Aedes aegypti: Eco-evolutionary Dynamics, Gene drives, and Sex-Ratios

by

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A Dissertation Presented in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Approved February 2026 by the
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May 2026

ABSTRACT

Mosquito-borne diseases pose a persistent global health threat. Traditionally managed with insecticides, approaches to combat this threat must address insecticide resistance and ecological concerns of the environment. Novel vector control strategies employing the release of sterile/irradiated, *Wolbachia*-infected, or genetically modified *Aedes aegypti* mosquitoes offer potentially more sustainable alternatives by reducing vector populations. However, the successful and safe implementation of these innovative technologies necessitates a thorough understanding of complex eco-evolutionary dynamics including: potential density-dependent feedback mechanisms, the role of sex ratios of breeders, and the impact of environmental factors like water depth and food availability on larval development; it is also imperative to integrate understanding of these dynamics into risk-benefit assessments and release strategies. Herein, this dissertation reviews the current status, successes, and limitations of these three mosquito modification approaches, in concert with original experimental investigations into (1) whether female *A. aegypti* can sense and alter offspring sex ratios in response to male-biased adult populations, a scenario relevant to many vector control interventions, and (2) how varying water depths and food availability affect *A. aegypti* larval development, survival, and adult fitness under controlled laboratory conditions.

The dissertation emphasizes critical eco-evolutionary questions that must be addressed through rigorous laboratory and field research – before, during, and after mosquito releases – to predict their efficacy, optimize release parameters, anticipate

potential ecological impacts (considering the mosquito's role in the ecosystem), and ensure their responsible deployment with community engagement. This work highlights the importance of considering species-specific traits, genetic variation, competitiveness, adaptation, long-term ecological consequences, the resilience of natural sex ratio regulation, the influence of larval environmental conditions (including water type and container characteristics) on adult traits, and the need for transparent communication and community involvement. Then this dissertation underscores the need for a holistic, eco-evolutionary informed framework to guide the development and implementation of modified mosquito-based vector control strategies for sustainable and impactful outcomes, further informing the minimal resource requirements for mosquito development.

DEDICATION

I dedicate this to:

My mother, Vanessa, who sacrificed her own education and career goals to raise me and continues to empower me to live a life of Honesty and Integrity above all else.

My sister, Denay, who has motivated me to be the best brother, friend, and role model I can be since her birth.

My belated and beloved Uncle Philip Gannon and Great Grandmother Ruby Bundy who we lost during my time in graduate school. They continue to motivate me to enjoy life, excel in all I do, and make the most of every opportunity.

My Great Grandfather Earl Bundy whose dedication, fortitude, leadership, selflessness, and vision blazed trails for my family.

The Black and African Canadian communities of Nova Scotia, as well as the Black and African American communities in the United States.

Finally, military children and dependents that are striving to find their calling and place of belonging.

ACKNOWLEDGMENTS

I thank my committee members Dr. James Collins, Dr. Krijn Paaijmans, Dr. Arianne Cease for their assistance, support, and time throughout my master's and PhD programs at Arizona State University.

I thank my committee member Dr. Scott Shaffer for introducing me to research as a curious undergraduate student and researcher at San Jose State University. I am grateful for his unwavering encouragement, guidance, and support throughout my academic career.

I thank Dr. Jane Maienschein, Dr. Kate MacCord, Dr. Matthew Chew, Dr. Keon McGuire, Mr. Jahmal Williams, and Dr. Jamie Alea for their feedback, insight, mentorship, and support throughout my graduate and undergraduate programs.

I thank Dr. Silvie Huijben, my colleagues in the Paaijmans' and Huijben's labs, the faculty/students/staff in my Biology and Society, the School of Life Sciences, and Dr. Shaffer's lab for the invaluable exchanges of advice and knowledge inside the lab and beyond.

I thank my mother (Vanessa), father (Derrick), and sister (Denay) for all their advice, guidance, laughter, love, and support. I thank Carl, Karen, Markara, Akeem, and Amiri Brown as well as their extended families for their unwavering love and support. I also recognize the consistent support of my grandparents, Aunt Donna, Uncle Philip, Aunt Danielle, the Köhler family (Grit, Uwe, Jonas, and Linus).

It takes a village to succeed; I appreciate all who've helped make this possible.

TABLE OF CONTENTS

Page

CHAPTER Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

CHAPTER 1

INTRODUCTION

Mosquito-borne diseases (MBDs), including malaria and dengue, continue to inflict a significant burden on global public health despite decades of vector control efforts. The increasing prevalence of insecticide resistance, coupled with the ecological harm associated with widespread insecticide use and the expansion of disease-endemic areas due to factors like globalization and climate change, necessitates the exploration of alternative, sustainable vector control measures. Among the most promising of these are strategies involving the release of modified *Aedes aegypti* mosquitoes, the primary vector for several life-threatening arboviruses such as dengue, Zika, and yellow fever. These strategies – utilizing sterile/irradiated insects, *Wolbachia*-infected mosquitoes inducing cytoplasmic incompatibility, and genetically modified strains designed for population suppression – offer the potential to reduce mosquito populations and consequently lower disease transmission. However, the transition from laboratory efficacy to successful and safe field implementation requires a comprehensive understanding of the intricate eco-evolutionary interactions that govern mosquito populations, their ecological roles, and their environments, including factors influencing sex ratios, density-dependent mechanisms, and the critical role of larval development conditions such as water depth, food availability, water type, and container characteristics.

This dissertation provides a critical review of these three innovative vector control technologies, highlighting their known successes and limitations. Furthermore, it presents original research investigating (1) the potential for female *A. aegypti* to adjust offspring

sex ratios in response to skewed adult sex ratios, a phenomenon with significant implications for the long-term efficacy of male-biased vector control strategies, and (2) the impact of varying water depths and food availability on *A. aegypti* larval development, survival, and adult fitness under controlled laboratory conditions, providing insights into the minimal resource thresholds for successful development. Recognizing the importance of community involvement and transparent communication, this work aims to contribute to a more informed and responsible deployment of modified mosquito-based vector control approaches, ultimately enhancing their effectiveness and minimizing potential unintended ecological and social ramifications for sustainable and impactful outcomes.

CHAPTER 2

ECO-EVOLUTIONARY CONSIDERATIONS TO ENSURE THE SAFE AND SECURE IMPLEMENTATION OF MODIFIED AEDES AEGYPTI MOSQUITO BASED VECTOR CONTROL APPROACHES

Abstract

Mosquito-borne diseases remain a significant public health threat worldwide and are commonly controlled or prevented using insecticidal applications. Rising concerns about insecticide resistance and the potential ecological harm they cause, in combination with a worldwide increase in the mosquito-borne disease burden, place an immense value on alternative, less invasive, and more sustainable mosquito control measures. Three promising approaches that are currently being explored in the laboratory and field include the release of sterile/irradiated, *Wolbachia*-infected, or genetically modified mosquitoes. While these methods demonstrate the potential for reducing infectious disease incidence, mainly through reductions in mosquito vector population sizes, several important factors are poorly understood. These range from (seasonal changes in) mosquito population abundance and metapopulation connectivity to the impacts of innovative technologies on local ecosystems. This chapter reviews the three technologies, their known successes and pitfalls. Then it focuses on critical eco-evolutionary questions that need to be tested in the field and laboratory—before, during, and after mosquito release—to anticipate the successes or failures of those innovative technologies.

Introduction

Mosquito-borne diseases (MBDs) are transmitted through the bites of infected mosquitoes. Malaria and dengue viruses are among the most widespread mosquito-borne infectious agents, and both continue to impact human populations across the world despite decades of vector control initiatives (1-9). For example, malaria cases continued to increase in 2021 and 2022, albeit less quickly than from 2019-2020 [1-3]. Dengue viruses are now endemic in approximately 129 countries and infections grew by ~2.8 million cases in 2019 compared to 2010 [4-10]. Increased global trade and travel, urbanization, climate change, and changing weather patterns are likely to contribute to the expansion of at-risk areas and cases [11-13].

Resistance to insecticides in front-line vector control tools such as bed nets, indoor residual spraying, and aerial spraying is also spreading rapidly. According to the 2020 World Malaria Report, 73 of 82 malaria-endemic countries that provided data identified insecticide resistance to at least one of the four most common insecticide classes (pyrethroids, organochlorines, carbamates, and organophosphates) [1]. Resistance to all four of the most commonly used insecticide classes existed in 28 countries; only 8 countries reported no resistance to any insecticide class [1]. As per the 2023 edition of the World Malaria Report, 78 of the 88 malaria endemic countries that provided data detected resistance to at least one insecticide class [3]. 29 of those countries detected resistance to all four of the most commonly used insecticide classes [3]. In addition to increasing resistance to insecticides, mosquitoes can circumvent vector control interventions through changes in behavior (resting and feeding), which includes outdoor

resting/biting and the ability to engage in opportunistic feeding on animals other than humans [14-15].

These factors facilitate the dispersal of mosquito populations and are concerning given current reliance on vector control to secure, protect, and improve public health. Affordable and effective drug treatments and vaccines are not available for most mosquito-borne diseases [16-17]. In response to this ongoing health challenge, various stakeholders including funders, industry, governments, scientists, and other public health entities, continue seeking effective methods to combat mosquito populations and reduce the transmission of mosquito borne diseases. In addition to improving traditional methods of vector control (e.g., by using new classes of insecticides for bed nets or indoor, outdoor, or aerial spraying), interest in three innovative vector control approaches—sterile/irradiated insects, and *Wolbachia*-infected mosquitoes inducing cytoplasmic incompatibility, and genetically modified strains designed for population suppression—continues to grow [8, 13, 16]. These three approaches are drawing increased attention because they can work in parallel with other vector control tools to reduce more efficiently the number of mosquito offspring in an area, thereby decreasing the size of local mosquito populations, and lower disease risk as well as nuisance biting [8, 18-19]. These approaches will be particularly helpful if they work despite evolutionary changes in mosquito feeding and resting behaviors that are observed under high insecticidal pressures.

Here this chapter reviews the three technologies, and their known successes and pitfalls. Given the specifics of how sterile/irradiated, *Wolbachia*-infected, and genetically modified mosquitoes function, their performance inevitably varies. This chapter then

focuses on critical eco-evolutionary questions that need to be addressed in the field and laboratory—before, during, and after mosquito release—to understand the successes or failures of those innovative technologies. Answering these questions may also help improve community acceptance because there is often lack of unanimous support from many communities and researchers due to concerns regarding their effectiveness, reliability, and novelty [20]. There is hesitancy to use these tools because of skepticism toward science and technology as well as a lack of data that convincingly illustrate sustained/long term results in the field. Some general questions that need to be answered include: *1) Can these tools succeed despite the diversity of mosquito populations, their behaviors, and evolutionary responses (natural and because of human activities)? 2) Will any mosquitoes (wild or released) adapt in response to or from the use of these tools? 3) Does the use of these mosquitoes pose a hazard to humans or the environment and what will ensure these tools are deployed safely?*

Three Technologies Garnering Attention for their Potential to Modify Mosquitoes: Important Eco-evolutionary Considerations for Monitoring the Efficacy of Novel Vector Control Tools

Sterile/irradiated mosquitoes

Release of sterile/irradiated mosquitoes is not new, but this method is becoming increasingly popular as stakeholders seek more economical and effective vector control tools that do not depend on insecticides, or large amounts of personnel and time to succeed [13]. Sterile/irradiated insect technologies release irradiated mosquitoes, which

commonly entails breeding mosquitoes and separating male and female pupae in controlled environments. Male mosquitoes are then sterilized, often via x-rays or chemosterilants before being released to mate with wild females rendering the resulting eggs inviable [21-25]. Accordingly, since radiation induces a unique and random array of dominant lethal mutations in each individual, the resulting sterility is multi-faceted; this genomic redundancy makes it statistically improbable for a wild population to evolve a singular resistance mechanism [21]. While these tools can reduce the number of viable mosquito eggs laid within a population, they do not directly reduce all disease risks from biting since this treatment only impacts eggs laid by females that mate with sterile/irradiated males. It is also worth noting that sterile/irradiated mosquitoes are not self-sustaining, and modelers recommend a minimum 2:1 ratio up to 9+ fold release rates of non-wild-type to wild-type mosquitoes (those indigenous to an area) to suppress wild mosquito populations [24, 26-27]. Despite attempts by modelers to predict how releasing a certain number of sterile insects should suppress mosquito populations, regular releases of sterile/irradiated mosquitoes in combination with integrated vector management techniques are often required to meet the goals of vector control programs [24, 27-28].

Notable field releases of sterile *Aedes aegypti* mosquitoes have occurred in Thailand (2016) [29], and Cuba (2020) [30]. Both releases entailed the separation of male pupae from females in a laboratory environment and irradiation the male pupae before they emerged as adults [29-30]. The project in Thailand during 2016 reported that use of sterile insect mosquitoes led to an 84% reduction in the mean hatch rate and 94% reduction in the mean number of female mosquitoes per household [29]. Cuba hosted a three-year vector control program designed to include a barrier zone and release of an

estimated 1.25 million male mosquito pupae or 0.05 million sterile male mosquitoes per day, which were sterilized using chemosterilants [30]. The ~ 39-week experiment in Cuba during 2020 focused on suppression of a local mosquito population using sterile mosquitoes [30], resulting in a mean of 0 mosquito eggs per trap after 17 weeks of releases. A mean of 0 mosquito eggs per trap was also collected during the final 3 weeks (weeks 37-39) of the those releases in Cuba compared to means ranging from 28-32 mosquito eggs per trap in untreated control areas [30]. Data from those releases of sterile mosquitoes in Cuba also shows releases can obtain reductions in local mosquito population numbers independent of other vector control tools and over a shorter period if conducted during winter months when mosquito populations are lowest [24, 30, 31].

Results of these field trials demonstrate that sterile insect technology is more complex than "simply releasing large numbers of modified mosquitoes in the wild." For experiments that use sterile insects to succeed, workers must mass-rear mosquitoes and release them into the wild. The sterile mosquitoes must then survive long enough to find a mate and outcompete nearby wild-type mosquitoes for multiple generations for the releases to succeed. The inherent complexities associated with released mosquitoes (i.e., outcompeting immigrants from other populations and mating with females) illustrates the importance of conducting regular releases of modified mosquitoes. One-time or short-term releases provide more opportunities for wild mosquito populations to rebound to previous levels [31]. The ability of wild mosquito populations to rebound highlights how immigration of wild mosquitoes to trial areas, and less frequent or large releases of modified mosquitoes, can influence the outcomes of vector control initiatives [24]. This underscores the fact that this tool's performance and the sustainability of its effects will

likely improve when an overwhelming number of sterile/irradiated mosquitoes are regularly released in each area.

It is important to replicate previous release experiments like those conducted in Cuba [30] and Thailand [29] to test whether experimental designs yield similarly positive results outside of the original trial areas and during seasons when mosquito populations have higher densities. Hence it is critical to test the efficacy of this approach under different environments and variables such as area, timeframe, degree of urbanization, vegetation density, and water availability. Furthermore, replication is necessary to assess the effectiveness of vector control projects that use newer technologies.

By promoting the sustainability and impact of projects that release sterile mosquitoes, it is possible to ensure they are one element of an integrated vector control intervention. This can be achieved by designing projects to include a variety of tools. For example, release of sterile insects in combination can be effective when paired with the staggered application of insecticide/larvicide on nearby areas where wild-type mosquitoes find refuge and treating potential mosquito resting areas like walls or doors can be effective. In addition to reducing room for error during projects, an integrated approach can help alleviate the pressure on one tool such as irradiated or sterile insects to perform perfectly. Improving the design of sterile insect release in that manner will also help to address potential issues such as logistical challenges or increased ecological competition from mosquitoes that are local to an area or that immigrate when the overall mosquito population decreases.

Wolbachia-infected mosquitoes

Unlike sterile/irradiated mosquitoes, this incompatible insect technique (IIT) entails infecting mosquitoes with *Wolbachia sp.*, a bacterium first described by Hertig and Wolbach in 1924 [8, 33]. These bacteria confer inherited cytoplasmic incompatibility, which prevents wild female mosquitoes that mated with the released *Wolbachia*-infected males from producing viable eggs [33]. While *Wolbachia*-infected mosquitoes can have reduced fecundity and fertility compared to wild populations [34], females can reproduce with males that are infected by the same *Wolbachia* [34-35]. As a result, these *Wolbachia* mosquitoes can suppress or replace existing mosquito populations [8, 35].

Results from recent field trials indicate that this method has the potential to further vector control by reducing mosquito related impacts that threaten human populations. For example, the release of *Wolbachia*-infected mosquitoes contributed to a 40.3% reduction in dengue virus transmission from wild *A. aegypti* in Malaysia from 2017-2018 [36]. Similar work in urban Singapore from 2016-2022 led to 98% reductions in the *A. aegypti* population, 88% reductions in dengue incidence over the course of a year, and a 69% decrease over a 3-year period compared to a control area [8, 37-40]. Releases conducted in an "ecologically isolated village" within Thailand during 2016 led to reductions of 84% in mean egg hatch rate and 97.3% in mean number of female mosquitoes per household [41]. Additionally, a *Wolbachia* release in two of Miami's urban neighborhoods contributed to a 78% reduction in *A. aegypti* females when comparing data from the center of their trial area to untreated areas [42].

Researchers credit the use of *Wolbachia*-infected mosquitoes with a 77% decrease in dengue over a 27-month period following a similar experiment in Indonesia [8]. Studies also report that this tool contributed to a reduction in symptomatic dengue and mosquito related hospitalizations in Brazil [42] and Australia [8, 10, 44-47]. *Wolbachia*-infected mosquitoes also contributed to the suppression of mosquito populations in California and Texas [33]. However, the ability of Wolbachia-infected mosquitoes to suppress mosquito populations will likely decrease if the rate of the releases slows in frequency or experiments conclude [33,38, 48].

The release of *Wolbachia*-infected mosquitoes clearly has the potential to reduce *A. aegypti* populations and therefore dengue transmission. Still, there are several critical eco-evolutionary questions that need to be addressed. As with the releases of sterile/irradiated mosquitoes, it remains important to continue investigating this vector control tool's performance in the field for longer timeframes (especially following the conclusion of trials). It is also essential to study the impact of *Wolbachia* on mosquito fitness (such as mating competitiveness), behavior, and the likelihood of evolutionary adaptation by mosquitoes, *Wolbachia*, or viruses like dengue in response to selection pressures caused by *Wolbachia*-infected mosquitoes [8, 49-50]. Given that *Wolbachia* is present in nature and is species-specific, it is essential to understand whether the strains carried by mosquitoes that are infected and released in lab-based colonies or trial areas can successfully compete against endemic strains in different regions of the world [33, 40].

Genetically modified mosquitoes and gene drive bearing mosquitoes

The term gene drive refers to phenomena (natural or engineered) that cause a certain allele(s) frequencies to increase in a population regardless of fitness costs at the individual level [51-53]. The gene drive mosquitoes discussed in this review are animals that researchers genetically modify to favor certain genes or phenotypes over others. In this case it is essential to maintain clear communication regarding vector control tools, especially when considering the necessity to gain public acceptance, awareness, and trust of this emerging technology [20]. While this approach has attracted attention, some information regarding the ability of gene drive and genetically modified mosquitoes to reduce mosquito populations, rates of biting, and disease transmission remains unknown. There is, for example, a lack of clear national and transnational regulations pertaining to using genetically modified mosquitoes and no generally accepted consensus about the knowledge stakeholders should possess before releasing mosquitoes into the wild [20]. This is partly due to the lack of experiments to test released mosquitoes in the field, which prevents stakeholders from gauging the effectiveness of these technologies outside of labs and small-scale pilot projects. The decentralized nature of research across the world and the diversity of mosquito populations, as well as the limited knowledge of mosquitoes, the distinctiveness of their local ecology, and public sentiments also contribute to the lack of standards specifying when and how to use these vector control tools in the field. There are, however, some pilot experiments despite these deficiencies.

Two projects associated with field releases of genetically modified mosquitoes use OX513A (first generation) and OX5034 (second generation) mosquito strains reared by Oxitec, a biotechnology company [54]. The mosquitoes have a fluorescent marker

gene that glows under red light and a self-limiting gene that prevents female offspring from surviving to adulthood [55-57]. Oxitec's self-limiting OX513A male mosquitoes produce inviable offspring when they mate with wild-type females [58]. Experimental data associated with releases of OX513A males in Jacobina, Brazil from 2013-2015 [59], raised some concern regarding the potential for mosquitoes and/or their genes to proliferate beyond the scope of trial areas where they are released [60-61]. This resulted in some apprehension that genetically modified mosquitoes could mate with other individuals in nearby populations, thereby impacting nearby ecosystems in unintended ways. However, a since published addendum to that article and an editorial report that genes of concern that OX513A mosquitoes unintentionally transferred to mosquitoes outside the trial area were lost from the population as the number of hybrid individuals with wild-type and OX513A genes decreased over time [60-61]. This finding suggests that while a few mosquitoes may travel beyond a trial area and mate, those genes are unlikely to be inherited for multiple generations. As a result, the addendum [61] and other published work [55] help assuage concerns that genes from OX513A mosquitoes could "escape" to nearby populations and make future vector control efforts more challenging.

Oxitec has since developed its OX5034 mosquitoes which are self-limiting because female offspring do not survive [62]. While male offspring of released OX5034 mosquitoes survive, half inherit the self-limiting gene and half inherit genes that are naturally susceptible to insecticides [62]. The prediction is that fewer males live to pass on their genes with every generation to such an extent that OX5034's self-limiting genes are not expected to remain in an environment by 10 generations after mosquito releases end [54, 63-64]. These genetically modified mosquitoes are also designed to only

decrease populations of target mosquito species [55-56]. Oxitec has released mosquitoes in various countries, including Brazil (2010; 2013-2015), the Cayman Islands (2009-2010), India (2017), Panama (2014), and Malaysia (2010) [58-73]. Oxitec's mosquitoes have demonstrated utility given their ability to reduce mosquito populations in the Cayman Islands and Bahia, Brazil, by ~96% and 70-90%, respectively [28, 67, 71]. Such performance in the field helped this technology garner attention in other countries like the United States where California, Florida [56], and Texas [56] have discussed and, in some cases, approved releases of these mosquitoes [56, 70, 74].

In addition, a different project in Brazil during 2018 used drones in release of insects carrying dominant lethal (RIDL) male mosquitoes; these RIDL males mate with wild females and their offspring die before adulthood [75, 76]. Results from this drone-facilitated release of 50,400 RIDL mosquitoes within a 20-ha trial area in Brazil during 2018 resulted in a 50% decline in viable eggs collected in the trial area compared to eggs collected from a nearby control area [75]. Replication is especially warranted to assess the effectiveness of vector control projects that use ingenious approaches like the experiment in Brazil. This experimental approach is innovative because it can be used to reach areas that are impassable or reach mosquito populations that potentially disperse from trial areas and hinder vector control. Incorporating innovative and less invasive tools like drones in remote areas, or *Wolbachia* in urban areas, could reduce environmental impacts while improving the sustainability of projects and address concerns related to expenses, logistics, personnel, and privacy (especially if it is possible to replicate the results observed in Brazil).

Other gene drive bearing technologies

Although Oxitec genetically engineers' mosquitoes to carry dominant lethal genes, its products are not the only approach to using genetically modified mosquitoes to combat mosquito populations [77]. CRISPR-based gene drives are another emerging vector tool that has attracted attention due to its potential to be more cost-effective, less invasive, and species-specific vector control tools [52, 78]. Although the efficacy of CRISPR-based gene drives in the field has not yet been established, lab experiments have yielded positive results that include facilitating a gene becoming 100% prevalent in and subsequently collapsing a mosquito population in 11 generations [79]. Researchers are also testing the possibility of using CRISPR's ability to genetically modify mosquitoes to reduce and in some cases block the ability of mosquitoes to transmit viruses like chikungunya, dengue, and Zika [80].

The connection between the occurrence of Oxitec's mosquito releases and the reduction of mosquito populations is similar to many projects that rely on regular releases of sterile/irradiated and *Wolbachia*-infected mosquitoes to control wild mosquito populations. Whether it is by design or unintended after lengthy field releases, the current self-limiting design of Oxitec's strains may prevent them from eradicating mosquitoes independent of other vector control methods. This limit supports the conclusion that these mosquitoes, by way of their current self-limited form have many advantages and are designed to decrease the population of a target species rather than eliminate all mosquitoes in an ecosystem [56, 80].

Important Eco-evolutionary Considerations for Monitoring the Efficacy of Novel Vector Control Tools

There are critical indicators that stakeholders should monitor and evaluate before, during, and after such releases of modified mosquitoes to ensure these tools are implemented in the most effective way. As such, recommendations are summarized in Table 2.1, followed by a discussion of each.

Table 2.1: Monitoring and evaluation criteria that can guide the effective release of sterile, *Wolbachia*-infected, or genetically modified mosquitoes

Table 2.1	
Before	• Recognize species and population-specific behaviors, genetics, and preferences. • Assess ecological and health risks of introducing/re-introducing mosquito populations. • Gauge and understand the responses of human communities, mosquito populations, and the larger ecosystem before deciding to use these tools. • Investigate how interconnected ecosystems are and how far mosquitoes can travel when seeking mates.

	• Compare the competitiveness of mosquitoes that will be released with existing wild-type populations.
Before and during	• Consider the probability of and rate at which released mosquitoes survive and mate successfully compared to individuals from wild, local mosquito populations? • How many mosquitoes need to be released and for what duration [81]?
During	• Can wild mosquito populations adapt to coexist with the released mosquitoes or discriminate against possible mates that are genetically modified, sterile, or carry *Wolbachia*? • How long are the releases of mosquitoes able to suppress target populations?
During and after	• Monitor gene flow and record if resistance to vector control tools develops. • Compare the performance of released modified mosquitoes with other vector control tools [81].
After	• Does the timeframe and success of using these tools vary between mosquito species and geographical landscapes?

	• Assess whether modified mosquitoes could be deployed/utilized to stop outbreaks of mosquito-transmitted diseases given the ability of *Wolbachia*-infected mosquitoes to reduce mosquito populations [56]. • Monitor mosquito populations after trials and assess whether alleles from released mosquitoes persist. • To what extent do overall mosquito populations and rates of disease transmission change. • How long can released mosquitoes suppress target populations, and does the timeframe and success of using this tool vary between mosquito species and across geographical landscapes? • Gauge and understand the responses of citizens, mosquito populations and the larger ecosystem before deciding to use these tools.

Before Releases

Prior to any releases, it is critical to understand the biology and ecology of the targeted local mosquito populations and to compare those with the life-history traits of

modified mosquitoes that are reared for releases (Table 2.1). This comparison is essential to ensure that released mosquitoes are capable of effectively competing with or outcompeting and/or replacing the target populations in trial areas (Table 2.1). It is also important to consider how reductions or eliminations of mosquito populations impact broader ecosystems. Such insight allows stakeholders to accurately analyze costs and benefits associated with any mosquito releases or vector control projects.

Species diversity and distribution

Many countries have populations of endemic and non-endemic mosquito species, some of which differ in habitat preferences. Mosquito population distributions and sizes are also impacted by numerous factors, including humidity, housing/living conditions, population density, seasonality, temperature, vegetation, water availability, etc. For example, *Culex quinquefasciatus* thrives in habitats with standing water rich with organic matter and waste that humans consider "dirty" [82-83]. In comparison, species like *A. aegypti* prefer the same clean and fresh water that humans need to survive and have settled near for millennia. It is these kinds of habitat and niche preferences between mosquito species and their behavior that allow both mosquito species to coexist near humans, although *Aedes* have developed a preference for human odor and blood-feeding that is evident from their house entering behavior [82]. As it pertains to vector control, understanding the abundance, distribution, and preferences of target mosquito species as well as other mosquito populations that are present in an area directly influences which strains or types of modified mosquitoes, vector control tools, frequency of releases, and interventions (i.e., community maintenance and vigilance) will be most effective. Since female mosquitoes are more likely to oviposit in locations that increase offspring fitness

[83], it is essential to assess mosquito abundance and distribution across breeding sites from season to season and across the year. This data is particularly important when vector control projects intend to reduce risks of biting, disease transmission, and track insecticide resistance or gene flow in an area.

Genetic variation

When considering mosquito populations for designing, conducting, and evaluating vector control projects, it is important to evaluate the complexity and extent of genetic variation within areas from a behavioral and genetic standpoint, as well as the existence of different subspecies, especially within a local area [12]. One way to accomplish this is by investigating whether any genetically distinct species or subspecies are present in trial areas (Table 2.1). If sampled mosquitoes differ significantly from a genetic point of view, additional studies can assess whether their behaviors, abilities to vector disease, and/or population densities differ from those that the vector control tools or modified mosquitoes are intended to combat [12]. It is critical to collect this information and use the data to inform localized vector control plans about target ecosystems because mosquito populations can be remarkably diverse, even in smaller nations, between or within cities, towns, and villages. For example, researchers who conducted a project in Panama recently determined that mosquito genomic variation in populations of *A. aegypti* were not randomly structured [84]. Other studies have also reported similar genetic variation in mosquito populations in Belgium [85] and Vietnam [86]. Research also shows that seasonal fluctuations and geographic variation can impact mosquito population dynamics [87-90]. These findings demonstrate that geographic regions may require the release of genetically modified mosquitoes compatible with the local

ecosystem or the use of multiple vector control techniques to ensure efficacy. Although, it is resource intensive to conduct such work before releases, the information will contribute to a more successful vector control program by recognizing species that are present in an area, population specific behavior, as well as gene flow and dispersal patterns that will impact the efficacy of vector control tools (Table 2.1).

In addition to understanding how mosquito evolutionary responses could impact the performance of released mosquitoes, it is equally important to study the population of mosquitoes planned for release and anticipate which variables like variable field temperatures, mosquito mating competitiveness, mosquito dispersal abilities (via flight or wind, etc.,) might affect the strains of mosquitoes used as well as where and how often mosquitoes are released [8, 91]. This prior planning can provide critical data that will directly inform which strain(s) of *Wolbachia* (for example) researchers require for releases. This may also indicate how frequently to release modified mosquitoes, how many mosquitoes to release, and how long releases should continue as well as when, and where to release modified mosquitoes to combat mosquito populations in an area [81].

Competitiveness

It is also important to ensure the modified mosquitoes intended for release are competitive with local populations. Whether vector control programs that use these tools aim for mosquito/disease transmission limitation or elimination, research has shown that mosquitoes may experience evolutionary responses to vector control tools such as developing and maturing in a shorter period [92-95]. Given such findings, it is beneficial to know which impacts could be expected if released mosquitoes and their offspring

prove to be less fit or less successful at finding mates (Table 2.1). Such effort is worthwhile considering a variety of traits from body/wing size, fecundity, larval development, longevity, and reproduction can impact mosquito fitness as well as mosquito behavior from flight and host seeking to feeding frequency and success [96-99]. Once researchers are familiar with life history traits and behaviors of the mosquitoes they plan to release, it becomes possible to make direct comparisons with those expressed by the wild-type mosquito populations within trial areas. It is also important to demonstrate the capacity to successfully mass rear the mosquitoes that vector control projects will release [28]. Only then will it be possible to ensure any mosquitoes that are reared in the lab are well suited for the field locations where they are to be introduced. Researchers will also be able to recognize variations that could help or hinder the impact of vector control programs [69, 100].

The suggested information presented in Table 2.1, could help inform risk benefit plans, assess whether the mosquitoes researchers plan to release are likely to achieve their goals, and test if releases of male and female mosquitoes, or only male mosquitoes, would be the most effective and affordable approach [53, 101]. Furthermore, stakeholders will be better positioned to understand the optimal survival rate of offspring and adult mosquitoes that is necessary to reduce local mosquito populations in trial areas. This will also inform us regarding how many mosquitoes (e.g., what ratio of modified to wildtype) should be released into trial areas and how often releases should occur to ensure reductions in mosquito population size (Table 2.1). This is essential when designing more sustainable vector control programs (such as those that do not require regular releases)

since mosquito populations tend to rebound to their previous sizes if releases of modified mosquitoes stop, and no other vector control tools continue to pressure mosquitoes in the trial area [39, 102].

Role of target organism in the local ecosystem

Before releasing modified mosquitoes in the wild, it is crucial to conduct thorough assessments and reassure stakeholders that releases of modified mosquitoes (sterile/irradiated, *Wolbachia*-infected, or genetically modified) will not adversely affect communities or ecosystems (Table 2.1). Some researchers hypothesize that mosquitoes are essential to the diet of migratory birds, or that caribou herds may migrate or travel in certain patterns (i.e., towards the wind direction) to avoid mosquito swarms [103]. Species of fish like the mosquitofish (*G. affinis*) as well as insects, arachnids, and amphibians that consume mosquitoes could face risks associated with the loss of their primary and secondary sources of food [103]. The loss of mosquito populations could also negatively impact crops or plants that benefit from mosquitoes as pollinators [103-104] as well as contributors to nitrogen and nutrient cycling (e.g., pitcher plants) [103, 105-106]. However, the genetic tools that help control mosquitoes generally target a specific species and can help reduce the risk that mosquitoes are completely removed from an ecosystem.

Although significant reductions or eradications of mosquitoes may be concerning, some research suggests that other species will fill the niches that mosquitoes occupy [103]. In addition, ecosystem services like flower pollination by mosquitoes may not be essential considering the complementarity of functional roles by insects that are likely

more significant [103). Eliminating harmful mosquitoes could also benefit human populations, by reducing healthcare expenditures and services, improving public health, and reducing school or work absenteeism [103]. More research is definitely needed to better understand the cost/benefits and ethical issues that will inform whether to proceed with field releases of modified mosquitoes. If viable, stakeholders can make informed decisions regarding the type of modified mosquitoes to release, what additional vector control tools to deploy, and how to continue a project and/or releases.

Informing plans for releases

Collecting the information outlined in Table 2.1 before releasing modified mosquitoes will help develop specific/localized or general experimental plans that detail costs and benefits of releases as well as contingencies regarding how released mosquito populations can be controlled [53]. Researchers can also use the information to form an assessment plan that outlines potential benefits, risks, and discloses whom the project could impact as well as which parts of the environment are likely to experience such effects [53]. It is also important that stakeholders develop clear and transparent community involvement and outreach plans with a focus on ensuring local capacity building to increase community education, involvement, and the likelihood the vector control program will succeed in a sustainable manner. Pending approval, the next steps would entail selecting trial sites where releases are most appropriate and likely to succeed given this fundamental information [53].

Community involvement

Although not an eco-evolutionary consideration, it is essential that proposed projects are well received with informed consent and support from communities, governments, and funders. Hence, this requires community engagement, transparent research, and consent from a variety of stakeholders, ranging from local citizens to elected and appointed government officials and review boards [107-109]. Accordingly, societal concerns from cultural, governmental, and citizen opinions should be voiced, heard, and respected [53]. Thus, fostering clear, inclusive, jargon-free dialogue and information exchange will reduce obstacles to understanding and help establish consensus stakeholders as to how vector control programs ought to proceed [53, 110].

During and after releases

In addition to understanding mosquito populations (local and reared for release) and gaining informed consent in the areas proposed for use of vector control tools prior to mosquito releases, it is imperative to monitor these indicators during and after releases, to analyze the performance and limitations of interventions. This also means monitoring target mosquito abundance and analyzing how any reductions or eliminations of mosquito populations impact broader ecosystems and conduct cost benefit analyses that compare the performance of released modified mosquitoes to the performance of other vector control tools (Table 2.1).

Gene flow and mosquito performance

Continued monitoring of gene flow and/or the transfer of *Wolbachia* between released and wild-type mosquitoes is essential [111]. This enables stakeholders to determine mosquito populations trends after the releases and to identify where any changes are occurring. Investigators can also use this as an opportunity to assess whether there is dispersal and/or horizontal transfer of genes, *Wolbachia* present, or sterility between released and wild-type mosquitoes [112-113]. Regularly conducting such surveillance via collections also allows for the detection of any genes escaping or entering the trial area, which can help mitigate the chances of unintended gene flow and impacts on nearby ecosystems (Table 2.1). Simultaneously, researchers can also monitor environmental indicators ranging from temperature and relative humidity to rainfall amounts, and water quality, all of which have implications for mosquito biology, community, and ecosystem health. Integrating these data points allows for a more nuanced interpretation of program outcomes and provides the necessary context to determine whether a project's success or failure was driven by the intervention itself or by external ecological shifts. This information is beneficial for confirming whether the vector control tools function as designed and sustainably limit mosquito populations without impacting broader ecosystems [12]. It is also imperative that researchers take steps to assess the performance of released mosquitoes as well as to assess mosquito fitness, mating behavior, and population connectivity in the areas that will be impacted by fieldwork [114]. This will include investigating how long it takes releases to reach and/or sustain their goals, the extent to which released mosquitoes coexist or replace

wild-type mosquito populations and overall impacts on mosquito populations, disease transmission, and broader ecosystems.

Failure to adequately consider, monitor, reflect upon, and understand these eco-evolutionary factors during and after releases of modified mosquitoes will limit understanding of when, where and why these vector control tools succeed or not. Without collecting this eco-evolutionary information, data or insight that could hinder the effectiveness of vector control projects aiming to reduce the impact of mosquito-borne diseases may be lost. In addition, it is essential that stakeholders clearly define the parameters for the success and failure of vector control programs or field trials planning to use these tools before projects begin. Thereafter, stakeholders should continue to monitor the ecosystem in which they release mosquitoes during the experiments/releases and after they "conclude/end."

Most critical considerations as per Table 2.1

Although stakeholders may find themselves unable to adhere to all the criteria contained in Table 2.1, there is critical data and information they should prioritize. It will always be essential to determine whether the goal of releasing mosquitoes is the reduction of a given mosquito population, the replacement of a mosquito population, or the eradication of a mosquito population. Attempts to eradicate mosquitoes are likely to encounter significant resistance due to concerns about ecosystem health and philosophical concerns if an organism is removed or driven to extinction. Releases of *Wolbachia*-infected mosquitoes are more likely to replace an existing mosquito

population over time, whereas sterile/irradiated mosquito releases can reduce existing mosquito populations provided they occur regularly.

Before releases, stakeholders should prioritize investigating how interconnected ecosystems are and how far mosquitoes can travel when seeking mates. Undertaking this work is essential before releases of mosquitoes begin. Understanding population connectivity and gene flow will help determine how many mosquitoes should be released. It will also inform stakeholders how often mosquitoes should be released and how long projects should continue. During and after releases, stakeholders should prioritize monitoring if the releases are achieving their goals and whether resistance to vector control tools develop. Early vigilance makes it possible to detect and ameliorate any inefficiencies before vector control projects that release mosquitoes are too far along. This can subsequently maximize the chances vector control projects that release mosquitoes will be successful.

Conclusion

Releasing modified mosquitoes into the environment raises complex ecological and evolutionary questions that must be considered and addressed. Without an understanding of these potential impacts, it is impossible to accurately design, conduct, and evaluate the success or failure of experimental plans or trials involving modified mosquitoes.

It is crucial for researchers to collect and analyze all relevant data regarding mosquito populations, modified mosquitoes, the performance of other vector control

tools, potential factors that can affect the success of releases such as environmental factors, and relevant short/long term impacts on ecosystems and human populations.

The way that projects use these vector control tools, share the data produced from these tools, and transparently communicate any successes or shortcomings of such experiments, will influence the extent to which these vector control tools are utilized globally. There is a scientific and socially responsible obligation to communicate the ethical and scientific imperative to consider the ecological and evolutionary implications of releasing modified mosquitoes before proceeding with such experimental trials. Open communication and transparency are essential for informed decision-making and public engagement in this area. There is only one opportunity to make a first impression with these tools. Therefore, it is important to take the discussed ecological and evolutionary information into account and ensure the best chance of success in areas where releases of mosquitoes occur.

Acknowledgements

We thank Drs. Arianne Cease, Scott Shaffer, and Silvie Huijben for the helpful discussions.

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CHAPTER 3

DO FEMALE AEDES AEGYPTI MOSQUITOES SENSE UNBALANCED SEX RATIOS AND ALTER THEIR NEXT GENERATION?

Abstract

This chapter investigates the potential for "female cryptic choice" and adaptive sex ratio adjustment in *Aedes aegypti* mosquitoes, specifically addressing whether females can counteract male-biased populations—a common outcome of modern vector control strategies like the release of genetically modified or sterile insects. While evolutionary theory and examples from haplodiploid species suggest females might influence offspring sex by controlling sperm release from the spermatheca, existing research does not clarify the prevalence of such adjustments in *A. aegypti* populations.

The laboratory experiments outlined in this chapter used lab reared *A. aegypti* (not sterile/irradiated or genetically modified) to test the impact of skewed parental sex ratios on F1 generation outcomes. Results indicate that *A. aegypti* females do not significantly adjust offspring sex ratios or egg production within a single generation in response to male dominance. Instead, the F1 generations consistently reverted toward a Mendelian 1:1 ratio, affirming the resilience of natural sex chromosome segregation. Significant sexual dimorphism in body mass was observed, highlighting sex-specific ecological pressures and the role of larval fitness in reproductive potential. These findings suggest that while vector control tools effectively disrupt populations in the short term, subsequent generations tend to naturally stabilize without regular intervention and vigilance, necessitating a focus on long-term population dynamics and the potential for genetic bottlenecks in mosquito management strategies.

Introduction

Since the 1960s, researchers investigated factors that could influence *Aedes aegypti* sex ratios. Those factors ranged from genetic mechanisms based on chromosomes and sex determining alleles [1-4] to density dependent feedback mechanisms and competition for resources or mates that can influence mosquito populations and the sex ratios within those population [5-6]. Researchers also noted *A. aegypti* sex ratios could be influenced by larval density [5] and mating mechanics such as sperm development, competition, migration, transfer, and viability [7-10]. Other research showed that age of eggs, time of hatching, and position of eggs when oviposited did not significantly affect offspring sex ratios [11]. However, it remains unclear whether female or male mosquitoes had a more significant impact on offspring sex ratios [1, 10, 14].

Researchers later identified a pair of sex determining alleles present in mosquitoes for which females were homozygous (mm), and males were heterozygous (Mm) [10-13]. Those genetic markers led to the conclusion that male *A. aegypti* were the sex determining gender [10-11]. Researchers then predicted that mosquito mating and normal segregation would most likely result in a 1:1 female/male sex ratio [10-11]. However, lab experiments often produced skewed sex ratios with fewer females, varying between 35-50% [14-15] and rarely reaching 30% or less in lab populations [10].

Previous studies concluded that males play a central role in offspring sex ratio determination [10-11]. However, sexual selection predicts that females may choose the male's sperm that will fertilize their eggs [16]. In the case of parasitoid *Hymernoptera trichogrammatidae,* females store sperm in a spermatheca, a sac controlled by a neural loop that can use muscle contractions to induce fertilization [17-18]. The spermatheca depends

on neural connections to function; it uses muscle contractions to fertilize or leave eggs unfertilized, which could make it possible for females to influence the sex of their offspring during oviposition via their decision to release or retain the sperm [18-20].

This "choice" has been termed "female cryptic choice" and could enable females to discriminate against males they mate with that lack certain traits or fail to express certain behaviors [21]. Phenotypic traits associated with fecundity such as body size, and wing beats are more likely to be favored by females when selecting a mate and such selection would increase the frequency of the genes that are responsible for those traits in mosquito populations. However, there is insufficient lab or field data to support whether sexual selection or cryptic choice allows females to impact offspring sex ratios.

Despite clear evidence supporting sexual selection, West and Sheldon [22] suggest that "evolutionary theory predicts adaptive adjustment in offspring sex ratios by females." While the possibility of such adjustment is uncommon in discussions focused on mosquitoes, offspring sex ratio adjustments have been observed in ants, bees, and wasps that are known for their haplodiploid sex determination. That mechanism of sex determination provides females with the ability to control whether they fertilize their eggs and as a result, the sex of those offspring [22-27]. Additionally, it is widely acknowledged that per haplodiploidy sex determination, fertilized eggs result in the development of females while unfertilized eggs produce males [24-26]. Thus, seems reasonable for females to choose whether or not to fertilize eggs despite the heterozygosity of sex determining chromosomes in male mosquitoes (Mm) and selective pressures on male mosquitoes to increase their mating success [16]. If true for mosquitoes and a female were to mate with multiple males, it seems reasonable that female mosquitoes can make reproductive choices

to ensure the continued survival of their populations despite attempts from genetically modified or sterile insect releases to reduce the number of fit males or the overall number of females in an area?

Despite the possibility of "female cryptic choice," most wild mosquito populations tend to have 1:1 female/male or male-biased sex ratios and data from field experiments have not demonstrated significant female biases among *A. aegypti* [6]. Yet, research has shown that density dependent feedback mechanisms [5] and delayed hatching [6, 28] could affect mosquito sex ratios and lead to more females than males during certain hatching events. Temperature has also been noted for its impact on the development of reptiles [18, 29] and influence on sex ratios via sperm incapacitation and/or male sterilization [18, 30-33]. The lack of clear experimental and field evidence regarding sexual selection or adaptive adjustment to the ratio of female and male *A. aegypti* mosquitoes motivates the present experimental study.

In addition to the possibility of female mosquitoes influencing the sex ratios of their offspring, there are compounding concerns regarding the use of tools like genetically modified mosquitoes, irradiated/sterile mosquitoes, and *Wolbachia*-infected mosquitoes to disrupt the sex ratios of target mosquito populations. These vector control tools often decrease mosquito populations and/or increase the ratio of male to female mosquitoes in a population, which can reduce human health risks since male mosquitoes do not consume blood meals. My experiment will test whether female mosquitoes can produce a female-biased population when reared under conditions where male mosquitoes dominate by replicating a situation that may occur when researchers release genetically modified or

sterile mosquitoes into an area to dramatically decrease the proportion of female to male mosquitoes.

Observing the sex ratios of control and experimental group's offspring will provide insights into whether females can adjust the sex ratios of their offspring to counteract male dominance. Analyzing the offspring sex ratios will inform whether an environment dominated by male-mosquito mosquitoes can result in a female-biased generation/population. Furthermore, dry mass measurements of offspring will provide information on the progeny's fitness. Overall, this work will inform the extent to which the aforementioned vector control tools may be needed by illustrating whether non-modified female mosquitoes can adjust the sex ratios of their offspring and/or produce more female than male mosquitoes. Here I test the hypothesis that *A. aegypti* will not be able to adjust the sex ratio of their F1 generations in response to skewed and male-biased sex ratios in one generation. I predict that female mosquitoes in populations with higher percentages of males will face more stress given the larger number of males trying to mate with them, which could reduce the number of eggs they lay.

Methods

Mosquito Rearing and Sex Separation

Mosquito eggs were hatched overnight in a 3.5L plastic container with 1.5L D.I water and 1/64tsp (.078mL) of fish food. The next day, all mosquito larvae were transferred to 3.5L containers with 1.5L of D.I water and 1/16tsp of fish food. The containers were refreshed daily with fresh D.I. water and fish food. Upon pupation, mosquito pupae were separated from larvae and each individual pupa was transferred via

pipette into an individual test tube with D.I. water. Mosquito sex was predicted based on size dimorphism since female pupae and adult mosquitoes are typically larger than males [34-36]. Pupae were monitored daily for mortality and emergence into adult females or males. This way, virgin adult females were kept separate from the males until assigned to experimental groups (i.e. sex ratio groups). Mosquito rearing and experiments described below were performed at 27°C and 80% relative humidity with a 12-hour light: dark cycle in walk-in climate chambers (Model No. ENV-G1HD-8X12, Darwin Chambers, Missouri, USA).

Combining Mosquitoes into Experimental Groups to Mate

Three experimental cages were prepared (one cage per sex ratio group) during each replicate to contain one of the following sex ratios: 100 females: 100 males (or 1:1 ratio, control group), 50 females: 150 males (1:3 ratio, experimental group), 20 females: 180 males (1:9 ratio, experimental group). On a designated day of the week and at a consistent time, virgin males and females were combined in their designated cages to initiate mating. Mosquitoes were allowed to mate freely for a standardized duration (7 days). A cotton pad soaked with 10% sugar water was left atop each cage to prevent dehydration and starvation. This was repeated six times (seven replicates total).

Blood Feeding

Four days after combining the mosquitoes into their experimental groups, all groups received a blood meal in accordance with the following standard lab procedures [37]. Three 3 mL reservoirs were filled with defibrinated human blood (2 mL per

reservoir) that was warmed to 37°C. The reservoirs were secured with plugs to prevent leakage and attached to Hemotek feeders [38]. Each reservoir's membrane was wet with a drop of DI water and affixed to the top of a cage. Each cage of mosquitoes was provided with its own blood meal for 40 minutes, and the reservoirs were relubricated with water and gently rocked every 10 minutes to stimulate feeding.

Mosquito Egg Collection

Three days after blood feeding, 20 females from each cage were aspirated into individual pre-labelled 15 mL Falcon tubes containing a piece of oviposition paper that was moistened with 2 mL D.I. water. The Falcon tubes were capped and stored in the same rearing chamber for three days (until the following Monday at noon) to allow the females to oviposit. Following the oviposition period (48 hours), females were transferred from the Falcon tubes to another cage. Each Falcon tube and the oviposition sheet it contained was held under a microscope to count the number of eggs and larvae present. Once all female mosquitoes were inside the new cage, they were placed in a -20°C freezer for 24 hours and sacrificed.

Egg Hatching and Larval Rearing

Three containers were pre-labeled to correspond to the 20 Falcon tubes per treatment group. All 60 Falcon tubes were submerged in their corresponding container, each containing 100 mL of D.I. water. The submerged tubes were subjected to a 60-minute vacuum while inside their container to facilitate egg hatching[39]. Simultaneously, three rearing trays were prepared and labelled with the mosquito line

information and oviposition paper submersion date. Following the vacuum treatment, the contents of each container (water, larvae, and oviposition paper) were carefully transferred to their designated rearing tray. The larvae were transferred to new 3.5L containers with 1.5L of D.I water and .078mL of fish food each day until pupation. The larvae were then reared to adulthood following standard protocols, ensuring each treatment group was maintained in separate containers throughout development. Pupae were collected and housed in cages labelled according to their parent's treatment/sex ratio group. Cotton pads soaked in sugar water were provided within each cage for adult mosquito sustenance. After all pupae matured into adults or died, the adult mosquitoes were sexed and frozen for subsequent analysis.

Adult Mosquito Dry Mass Measurement

Following mosquito emergence and freezing, the first approximately 25 males and 25 females aspirated from each cage (once all pupae matured or died) were carefully collected and transferred to pre-labelled Eppendorf tubes. The collected mosquitoes were maintained whole to ensure accurate dry mass measurement. Eppendorf tubes were placed in an oven at 40°C for four hours to desiccate the mosquitoes. After drying, the Eppendorf tubes were removed from the oven, and the dry mass of each mosquito was measured using a microbalance.

Data Analysis

Statistical analyses began by investigating whether the data (raw or transformed via natural log) satisfied Levene's test of equality of error variances. Univariate general linear

models (one-way ANOVAs) assessed Total number of eggs laid per sex ratio, mean number of eggs (including female mosquitoes that laid no eggs), mean eggs laid (excluding female mosquitoes that did not lay eggs), total number of eggs vs. total number of adults, and F1 adult sex ratios. Tukey HSD (equal variances assumed) discerned differences between groups. A two-way ANOVA assessed F1 generation adult dry body mass. When the ANOVAs were significant, but failed to meet Levene's test, Games-Howell post hoc tests (equal variances not assumed) were used to identify differences between groups. All analyses considered $p < 0.05$ as statistically significant.

Results

Study Limitations

This section presents the findings on egg laying, adult emergence rates, and adult body mass. Afterwards, it presents a limitation in the study design that potentially affects the interpretation of the results.

Blood Meal Limitations

While females in each experimental group were provided with access to blood meals after being assigned to sex ratio groups, it is not possible to guarantee that each female fed equally (or took a sufficient bloodmeal). This potential variation in blood meal size could have influenced egg production and might explain some of the observed inconsistencies such as why some females did not oviposit. Future studies could benefit from incorporating methods to standardize blood meal intake. To account for this

possibility that not all females took an equal or sufficient blood meal to fertilize eggs, two figures were developed, one where the mosquitoes that did not lay eggs were excluded from the analysis (Figure 3.3; assuming those did not take a sufficient blood meal) and another where they were included (Figure 3.2). In both figures, females from the 100:100 sex ratio group laid the most eggs. However, the results are inconsistent for the 20:180 and 50:150 sex ratio groups with those groups being associated with the lowest mean eggs per female in one of those two figures.

Total Number of Eggs Laid Per Sex Ratio

Levene's test confirmed homogeneity of variances among groups ($p = 0.239$). The total number of eggs laid by the 20 females from each group that were allowed to oviposit for 48 hours was not significantly influenced by the adult sex ratio they were exposed to (Figure 3.1; one-way ANOVA, $F(2,21) = 1.176$, $p = 0.328$). While females in the control group (1:1) tended to lay the most eggs across replicates (four out of seven), this difference was not statistically significant compared to the other groups.

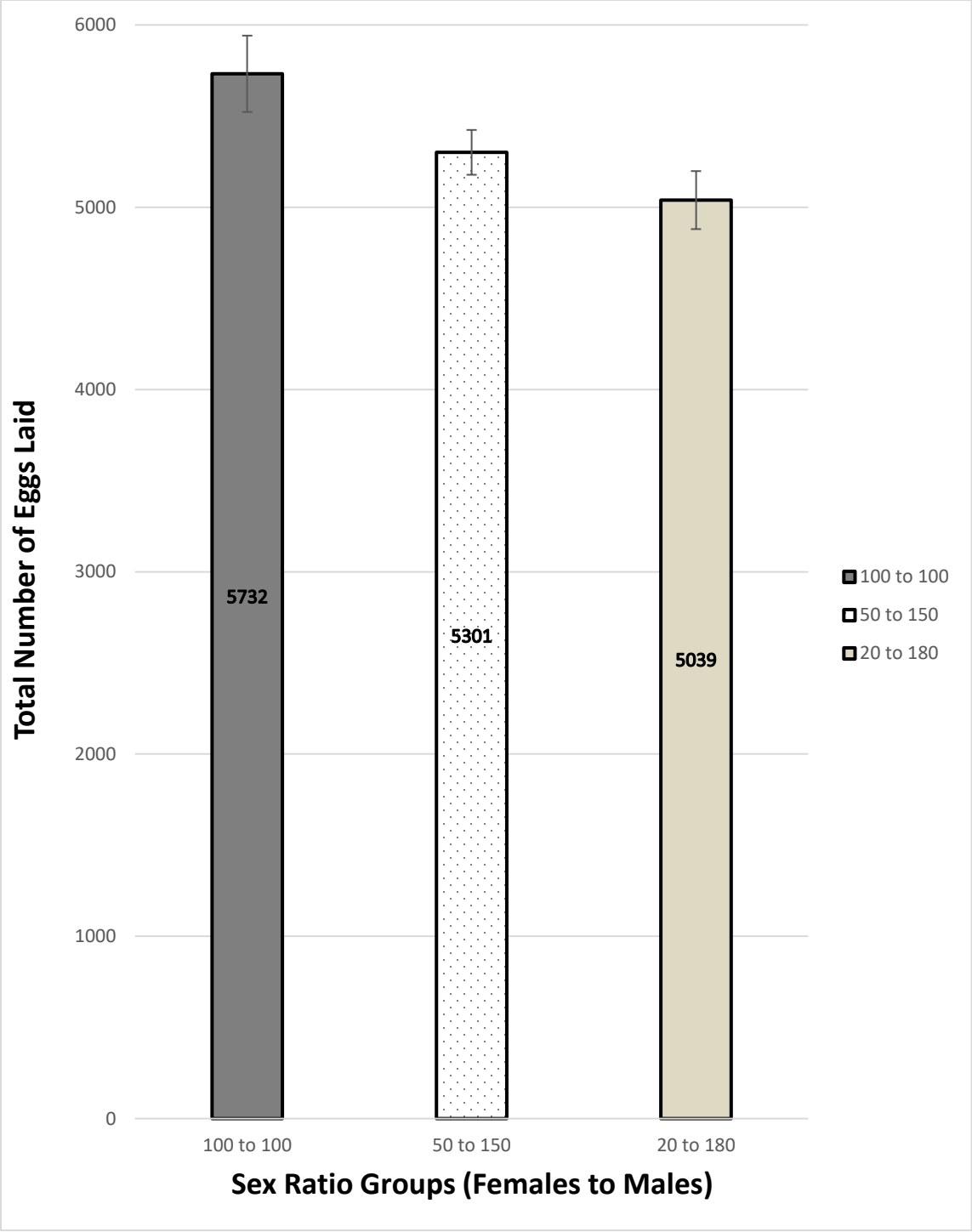

Figure 3.1: The Total Number of Eggs Laid by All The 20 Females Allowed to Oviposit from Each Sex Ratio Group's Seven Replicates. The Total Number of Eggs Laid by Female *A. Aegypti* Was Not Significantly Influenced by The Adult Sex Ratio ($p > .05$). Error Bars Represent the Standard Error From Each Group's Mean.

Mean Number of Eggs (including female mosquitoes that laid no eggs)

Levene's test confirmed homogeneity of variances among groups ($p = 0.249$). Despite some females laying no eggs, adult sex ratio did not significantly affect mean egg production per female (Figure 3.2; one-way ANOVA, $F(2,21) = 1.061$, $p = 0.364$). The control group (1:1) exhibited a trend toward higher average egg production compared to the other groups (four out of seven replicates with the highest mean), but this difference was not statistically significant.

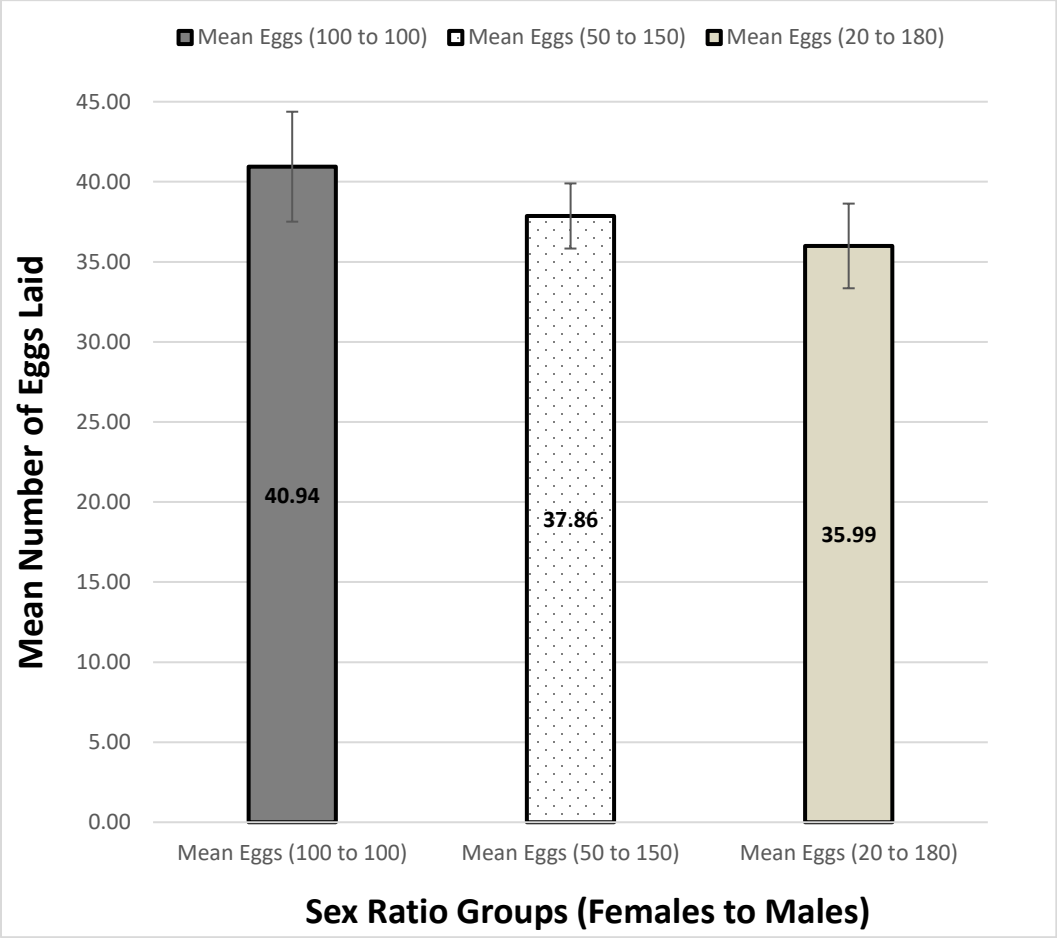

Figure 3.2: The Mean Number of Eggs Laid by All Females from Each Sex Ratio Group's Seven Replicates (Including Females That Laid 0 Eggs). The Mean Number of Eggs Laid by Female *A. Aegypti* Was Not Significantly Influenced by The Adult Sex Ratio ($p > 0.05$). Error Bars Represent the Standard Error from Each Group's Mean.

Mean Eggs Laid (Excluding Females that Did Not Lay Eggs

Levene's test confirmed homogeneity of variances among groups ($p = 0.434$). Even when excluding females that laid no eggs from mean egg production per female analyses, the overall pattern remained consistent, with no significant effect of adult sex ratio on mean egg production per female (Figure 3.3; one-way ANOVA, $F(2,21) = 0.045$, $p = 0.956$). Although the control group (1:1) exhibited the highest average egg production in three replicates, no consistent pattern emerged across treatments (other groups had the highest mean number of eggs laid in two replicates each). However, it is important to consider that some females did not oviposit, potentially due to factors not captured in this study design.

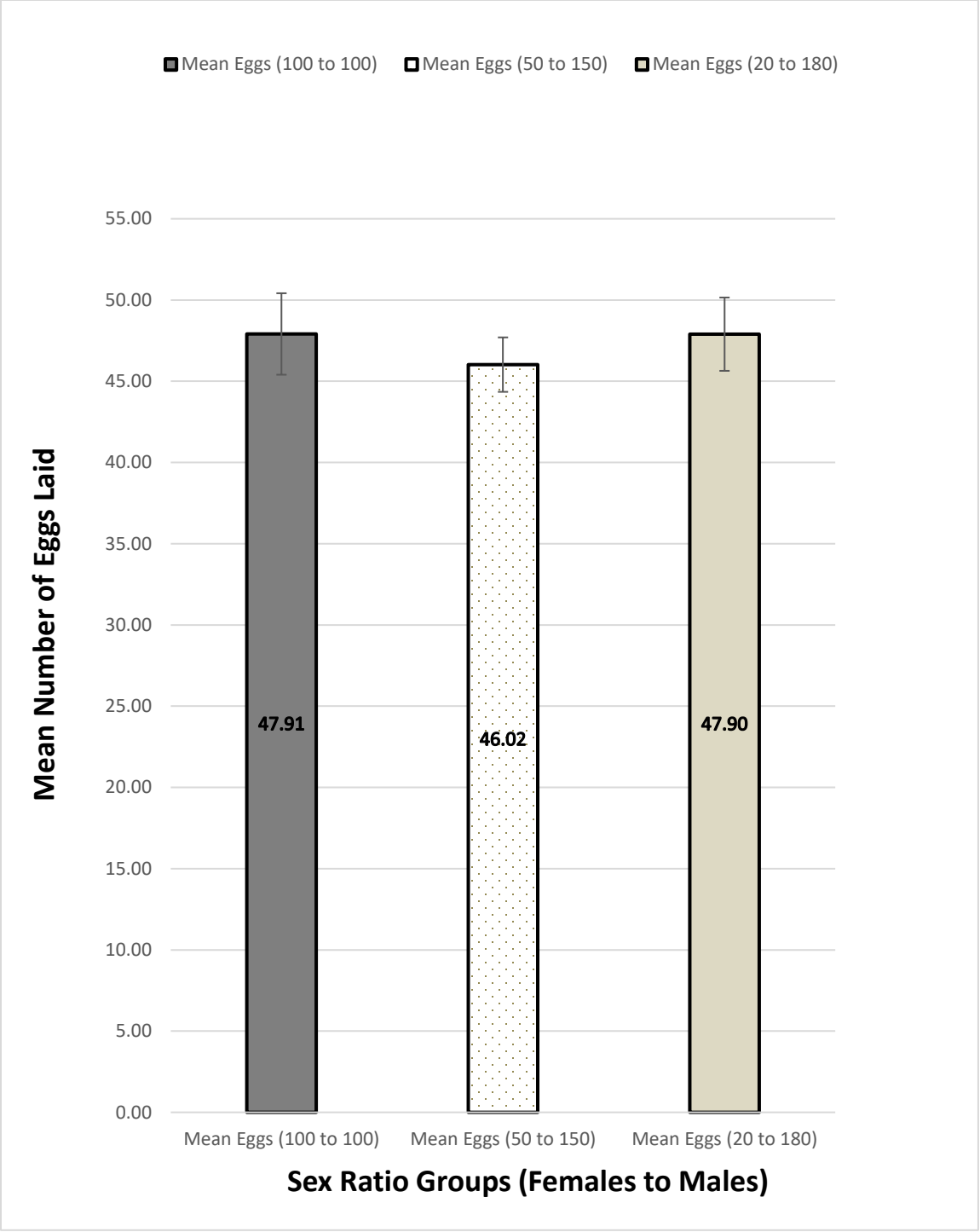

Figure 3.3: The Mean Number of Eggs Laid by All Females from Each Sex Ratio Group's Seven Replicates (Excluding Females That Laid 0 Eggs). The Mean Number of Eggs Laid by Female *A. Aegypti* Was Not Significantly Influenced by The Adult Sex Ratio ($p > 0.05$). Error Bars Represent the Standard Error from Each Group's Mean.

Rearing the F1 Generation:

Approximately 70.2% of hatched eggs successfully matured into adults (Figure 3.4). These data were not analyzed further as the primary focus of these experiments was on egg laying and adult body mass. A total of 2,148 *A. aegypti* eggs laid by females during the sex ratio experiments were hatched. Of those, 1,507 (approximately 70.2%) matured to adults and 779 of the adults were identified as males and 728 were identified as females (Figure 3.4).

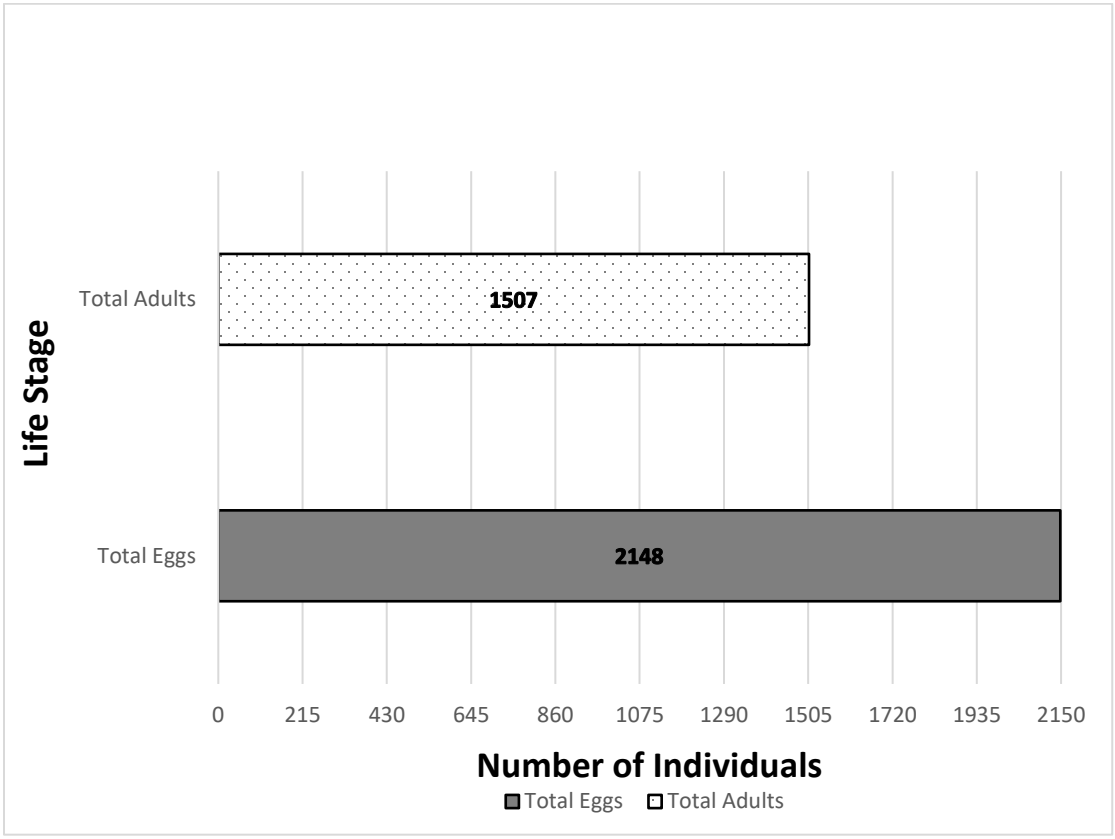

Figure 3.4: This Figure Illustrates the Total Number of *A. Aegypti* Eggs And Compares Those Data With the Total Number of *A. Aegypti* Adults That Developed.

Larval density limitations:

The F1 generation was reared in groups of different sizes compared to the parental generation. Since larval density and food availability are known to affect development time, survival, and adult size [40-41], this difference in rearing protocols could have influenced the adult body mass results. Future studies should maintain consistent larval rearing densities across treatment groups.

F1 Adult Sex Ratios:

There were no significant differences in F1 sex ratios between the groups (Figure 3.5; one-way ANOVA, $F(2,18) = 1.672$, $p = 0.216$). Levene's test confirmed homogeneity of variances ($p = 0.432$). Females exposed to the 1:3 and 1:9 sex ratio produced an F1 generation that approached the expected 1:1 sex ratio discussed earlier (Figure 3.5).

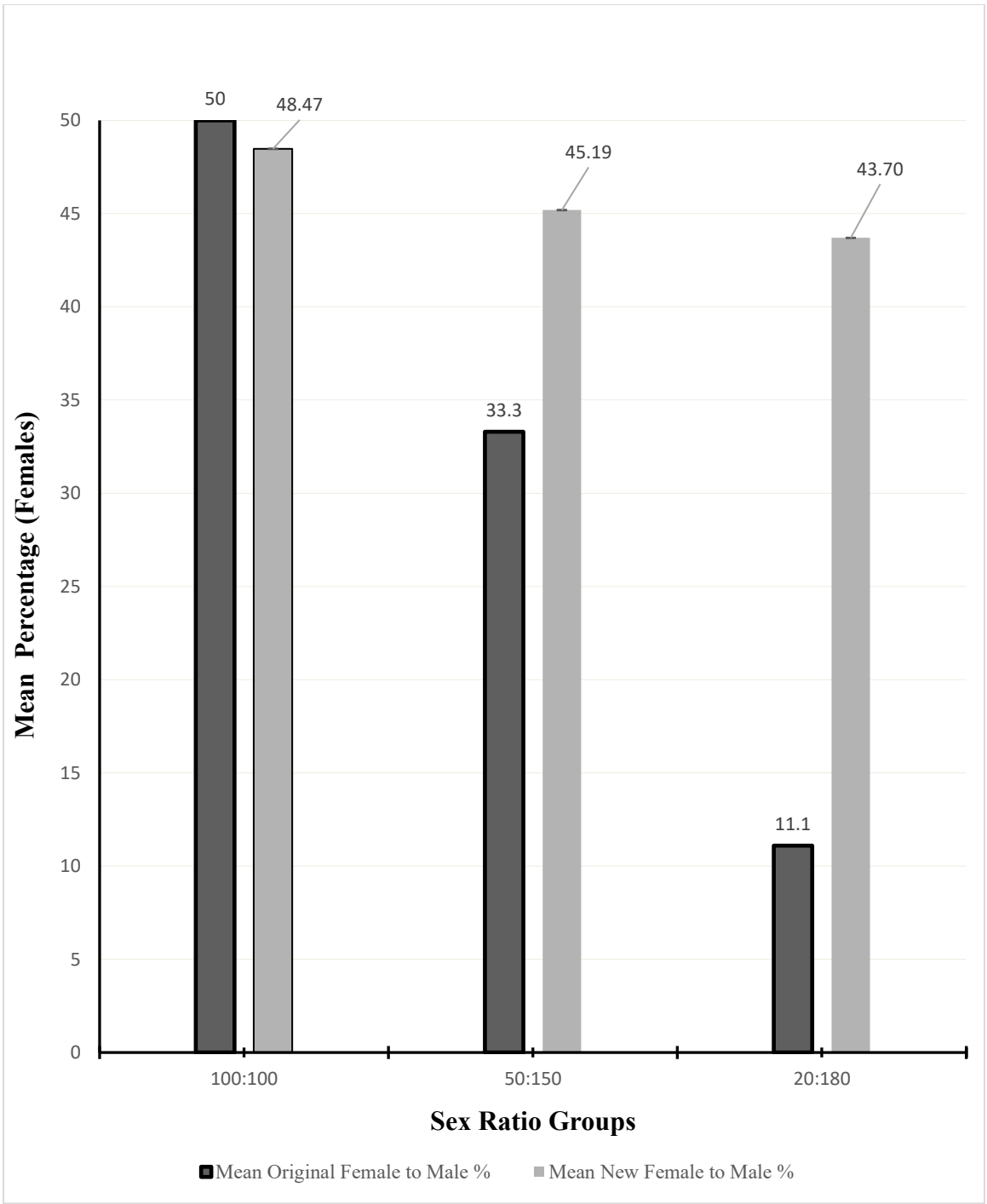

Figure 3.5: A Depiction of the Original Mean Female *A. aegypti* Percentage (1:1, 1:3, 1:9 Sex Ratio Groups) And The "New" Mean Female *A. aegypti* Percentage (F1 Generation). No Significant Difference Identified Between The "New Mean Female to Male Percentage" ($p > 0.05$). All New Female to Male Percentages Were Close to The Expected 50% Male/Female Sex Ratio.

F1 Generation Adult Dry Body Mass:

Natural log (ln) was used to transform the mean adult body mass. The corrected univariate general linear model included all main effects, interactions, and was significant (*Figure 3.6; two-way ANOVA, F (5,1071) = 349.800, $p < 0.001$*). However, it did not meet Levene's test of equality of error variances (*Figure 3.6; $p < 0.001$*). Analyses of the three sex ratio groups using the Games-Howell test showed the dry body mass of adult mosquitoes differed significantly between male and females in each sex ratio group. (*Figure 3.6; F (1,1075) = 976.558, $p < 0.001$*). These analyses also indicate interactions between sex and sex ratio groups (F (2,1071) = 13.133, $p < 0.001$*)*.

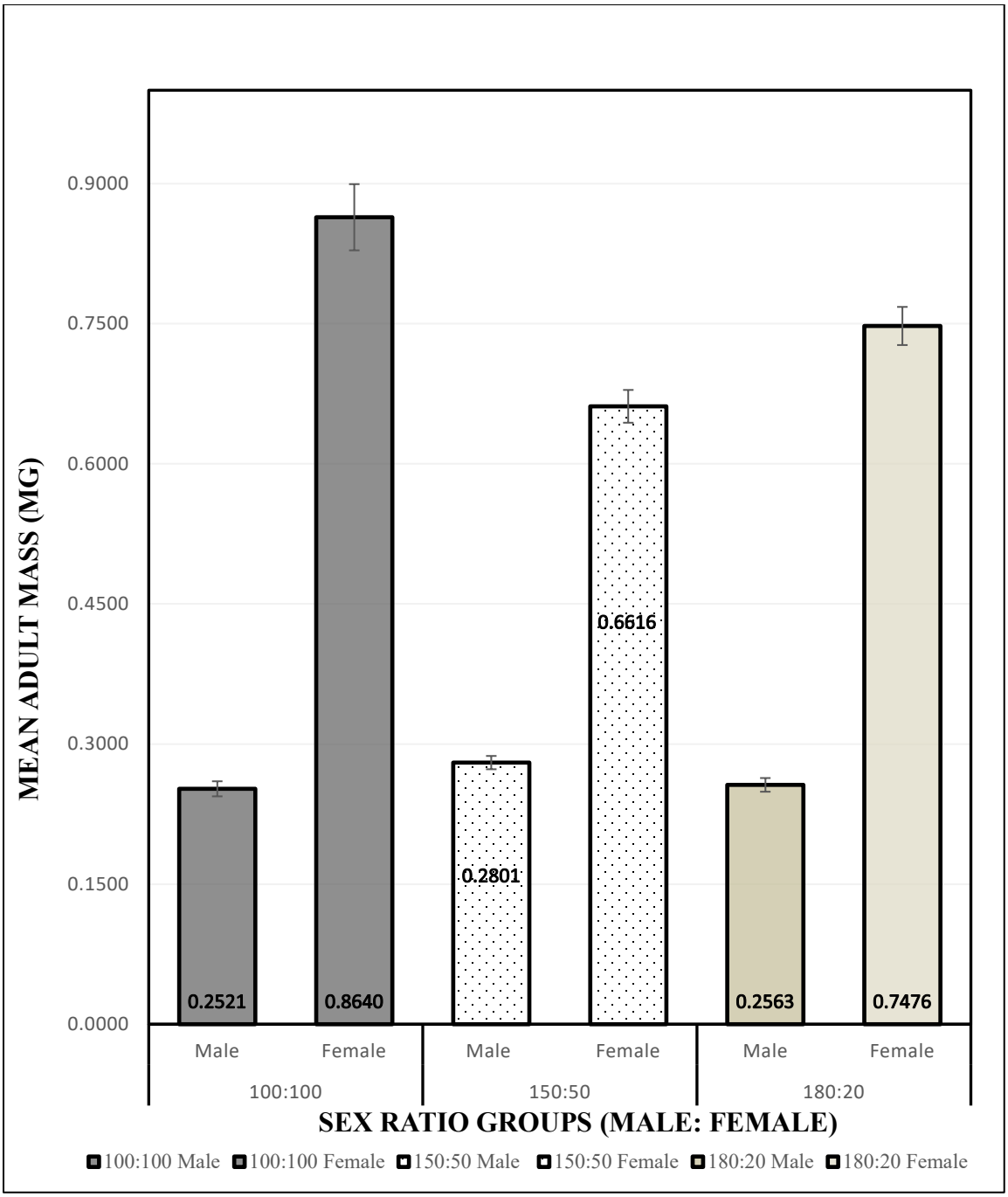

Figure 3.6: The Mean Dry Mass of F1 A. Aegypti (According to Their Parent's Sex Ratio Group). Significant Differences in Mean Dry Mass Were Identified Between Males in the 1:1 And 1:3 Sex Ratio Groups as Well as Males in the 1:1 And 1:9 Sex Ratio Group. Significant Differences in Mean Dry Mass for Females in the 1:1 And 1:3 Sex Ratio Groups as Well as the 1:3 And 1:9 Sex Ratio Group. Error Bars Represent the Standard Error from Each Group's Mean.

Following a two-way ANOVA, a Games- Howell post-hoc test discerned differences among three groups without assuming equal variance (Figure 3.6; ($F(1,1075) = 976.558$, $p < 0.001$)). That analysis revealed significant differences in mean dry mass between male and female mosquitoes within each sex ratio group. The analyses also indicate significant differences in mean dry mass between females in the control group (1:1) and the (1:3) male-biased sex ratio group (Figure 3.6; $p < 0.05$). It also identified a significant difference in mean dry mass between female mosquitoes from the control group (1:1) and the (1:9) male-biased sex ratio group (Figure 3.6; $p < 0.05$).

These findings supported findings from an initial two-way ANOVA and Tukey's post hoc test that hinted at differences between groups but did not meet Levene's test of equal variances. Tukey's post hoc test revealed significant differences in mean dry mass between male mosquitoes and female mosquitoes (Figure 3.6; $p < 0.001$) as well as significant differences between females from the control group (1:1) and the (1:3) male-biased sex ratio group (Figure 3.6; $p < 0.001$). Tukey HSD also identified a significant difference in mean dry mass between female mosquitoes from the control group (1:1) and females from the (1:9) male-biased sex ratio group (Figure 3.6; $p = 0.018$). A significant different in mean dry mass was also noted for female mosquitoes in the 1:3 and 1:9 sex ratio groups (Figure 3.6).

Discussion

The experiments tested the ability of female *A. aegypti* mosquitoes to counteract biased sex ratios experienced as adults within one generation. There was no significant difference in the number of eggs laid by females from any of the three sex ratio groups (Figure 3.1). Additionally, none of the groups produced a significantly larger number of female offspring compared to others (Figure 3.5). These findings support the hypothesis that female *A. aegypti* are unable to reverse skewed sex ratios within a single generation.

The F1 generation was reared simultaneously in three groups based on the sex ratio their parents were exposed to (i.e. 100 females: 100 males; 50 females: 150 males; 20 females: 180 males). However, the F1 generation was not reared in groups of 200 like their parents. It is also important to note this difference in rearing because the environmental conditions mosquitoes experience as larvae can influence larval development and conspecific growth [40, 42], how many adults are produced, as well as the traits and fitness of those adults [34, 40, 41, 43,44]. Reduced larval diet is known to result in longer development time [43, 45- 61]**,** lower larval survival [45, 47-48, 60-64), decreased lifespans [40, 43, 55, 64], smaller adult body size [40, 45, 47, 49, 52-53, 56, 58-60, 62-67). Research also suggests that larval behavior and the rate at which larvae develop may also impact viral development in *A. aegypti* [40, 68]. Developing more consistent rearing protocols and investigating the ways environmental conditions (including diet, food availability, habitat, temperature and thermal variance, water availability and depth, etc....) as well as larval and pupal development can be instrumental to furthering understanding of adult mosquito fitness and populations.

The F1 generation of all treatment groups exhibited sex ratios that approached a 1:1 sex ratio compared to the male-biased sex ratios experienced by their parents e.g. 48% males (100:100 ratio), 45% males (50:150 ratio), and 43% males (20:180 ratio) (Figure 3.5). This shift suggests that random mating and normal sex chromosome segregation mechanisms tend to restore a balanced sex ratio over time [10-11]. These data also illustrate that short-term exposure to male biased populations is insufficient to significantly alter the sex ratio of mosquito populations contrary the expected 1:1 sex ratio (Figure 3.5). These results further support the hypothesis that female *A. aegypti* are unable to independently reverse biased sex ratios within one generation and highlight the resilience of natural sex ratio regulation in *A. aegypti* populations. While this study focused on laboratory settings, the findings have implications for vector control strategies that rely on manipulating mosquito sex ratios. Technologies like *Wolbachia*-infected, gene drive, and genetically modified mosquitoes often aim to reduce mosquito populations and/or the number of female mosquitoes to lower disease transmission rates. Experimental results in this chapter suggest that even with a skewed sex ratio in the initial release, subsequent generations may naturally revert to a more balanced ratio through regular mating patterns (Figure 3.5). This highlights the importance of considering long-term population dynamics when evaluating these vector control methods.

Potential implications of sex ratio and population size manipulation

Beyond immediate impacts on sex ratios, it is critical to consider the genetic implications of altering sex ratios and reducing mosquito populations in lab settings as

well as in the field. Interventions that significantly reduce mosquito populations could have unintended consequences on the broader ecosystem. Additionally, decreasing population size without introducing new genetic material can increase the risk of genetic bottlenecks, leading to reduced genetic diversity. This can have several negative consequences, including increased inbreeding, reduced adaptability, and higher vulnerability to environmental pressures [69]. Furthermore, smaller populations are more likely to experience stronger effects of genetic drift, potentially leading to the fixation of deleterious mutations or resistance genes if they are already present [69]. While vector control will likely benefit from reductions in the fitness of mosquito populations and increased genetic diseases in nuisance populations, bottlenecks could also result in an increased frequency of resistance genes if they were already established in that population pre-intervention.

Sexual dimorphism and body size

Male mosquitoes are often under pressure to mature quickly so they have higher frequency being the first to inseminate a female mosquito, most of which only mate once in their lifetime but go through multiple feeding and egg-laying cycles [34]. Yet, mosquitoes with large body sizes are known to have more time to find food, illustrating that larger body sizes tend to be associated with more fit and well-nourished mosquitoes [36, 47, 70-71]. Unlike males, female reproductive potential is directly influenced by larval mass and favors individuals with more energy [34, 47, 67, 72]. Body size is also known to impact the size of female blood meals and the number of viable eggs produced. Females with larger body sizes also tend to have increased reproductive success.

The observed mean body mass results (Figure 3.6, $p < 0.05$) were significant and confirm recognized reports of sexual dimorphism in *A. aegypti* body size, with females being larger than males [34, 47, 70-71]. These data show a significant difference between mean male body mass of the 100:100 and 50:150 sex ratio group (Figure 3.6, $p < 0.001$). Larger body size in males may be advantageous for faster development and increased mating success, particularly in competitive environments [34]. Conversely, these data also illustrate a significant difference between mean female body mass of the 50:150 and 100:100 sex ratio group (Figure 3.6, $p < 0.001$). Female reproductive potential is directly linked to body size, with larger females having greater energy reserves for egg production [34, 47, 67, 72]. These findings highlight the importance of considering sex-specific ecological pressures when evaluating the impact of interventions on mosquito populations.

Future studies should investigate whether female mosquitoes are impacted by other ecological-evolutionary conditions. For example, one could study larvae, pupae, and adult mosquitoes in crowded (high density cages or swarms) and uncrowded areas (low density cages or flight rooms) to understand whether the density of individuals in their environment (crowdedness) affects mosquito behaviors or fitness. Research could also explore the potential for deviation from the ~1:1 ratio Mendelian sex ratio found in *A. aegypti* by conducting the experiments outlined in the present study for multiple generations, though a change is unlikely without the use of *Wolbachia*-infected or genetically modified mosquitoes. Furthermore, examining the effects of larval density and resource availability on offspring sex ratios and body size would be valuable. Finally,

long-term field studies are necessary to assess the efficacy and potential ecological consequences of vector control strategies that manipulate mosquito sex ratios.

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CHAPTER 4

IMPACT OF WATER DEPTH AND FOOD AVAILABILITY ON *AEDES AEGYPTI* DEVELOPMENT: HOW MUCH IS ENOUGH?

Abstract

This chapter investigates the ecological determinants of *Aedes aegypti* larval development, focusing on the interplay between water depth, larval density, and food availability. While previous research emphasizes the role of container size, this study utilizes "Refreshed" and "Not-Refreshed" experimental models to simulate stable versus ephemeral breeding sites (e.g., irrigation pipes versus discarded tires). Results demonstrate that larval density and food availability exert a significantly more pronounced impact on developmental success and adult fitness than water depth alone.

In Refreshed environments, high larval density and limited food significantly extended the duration to pupation and adulthood, likely due to increased physical interference and resource competition. Conversely, in Not-Refreshed environments, water depth became a critical survival threshold; depths below 1.0cm often resulted in total mortality due to desiccation and the accumulation of metabolic toxins/bacterial films before maturation. Interestingly, despite these stressors, surviving individuals in most groups maintained a consistent sex ratio, though significant sexual dimorphism in adult dry mass persisted across all treatments. These findings highlight the extreme resilience and adaptability of *A. aegypti* in "dirty" or ephemeral standing water. Understanding these minimal developmental requirements provides vital insights for vector control, particularly in arid regions where rapid evaporation rates limit the window for mosquito maturation and disease transmission.

Introduction

Aedes aegypti larvae usually develop within shallow ephemeral pools of water where eggs are deposited [1]. Here they balance respiration with food consumption or do both simultaneously [2]. Although details of *Aedes* oviposition and development are not well understood, desiccation has clear implications for larval fitness and fecundity [3-4]. Water depth is thought to influence mosquito behavior because females prefer to lay eggs in pools (e.g. containers) that are unlikely to dry while larvae are developing [4]. Data from experiments comparing the development of larvae in glass columns with depths of 10cm, 25cm, 50cm, 75cm, and 100cm show that the depth of water in a container (i.e., water depth) has a greater impact on female larvae than males, because females require longer development times [2]. Audet [2] also showed that the time required for larvae to meet threshold mass for metamorphosis increases with greater water depth, suggesting that water depth has a greater impact on larval development time than on adult mass and wing length.

Experiments conducted in plastic beakers illustrate that the differences between deep (4.4cm or 50ml) and shallow water containers (2.3cm or 25ml) are insufficient to detect significant changes in larval feeding behavior [5]. These conclusions are also supported by research showing that increased water depth can result in longer pupation time, greater mortality, increased risks of disabled offspring, and smaller adults [2]. Researchers sampling larvae across urban, suburban, and rural sites in Sri Lanka, also report lower larval abundance in containers with more than 150ml of water and containers with less than 10ml of water compared to containers with 50-100ml of water

[6]. These findings support Skiff and Yee's [5] suggestion that future research should assess whether containers with much shallower depths lead to changes in larval behavior.

In addition to water depth, both food availability and density of mosquito larvae influence *A. aegypti* larval development and adult traits [7-8]. *A. aegypti* tend to oviposit in habitats that already contain larvae and pupae. The presence of conspecific larvae is thought to indicate a place that is conducive to larval development [9]. However, higher *A. aegypti* larval densities and greater competition for resources can increase larval development rates [7, 8, 10], but significantly decrease larval survival regardless of container size [7, 10]. These conditions also result in smaller females [7], smaller pupae and adults [2, 7, 8, 11-12], and shorter adult lifespans [7, 11, 13]. These trends are hypothesized to occur because high larval densities can interact with factors like large container sizes, food availability/quality, interference from other larvae (i.e., competition for resources, physical contact/collisions, growth retardant factors, etc.) and energy requirements for foraging to influence development [8]. Data also shows container size can interact with density to create a significant compounding effect on productivity [7, 14]. Greater conspecific density can also be affected by lower amounts of food per individual resulting in lower survival rates [9]. There are also suggestions that larvae produce a growth retardant factor that impacts conspecific growth, especially when late instar larvae hinder earlier instars [7, 14]. Although starvation does not always block larval development, it can hinder the process of pupation and prevent growth since fourth instar larvae cannot always pupate if they are severely starved [2, 15]. Qureshi *et al.,* [7] suggest studying larval responses to varied diet, density/competition, and space conditions in more detail.

Evaluating how minimum water depths are required for mosquito development can inform mitigation strategies that help abet vector control initiatives. It can also improve general knowledge of mosquito development and help reduce vector-borne threats such as chikungunya, dengue, malaria, west-Nile virus, and Zika [16-18]. This information is critical for vector control and public health initiatives in regions with intermittent or seasonal rainfall. Similarly, these experiments can model how excess rainfall or waterflow that accumulates in small containers can impact mosquito development and potentially their abilities to mature and spread diseases. Mosquito larvae in those environments likely have shorter windows to mature before their aquatic habitats are designated in comparison to breeding sites in areas with increased humidity and/or precipitation. Here a combined approach assesses how more extreme water depths in containers (i.e., shallow waters of 0.5cm, 1.0cm, 2.0cm) and different larval densities and food availability impact larval development, survival, and adult fitness. Based on the information provided in this introduction, this chapter predicts that larvae will develop more quickly in containers with more food, smaller larval densities, and lower water depths. This chapter also hypothesizes larvae survival will be greater in containers with smaller water depths.

Methods

Mosquito rearing and experiment preparation

A. aegypti eggs (Maricopa County 1 strain) were hatched in a 3.5L plastic container with 1.5L D.I. water. Within 6 hours all newly emerged mosquito larvae were

transferred to their assigned Niubee containers; Model: NBPN-0051A; Dimensions: 71.12cm D x 7.112cm W x 11.176cm H [19].

Three different water depths (0.5cm, 1.0cm, and 2.0cm) were tested, as well as different larval density x food availability combinations in both “Refreshed” and “Not refreshed” containers. This design is intended to assess the minimum amount of water required for mosquitoes to develop successfully.

Refreshed containers were manipulated daily as follows: immature mosquitoes were counted, D.I. water in each container was replaced (maintaining the appropriate water depth based on the experimental group), and fresh fish food was weighed using a VWR analytical balance (model: 120B2) [20] and added to each container (ensuring the appropriate amount of food per larva per day- based on the experimental group and number of larvae remaining) (see Table 4.1). In the Not Refreshed treatment, larvae were placed in containers with 10 days of fish food based on the initial number of individuals in each treatment group (Table 4.1). Adults (if present) were counted each day, but the water and food were never replaced and/or added. This was done to mimic more natural environments like containers with standing water and puddles where water evaporates, but water levels and food resources are not replenished.

For each experimental group, three different water depths were tested (0.5cm, 1.0cm, and 2.0cm). The first group (A) had the same number of larvae and the same food availability across the different water depths (Hereafter referred to as “Standard (larvae and food)”). The second group (B) differed in the number of larvae per container (to scale larval density to the water volume in each container), but with the same total food availability across the different water depths (Hereafter referred to as “More larvae and

standard food"). The third group (C) was similar to group B, but food availability was altered to correct for the number of larvae (the amount of food was scaled to the larval density in each container; hereafter referred to as "More larvae and more food treatment." The experiment was replicated four times; the Refreshed and Not Refreshed treatments ran concurrently at 27°C and 80% relative humidity under a 12-hour light cycle.

The following response variables were modeled: Duration to pupation (Refreshed), and Time to reach adulthood were transformed via natural log and a three-way ANOVA was conducted to test for the effects of predictors like water depth, amount of food, and number of larvae on mean time to reach adulthood amongst the Refreshed groups.

Experimental groups and trials

Larvae and pupae remained together in their assigned containers until they emerged as adults (Table 4.1) when they were counted and sexed. Subsequently, adult mosquitoes were dried in a Fisher Isotemp Gravity Oven 60L (Model: 42264884) [21] at 40°C for 12 hours (6 hours across two days) to desiccate the mosquitoes. Following drying, the dry mass of each fully intact mosquito was measured using a Sartorius Superrange-microbalance with manual metal ring draft shield (Model: MSA2.7S-OTR-DF) [22].

Table 4.1: Water Depth Experimental Groups

Shown are Refreshed and Not Refreshed experimental groups. Each day, the "Refreshed" groups were transferred to new, clean containers with the appropriate amount of food and water (based on the number of larvae remaining in each container). The Not Refreshed groups were not transferred to new containers, and they were not given more food or water [see the text for more details].

food	*0.3*	*Mg/larva/day*
Baseline density	*40*	*Larvae per container*

	Experiment A		**Experiment B**		**Experiment C**	
Depth	**Standard (larvae & depth)**		**More larvae & standard food**		**More larvae & more food**	
	# Larvae	***Amount of food (mg)***	***# Larvae***	***Amount of food (mg)***	***# Larvae***	***Amount of food (mg)***
0.5cm	40	12	40	12	40	12
1.0cm	40	12	80	12	80	24
2.0cm	40	12	160	12	160	48

Data analysis

Mean duration to pupation (in number of days), mean time to adult emergence (in number of days), mean survivorship to adult (in percentage), adult sex ratio, and adult dry mass were analyzed using IBM SPSS 29. Statistical analyses began by investigating whether the data (raw or transformed via natural log) satisfied Levene's test of equality of error variances. Univariate general linear models (three-way ANOVAs) assessed the amount of food, number of larvae, and water depth as the factors. Tukey HSD (equal variances assumed) discerned differences between groups. When the ANOVAs were significant, but failed to meet Levene's test, Games-Howell post hoc tests (equal variances not assumed) were used to identify differences between groups. All analyses considered $p < 0.05$ as statistically significant.

Results

Duration to Pupation

Natural log (ln) was used to transform the mean number of days to pupation. The corrected univariate general linear model included all main effects, interactions, met Levene's test of equality of error variances (Figure 4.1; $p = 0.318$), and was statistically significant (Figure 4.1; three-way ANOVA, F (6, 21)) = 14.6, $p < 0.001$). Results from Tukey HSD indicate that the mean number of days required for pupation increased significantly when larval density increases (Figure 4.1; $p < 0.001$). Containers in experiment A (lowest density of larvae) required less time to pupate than experiments B and C which had higher larval densities (Figure 4.1; $p < 0.001$*)*. Containers with more larvae and standard amounts of food required more time to pupate than containers with the standard number of larvae and standard amount food (Figure 4.1; $p < 0.001$). Containers with more larvae and standard amounts of food also required more time to pupate than containers with more larvae and more food (Figure 4.1; $p < 0.001$). Tukey HSD also indicated interactions between the experimental groups and water depth (F (2,21) = 4.715, $p = 0.020$), which shows that mean duration to pupation was not significantly impacted by water depth or the amount of food per larva alone.

A three-way ANOVA examined the effects of water depth, amount of food, and number of larvae on mean duration to pupation amongst the Not Refreshed groups (Figure 4.2). The corrected univariate general linear model for the Not Refreshed groups included all main effects, interactions, and met Levene's test of equality of variances (Figure 4.2; $p = 0.065$). However, the results were not statistically significant (Figure 4.2; three-way ANOVA, F (6, 21)) = 1.96, $p = 0.118$). This result indicates the mean number of days

required for pupation in the Not Refreshed groups was not significantly influenced by the treatment group (Experiment A, B, C) in which pupae were assigned.

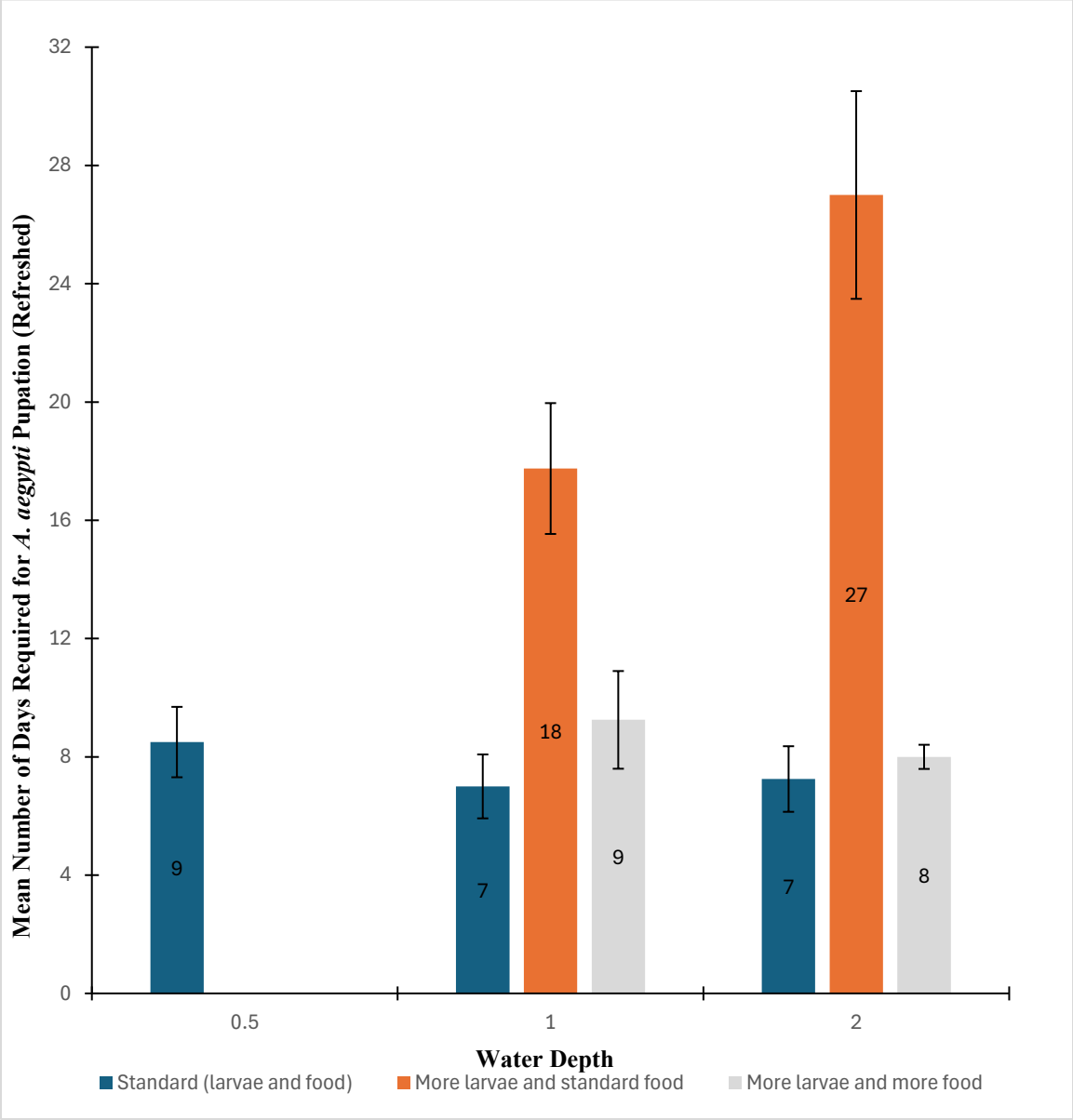

Figure 4.1: Mean Number of Days Required for *A. aegypti* Pupae in Refreshed Groups to Develop into Adults. Error Bars Represent Standard Error. Containers With Standard Number of Larvae Required Significantly Less Time to Pupate Than Containers With More Larvae and Standard Amounts of Food ($p < 0.001$), and Containers With More Larvae and More Food ($p < 0.001$).

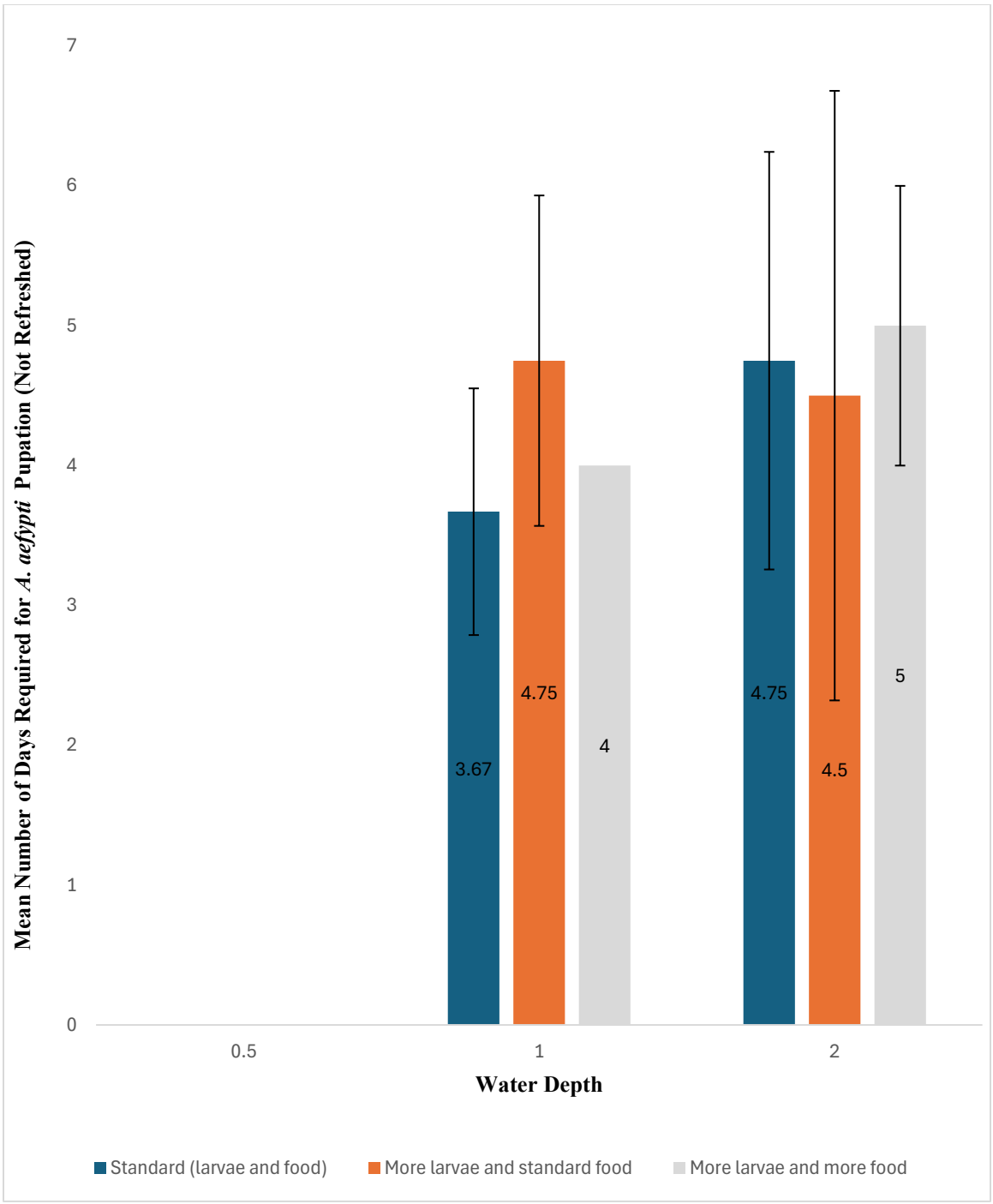

Figure 4.2: Mean Number of Days for *A. aegypti* Pupae in Not Refreshed Groups to Complete Developing Into Adults. Error Bars Represent Standard Error. The Standard 0.5cm Water Depth Is the Only Group Where No Pupation Occurred (Figure 4.2). As A Result, the 0.5cm Water Depth Group Has a Lower Mean Number of Days Required For Pupation Than All Other Groups (In Number of Days).

Duration to reach Adulthood

The corrected univariate general linear model included all main effects, interactions, and was statistically significant (Figure 4.3; three-way ANOVA, F (6, 1581)) = 291.645, $p < 0.001$). However, these data did not meet Levene's test of equality of error variances (*Figure 4.3;* $p < 0.001$). A Games-Howell post-hoc test discerned pairwise differences among the three experimental groups without assuming equal variance (Figure 4.3; (F (2, 1585) = 552.291, $p < 0.001$)). Both tests indicated that the mean number of days required to reach adulthood was significantly influenced by the treatment group in which pupae were assigned. Duration to reach adulthood increased when the amount of food remained unchanged and the number of larvae in a container increased.

Both Tukey HSD and Games-Howell tests indicated mean duration to adulthood was significantly impacted by the number of larvae in all groups (Figure 4.3; $p < 0.001$). Those tests suggested mean duration to reach adulthood was significantly impacted by water depth. *A. aegypti* in containers with low water depth had a significantly shorter duration to adulthood than individuals in containers with high water depth (Figure 4.3; $p < 0.001$). Individuals from containers with medium water depth required a significantly lower duration to adulthood than containers with high water depth (Figure 4.3; $p < 0.001$). Mean duration to reach adulthood was also significantly impacted by the amount of food larvae were provided each day. Individuals from containers with standard amounts of food per larva per day had a significantly shorter duration to adulthood than individuals in containers with standard amounts of larvae and more food per larvae per day (Figure 4.3; $p < 0.001$).

Individuals from containers with standard amounts of food per larva per day had a significantly shorter duration to adulthood than those in containers with more larvae and more food per larva per day (Figure 4.3; $p = 0.002$). Tukey HSD indicated interactions between the experimental groups and water depth ($F(2,1581) = 97.099$, $p < 0.001$) and between the experimental groups and number of larvae ($F(1,1583) = 171.761$, $p < 0.001$).

Natural log (ln) was used to transform the mean number of days to reach adulthood within the Not Refreshed groups. The corrected univariate general linear model included all main effects, interactions, and was significant (Figure 4.4; $F(6,213) = 241.080$, $p < 0.001$). However, it did not meet Levene's test of equality of error variances (Figure 4.4; $p < 0.001$). The Games-Howell test suggested significant differences between all experimental groups (Figure 4.4; $F(2,217) = 53.189$, $p < 0.001$). Those results indicated that containers with more larvae and standard food required more days to reach adulthood than standard groups ($p < 0.001$) and groups with more larvae and more food ($p < 0.001$). Tukey HSD also indicated interactions between the experimental groups and water depth ($F(6,213) = 209.970$, $p < 0.001$) and between the experimental groups and number of larvae ($F(4,215) = 12.774$, $p < 0.001$).

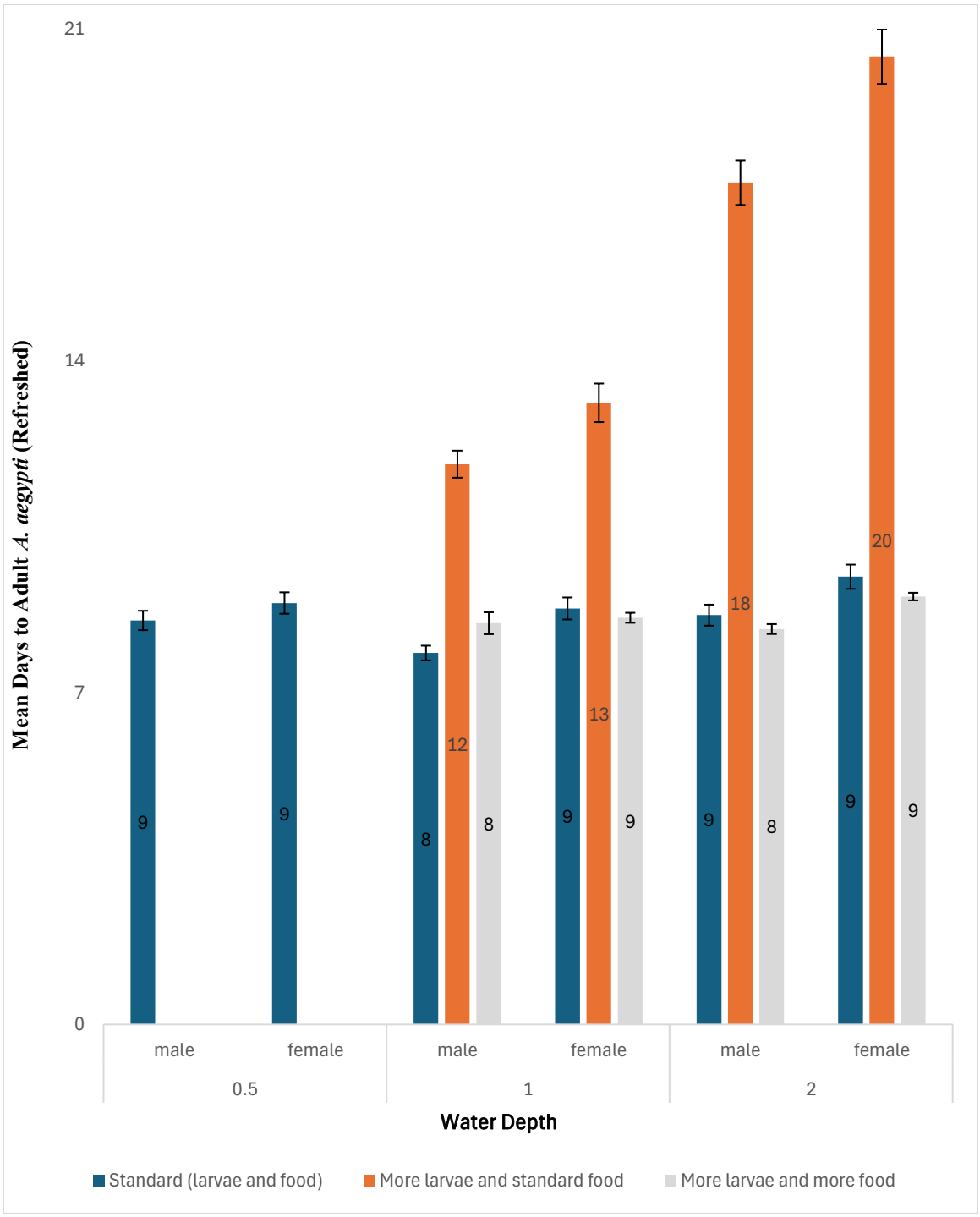

Figure 4.3: Mean Number of Days Required for *A. aegypti* Larvae in The Refreshed Groups to Emerge as Adults. Error Bars Represent Standard Error. Mean Time to Adulthood Was Significantly Influenced by the Treatment Group to Which Pupae Were Assigned ($p < 0.001$).

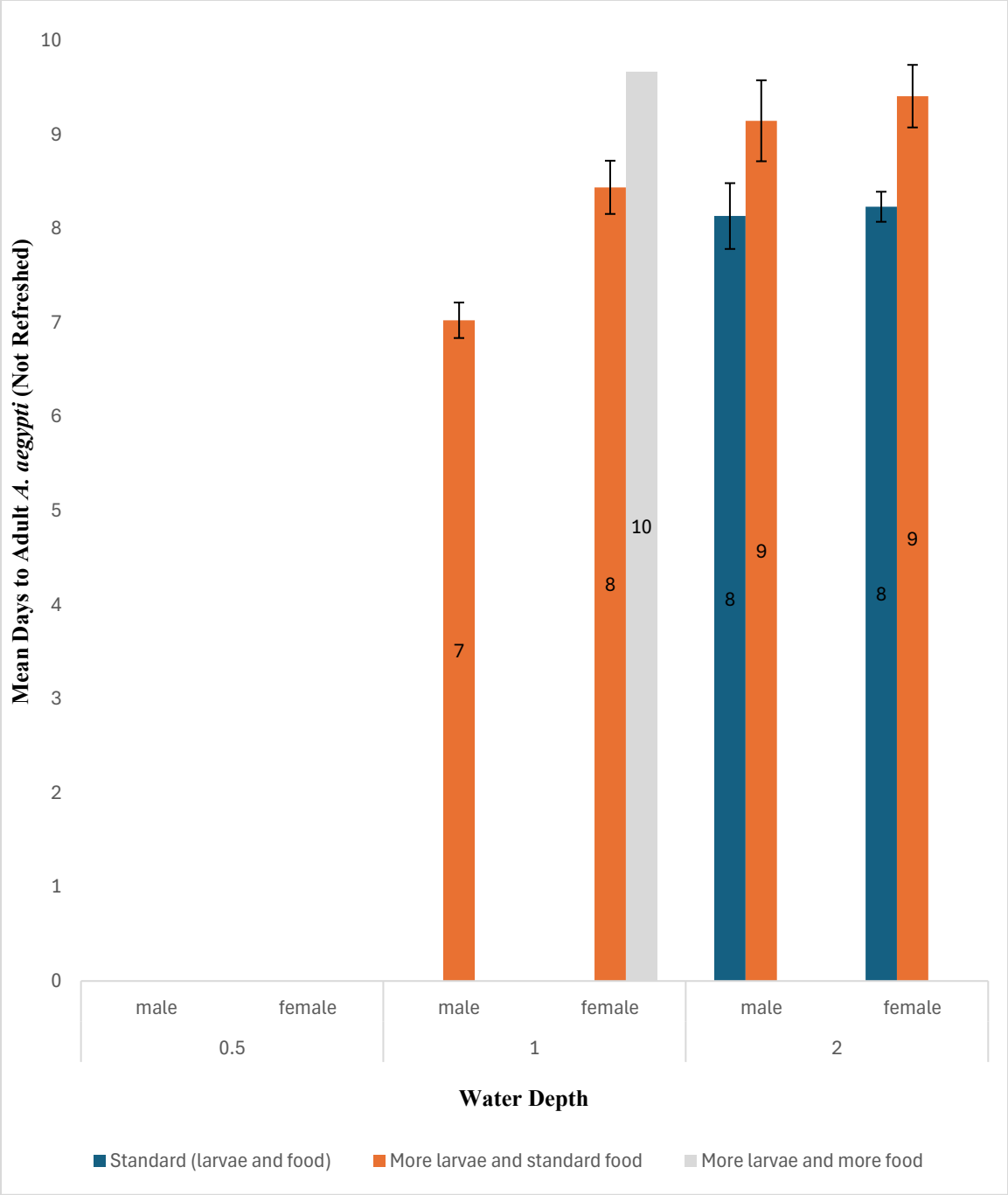

Figure 4.4: Mean Number of Days Required For *A. aegypti* Larvae in the Not Refreshed Groups to Emerge as Adults. Squares Represent Males and Circles Represent Females. Error Bars Represent Standard Error. No Adults Matured in Groups With Zero Mean Days to Adulthood. Mean Time to Adulthood Was Significantly Influenced by the Treatment Group to Which Pupae Were Assigned ($p = 0.001$)

Mean Survivorship to Adulthood

A univariate general linear model examined the effects of water depth, amount of food, and number of larvae on mean survivorship to adulthood amongst the Refreshed groups. The corrected model included all main effects, interactions, and met Levene's test of equality of error variances (Figure 4.5; $p = 0.634$). However, the data were not statistically significant (Figure 4.5; three-way ANOVA, $F(6, 21)) = 2.47$, $p = 0.058$). No interactions were identified. The transformed data also satisfied Levene's test of equality of error variances (Figure 4.5; $p = 0.493$) but results were not significant (Figure 4.5; three-way ANOVA, $F(6, 21)) = 2.367$, $p = 0.066$). Kruskal-Wallis tests on the non-transformed and transformed mean survivorship to adulthood among the three experimental groups indicated the results were not significant ($p = 0.240$).

Tukey HSD indicated that containers with the lowest number of larvae (N=40) had a greater mean survivorship to adulthood than containers with the greatest number of larvae (N=160) (Figure 4.5; $p = 0.039$). Additionally, larvae in containers with medium number of larvae (N=80) had a greater mean survivorship to adulthood than those in containers with the greatest number of larvae (Figure 4.5; $p = 0.029$). This makes sense considering containers with lower densities of larvae have reduced competition for resources, which should foster more successful development for greater amounts of larvae.

Similarly, Tukey HSD illustrates larvae in containers with the lowest water depths (0.5cm) had greater mean survivorship to adulthood than larvae in containers with the greatest water depths (2cm) (Figure 4.5; $p = 0.011$). Larvae in containers with medium water depths (1cm) also had higher mean survivorship to adulthood than larvae in

containers with the greatest water depths (Figure 4.5; $p = 0.024$). Tukey HSD identified no significant differences between the amount of food and the Refreshed groups.

A three-way ANOVA also examined the effects of water depth, amount of food, and number of larvae on mean survivorship to adulthood amongst the Not Refreshed groups. The data were statistically significant (Figure 4.6; three-way ANOVA, $F(6, 21)) = 3.614$; $p = 0.013$), but the data did not meet Levene's test of equality of error variances (Figure 4.6; $p = 0.002$). Results from a Games-Howell post hoc test on the three experimental groups (A, B, and C) were not significant (Figure 4.6; $F(2,25) = 1.957$, $p = 0.162$). The transformed data also failed to meet Levene's test of equality of variances (Figure 4.6; $p = 0.004$) and the Games-Howell post hoc test was not significant (Figure 4.6; $F(2,25) = 1.620$, $p = 0.218$).

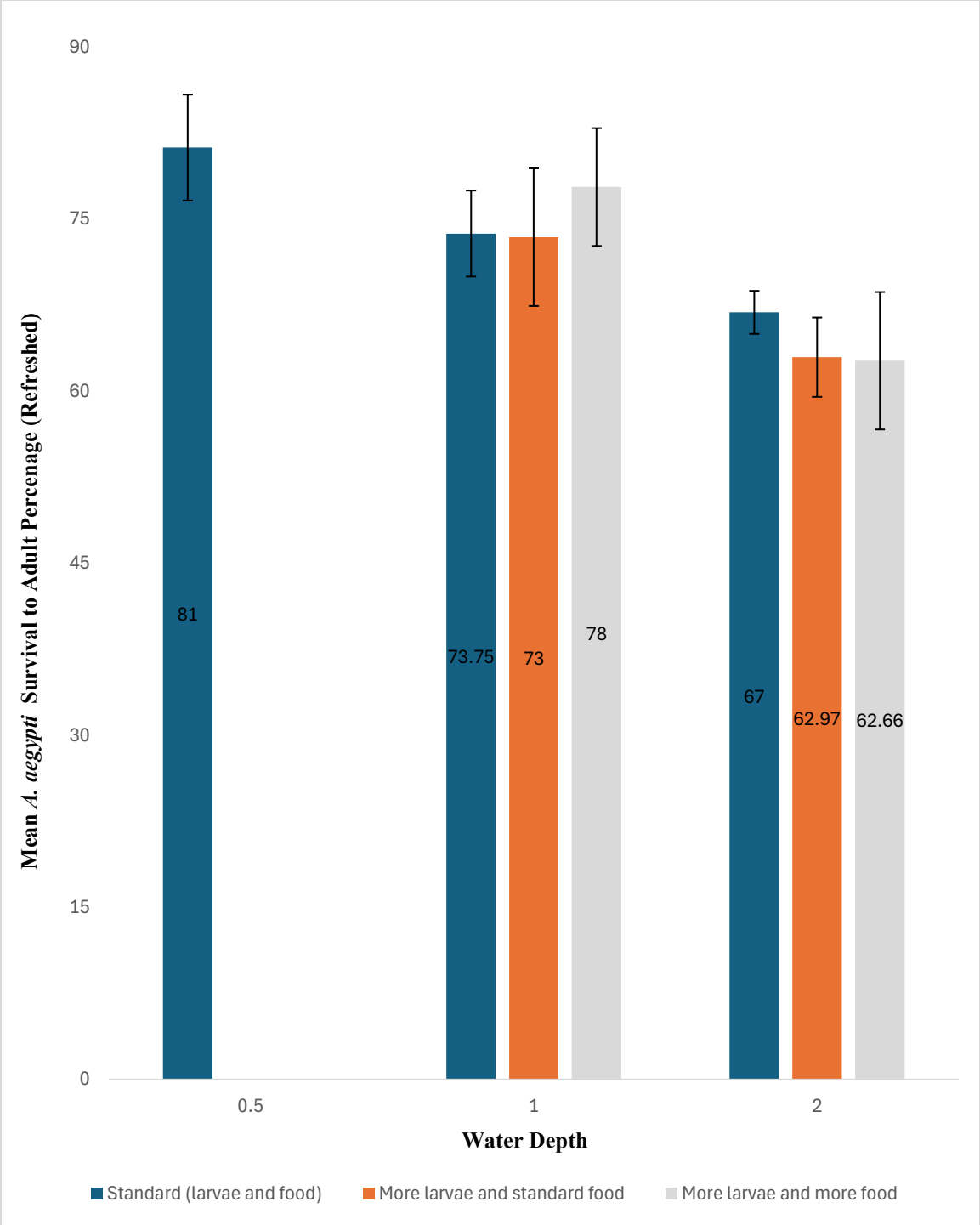

Figure 4.5: Mean Percent of *A. aegypti* Survival to Adulthood in Refreshed Groups. Error Bars Represent Standard Error. Mean Adult Survival Was Not Significantly Influenced by the Treatment Group to Which Pupae Were Assigned.

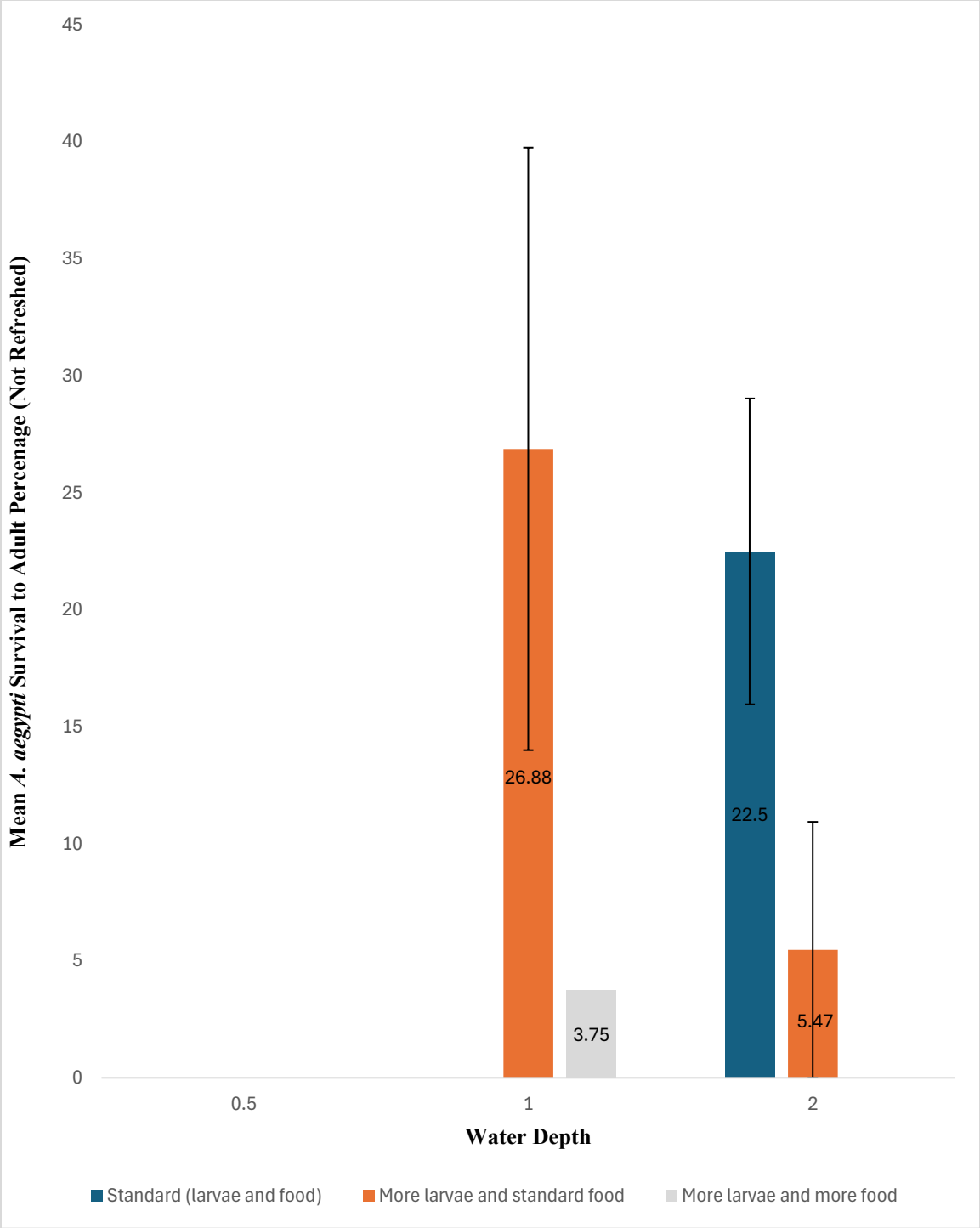

Figure 4.6: Mean Percent of *A. aegypti* Survival to Adulthood in Not Refreshed Groups. Error Bars Represent Standard Error. No Adults Matured in Groups With Zero Mean Days to Adulthood. Mean Adult Survival Was Not Significantly Influenced by the Treatment Group to Which Pupae Were Assigned.

Sex Ratio (Percentage)

A three-way ANOVA examined the effects of water depth, amount of food, and number of larvae on mean percent female (sex ratio) amongst the Refreshed groups. The corrected model included all main effects, interactions, and met Levene's test of equality of variances (Figure 4.7; $p = 0.116$). However, the results were not statistically significant (Figure 4.7; three-way ANOVA, F (6, 21)) = 1.096, $p = 0.397$*).* Transforming the data also led to results that were not statistically significant (Figure 4.7; three-way ANOVA, F (6,21)) = 1.148, $p = 0.370$).

A three-way ANOVA investigated the effects of water depth, amount of food, and number of larvae on mean sex ratio amongst the Not Refreshed groups. The results were significant (Figure 4.8; three-way ANOVA, F (6, 21), F = 2.911, $p = 0.032$). However, the data did not meet Levene's, test of equality of error variances ($p = 0.002$). Analyses of the three experimental groups (A, B, and C) using the Games-Howell test were not significant (Figure 4.7; F (2,25) = 2.108, $p = 0.143$). The transformed data were significant (Figure 4.8; three-way ANOVA, F (6, 21), F = 12.262, $p < 0.001$) and suggested interactions between the experimental groups and water depth (F (2,21) = 12.007, $p < 0.001$). However, these data also failed to meet Levene's test of equality of variances (Figure 4.8; $p < 0.001$). Results from a Games-Howell post hoc test on the three experimental groups (A, B, and C) were not significant (Figure 4.8; F (2,25) = 5.863, $p = 0.008$). It is worth noting Games-Howell indicated groups with more larvae and standard food had a higher percentage of females than those with more larvae and more food ($p = 0.005$*)*.

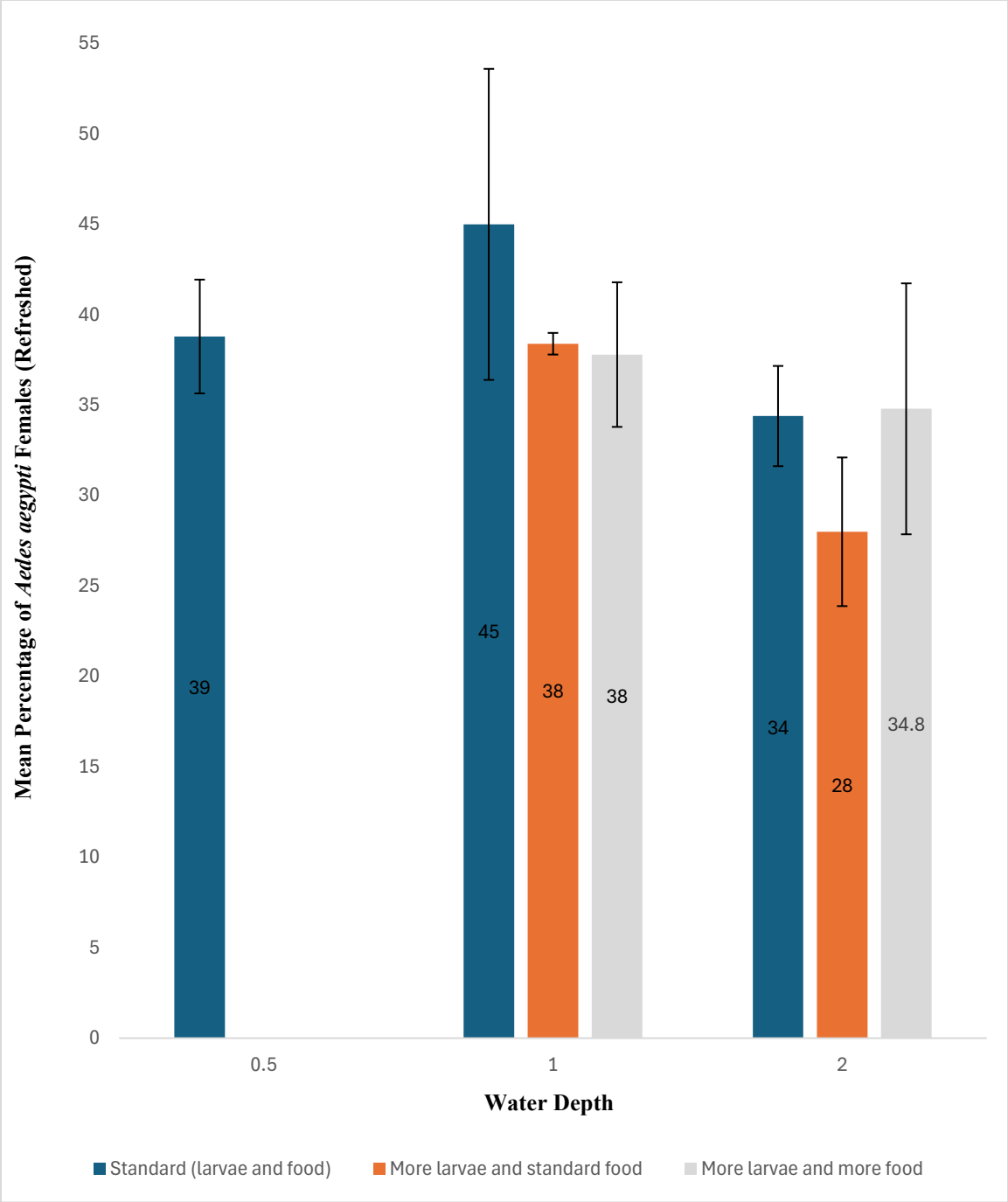

Figure 4.7: Mean Percentage of *A. Aegypti* Females That Emerged From Each Refreshed Group. Error Bars Represent Standard Error. Circles Represent Females. Mean Percentage of *A. aegypti* Females Was Not Significantly Influenced by the Treatment Group to Which Pupae Were Assigned.

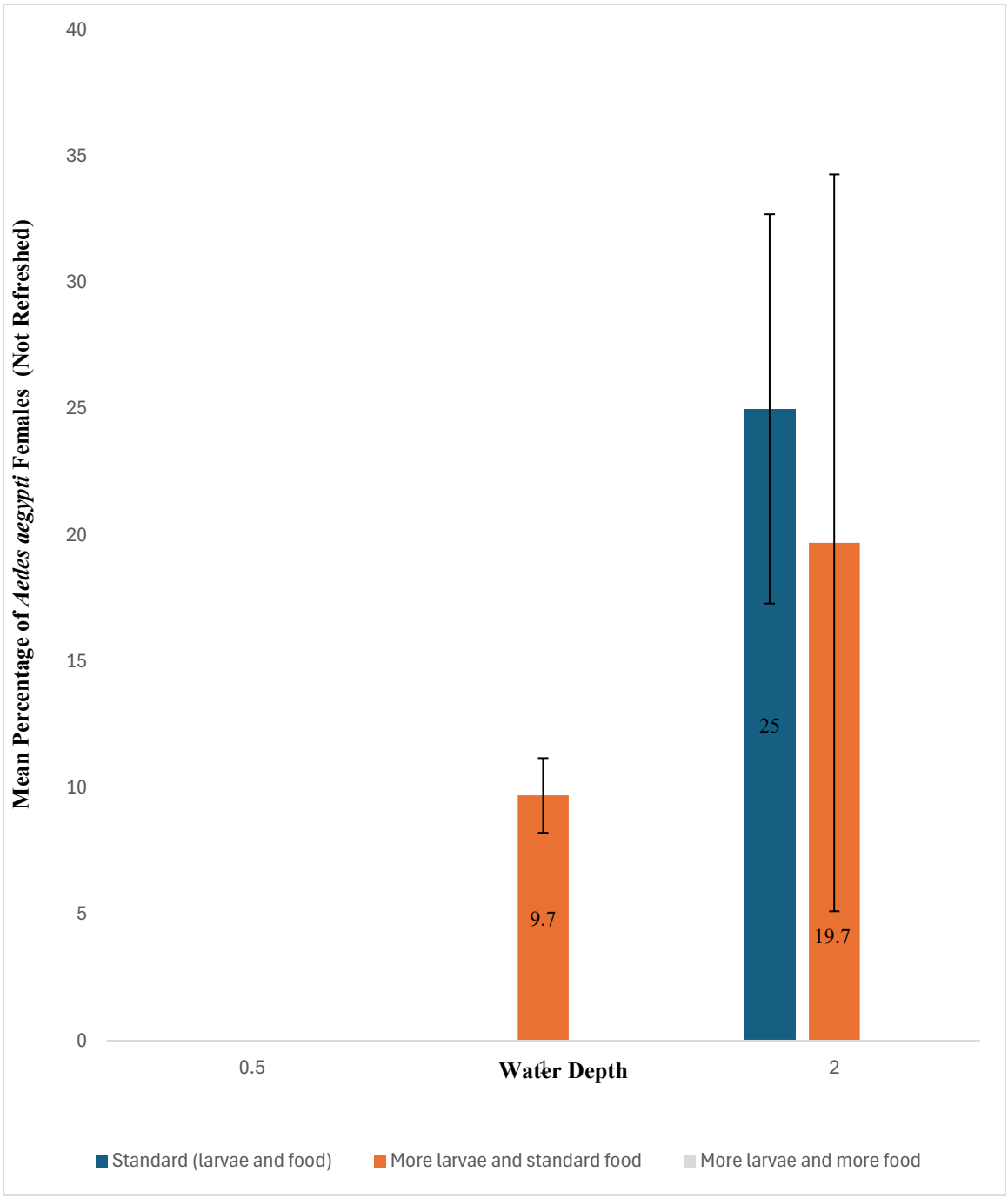

Figure 4.8: Mean Percentage of *A. aegypti* Females That Emerged From Each Not Refreshed Group. Error Bars Represent Standard Error. Circles Represent Females. No Adults Matured in Groups With Zero Mean Days to Adulthood. Mean Percentage of *A. aegypti* Females Was Not Significantly Influenced by the Treatment Group to Which Pupae Were Assigned.

Adult Dry Mass

A three-way ANOVA examined the effects of water depth, amount of food, and number of larvae on mean adult dry mass amongst the Refreshed groups. The corrected univariate general linear model included all main effects, interactions, and the results were statistically significant (Figure 4.9; $F(6,1234) = 37.607$, $p < 0.001$). However, the results did not meet Levene's test of equality of error variances (Figure 4.9; $p < 0.001$). Analyses of the three experimental groups using the Games-Howell test showed significant differences between all groups (Figure 4.9; $F(2,1262) = 83.658$, $p < 0.001$). A significant difference was identified between mean dry mass of the standard group and the more larvae and standard food group ($p < 0.001$). A significant difference was identified between dry mass of the standard group and the more larvae and more food group ($p = 0.010$). A significant difference was also found between mean dry mass of the more larvae and standard food group and the more larvae and more food group ($p < 0.001$).

After transforming the original mean adult dry mass data, the results met Levene's test of equality of error variances (Figure 4.9; $p = 0.009$) and were significant (three-way ANOVA; $F(6, 1234) = 42.666$, $p < 0.001$). Tukey HSD indicated significant differences between in the standard group and the more larvae and standard food group ($p < 0.001$). Tukey HSD suggested significant differences between the standard group and the more larvae and more food group ($p = 0.001$). A significant difference was also found between mean dry mass of mosquitoes in the more larvae and standard food group and the more larvae and more food group ($p < 0.001$). Tukey HSD identified a significant difference in mean dry mass between the 1.0cm and 2.0cm water depth ($p < 0.001$). Tukey HSD

suggested significant differences between the number of larvae in groups with small density and medium density ($p < 0.001$). Tukey HSD identified significant differences between the number of larvae in groups with medium density and high density ($p < 0.001$). Tukey HSD also found a significant difference in mean dry mass between the amount of food: small and medium ($p < 0.001$), small and large ($p < 0.001$), medium and large ($p = 0.002$).

Tukey HSD also indicated interactions between the experimental groups and water depth (Figure 4.9; $F (6, 1258) = 35.550$, $p < 0.001$). A significant difference was found between mean dry mass of mosquitoes in the standard group and the more larvae and standard food group ($p < 0.001$). A significant difference was identified between mean dry mass of mosquitoes in the standard group and the more larvae and more food group ($p = 0.004$). A significant difference was also found between mean dry mass of mosquitoes in the more larvae and standard group and the more larvae and more food group ($p < 0.001$).

Tukey HSD also indicated interactions between the experimental groups and the amount of food (Figure 4.9; $F (3, 1261) = 60.452$, $p < 0.001$). A significant difference was found between mean dry mass of mosquitoes in the standard group and the more larvae and standard food group ($p < 0.001$). A significant difference was also found between dry mass of the standard group and the more larvae and more food group ($p = 0.005$). A significant difference was identified between mean dry mass of mosquitoes in the more larvae and standard group and the more larvae and more food group ($p < 0.001$).

A three-way ANOVA examined the effects of water depth, amount of food, and number of larvae on mean adult dry mass amongst the Not Refreshed groups. This data did

not meet Levene's test of equality of error variances (Figure 4.10; $p < 0.001$). However, the results were significant (three-way ANOVA; F (6, 276) = 52.330, $p < 0.001$). After transforming the data, the results met Levene's test of equality of error variances (Figure 4.10; $p = 0.203$) and were significant (Figure 4.10; three-way *ANOVA*; F (2, 200) = 8.993, $p < 0.001$). Tukey HSD indicated significant differences between mean dry mass of individuals in the standard group and individuals in the more larvae and standard food group ($p = 0.001$). Tukey HSD indicated significant differences between individuals in the standard group and the more larvae and more food group ($p < 0.001$).

A three-way ANOVA also examined the effects of water depth, amount of food, and number of larvae on the transformed mean adult dry mass amongst the Refreshed and Not Refreshed groups. The results met Levene's test of equality of error variances ($p < 0.199$) and were significant (Figures 4.9, Figure 4.10; F (2, 1441) = 93.661, $p < 0.001$). There were significant differences between all groups ($p < 0.05$). A significant difference was identified between the mean dry mass of mosquitoes in the standard group and the more larvae and standard food group ($p < 0.001$). A significant difference was found between mean dry mass of mosquitoes in the standard group and the more larvae and more food group ($p = 0.022$). A significant difference was also found between mean dry mass of mosquitoes in the more larvae and standard food group and the more larvae and more food group ($p < 0.001$).

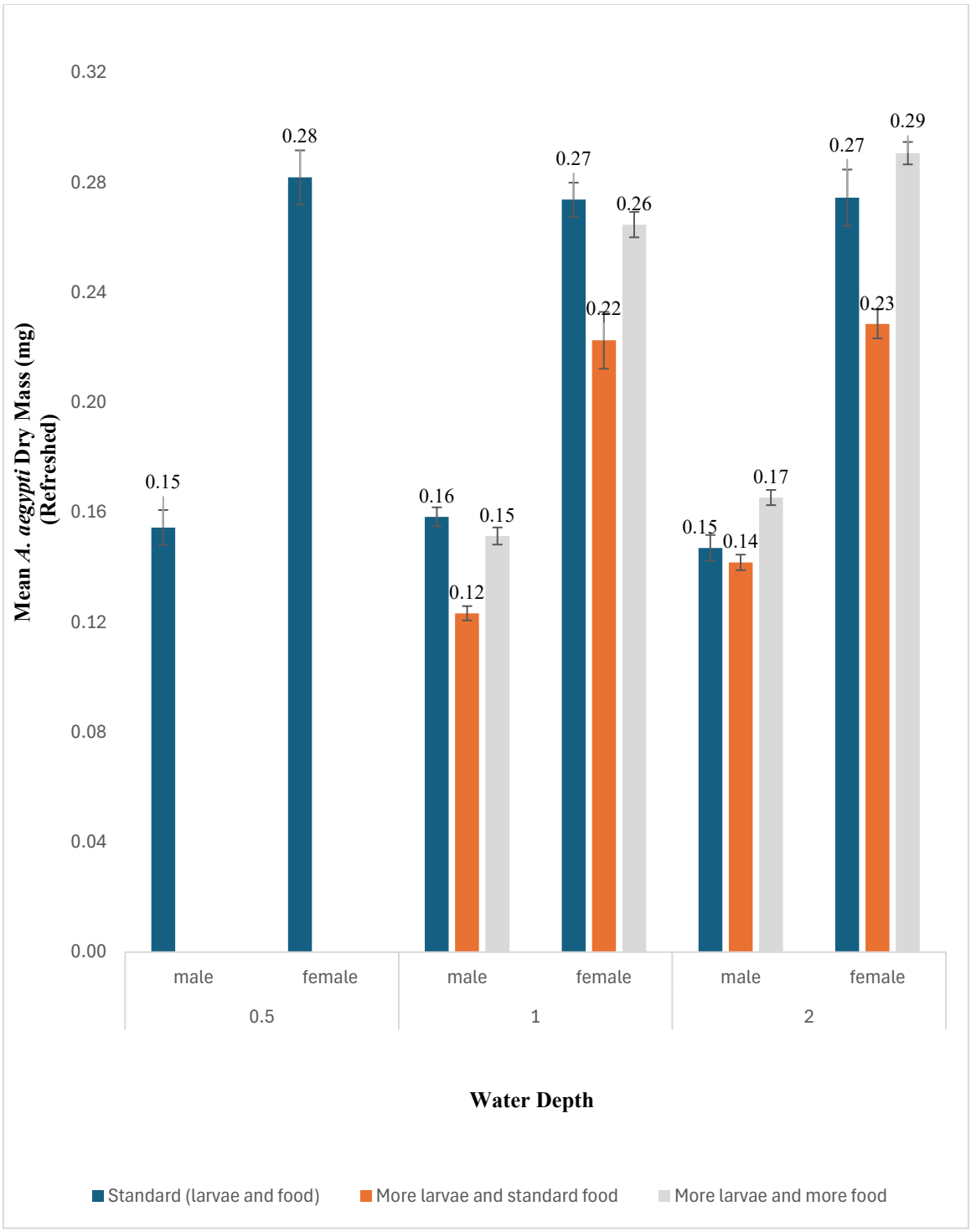

Figure 4.9: Mean *A. aegypti* Adult Female and Male Dry Mass for Each Refreshed Group. Error Bars Represent Standard Error. Squares Represent Males and Circles Represent Females. Mean Female Mass Was Greater Than Mean Male Mass in All Groups ($p < 0.001$).

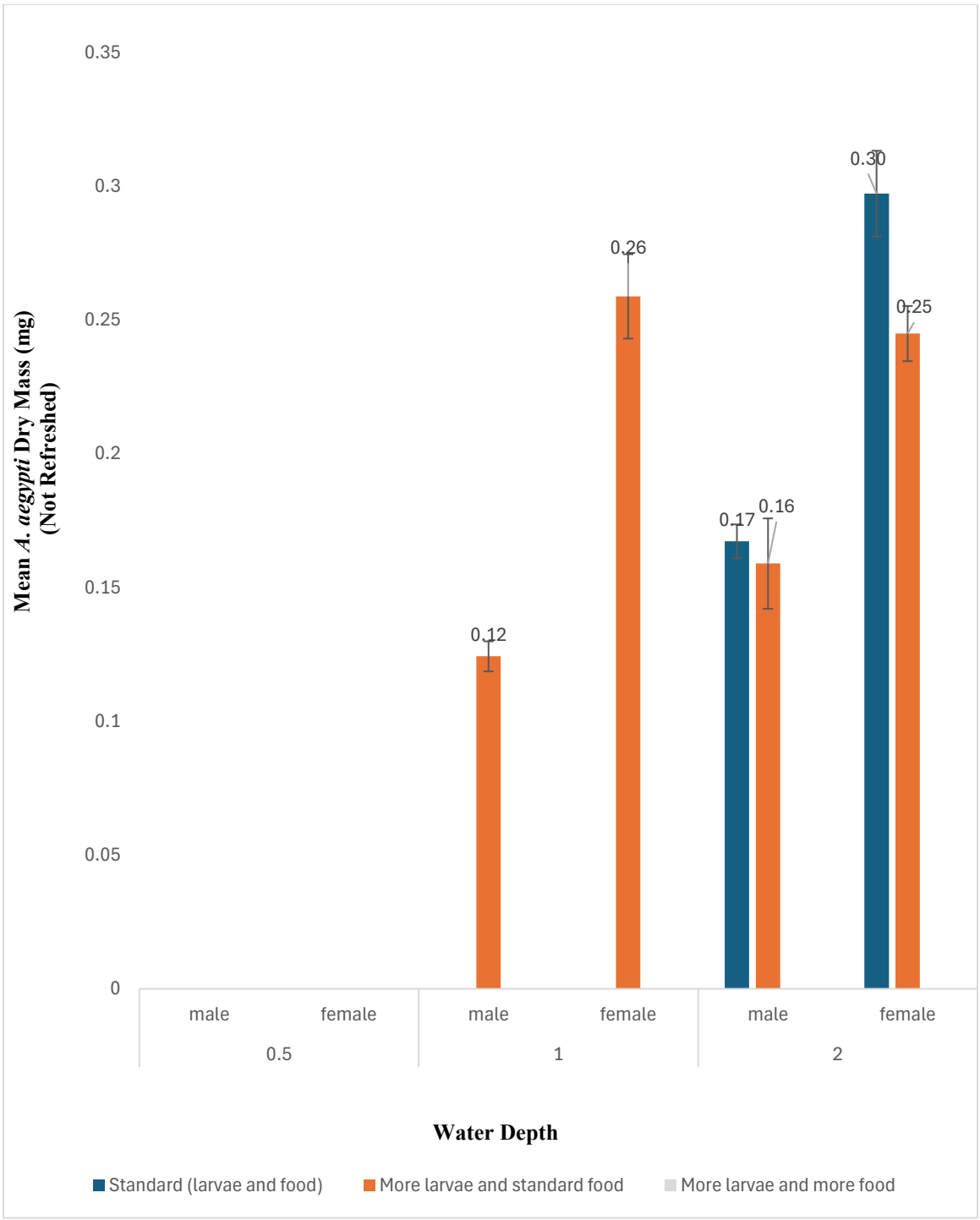

Figure 4.10: Mean *A. aegypti* Dry Mass for Each Not Refreshed Group. Error Bars Represent Standard Error. Squares Represent Males and Circles Represent Females. No Adults Matured in Groups With 0mg Dry Mass. Mean Female Mass Was Greater Than Mean Male Mass in All Groups ($p < 0.001$).

Discussion

This chapter explored how environmental factors like water depth, food availability, and larval density influence mosquito development in Refreshed and Not-refreshed containers that mimic common *A. aegypti* breeding sites and aquatic habitats. Overall, this research highlights that larval density and food availability have a more pronounced impact on mosquito development than water depth, especially in environments with replenished resources. This aligns with the observation of larger mosquito populations in resource-rich areas. While refreshed conditions foster a healthier aquatic environment with less bacterial growth, reduced toxin build-up, and likely contribute to higher survival, the ability of mosquitoes to mature even in challenging, un-refreshed environments underscores their adaptability and the persistent health risks they pose.

These findings suggest that water depth plays a more critical role in Not Refreshed groups where evaporation was a factor, leading to increased larval stress, slower development, and higher mortality due to limited resources and potential metabolic toxins in the water environments. This work illustrates how a container can shift from an environment that supports larval development to one that results in higher mortality. This chapter's results also demonstrate the resilience of *Aedes aegypti* larvae, which can develop in fetid standing water when essential conditions are met.

For the Refreshed groups, which mimic environments with regular water and nutrient replenishment (e.g., drains, drying flowerpots, gutters, irrigation pipes, puddles, waterlogged tires/litter), this chapter's findings showed the number of larvae in a container significantly impacted both the duration to pupation and duration to adulthood. Data

indicated that increased larval density results in a greater number of days for pupation (Figure 4.1). Specifically, lower larval densities led to significantly faster *A. aegypti* development. These results mirror previous experiments, showing that individuals in large containers with high larval densities and reduced amounts of food required one to three more days to pupate than individuals in smaller containers [7]. This is not a surprise considering crowded environments increase the number of collisions between larvae can cause stress [2], increase the amount of time required to feed, and impact their ability to obtain sufficient nourishment [7]. Slower larval development in containers with higher densities of *A. aegypti* lends support to the hypothesis that larvae can produce a chemical retardant that interferes with conspecific growth [7, 14]. This further supports the conclusion that reduced food availability coupled with increased competition resulted in a longer larval development [7-8, 10, 22, 23]. These data also suggest that food availability and/or the lack thereof (i.e., the combination of larval population size and food available per larva) likely have a greater impact on *A. aegypti* pupation than the low water depths this chapter evaluated. Research also indicates that interactions between the availability of resources, intraspecific density, and the competition that results from greater densities influence the time required for *A. aegypti* larvae to develop [11, 24]. This is similar to conclusions from a prior study that associated high density environments with greater effects of container [7].

Water depth and food amount had no significant impact on duration to pupation, but both water depth and food significantly influenced the time to reach adulthood. Larvae in lower water depths and with standard food amounts developed faster to adulthood. Adult

mass was significantly influenced by the amount of food, with standard food amounts resulting in lower adult mass compared to higher food and larval densities. Water depth and larval amount did not significantly impact adult mass. Neither adult survivorship nor sex ratio were significantly influenced by the treatment groups in the Refreshed conditions.

Not Refreshed groups simulated ephemeral environments with limited water and nutrient replenishment (e.g., puddles, discarded tires, containers that dry out). Previous research shows *A. aegypti* females oviposit in ephemeral pools of water that already have larvae and pupae; the presence of conspecific larvae is also thought to demonstrate that a location is conducive to larval development [9]. Not surprisingly, factors such as pool size, food accessibility/availability, and larval density impact larval development [7, 14]. Massing effects like overcrowding and intraspecific competition also influence how quickly and successfully larvae develop [7, 14]. Not Refreshed treatment groups did not significantly influence the mean duration to pupation or the mean number of days required to reach adulthood in the Not Refreshed groups. This suggests that in these harsher, un-replenished environments, the varied conditions within this chapter's experimental design did not lead to significant differences in developmental time. Still, the absence of adults in many groups with less than 1cm of water demonstrates the profound impact evaporation/water loss can have on mosquito development. Considering these observations occurred despite the laboratory's high relative humidity environment, larval survival would be further reduced in natural environments where lower relative humidity levels prevail. Under such conditions, increased evaporation rates shorten the window available for *A. aegypti* to complete development in small water bodies. This environmental pressure

serves as a key factor in explaining the lower mosquito populations in some arid regions. These lab observations have a higher relative humidity than arid regions across Maricopa county, deserts in southwestern North America, and the Sahel where lower relative humidity accelerates water loss Data for survivorship and sex ratio in these groups often did not meet the assumptions for statistical analysis (Levene's test), indicating high variability or non-normal distribution, which could indicate the extreme stress these environments impose. This is not surprising since published data suggest diet greatly influences insect development and nutritional limitation can hinder *A. aegypti* survival [24]. Similar to survivorship and sex ratio, the data for adult mass in the Not Refreshed groups did not meet the assumptions for statistical analysis.

Duration to Reach Adulthood

The Refreshed group with more larvae and standard food required significantly longer to reach adulthood than all other groups (Figure 4.3). The higher density of larvae creates more competition for resources and stress from crowding and searching for food than existed the other experimental groups [2,7, 11, 15]. Increased water depths also increase the amount of energy required for larvae locate food and filter feed. It makes sense that larvae with less access to food and increased energy expenditure will require longer to develop.

The Not Refreshed (0.5cm and 1.0cm standard larvae/food groups as well as the 2.0cm more larvae and more food) groups had a lower mean time to reach adulthood than

the other Refreshed and Not Refreshed groups (Figure 4.4). No adults matured in those groups; water in the 0.5cm and 1cm groups evaporated before adult mosquitoes could develop and a layer of bacterial film formed atop the water surface in the 2.0cm groups, which hindered development and likely explains the lack of adults in those Not Refreshed groups (Figure 4.4). Only females matured in the Not Refreshed 1.0cm corrected surface area and food group which explains the lack of data on males (Figure 4.4). Tukey HSD indicated interactions between the experimental groups and water depth (Figure 4.4, $p < 0.001$). It would make sense that *A. aegypti* larvae sense when their environment is desiccating and prioritize maturing more quickly over consuming more food in an unstable environment. Tukey HSD also suggested a significant difference between the experimental groups and number of larvae (Figure 4.4, $p < 0.001$*)*. This chapter's experimental results supported that suggestion by showing containers with more larvae and standard food required more days to reach adulthood than standard groups (Figure 4.4, $p < 0.001$*)* and groups with more larvae and more food (Figure 4.4, $p < 0.001$*).* Although not found to be significant, each group's females appear to require a greater mean number of days to reach adulthood than males from the same group (Figures 4.3 and 4.4, 11). This supports prior conclusions where males experience selective pressure to mature faster than females to increase the chances that they find and mate with a female; the latter normally only mate once [2].

Mean Survivorship to Adulthood

Data illustrate that mean survivorship to adulthood in the Refreshed groups was not significantly influenced by the treatment group (*Figure 4.5; p* = 0.058). A three-way ANOVA also examined the effects of water depth, amount of food, and number of larvae on mean survivorship to adulthood amongst the Not Refreshed groups. These data were not significant ($p = 0.218$).

Results show no survivorship to adulthood in the Not Refreshed (0.5cm and 1.0cm standard larvae and food groups as well as the 2.0cm more larvae and more food group) (Figure 4.6). This could be due to high mortality rate in these groups from water evaporation (Not Refreshed 0.5cm standard larvae and food group), cannibalism and evaporation (Not Refreshed standard larvae and food group), and water pollution that likely hindered development (Not Refreshed 2.0cm corrected surface area and food group). Research indicates that larvae (especially first instar larvae) can die from asphyxiation if pollution facilitates the formation of films in their aquatic environment that hinder larval gas exchange [24-25]. Hence, the aquatic environment and larval experiences during that portion of their life cycle, as well as diet availability and larval density, influence the number of adults produced, fitness of those adults [7, 13, 26] and development time to adulthood and adult size [23, 27-28]. Greater larval densities reduce larval survival [7, 11 22, 23, 29].

Mean Female Sex Ratio

Since the 1960s, researchers have hypothesized the XX-XY sex mechanism is likely to result in 1:1 sex ratio (Hickey & Craig, 1966). Analyses of mean survivorship to adulthood data reveal no significant difference between groups in the Refreshed treatments (Figure 4.7, $p = 0.397$*)*. Data for the Not Refreshed groups appeared significant following data transformation ($p < 0.001$*)* despite lack of adherence to Levene's test and suggested interactions existed between the experimental groups and water depth (Figure 4.7, $p < 0.001$*)*. However, the results of a subsequent Games-Howell post hoc test were not significant (Figure 4.7, $p = 0.008$) the Not Refreshed groups (0.5cm and 1.0cm standard larvae and food groups as well as the 2.0cm more larvae and more food groups) had a lower mean female sex ratio than other groups due to high mortality rates and the lack of more food/water.

Mean Adult Dry Mass

Mean adult *A. aegypti* dry mass in the Refreshed treatment was significantly influenced by the treatment group in which pupae were assigned (Figure 4.9*;* $p < 0.001$). Water depth and larval density did not significantly impact mean adult dry mass. However, the allocation of food per larva did significantly impact mean adult dry mass where individuals from containers with standard amounts of food had a significantly greater mean dry mass than containers with more food and standard larvae ($p < 0.001$) and containers more food and more larvae (Figure 4.9*;* $p = 0.010$). Additionally, individuals from

containers with more larvae and more food had a significantly greater mean dry mass than individuals from containers with more larvae and standard food ($p < 0.001$). Tukey HSD suggested density and amount of food have an impact since individuals from containers with more larvae and standard food have significantly lower mean dry mass than individuals from other groups ($p < 0.001$).

Females from Refreshed groups had a greater dry mass than males in all Refreshed treatments (Figure 4.9). This suggests that the difference between the size of female and male *A.* aegypti is the result of sexual dimorphism [24]. Males tend to emerge before females and have a smaller body size ([24]. None of the Refreshed group's females had a dry mass that was significantly different from the dry mass of females in the other Refreshed groups (Figure 4.9). None of the Refreshed groups had a mean male dry mass that that is significantly different from the dry mass of males in the other Refreshed groups (Figure 4.9).

A three-way ANOVA examining the effects of water depth, amount of food, and number of larvae to predict mean dry mass within the Not Refreshed groups was significant ($p < 0.001$). Again, significant differences were identified individuals in the standard group and the more larvae and standard food group ($p = 0.001$) and between individuals in the standard group and the more larvae and more food group ($p < 0.001$).

Significance of Water Depth

This chapter demonstrates that larval density and food amount have a greater impact on mosquito development than water depth. This finding was consistent for the Refreshed groups, where larval density and food availability appeared to have a greater impact on mosquito development than water depth alone. The Refreshed groups mimicked containers that receive an influx of water daily such as: irrigation pipes, sewer ducts, lakes, flowerpots, etc. Hence, adding more water to a container or replacing the existing water likely reduces the potential toxicity of the aquatic environment, while adding food replenishes nutrients that the larvae consumed. Providing sufficient fresh food daily likely reduces the stress larvae experience through competition for resources. Access to adequate food also reduces the risk that a larvae will be cannibalized by other hungry larvae or have their growth stunted due to competition with other larvae over resources [7]. These conclusions help clarify why survival to adulthood was greater in Refreshed groups, even if development took longer. This also illustrates why mosquito populations are often greater in areas where water and resources are more plentiful such as tropical/subtropical areas, wetlands, lakes, etc.

Water depth was also critical in Not Refreshed groups where water tended to evaporate before all larvae and pupae can mature (i.e., containers, ditches, potholes, and puddles in arid areas), resulting in fewer adults. Larval stress increases due to high larval densities, low food availability and quality [2]. This often results in longer time requirements for larval and pupal development, greater larval mortality, smaller pupal size, and mass, as well as smaller adult size [2]. Not Refreshed groups were models for

ephemeral streams, puddles, overturned pieces of plastic, used tires, and other reservoirs in areas with high rates of evaporation that contain water/nutrients from a single rain/water event and dry with time. The lack of refreshed water also results in the buildup of metabolic waste products that enhance toxicity within the containers, which can hinder mosquito development. The lack of access to quality food and/or nutrition also shows that starvation impacts larval growth and pupation [2, 15]. Once all food resources are exhausted, larvae resort to cannibalism to continue maturing. It is also possible that greater water depths create more challenging conditions for *A. aegypti* to gain adequate nutrition via feeding on the bottom or side of containers and successfully return to the surface to respire [2], especially in dirty containers like the Not Refreshed 2.0cm corrected surface area and food group where bacterial films developed atop the water and less light penetrated the container. Ultimately, these conditions resulted in higher mortality in the Not Refreshed groups. The ability for mosquitoes to fully mature in most Refreshed and Not Refreshed groups illustrates how mosquitoes can still mature and pose health risks in areas with highly ephemeral bodies of water in deserts or tropical areas. Additionally, these experimental results illustrate how *A. aegypti* larvae can also develop in "dirty" standing water within solid waste and drainage infrastructure provided those aquatic environments have appropriate conditions especially regarding temperature and organic material [31].

Future studies could expand on the findings in this chapter by replicating this work in cages or a flight room. This improves understanding of whether larval density and/or water depth significantly impact the containers where females choose to oviposit. This might also determine whether the presence of adult mosquitoes results in faster pupation

and larval development while also demonstrating whether males continue to mature more quickly than females. Researchers could also assess if exposure to water levels significantly impacts *A. aegypti* reproduction and longevity [24]. Future experiments could also replicate this chapter's experiments using different diets (food), types of water (DI, Tap, brackish, etc..), and types of containers (dimensions, size, material, color) to better mimic field conditions to gain a more wholistic understanding of how containers, diet, density, and water depth impact mosquito development and fecundity. Understanding the minimal conditions development would further vector control and public health efforts.

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CHAPTER 5

CONCLUSION

The development and application of modified *Aedes aegypti* mosquitoes for vector control represent a significant advancement in the fight against mosquito-borne diseases. The successes observed in laboratory and initial field trials for sterile/irradiated, *Wolbachia*-infected, and genetically modified mosquitoes offer compelling evidence for their potential to reduce vector populations and disease transmission. However, this dissertation underscores that realizing the full potential of these technologies while ensuring their safety and sustainability relies on a deep understanding of the complex eco-evolutionary landscape in which they are deployed, coupled with robust risk-benefit assessments and well-informed release strategies. The critical questions outlined regarding species-specific traits, genetic variation, competitiveness, and adaptation underscore the complexity of gene flow and its long-term ecological impacts. Central to these questions is the importance of mosquito populations within an ecosystem and the necessity of optimizing release parameters to control *A aegypti* populations.

These chapters demonstrate the resilience of natural sex ratio conservation and regulation in laboratory *A. aegypti* populations. Additional findings indicate that larval environmental conditions, specifically larval density, food availability, and water depth significantly influence *A. aegypti* development and fitness. Those results help discern minimal resource requirements for *A. aegypti* populations in environments with small,

isolated, and ephemeral bodies of water. Additionally, this dissertation highlights the necessity for comprehensive research before, during, and after mosquito releases.

Furthermore, future research replicating the present experiments with variations in diet, water type, and container characteristics will further refine understanding of these crucial larval development parameters. Additionally, transparent communication and genuine community engagement are paramount for the ethical and effective implementation of these novel tools. Moving forward, a holistic, eco-evolutionary informed framework is essential for guiding the design, implementation, and monitoring of these vector control strategies. This includes rigorous field evaluations, comparative analyses with traditional methods, continuous assessment of potential evolutionary responses in both target and non-target organisms, consideration of genetic bottlenecks, detailed studies on larval ecology encompassing diverse environmental conditions, and a commitment to open dialogue with all stakeholders. By prioritizing these considerations, the scientific community and public health stakeholders can work towards the safe, secure, and effective integration of modified mosquito-based approaches into sustainable vector control programs. Ultimately, this will contribute to a significant reduction in the global burden of mosquito-borne diseases while minimizing ecological disruption and fostering public trust, informed by a comprehensive understanding of mosquito developmental ecology.

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CHAPTER 2

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BIOGRAPHICAL SKETCH

Va'Trelle Stokely (Bundy) is a Biology PhD candidate at Arizona State University (ASU), specializing in Biology and Society. He is a rising medical student. Born in a historic Black community in Nova Scotia, Canada, and deeply connected to the province through extensive family ties. Va'Trelle spent his formative years in cities across the United States as the child of an active-duty (now veteran) serviceman. His commitment to global engagement is evident through his selection as a U.S. Student Ambassador to Germany via the prestigious CBYX (Congress-Bundestag Youth Exchange) program.

An alumnus of San Jose State University's Humanities Honors Program and a McNair Scholar, Va'Trelle is a three-time recipient of Louis Stokes Alliance for Minority Participation (LSAMP) stipends. He earned his bachelor's degree in biology with minors in Chemistry and Humanities. Demonstrating his interdisciplinary interests, he is a member of the American Association for the Advancement of Science (AAAS) and the Canadian Black Scientists Network. Va'Trelle possesses strong communication skills, being fluent in English and German, and proficient in conversational French and Spanish. Further expanding his technical expertise, Va'Trelle completed Penn State's Microelectronics and Nanomanufacturing Certificate Program, earning an ASTM International Workforce Certificate for Health and Safety in Nanotechnology.

During his graduate studies at ASU for the past four years, Va'Trelle has served as a dedicated Teaching Assistant/Associate for courses such as "Biology and Society" and "History of Medicine." Simultaneously, he has excelled as a graduate student researcher on campus and a Global Drylands Center-Gobabeb Research Opportunity Fellow in Namibia. For the past six years, Va'Trelle has also shared his knowledge and expertise as a Master Tutor for The Princeton Review and Tutor.com, qualified to teach a range of subjects including: English, Earth Science, Geography, Social Studies, U.S. History, and World History.

Va'Trelle has actively contributed to his communities in various capacities. He participated in Boy Scouts throughout his childhood. As a university student, he has supported the Arizona Game and Fish Department with chronic wasting disease surveillance and served as a valuable panelist for student success centers and seminars. Va'Trelle also worked as a Case Investigator/Contact Tracer/Epidemiology Intern for ASU and the Maricopa County Department of Public Health. His commitment to public health is further underscored by his certificate in controlling vector-borne diseases from the esteemed London School of Tropical Medicine & Hygiene and the Liverpool School of Tropical Medicine.

Demonstrating his leadership and commitment to graduate student representation, Va'Trelle served as a graduate student representative for ASU's Charter Initiative Task Force and as an online student representative for the School of Life Sciences Executive Board. He further volunteered his time and insights as a graduate student representative for the School of Life Sciences Seminar Committee, as a grant reviewer, and as a judge for numerous academic symposia and poster competitions.