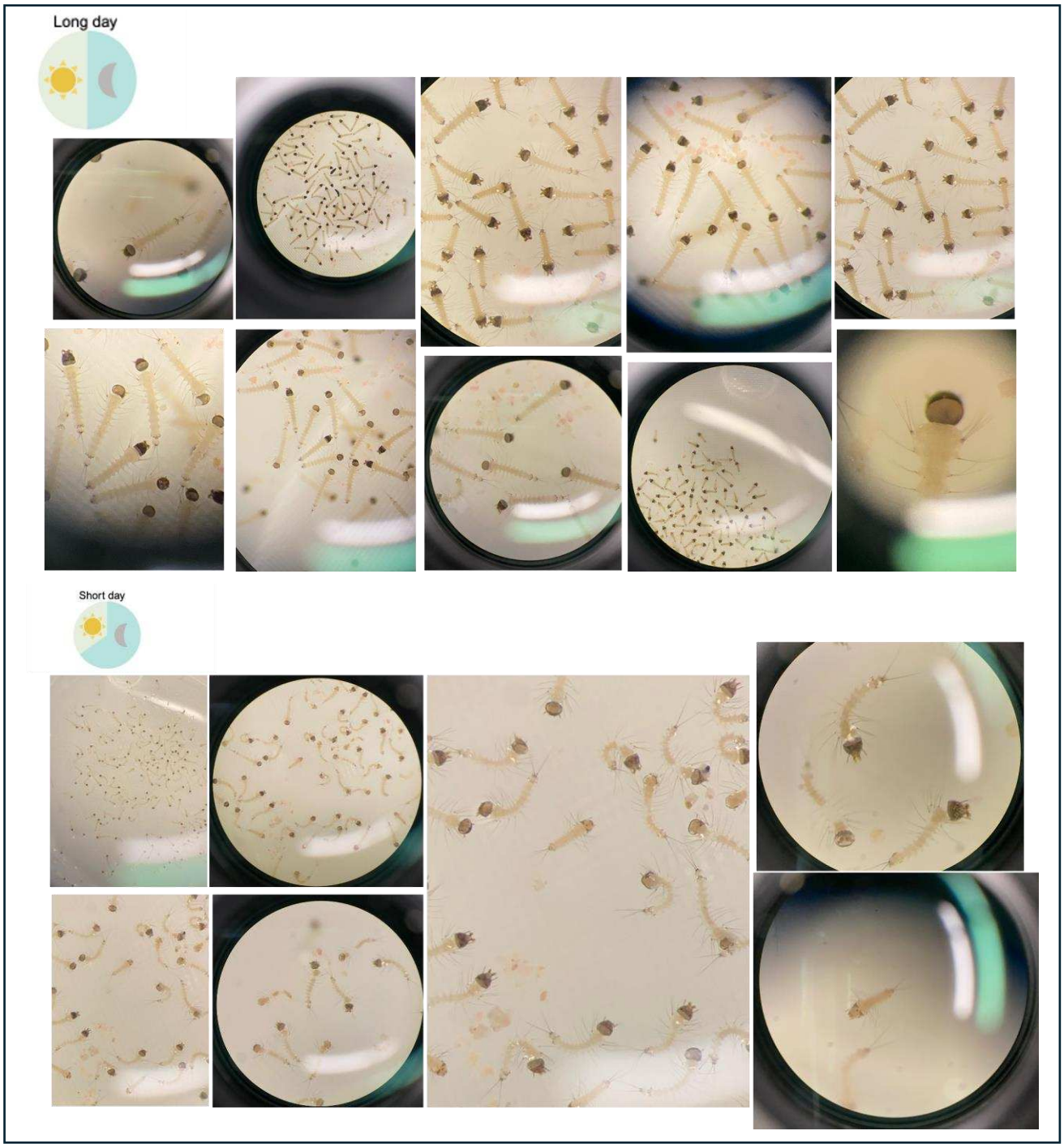

Figure 4.2.2.2. Morphology of first larvae stage (L1s) after exposure to -20 °C for 35 days. A) shows the regular colony and **B)** diapause colony.

Inducing diapause before cryopreservation could be a valuable strategy for preserving mosquitoes. By leveraging the natural adaptations that occur during diapause, researchers can enhance the cryopreservation outcomes, potentially leading to higher recovery rates and better developmental stability post-thaw. This approach could provide significant advancements in maintaining mosquito colonies for research and vector control programs.

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APPENDIX A: Short-Term Freezing of *Anopheles stephensi* (Mosquito) Larvae - Protocol

I. **Materials**

a. Mosquito eggs on egg paper

b. Insectary chamber, capable of programable temperature, light, and humidity

c. Distilled water

d. Fish food

e. Small hatch bin (a solid, polycarbonate or greater strength container, that can hold water to a depth of 5 or more cm, with dimensions approximately 10 cm x 20 cm)

f. Two (or more) Large Hatch bins (dimensions approximately 20 cm x 40 cm)

g. Plastic transfer pipettes

h. Strainer

i. Gloves

j. Small (2 oz) disposable cups

k. Insect rearing cage with nylon mesh screen and port

l. Scissors

m. Circulating water pump

n. Glass insect feeder

o. Parafilm

p. Filter paper

q. Defibrinated calf blood

r. Microscope slides

s. Olympus SZX16 Advanced Stereo Microscope (or similar)

t. Penetrating cryoprotective agents (see examples, section III.A

u. Non-penetrating cryoprotective agents (we use 0.5 M Trehalose)

v. Square culture (petri) plates

w. FalconTM Cell Strainers, 70 um (or similar)

x. Thermoelectric Laboratory Cold Plate (AHP-1200CPV or similar)

y. Kim wipes

II. **Phase I: Establish and maintain mosquito colony**

A. **Phenotypic Analysis of developing mosquitoes (Figure 1)**

Larvae develop from eggs to first instar larvae (L1) around 2-3 days after transferring egg paper. L1 develop into L2/L3 over the next 4 days (approximately). During that time, they show the ability to hatch, feed and further develop. L4 transition into pupa and emerge as adults approximately 2 days after pupation.

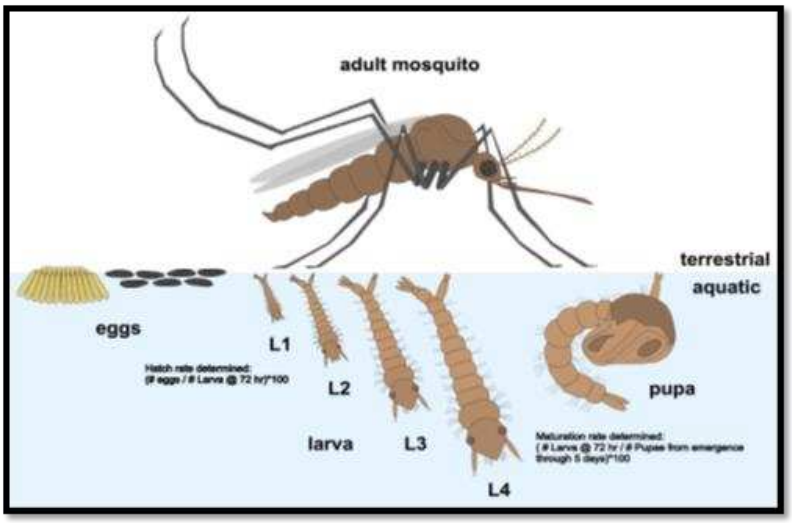


Figure 1: *An. stephensi* life stages showing hatch and maturation rate data collection points.

B. **Husbandry (modified from MR4 Methods in Anopheles Research. MR4 M*ethods in Anopheles Research Laboratory Manual*, BEI Resources, 2015)**

1. **Hatch eggs**
    - Obtain egg paper with referenced eggs from BEI or other qualified source.
    - Take clean small hatch bin, label with date, and dispense ~500mL of distilled water. Dispense slowly to avoid bubbles (eggs will not hatch in a synchronized manner).
    - Put on gloves and place the egg paper into the water.
    - Add a pinch of fish food and cover the pan
    - Place pan in insect chamber and maintain under controlled conditions of temperature (28 °C), relative humidity (70%) and light (12:12 L:D diurnal cycle).
2. **Larvae splitting and feeding**
    - Move larvae into a larger bin using a strainer, roughly 24 hours after eggs are hatched. Follow the same instructions as far as pouring DI water slowly; from here tap water can be used.
    - Make sure to thoroughly rinse out the small bin where eggs were hatched.
    - For the following days, make sure that bins are not over saturated with larvae (~ 60% saturation). Higher than that, larvae will stop growing. So split as much as necessary
    - Add food and cover bin.

- During this period, changing the water may be necessary to avoid mold and other microbial growth. Use strainers and transfer into a fresh bin if necessary

3. **Picking up pupae**
    - Pour an ounce of tap water into a clear, plastic, disposable 2oz (about 59.15 ml) cup.
    - Use a transfer pipette or a small mesh-made-scoop to collect the pupae into the cup.
    - Transfer the cup into an insect rearing cage by placing cup through top port. Add sugar and water cups through the port.
    - Males will emerge first, so it is important to pick up pupae for several days (~5) to ensure females emerging from pupae are also picked.
4. **Blood feeding mosquitoes (glass feeder protocol)**
    - The optimal time to blood feed mosquitoes is 3-5 days post-eclosion
    - Remove sugar and water 12-16 hrs. prior to blood feed (Note: the colony will significantly die off if starved for more than 24 hrs.)
    - Fill water basin with tap water and attach heating element/pump. Turn on and pre-warm water to 37 – 42C.
    - Prepare glass feeders. Use scissors to cut the length of parafilm or hog gut sufficient to cover mouth of glass feeder. (Note: hog gut is often stored frozen. Allow time to thaw before use or thaw in tap water). Stretch tightly across mouth without tearing and use rubber band to hold membrane in place. (Note: If hog gut is used, be careful not to let it dry out. A petri dish with wet paper

towels may be used for this purpose. Do not place prepared glass feeders in petri dish with standing water as the water may enter the feeder through the membrane and dilute the blood.

- Briefly turn off the water pump and use plastic tubing to connect all feeders in a circuit that leads back into the water bath. (Wet each end of each piece of plastic tubing before use to ensure easier fit.) Turn pump back on and ensure A) a steady flow of water around circuit and B) no dripping. Do not, however, place feeders on top of cartons yet, as mosquitoes may begin to drink and fill up on water.
- Use transfer pipette to measure appropriate amount of blood into well of glass feeder. 1-2 ml per feeder is more than enough.
- Place feeders on top of cartons and begin feeding. Optional: tear off a long strip of tape to hold glass feeder firmly in place against roof of mosquito carton.
- Visually inspect mosquitoes to see if they are feeding. Look for females clustered around feeder with bright red full bellies. Ensure that the temperature of the water is approximately 37 C. Some variability in temperature is permissible but note that parafilm may melt/deform if bath is run too hot.
- Allow mosquitoes to feed for up to one hour. Return all mosquito cartons to insectary and disassemble water bath apparatus.
- Let glass feeder and membrane soak in 10% bleach solution for at least 15 minutes. Dispose of membrane and rubber band in trash. Wash glass feeder and let dry.

- Collect eggs onto filter paper and continue the process to maintain colony.

C. **Obtaining and counting larvae**

1. At 16-18 hours post-egg hatching, remove *An. stephensi* larvae hatch bin.
2. Transfer a small amount of water from the hatching habitat to a new bin.
3. Cut the tip of a plastic bulb pipette with scissors, approximately 1cm above the tip, to widen the inlet area of the pipette.
4. Remove 7-20 larvae at a time by pipetting, and count larvae through the clear walls of the pipette.
5. Transfer to a new bin containing hatch bin water.
6. After larvae are counted, transfer them back to the original habitat, along with the water from the new hatch bin.

D. **Phenotypic Characterization of Larvae at each Instar by Advanced Stereo Microscope (Larval Microscopy)**

1. Cut the tip of a plastic bulb pipette with scissors, approximately 1cm above the tip, to widen the inlet area of the pipette.
2. Remove 5-7 larvae from their hatch bin, and transfer in one drop to a glass microscope slide.
3. Analyze larvae using an Olympus SZX16 Advanced Stereo Microscope, at variable magnification settings and determine (a tabulation might help):
    a. Microscope settings
    b. Photographic and descriptive phenotypic characterization of larvae at each instar:

c. Number of larvae at first instar (L1)

d. Characteristics of L1 larvae and on through pupation

III. **Phase 2: Freezing and Recovery of L1 Larvae**

Note: for each of these initial conditions, 150-400 larvae are used per condition.

Maturation rate (L1-pupae) is determined for evaluation during initial experiments.

A. **Cryoprotectant Selection**

Prepare cryoprotectants (or Cryoprotective Agents, CPAs) in nano pure water at room temperature and determine toxicity of cryoprotectants to the L1 larvae. Potential cryoprotectants are listed below.

Potential Cryoprotectants

1. 1.5 M ethylene glycol (EG),
2. 1.5 M methanol (ML),
3. 1.5 M dimethyl sulfoxide (DMSO), or
4. 1.5 M methyl acetamide or methyl formamide (MA or MF)

B. **Determination of the Toxicity of Cryoprotectants**

1. Collect 50 or more L1 larvae from the insectary bin using a transfer pipette with a blunt end and transfer onto a filter dish.
2. Immerse the larva in 1.5 M EG, 1.5 M ML, 1.5 M DMSO, or 1.5 M MA/MF, or water alone (control) while in the filter dish for 1, 2, 3, 4, 5, 6, or 8 h at room temperature (22-25°C).
3. After the appropriate exposure time, drain respective CPA from each dish and rinse larva by immersion in tap water while they remain in the filter dish.

4. Continue Step three with two additional washes, or empirically based on sheen of the L1 larvae.

5. Culture the L1 larvae in water with fish food (as described in Section 1.B) and assess for survival based morphological changes from L1-L4 larvae.

6. Determine the percent survival (Percent survival = # of pupae collected/initial egg count) for each time point for a given CPA solution.

7. Record a minimum of three replicates of each to assess toxicity.

C. **Freezing larvae**

In this example, a mixture of 7M methyl formamide (MF) in 0.5 M of trehalose was chosen as the optimum CPA. Further, our studies with newly emerged versus late L1 stage larvae supported using 14–24-hour old L1 larvae for freezing. A diagram of the protocol for this process is provided (**Figure 2**).

1. Prepare the CPA solution of 7 M MF in 0.5 M trehalose in a square culture dish and place dish on cooling table set at 28°C.

2. Place 14-20 hr emerged L1 larvae in clean water. Count larvae.

3. Transfer larvae to a nylon mesh basket submerged in clean water that is in a square culture dish set on the cooling table. Use ~ 200 L1 larvae per condition group for optimum data sets.

4. Begin slow cooling on the cooling plate from 28°C to 0°C over 30 minutes.

5. Retract the basket from the water, blot dry the L1 Larvae with a Kim wipe, and transfer the basket to the culture dish containing the CPA solution.

6. Hold for 5 minutes at 0°C

7. Begin rapid cooling of L1 Larvae on cooling table, and cool to -15°C.

8. Leave L1 larvae on cooling table (10 min – 2 hr), or transfer to -20°C freezer (longer time periods) until warming.

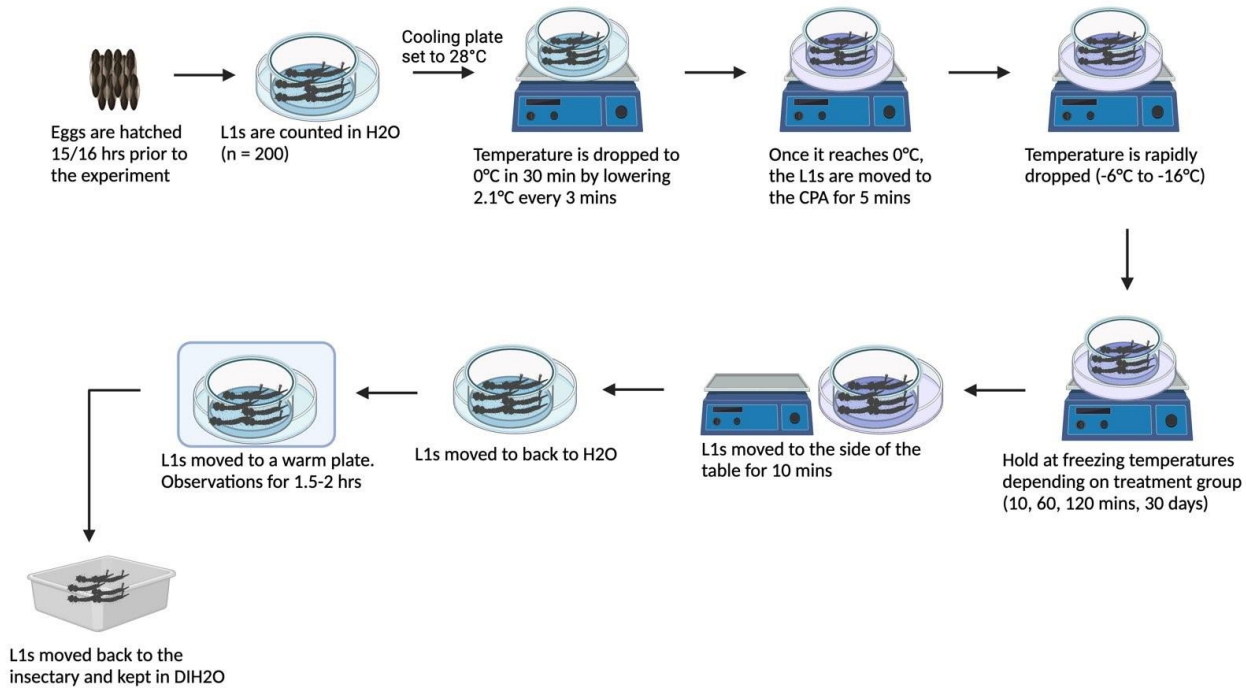


Figure 2. Diagram of L1 larvae freezing process

D. **Warming larvae**

The current warming method is described below. We note that additional warming trials may need to be conducted for further optimization; viability using this method and maintaining freezing conditions for 10 min is 60%.

1. Remove larvae from the cooling table and hold on the side of the table, at 4°C for 10 min.
2. Remove the mesh basket and submerge into a square culture dish containing water held at 4°C and incubate for an additional 5 min.

3. After this time, move the L1 larvae to a square dish with water held at 28°C for 1-2 hrs. Perform initial viability assessment by watching for movement (including mid-gut movement) using a dissecting microscope.

4. Return L1 larvae to husbandry conditions for recovery and development through life stages (**Figure 1**).

5. Determine recovery and analysis of viability as described below.

E. **Viability studies**

1. Determine the number of pupae that emerge from each freezing incubation time point; calculate the percent recovery as done for percent survival, based on number of L1 larvae from each time point.

2. Determine the percentage of recovered pupae that survive and develop into adults.

3. Determine the ratio of males to females (sexual differentiation) in adult mosquitoes.

4. Perform phenotypic similarity analyses (via microscopy; section I.C. of this protocol)

5. Determine the number of eggs subsequently produced from adults maturing after recovery from cryopreservation.

6. Determine the number of larvae (F2) hatching from eggs produced by adults that matured after recovery from freezing to confirm the colony has returned to healthy, normal husbandry conditions

APPENDIX B: DRY ICE VAPOR PROCEDURE FOR CRYOPRESERVATION

Purpose: This procedure describes a process for the cryopreservation of L1 *Anopheles stephensi* larvae and the evaluation of the new progeny from the cryopreserved larvae.

Scope: This procedure applies to the process of rearing, cryopreserving, and evaluating *Anopheles stephensi* L1 larvae.

Responsibilities: Adherence to precautions and laboratory safety needs when working with the chemicals described in this protocol is required.

Reagents, Materials and Equipment: All reagents, materials, and equipment required are standard laboratory grade, equipment except when noted in the method, and except for the requirement of a thermocouple sensor and thermocouple thermometer. For this, a standard thermocouple thermometer and wire sensors are required (no specific brand is necessary for performing these studies).

Procedure

I. Colony establishment and maintenance

A. Rearing method

1. Obtain *A. stephensi* eggs from BEI Resources (NIAID, NIH: *Anopheles stephensi*, Strain STE2, MRA-128, contributed by Mark Q. Benedict).
2. Remove egg paper from the habitat.
3. Transfer paper to 4 L of water and add fish food (for larvae to feed on) to the habitat (initial conditions and concentration of food may vary based on yield).
4. Follow feeding schedule and removal of pupae using this reference as a guide https://www.yumpu.com/en/document/read/49409322/methods-in-anopheles-research-mr4.

5. For colony maintenance, feed *Anopheles stephensi* mosquitoes defibrinated calf blood, with sugar and water provided ad libitum.
6. Rear larvae (***note 1***) under controlled conditions of temperature (28°C), relative humidity (70%) and light (12:12 L:D diurnal cycle).
7. Count pupae at emergence over a 5-day period (day 9-13) (***note 2***).

   Percent Maturation Rate = Percent of larvae converted to pupae = (number of pupae/number of L1 larvae) x (100)
8. Maintain adults under the same controlled conditions.

B. Determining the total number of larvae (*note 3*) - Larvae develop from eggs to first instar larvae (L1) around 2-3 days after transferring egg paper. L1 develop into L2/L3 over the next 4 days (approximately).

1. At 72 hours, remove *An. stephensi* larvae hatch bin.
2. Transfer a small amount of water from the hatching habitat to a new bin.
3. Cut the tip of a plastic bulb pipette with scissors, approximately 1cm above the tip, to widen the inlet area of the pipette.
4. Remove 7-20 larvae at a time by pipetting, and count larvae through the clear walls of the pipette.
5. Transfer to a new bin containing hatch bin water.
6. After larvae are counted, transfer them back to the original habitat, along with the water from the new hatch bin.

C. Phenotypic characterization of larvae at each instar by advanced stereo microscope (Larval Microscopy)

1. Cut the tip of a plastic bulb pipette with scissors, approximately 1cm above the tip, to widen the inlet area of the pipette.
2. Remove 5-7 larvae from their hatch bin, and transfer in one drop to a glass microscope slide.
3. Analyze larvae using an advanced Stereo Microscope (Olympus SZX16 or similar), at variable magnification settings and determine (a tabulation might help):
    a. Microscope settings.
    b. Photographic and descriptive phenotypic characterization of larvae at each instar.
    c. Number of larvae at first instar (L1).
    d. Characteristics of L1 larvae.

II. Cryopreservation of L1 larvae (*note 4*)

A. Procedure

1. Prepare 7M methylformamide with 0.5M trehalose in tap water at room temperature.
2. Transfer 12 mL CPA solution into a 60 mm petri dish.
3. Set dishes containing CPA solutions on ice in a Styrofoam box with a lid.
4. Add dry ice to a well-insulated box so that the dry ice is flush with the base of the ladder (***note 5***).
5. Measure the temperature of each rung on the ladder using a thermocouple, to determine the rung for which -12°C to - 16°C is maintained when the lid of the box is closed (refer to figure, ***note 5***).

6. Prepare a 60 mm dish with tap water at room temperature; add a nylon mesh basket (***note 6***).
7. Using a 15 µL pipette (***note 7***), transfer L1 larvae into the nylon mesh basket in the water and transfer dish to ice bath.
8. Insert a thermocouple into one of the dishes containing a basket of L1 to monitor the change in temperature. When the temperature of the dish containing L1 reaches 0°C and is stable for 5 min, lift the basket out of the water, gently blot dry excess fluid from the basket using a Kimwipe, and immediately transfer the basket to the CPA solution on ice.
9. Incubate larva in the CPA solution for 5 minutes on ice.
10. Lift baskets, blot dry, and transfer nylon mesh containing the treated L1 to the ladder above dry ice at the -12°C to -16°C position.
11. Hold at this temperature for 5 min (***note 8***).
12. Transfer L1 back to the cold water on ice and immediately move the dish containing the L1 mesh basket to the benchtop; hold on the benchtop until the solution in the dish reaches room temperature.
13. Return L1 to standard culture conditions, rearing and collecting data for analysis and as needed for colony maintenance as described in Section I of this SOP.

B. Recovery and quality control analyses of new progeny from cryopreserved L1 larvae (*note 9*)

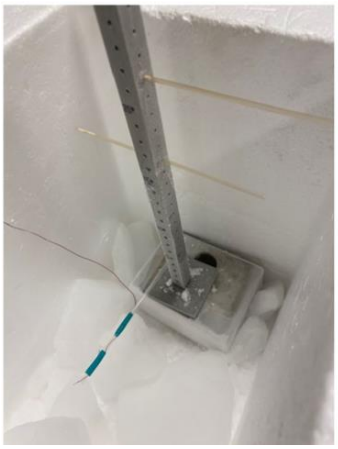
1. Determine the percentage of freeze-thawed larvae that survive and pupate in reference to a control group (see Section I.B for counting method).
2. Determine the number of viable adult mosquitoes generated following a single freeze-thaw cycle in reference to a control group.
3. Maintain adults (see Section I.A) through development of F2 progeny.
4. Perform phenotypic similarity analyses (via microscopy) and note any differences between groups.
5. Determine the ratio of males to females as F0 adults die off from each group.
6. Determine the number of F1 pupae in each group.
7. Determine the ratio of males to females as F1 progeny adults die off in each group (***note 10***).

III. Notes

1. Emergence of L1 larvae generally begins on day 3; each instar stage lasts about 2 days.
2. Emergence of pupae generally begins on day 9; most are transferred over the next 2 days.
3. Only use this method when counting larvae is required as part of an experimental process as it is stressful to the colony.
4. 100 or more L1 larvae should be used to establish and confirm applicability of this method in each unique laboratory setting. Once confirmed, 400 or more L1 larvae should be used to ensure enough surviving larvae to establish a colony after recovery from cryopreservation. Data from initial confirmation

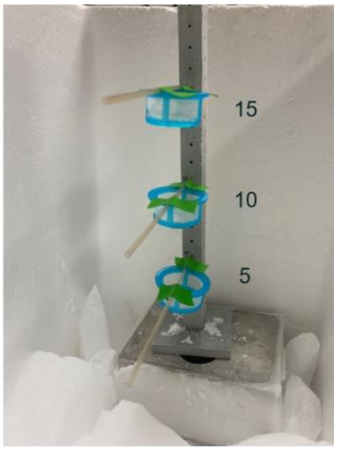
conditions should be further analyzed for quality control purposes (see section III for further details).

5. A thick Styrofoam box or insulated cooler may be used. The base of the ladder is approximately 2 inches high with rungs approximately 2.5 cm apart from just atop the dry ice to within 5 cm of the top of the box as shown in the figure below.



Position over dry ice as determined with thermocouple sensor and thermometer (Left) and subsequent placement of nylon mesh baskets (Right)

5 = -28C
10 = -16C
15 = -2.5C
Higher positions were above 0C



6. In our studies, we used nylon mesh baskets – FalconTM Cell Strainers 70µm, Fisher catalog #08-771-2.
7. The experimenter may have to cut the bore / tip of the pipette for larger sized L1.
8. We did not exceed 5 minutes in our current study. Longer incubations with survival are expected.

9. Quality control data that is collected from baseline colonies and assessed in comparison to recovered progeny from cryopreserved L1 is used to confirm reproducibility and establish acceptable margins during study optimization.
10. A large F1 colony may be expected based on the number of starting L1, and high viability. Determine the sex ratios in a percentage (10-20%) of F1 when progeny exceed 500.

APPENDIX C: ADDITIONAL ACADEMIC ACOMPLISHMENTS

During my first-year rotation in Dr. Erin Nishimura lab's, I worked with Dr. Robert Williams to determine the expression and functions of ELT-2 and ELT7 in *C.elegans* by RNA-sequencing and microscopy. I was involved in validating the knockdown of RNAi targets in single and double RNAi conditions via microscopy. Gratefully, my work was rewarded with co-authorship on a manuscript titles "Transcriptome profiling of the *Caenorhabditis elegans* intestine reveals that ELT-2 negatively and positively regulates intestinal gene expression within the context of a gene regulatory network" in the journal of Genetics.

As a member of Dr. Rushika Perera's lab, I worked in the project to determine the presence of fatty acid synthase (FAS) in Aedes aegypti mosquitoes. I was involved in in performing PCR and gel electrophoresis. This work resulted in co-authorship on a manuscript titled "Expression of fatty acid synthase genes and their role in development and arboviral infection of *Aedes aegypti*" in the journal of Parasite and Vectors. In another project, investigating the role of Acyl-coA thioesterases during dengue virus 2 infection *in vitro*, I was involved in performing plaque assays. This work also resulted in my co-authorship on a manuscript titled "Acyl-Coa Thioesterases: A Rheostat That Controls Activated Fatty Acids Modulates Dengue Virus Serotype 2 Replication" in the Journal of Viruses.

In Fall 2023, I worked in an education research project titled "Using Scientists Spotlights in a STEM course to support scientific identity within the context of Diversity and Social Justice" with Dr. Carolina Mehaffy. I was involved in curating a database

exhibiting diverse scientists, including information about personal life and professional accomplishments showcased to students enrolled in MIP 300.