LARVAL NUTRIENT-MEDIATED TRANSGENERATIONAL EFFECTS IN *AEDES AEGYPTI* MOSQUITOES

By

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To my pack for the continued support, constant love, and joy you’ve brought my life

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The environmental conditions experienced by parents can influence offspring phenotype generating parental effects. While there is evidence of transgenerational effects in mosquitoes, little is known regarding the conditions leading to these effects, mechanisms responsible, and how they influence host-parasite interactions between mosquitoes and the pathogens they transmit. Here we consider how parental larval nutrition influences offspring life histories and infection with dengue virus in the mosquito, *Aedes aegypti.*

By varying the input of invertebrate carcasses and oak leaves, both parents and offspring were reared under either high or low food. Life history parameters (female and male development time, female size, survivorship) were measured. Females were allowed to ingest dengue infected blood and tested for susceptibility to dengue infection, viral titer, and viral dissemination. Parental nutrition influenced female development time, but not male development time, and viral titer at 14 days, but not likelihood of infection with dengue virus.

To determine whether the maternal and/or paternal nutrition during the larval stage generates parental effects on offspring, parents were reared in high or low food

(yeast:lactalbumin), and mated in four sex-by-parental diet crosses. Maternal and paternal larval nutrition influenced offspring life history traits, with female development time contributing most to this effect. Viral traits were unaffected. Offspring from dissimilar nutrient condition parents developed the most quickly relative to other treatments.

Further testing considered whether parental larval nutrition influences reproductive output and resource allocation by measuring egg number, size, lipid content, and protein content using the four sex-by-parental diet mating crosses. Maternal nutrition affected egg number with high food females laying two times more eggs than low food females. Paternal nutrition affected eggs, with low nutrient females laying more lipid-rich eggs than high nutrient females, when both were mated with high food males.

Results suggest that carryover environmental stress from the parental generation can influence life histories and arbovirus infection. Such effects can be derived from both the maternal and paternal nutrient condition, in context dependent and sex specific ways. Lastly, variable reproductive output and lipid allocation to eggs are potential ways parents may influence life histories of *Ae. aegypti* offspring.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Introduction

Traditionally, our understanding of phenotype has been that it is the physical manifestation of genes resulting from the interaction between the organism's underlying genotype and the environment in which it lives. The genotype of the organism comes from genes inherited from its parents and additionally any mutations that may have occurred. The direct inheritance of genetic information to offspring from parents is the basis of Mendelian genetics and is referred to as 'hard inheritance'. However, significant research has demonstrated that offspring phenotype is not merely a reflection of the interaction between the organism's genotype and its environment. Rather the parental environment can influence offspring phenotype leading to what is often referred to as non-Mendelian inheritance. This type of inheritance is typically referred to as 'soft inheritance,' a phrase first described by Ernst Mayr (1982). Direct parental effects on offspring fitness combined with soft inheritance introduce additional sources of phenotypic variation in offspring that cannot be attributable solely to offspring genotype and its environment.

The literature surrounding soft inheritance is full of various terminology describing similar phenomena. These terms include and are not necessarily limited to: soft inheritance, non-Mendelian inheritance, parental effects, transgenerational effects, inherited environmental effects, epigenetic effects, and parental conditioning. Authors may also specify the parent from which these effects originate leading to additional terminology that must be considered by prefacing the aforementioned terms with maternal or paternal. Terminologies describing the mechanisms involved in these

effects include transgenerational epigenetic inheritance and genome imprinting. Youngson and Whitelaw (2008) provide useful definitions of several of these terms.

For the purposes of this research, the term transgenerational effects and parental effects will be used interchangeably to describe the factors influencing offspring phenotype beyond offspring genes and offspring environment. Additionally, the term maternal effects will be used to describe transgenerational effects derived from the mother and paternal effects will be used to describe transgenerational effects derived from the father. Transgenerational effects can be considered a type of phenotypic plasticity inherited by offspring arising from the experiences of the parental generation (Carrière 1994). The ability of the environment to influence phenotype leading to transgenerational effects has implications in terms of how we understand the biology and development of organisms (Guerrero-Bosagna and Skinner 2012). Transgenerational effects arise from parental contributions to offspring including transfer of nutrients, cytoplasmic inheritance, transmission of pathogens and antibodies, imitative behavior, and direct interactions between offspring and parents and kin (Mather and Jinks 1971). These effects also have been demonstrated to cause delayed life history effects influencing populations (Beckerman et al. 2002).

Transgenerational effects have become increasingly recognized as important ecological factors influencing subsequent generations and responses to selection (Uller 2008) and maternal effects have been demonstrated to influence both the rate and direction of the evolution of characters (Bernardo 1996a). Maternal effects produce a time lag in response to selection which can influence the direction and rate of evolution even after selection ceases (Kirkpatrick and Lande 1989). Transgenerational effects on

development enable evolution by influencing offspring developmental strategies and phenotype with underlying genetically inherited components (Badyaev and Uller 2009). Transgenerational effects themselves are subject to evolution since they require offspring ontogenies be sensitive to parental input and the degree of sensitivity changes the size of the effect (Badyaev and Uller 2009). While there is evidence of complex transgenerational effects in mosquitoes, little is known regarding the conditions leading to these effects, the underlying mechanisms responsible, and whether they influence the host-parasite interactions between mosquitoes and the pathogens they transmit.

Background and Significance

Transgenerational effects occur when the environmental conditions experienced by one or both parents influence offspring phenotype. Transgenerational effects may come from either parent; however maternal effects have been studied more frequently. Transgenerational effects have been demonstrated in nearly all major taxa which demonstrate how important they can be in shaping ecology and evolution (Uller 2008, Bonduriansky and Day 2009). Transgenerational effects have been demonstrated at many levels of organization and have been seen in single celled organisms, plants, and multicellular animals (Mousseau and Fox 1998a). In organisms with parental care, some transgenerational effects are obvious such as a mother providing milk to her offspring, offspring mimicry, or teaching specific adaptive behavioral skills. Many behavioral studies on organisms with direct parental care, including humans, have been conducted in the field. There is significant evidence demonstrating that transgenerational effects, especially maternal effects, occur in organisms without direct parental care including some insects. Additionally, transgenerational effects due to highly context dependent

environmental cues may influence life histories of descendants for several generations (Plaistow et al. 2006) further demonstrating the importance of these effects.

There have been more than 70 insects, representing a wide range of taxa, for which environmental conditions have been identified that induced maternal effects on life history traits (reviewed in Mousseau and Dingle 1991a). Investigation surrounding maternal effects and life history traits may prove to have significant implications for managing insect populations (Mousseau and Dingle 1991a). Future studies are needed that address the evolutionary dynamics of transgenerational effects particularly in important vector mosquitoes (Otti and Sadd 2008). Transgenerational effects may influence the life history and evolution of Culicidae and ultimately the pathogens that they vector (Otti and Sadd 2008). Consequently, understanding parental effects on Culicidae is one component that may influence control.

Transgenerational Effects on Offspring

Parents are able to influence the condition of their offspring both directly, through genetic contribution, and indirectly as a function of the environment leading to parental effects. Maternal effects occur within generations and among generations (Mousseau and Dingle 1991b). Environmental effects associated with parental effects on progeny are seen across a broad taxonomic range (Rossiter 1996). Clear strong maternal effects are readily seen in mammals; however, evidence suggests that maternal effects play a larger role in contributing to evolutionary processes and adaptive divergence in many systems than is currently acknowledged (Räsänen and Kruuk 2007). While it is difficult to determine the influence of maternal effects on the rate and direction of evolution, it is clear that maternal effects play a role in evolution at ecological time-scales (Räsänen and Kruuk 2007). Räsänen and Kruuk (2007) summarize a few examples from different

taxonomic groups, including amphibians, mammals, and birds, of rapid phenotypic changes due to maternal effects. For instance, in the moor frog *Rana arvalis*, maternally mediated adaptation to acidic geographic environments has occurred within the past 100 years indicating accelerated evolutionary change (Räsänen et al. 2003a, b). Environmentally-based maternal effects also have immediate ecological consequences and influence many life history parameters that ultimately affect offspring mortality (Rossiter 1991).

Brief overview of transgenerational effects in insects. Maternal effects influence a variety of factors including but not limited to diapause, dispersal, development time, growth rate, resistance to stressors, and survival (Mousseau and Dingle 1991a). The maternal effects seen may be in response to a variety of environmental stimuli such as photoperiod, temperature, nutrition, parasites and maternal age (Mousseau and Dingle 1991a). Here I summarize some key examples of maternal effects in insects researched since the Mousseau and Dingle (1991a) review.

There has been significant research demonstrating the importance of transgenerational effects on influencing life history traits including body size, survivorship and competitiveness. In insects, maternal effects may permit adaptive responses or programming of life cycles for phenotypically plastic traits in response to environmental conditions (Mousseau and Dingle 1991b; Reinhold 2002). Body size of the seed beetle *Callosobruchus maculatus*, which is partially a density dependent plastic trait, can be inherited by progeny via a non-genetic maternal effect (Fox and Savalli 1998). As a result, reduction in body size due to high levels of larval competition in the maternal environment persists in progeny and in F2 progeny allowing for

maturation at smaller sizes (Fox and Savalli 1998). In house flies, older females produce more competitive larvae than younger females (McIntyre and Gooding 2000). This is thought to be adaptive since fly populations in temperate regions increase throughout the summer, increasing the likelihood that late season offspring will experience higher larval densities and those from older females will be more competitive (McIntyre and Gooding 2000). In *Coenagrion pulla* damselflies, when mothers are infected with parasitic water mites associated with higher juvenile mortality, larger offspring are produced which may mediate offspring mortality and increase maternal fitness (Rolff 1999). In the midge, *Chironomus tepperi,* offspring of nutritionally stressed parents developed more quickly than those from high nutrient parents (Colombo et al. 2014). These examples suggest that environmental cues experienced by parents can influence the life history trajectory of offspring to improve fitness as well as competitiveness in challenging environments.

Additionally, important physiological processes like diapause can be influenced by the conditions experienced by parents. The mechanism of maternally induced egg diapause is well understood in the silkworm *Bombyx mori*. In *Bombyx mori* females experiencing low temperatures and low light during the pupal stage laid diapausing eggs due to the secretion of diapause hormone from the neurosecretory cells of the suboesophageal ganglion (Fukuda 1951, 1952). Secretion of diapause hormone, which is a neuropeptide, stimulates expression of the trehalase gene and synthesis of trehalase in developing ovaries leading to hyperglycogenism in the maturing eggs, which is required for diapause initiation (Yamashita 1996). Starvation of females through host deprivation causes a significant increase in the number of diapausing eggs

laid by *Nansonia vitripennis* (Saunders 1966). In the tsetse fly *Glossina morsitans* diet composition and blood meal quality influenced offspring size and likely survival (Langley et al. 1978). These studies suggest that nutrition obtained by the female influences her offspring. In *Peripsocus quadrifasciatus* offspring diapause is influenced by the geographic location of the population in addition to photoperiod, altitude and temperature experienced by the mother (Eertomoed 1978). In *Calliphora vicina*, a blow fly, larval diapause is displayed when adult females experience short-day lengths (McWatters and Saunders 1996). In this species, the father, also, in part, influences the duration of diapause (McWatters and Saunders 1996). When *C. vicina* males from southern populations mate with females from northern populations the length of offspring diapause is shortened compared to offspring of two parents from northern populations, a demonstration of a paternal effect in insects (McWatters and Saunders 1996). Physiological processes can be influenced by mothers and fathers leading to complex transgenerational effects in offspring.

Transgenerational immune effects in response to the maternal environment have also been identified in insects. In *Plodia interpunctella*, there is evidence of transgenerational immune priming to a DNA granulosis virus in that offspring of moths exposed were less susceptible to the virus (Tidbury et al. 2010). The mechanism(s) involved in transgenerational immune priming to DNA virus in this system are unknown (Tidbury et al. 2010). Additional studies on this moth species suggest that poor resources in the maternal environment led to increased resistance against granulosis virus in offspring (Boots and Roberts 2012). It was hypothesized that the increased resistance may be due to the poor environment cueing higher disease risk for offspring

(Boots and Roberts 2012). In the same species, poor parental food led to reduced phenoloxidase activity, an important component of the encapsulation immune response (Triggs and Knell 2012). In the blowfly *Protophormia terraenovae*, offspring of parents exposed to the heavy metal pollutant copper had stronger encapsulation responses to antigens (Pölkki et al. 2012). This research demonstrated changes in innate immunity in offspring even when the environmental challenge is removed (Pölkki et al. 2012). In the moth, *Manduca sexta,* when challenged by a parasite, eggs from immune-challenged parents had significantly higher transcription of immune-related genes than those from parents that were not challenged (Trauer-Kizilelma and Hilker 2015). These examples demonstrate that transgenerational immune effects in insects have been linked to a variety of environmental conditions including parental nutrition, direct exposure to virus, and exposure to environmental contaminants.

It is clear that the environment experienced by the mothers in particular can have significant consequences for the survivorship and competitiveness of offspring. In many species of insects early developmental conditions experienced by the mother were less influential on offspring success than environmental conditions experienced later in the mother's life (Mousseau and Dingle 1991a). There is also some evidence of paternal effects in insects. Environmental cues experienced by parents can influence the phenotypic traits of offspring, which may, in some instances, provide selective advantages for their offspring (e.g., diapause). These cues can influence physiological features including diapause, life history traits such as survivorship, and even relationships between insects and pathogens.

Parental Effects in Mosquitoes

In mosquitoes (Diptera: Culicidae) the literature about transgenerational effects has primarily focused on how maternal photoperiod and temperature influence egg diapause in the next generation. Additional studies have been conducted considering parental food, oviposition site selection, the maternal immune environment, infection with parasites and viruses, and pesticide exposure. These studies suggest that parental effects in mosquitoes are highly variable and alter the life history of offspring in complex ways. While studies have considered parental effects in mosquitoes, little has been done to see how the parental environment influences susceptibility to arboviral infection and vector competence. Vector competence describes susceptibility to infection, viral replication, and transmission.

Diapause

Diapause is an incredibly important physiological response allowing for mosquitoes to avoid risk due to unfavorable environmental conditions. Photoperiod and temperature are two environmental conditions that have been shown to influence whether or not females lay eggs that diapause. The exact response to these stimuli is often context dependent and varies depending upon the environmental conditions experienced, the species involved, and the specific population.

Maternal influence on diapause can significantly vary depending on the photoperiod females experienced (Vinogradova 1965, Anderson 1968, McHaffey and Harwood 1970, Wilson and Horsfall 1970, Mori and Oda 1981). Generally, when female *Aedes vexans*, *Ae. togoi*, *Ae. atropalpus, Ae. dorsalis*, and *Ae. albopictus* experienced short day lengths they laid more diapausing eggs than females experiencing long day lengths (Vinogradova 1965, Anderson 1968, McHaffey and Harwood 1970, Wilson and

Horsfall 1970, Mori and Oda 1981, Lounibos et al. 2011). In *Ae. atropalpus* whether or not females lay diapausing or non-diapausing eggs is influenced by the L:D ratio experienced specifically during the fourth larval instar and pupal stages (Anderson 1968) demonstrating that in some cases maternal effects can be stage dependent. Paternal photoperiod did not directly influence offspring egg type in *Ae. atropalpus* (Anderson 1968). Additionally, photoperiod can influence not only whether or not a female will lay eggs that diapause but also some of the characteristics of the eggs. In a temperate *Ae. albopictus* population, there were significant differences in surface lipids, in eggs laid by females that were exposed to short days compared to those exposed to long days (Urbanski et al. 2010b). Surface lipids provide protection against desiccation which would be beneficial during winter months (Urbanski et al. 2010b). Specifically, eggs laid by females experiencing short days underwent diapause, had higher surface lipid quantities, and were more desiccation resistant than those laid by females experiencing long days (Urbanski et al. 2010b).

In mosquito species populations, the maternal environment may be more influential than the environment experienced by offspring in the induction of diapause in the egg stage (Pinger and Eldridge 1977). In *Psorophora ferox* the photoperiod experienced by the mother influenced egg diapause more than the photoperiod experienced directly by the eggs (Pinger and Eldridge 1977). As a result, conditions experienced by the mother can be an excellent predictor of diapause in eggs. Depending upon the environmental conditions experienced, *Ae. albopictus* may lay eggs ranging from complete diapause to non-diapause with some in between allowing for increased plasticity in the egg hatching response (Mori and Oda 1981). For instance,

females may lay eggs that are in a weak diapause condition so that given favorable conditions they may hatch (Mori and Oda 1981). In *Ae. atropalpus*, regardless of the photoperiod experienced by the mother, eggs laid at 30°C did not diapause (Kalpage and Brust 1974) suggesting temperature is an important factor as well in this species. In *Ae. dorsalis* maternal effects were not seen in females exposed to longer photoperiods (McHaffey and Harwood 1970) demonstrating that specific conditions may trigger maternal effects.

Several studies on *Ae. triseriatus* found conflicting results surrounding whether the maternal environment or the embryonic environment influences diapause (Baker 1935, Love and Whelchel 1955, Kappus and Venard 1967, Anderson 1968). In a laboratory study, adult female *Ae. triseriatus* exposed to 15 hours of daylight during the midwinter produced offspring that developed and pupated in complete darkness (Love and Whelchel 1955). Female *Ae. triseriatus*, from the lab derived colony, reared in darkened rooms during the midsummer produced progeny that did not develop until the photoperiod lengthened (Love and Whelchel 1955). This study suggests that shortening photoperiod in the fall stimulates females to lay eggs that will diapause during the winter (Love and Whelchel 1955). Other studies argue that diapause in eggs of *Ae. triseriatus* results from conditions experienced during embryogenesis and not conditions experienced by their mothers (Baker 1935, Kappus and Venard 1967, Anderson 1968). It is possible that discrepancy obtained by Love and Whelchel (1955) were due to use of unnatural complete darkness conditions (Anderson 1968) or due to differences between northern and southern populations of *Ae. triseriatus* (Kappus and Venard 1967) and it is likely that the maternal effect is not seen in this species. Rather Shroyer and Craig

(1980) demonstrated that eggs are photosensitive in *Ae. triseriatus*, substantiating evidence that egg diapause in this species is not maternally derived.

Maternal effects in response to temperature can be variable. In *Ae. togoi* lower temperatures led to higher numbers of diapausing eggs (Vinogradova 1965). In *Ae. vexans*, eggs laid by females experiencing short days showed higher variation in hatching than eggs from females experiencing long days, but these effects depended on environmental temperature (Wilson and Horsfall 1970). A study using three different geographic populations of *Ae. atropalpus* found that the maternal induction of diapause in offspring was influenced by temperature in the mosquitoes from two southern locations (Tallulah Gorge, Rabun County, Georgia, U.S.A; and Lago San Diego, Metapan, El Salvador) but not in the mosquitoes from a northern location (Ontario, Canada) regardless of the effect of photoperiod (Beach 1978). Temperate and tropical populations of *Ae. albopictus* females lay eggs that display differences in diapause which may be attributed to photoperiod cues experienced by the mothers leading to differential gene expression (Urbanski et al. 2010a). Intraspecific variation in the influence of maternal effects in offspring at a population level may occur and be, in part, responsible for observed geographic differences in diapause responses (Beach 1978).

These studies demonstrate the complexity of transgenerational effects in mosquitoes as they relate to photoperiod and temperature. In some cases, the conditions experienced by mothers are more important in predicting offspring response than those experienced directly by offspring. However, in other instances, the combined environments of parents and offspring both matter. In mosquitoes, transgenerational effects of parental photoperiod and temperature can influence whether or not eggs enter

diapause, hatching responses, and other characteristics such as egg volume (Lacour et al. 2014) and clutch size (Grech et al. 2007).

Oviposition Behavior and Timing

Choice of oviposition site can greatly influence offspring success and is a behavioral strategy that influences hatching success and larval performance (Resetarits 1996). Mosquitoes may display oviposition site preferences in response to a variety of physical and chemical cues many of which are reviewed by Bentley and Day (1989). Several examples of oviposition site selection follow. In *Ae. aegypti*, a laboratory study demonstrated that under low humidity and high sugar concentrations, females delayed oviposition to protect eggs from environmental hardships (Canyon et al. 1999). In *Ae. togoi* mosquitoes, a species whose larvae develop in rock pools, females avoided ovipositing in sites with salinities greater than 40 g NaCl/L (Trimble and Wellington 1978). Female *Ae. togoi* may avoid areas of high salt concentration since they are indicative of pools which may not retain water long enough for larvae to complete development (Trimble and Wellington 1978). A series of artificial pond experiments found that *Culex quinquefasciatus* and *Ochlerotatus australis* females were more likely to oviposit in water containing conspecific larvae (Mokany and Shine 2003). In terms of tadpole predator response, females *Cx. quinquefasciatus* did not avoid areas containing tadpoles, however, *Oc. australis* females did likely because anuran tadpoles are a common predator of this species (Mokany and Shine 2003). The examples provided demonstrate female oviposition site selection in response to a variety of different stimuli.

Parental Nutrition Effects

Parental nutrition is an important contributor to transgenerational effects in various taxa. In *Anopheles stephensi*, parental food conditions during the larval stage

influenced offspring fecundity but did not significantly influence emergence time, size, or survival (Grech et al. 2007). *Anopheles stephensi* offspring of parents raised in low food conditions produced more eggs than offspring from parents reared in high food conditions (Grech et al. 2007). Parental effects on blood meal size as indicated by haematin mass were also seen but only for offspring in low food conditions, evidence for nutrient-dependent parental effects (Grech et al. 2007). Female offspring experiencing low food conditions took larger blood meals if their parents also experienced low food conditions (Grech et al. 2007). These results may suggest an adaptive response to compensate for poor maternal provision and resource acquisition as larvae (Grech et al. 2007). Another lab experiment demonstrated that starvation of *Ae. atropalpus* females during the fourth larval instar was important along with photoperiod and temperature in determining whether or not they laid diapausing eggs (Beach 1978). These studies demonstrate that parental nutrition may also be an important contributor to transgenerational effects in mosquitoes and could influence host-parasite interactions as well.

Maternal Immune Effects and Infection

A few studies have considered how the maternal environment influences offspring immunity in mosquitoes. Melanization response in mosquitoes may be used as an indicator of transgenerational immune priming (Voordouw et al. 2008). In *Ae. aegypti* stimulation of the maternal melanization response had no effect on offspring melanization responses or fitness correlates (Voordouw et al. 2008). The authors were unable to detect significant differences between fitness in offspring of immune challenged mothers and offspring of unchallenged mothers, however, they concluded that fitness costs or benefits may occur if offspring were under more competitive

environments (Voordouw et al. 2008). Additionally, literature suggests that transgenerational immune priming may be more important for some immune traits over others (Voordouw et al. 2008). In *Anopheles gambiae* the maternal environment can shape offspring life history and susceptibility to malaria (Lorenz and Koella 2011). In *An. gambiae* offspring of females infected with the microsporidian parasite *Vavraia culicis*, a biological control candidate, were less likely to become infected with the malaria parasite *Plasmodium berghei* than the offspring of females that were not infected with *V. culicis* (42% and 70% infected respectively) (Lorenz and Koella 2011). Although definitive evidence is lacking, lower susceptibility to *P. berghei* infection may be related to an altered immune status. A study on maternal infection in *Anopheles coluzzii,* demonstrated that offspring of mothers infected with *Plasmodium falciparum* had greater numbers of oocysts in the gut post-infection relative to those from uninfected mothers (Vantaux et al. 2014). The authors determined that increased parasite intensity in offspring may have been due to mothers investing more energy in immune defenses and consequently producing lower quality, less resistant offspring (Vantaux et al. 2014). These studies demonstrate that environmental conditions experienced by mothers may or may not influence offspring immunity, and that maternal effects on offspring immunity can be adaptive or maladaptive when they occur. Additionally, these studies demonstrate the importance of considering multiple aspects of the immune response when trying to understand transgenerational effects on offspring immunity.

Vertical transmission of pathogens directly to offspring is another parental effect that has been demonstrated in mosquitoes. Vertical transmission has been identified in many important vector species as well as viruses. The first successful known

experiment demonstrating transovarial transmission of an arbovirus was conducted by Marchoux and Simond (1905) on *Ae. aegypti* using yellow fever virus. One laboratory study conducted on *Ae. triseriatus* found that La Crosse virus can persist for four years or longer in the absence of a horizontal transmission host due to transovarial transmission (Miller et al. 1977). Another experiment found transovarial transmission rates of 2.7% in offspring from *Ae. albopictus* parents experimentally infected with La Cross virus (Tesh and Gubler 1975). A study conducted on wild caught Californian *Culex* species determined that after intrathoracic inoculation, *Cx. tarsalis* had the highest filial infection rates followed by *Cx. pipiens quinquefasciatus* and *Cx. pipiens pipiens* yielded no detectable virus (Goddard et al. 2003). In addition to lab studies, all four serotypes of dengue virus have been found in nature to be transovarially transmitted in *Ae. aegypti* and *Ae. albopictus* through identification of the virus in larvae and/or males (Khin and Than 1983, Hull et al. 1984, Ibáñez-Bernal et al. 1997, and Kow et al. 2001). In mosquitoes, vertical transmission of viruses to offspring is one way that viruses can persist in environments that are unfavorable for horizontal transmission.

Pesticide Exposure

Mosquito control efforts often involve the use of pesticides and it is likely that mosquito larvae are exposed to sublethal doses. In *Ae. aegypti*, when parents were exposed to sublethal doses of *Bacillus thuringiensis var israelensis* (Bti), significant changes were seen in offspring (Wang and Jaal 2005). Offspring of parents that were exposed to Bti had reduced survival rates and increased development times (Wang and Jaal 2005). Additionally, the sex ratio of offspring shifted from that of the control group (approximately 50:50 of males:females) so that fewer female offspring were produced (approximately 60:40 of males:females) (Wang and Jaal 2005). These changes in

survival and development of offspring in response to parental exposure to sublethal pesticides may influence how offspring interact with viruses.

Summary of Parental Effects

From the examples in insects provided, it is clear that both biotic (e.g. nutrition, maternal age, parasites) and abiotic factors (e.g. photoperiod, temperature, pesticides, altitude) influence maternal effects on offspring. As a result of these effects, the life history trajectory of offspring may be influenced (e.g. egg size and hatchability, diapause, competitiveness, survivorship, and susceptibility to parasitic infection). Studies of parental effects in mosquitoes have largely focused on the influence of the maternal environment on egg diapause. Exceptions include a few studies on the influence of parental nutrition and maternal immune condition on offspring. The results from these studies suggest that there are a wide range of factors that likely influence whether or not parental effects occur and to what extent they influence offspring.

Environmental conditions experienced by parents can influence offspring phenotype generating parental effects. Factors influencing phenotype of offspring may have significant implications in terms of mosquito life history traits and the ability of mosquitoes to transmit arboviruses that influence public health. Parental nutrition has been identified as an environmental factor that leads to transgenerational effects in several different insects (Saunders 1966, Langley et al. 1978, Rossiter 1991, Rossiter 1996, Gould 1988, Bonduriansky and Head 2007, Grech et al. 2007, Freitak et al. 2009, Boots and Roberts 2012, Valtonen et al. 2012, Triggs and Knell 2012). There is also research demonstrating transgenerational effects on offspring immunity in insects (Tidbury et al. 2010, Lorenz and Koella 2011, Boots and Roberts 2012, Pölkki et al. 2012, and Triggs and Knell 2012). Larval nutrition has been linked to immunity in the

adult stage in several insect species including mosquitoes (Suwanchaichinda and Paskewitz 1998, Okech et al. 2007, Fellous and Lazzaro 2010, Boots and Roberts 2012, Telang et al. 2012).

Consequently, it is possible that there is a significant relationship between parental larval nutrition and offspring life histories and immunity due to transgenerational effects in some mosquito species. These potential changes in immune state would likely influence vector competence as well. Given that much of the research on parental effects in mosquitoes has focused on maternal effects on diapause, this research aims to improve our understanding of the conditions that lead to transgenerational effects in mosquitoes and how they are related to offspring immunity and vector competence. *Aedes aegypti* is one of the most important arboviral vectors in the world given its role in transmitting dengue, yellow fever, chikungunya, and Zika. Additionally, it is relatively easy to rear in the laboratory and larvae are likely to experience nutrient limitations associated with the container systems they inhabit as larvae (Washburn 1995). These advantages provide a good starting point for investigating parental nutrient effects on offspring life histories and infection with an arbovirus. From here further studies can apply these concepts to other mosquito:pathogen systems and expand our understanding of parental effects in vector mosquitoes. Improving our understanding of parental effects in vector mosquitoes given the importance of these effects in terms of how populations respond to natural selection.

CHAPTER 2
THE LARVAL DIET OF PARENTS INFLUENCES LIFE HISTORY TRAITS AND DENGUE VIRUS INFECTION OF OFFSPRING IN *AEDES AEGYPTI*

Introduction

Parental effects occur when the environmental conditions experienced by parents influence offspring phenotypes. These effects can be considered a type of inherited phenotypic plasticity (Carrière 1994) and their importance is clear in organisms displaying parental care (Mousseau and Fox 1998b). However, parental effects influence the ecology and evolution of a wide range of taxa (Mousseau and Fox 1998b, Uller 2008, Bonduriansky and Day 2009). Adaptive parental effects occur when environmental conditions experienced by parents enhance offspring fitness (Marshall and Uller 2007). Maladaptive parental effects occur when environmental conditions result in poorer quality offspring (Vijendravarma et al. 2010). The conditions leading to parental effects vary across species and traits considered. In insects, parental nutrition has been linked to parental effects in several orders (Rossiter 1996, Grech et al. 2007, Freitak et al. 2009, Valtonen et al. 2012, Triggs and Knell 2012). One offspring trait that has been linked to parental effects is immunity (Freitak et al. 2009, Triggs and Knell 2012, Pigeault et al. 2016). These studies demonstrate the importance of considering parental nutrition effects on immunity in other systems.

Container mosquitoes have received considerable attention in ecological studies partially because they vector arboviruses including dengue, chikungunya, yellow fever and Zika. Dengue alone is estimated to infect 390 million people per year, with 96 million cases with clinical manifestations (Bhatt et al. 2013). The term ‘container’ refers to the natural and artificial larval habitats these mosquitoes rely upon. Allochthonous detritus inputs to these containers (including plant detritus and invertebrate carcasses)

serve as food for microorganisms upon which developing larvae feed. The quantity and type of detritus in a container affect larval growth and development as does competition between conspecifics and heterospecifics (Murrell et al. 2011). Nutrient limitation is likely common in container systems (Washburn 1995).

In mosquitoes, larval nutrient limitation is associated with reduced survival to adulthood, longer development, smaller size, and reduced adult fitness (e.g. Triggs and Knell 2012, Pigeault et al. 2016, Bhatt et al. 2013, Murrell et al. 2011, Washburn 1995, Telang and Wells 2004, Muturi et al. 2011). Nutritionally deprived females often lay fewer eggs (Telang and Wells 2004) and have reduced fecundity (Briegel 1990). Larval nutrition is positively associated with body size and metabolic reserves (Briegel 1990). During nutritive stress teneral protein, lipid, and carbohydrate reserves acquired during larval development can be mobilized to promote survival (Briegel 1990, Farjana and Tuno 2013). Larger adult mosquitoes generally live longer (Hawley 1985, Takken et al. 1998), take larger blood meals (Klowden and Lea 1978), and have higher total fecundity (Briegel 1990) than smaller mosquitoes. However, after an initial blood meal, survival may no longer be associated with size (Lyimo and Takken 1993). Body size, adult longevity, and blood feeding behavior are associated with vector potential (Farjana and Tuno 2013, Hawley 1985, Scott and Takken 2012). *Aedes aegypti* and *Anopheles gambiae* often take multiple blood meals per gonotrophic event, a behavior associated with the high transmission of pathogens by these species (Scott and Takken 2012). The ability of the vector to survive the extrinsic incubation period of a pathogen (period of development in the vector) and to then engage in subsequent blood feeding is necessary for transmission.

Larval nutrition within a generation has been shown to influence vector competence of mosquitoes in complex ways. Vector competence refers to susceptibility to infection, replication, and transmission of pathogens. Larval nutrient limitation is associated with increased vector competence in *Aedes albopictus* (Zhang et al. 1993), *Aedes triseriatus* (Grimstad and Walker 1991), and *Culex tritaeniorhynchus* (Takahashi 1976); both increased (Muturi et al. 2011) and decreased in vector competence in *Ae. aegypti* (Nasci and Mitchell 1994); and no effect on vector competence in *Culex tarsalis* (Dodson et al. 2011). The influence of larval nutrition on vector competence is associated with barriers to infection and innate immunity (Grimstad and Walker 1991, Telang et al. 2012). This is important because physical and physiological barriers to infection influence dissemination of the virus into secondary tissue and transmission potential. Given the importance of larval nutrition in influencing adult mosquito phenotypes and fecundity, a logical step is to consider how larval nutrition experienced by parents influences subsequent offspring.

The relationship between larval nutrition, immunity, and parental effects is important to consider for mosquitoes that transmit pathogens. In the mosquito *Anopheles stephensi* parental food during the larval stage did not significantly affect offspring emergence time, size, or survival but did influence offspring fecundity (Grech et al. 2007). In *An. gambiae*, daughters of females reared in nutrient-deprived conditions as larvae were more likely to be infected with the malaria parasite, *Plasmodium berghei* (Lorenz and Koella 2011). However, maternal infection with a microsporidian, in *An. gambiae* resulted in reduced susceptibility to malaria in daughters possibly due to immune priming (Lorenz and Koella 2011). These studies demonstrate that interactions

between the parental environment and offspring phenotype are complex. Parental effects can influence host-parasite interactions in important vector species and consequently should be considered in research (Otti and Sadd 2008), especially for emerging arboviruses, which remain under-investigated.

The purpose of this study is to investigate whether larval nutrition experienced by parental *Ae. aegypti* influences offspring (1) life history traits (growth, development, survival to adulthood) and (2) susceptibility to dengue-1 virus infection. Though we focus on *Ae. aegypti* and dengue, due to the importance of this mosquito as a vector and the global burden of dengue, the concepts improve our general understanding of the potential role of parental effects on arbovirus mosquito vectors.

Materials and Methods

Source and Rearing of Parental Mosquitoes

Aedes aegypti used were from an established colony of wild-caught mosquito larvae collected from Key West, FL in 2012. We used *Ae. aegypti* from Key West as this population was responsible for dengue-1 virus transmission in 2009 and 2010 (CDC 2010). Insectary conditions were set to 28±0.5°C and a 12:12 (light:dark) hour photoperiod and sustained throughout the experiment. Colony mosquito larvae were reared in enamel pans (24 by 36 by 5 cm) in 1.5 L of water in cohorts of ≈ 200 mosquitoes on an equal mixture of brewer's yeast and lactalbumin given in 0.2g increments two to three times per week. Pupae were transferred to water-filled cups and placed into plastic cages (45.7 x 45.7 x 45.7 cm, BugDorm, MegaView Science Co. Ltd. Taichung, Taiwan) to emerge as adults. Adults were maintained on a 20% sucrose solution from cotton wicks and blood fed on live chickens approximately once per week.

Chicken care followed the animal use and care policies of the University of Florida's Institutional Animal Care and Use Committee (IACUC Protocol 201003892).

To reduce the possibility of parental effects generated in the wild, the F4 generation was used as the parental (P) generation for this study (Mousseau 2000). P larval nutrition treatments included natural sources of plant and invertebrate detritus, and microorganisms. Ten 2.0 L cylindrical plastic containers (15.5cm x 17.1cm, ht. x dia.) for each treatment group were used to rear the P larvae. Each container received 2.0 L of tap water and 10 mL of tire water inoculum (collected from tires in at the Florida Medical Entomology Laboratory (FMEL) campus) with associated microorganisms. Each container received senescent live oak leaves (*Quercus virginiana*), a predominant host tree for container habitats in Florida, and dead field crickets (*Gryllus sp.*) in amounts varying by treatment (low nutrition, 4 g oak leaves+0.06 g crickets; high nutrition, 12 g oak leaves+0.2 g crickets). The oak leaves were collected from the FMEL campus and dried for 24 hours at 70°C. The crickets were obtained from the local pet supply store and dried for 48 hours at 60°C using established methods (Daugherty et al. 2000).

Contents were incubated in containers for four days prior to use to allow microbial populations to establish. P generation eggs were hatched in containers with 1.0 L tap water and 0.2 g of an equal mixture of brewer's yeast and lactalbumin. After 24 hours, larvae were removed from nutrients, rinsed, and 200 were added to each experimental container (0.1 larvae/ml). Larval density approximated the mean natural density in Florida containers occupied by *Ae. aegypti* and competitor *Ae. albopictus* (N=790, mean±SE, 0.17±0.02, range 0.00083-3.08 larvae/ml) (Alto et al. 2005).

Supplemental food consisting of half the initial amount was added every 7 days. P larvae were checked daily for pupation. Pupae were transferred to plastic vials containing water and sealed with cotton until emergence as adults. As adults, mosquitoes were sexed and placed in treatment specific cages containing a water-filled cup lined with paper towel for an oviposition substrate. No more than 50 mosquitoes were added to each cylindrical cardboard cage (9.7 x 9.7 cm ht. x dia.). Each cage contained approximately a 1:1 male to female ratio, and females within a 3-day age span.

The mosquitoes were given 8-11 days to mate and were sustained on a 20% sucrose solution. After the mating period, sucrose was replaced with water for two days to increase propensity to blood feed. Mosquitoes were then offered a bovine blood meal warmed to 37°C using an artificial membrane feeding system (Hemotek®). Feeding rates were 73.7% for high food females and 64.0% for low food females. Fully-engorged females were held in cages for 7 days during which gravid females laid eggs. Eggs were removed after 7 days and maintained in a humid environment until hatching (see Offspring rearing). Two high food and one low food parental treatment containers were lost due to water quality issues. See Figure 2-1 for parental treatments.

Offspring Rearing

Four days prior to hatching the offspring (F1 generation), experimental containers were set up for each F1 treatment group. Hatching was staggered to ensure larvae were similar in age. Experimental containers were the same as those for the parental treatment groups. A total of four different experimental replicate treatment groups were set up. The F1 generation eggs from each parental experimental replicate were hatched and 200 of the resulting larvae were reared in low nutrients and 200 were reared in high

nutrients using the same methods as the P generation. The treatment manipulation allows for similar genetic diversity between the offspring treatment groups derived from each parental treatment group.

F1 larvae were checked daily for pupation. Pupae were transferred to sealed vials until emergence as adults. Development was measured as the time in days from hatch to adult emergence. Survivorship to adulthood was recorded for each experimental replicate calculated from the initial cohort size. Upon emergence, adults were sexed and transferred into treatment specific cages. Adults were maintained on a 20% sucrose solution for 8-10 days post-emergence. Forty-eight hours prior to blood feeding, the sucrose solution was replaced with water to improve feeding on infectious blood. Females were offered blood infected with dengue-1 virus isolated from Key West, FL, see GenBank: JQ675358.1, using the Hemotek® feeding system described by Alto and Bettinardi (2013). See Figure 2-1 for Offspring nutrient treatments.

Mosquito Infection with Dengue Virus

Dengue virus was propagated for blood meals following previously established methods (Alto et al. 2008, Alto and Bettinardi 2013). Briefly, monolayers of African Green monkey kidney (Vero) cells, in tissue culture flasks (175 cm2), were inoculated with 250 µL of dengue at a multiplicity of infection of 1.2 followed by one-hour incubation at 37°C in a 5% CO2 atmosphere. Post-incubation, 25 mL of media (199 media, 10% fetal bovine serum, 0.2% antimycotic, and 0.2% penicillin-streptomycin) were added to each flask. After 7 days, the media (containing dengue) were combined with defibrinated bovine blood (Hemostat, Dixon, CA) in a 1:1 ratio.

F1 females were offered dengue infectious blood for one hour. Samples of the infectious blood meal were taken prior to blood feeding to determine dengue titers using

quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Post blood feeding females were cold anesthetized and fully engorged females were separated and housed in cages. Fed F1 females were held individually for either 24 hours, 3 days, or 14 days, where 14 days is the approximate extrinsic incubation period of dengue in *Ae. aegypti* at 28°C (Chan and Johansson 2012). After the incubation period, females were individually stored in 2.0 mL centrifuge tubes at -80°C until assayed to determine infection status, body viral titer, and (in 14 day mosquitoes) dissemination status. We did not test for disseminated infections in samples from the 24 hour and 3 day time points because dissemination is unlikely at these time points (Chan and Johansson 2012).

Determination of Infection Status

F1 blood fed females were dissected using sterilized forceps to separate the body, legs, and wings. Wing length was measured, in millimeters from alula to wing tip, to approximate mosquito body size (reviewed in Armbruster and Hutchinson 2002). Bodies were homogenized in 1.0 mL of TRI Reagent® (Molecular Research Center, Inc., Cincinnati, OH) with 3 glass beads at 25 Hz for 3 minutes using a Qiagen® Tissue Lyser. Total RNA was extracted following the TRI Reagent® protocol and stored at -80°C until qRT-PCR assays could be completed. RNA was then extracted from leg tissues of mosquitoes with dengue positive bodies as determined by qRT-PCR. Viral dissemination into the hemocoel was measured at 14 days as indicated by infection of body and leg tissues (Turell et al. 1984). Disseminated infection is regarded as a state of advanced infection and a prerequisite for transmission. Quantitative RT-PCR for dengue was performed using the SuperScript® III Platinum® one step qRT-PCR kit (Invitrogen Life Technologies, Carlsbad, CA) and fluorogenic probes (TaqMan®,

Applied Biosystems, Foster City, CA). Dengue-1 specific primers and probes were designed by (Callahan et al. 2001) and can be found in Table 2-1. The PCR reagent quantities and protocol used are described in (Buckner et al. 2013). Plaque assays, based on methods established in (Alto et al. 2008), were used to calculate dengue titer in plaque forming unit equivalents (PFUe)/mL in mosquito tissues.

Statistical Analyses

Multivariate Analysis of Variance (MANOVA) was used to determine the effect of parental nutrient environment on high and low food offspring life history traits: male development time, female development time, and percent survivorship (PROC GLM, SAS v.9.3). Standardized canonical coefficients were used to measure the relative contribution of each life history trait to significant treatment effects and their relationship to one another (positive or negative) (Scheiner 2001). Dengue infection status and dissemination status was determined on an individual female basis and results from individual containers were pooled by treatment due to poor feeding rates. The effect of parental larval nutrition on offspring infection was analyzed using maximum likelihood categorical analyses of contingency tables (PROC GENMOD, SAS v. 9.3) based on the number of mosquitoes infected or uninfected as well as absence or presence of disseminated infection dengue and proportion of infected with dissemination results. In order to satisfy requirements for parametric testing, a Log10 transformation was performed on viral titer data. Log10 viral titer results were analyzed using generalized linear mixed models (PROC GLIMMIX, SAS v. 9.3). Significant effects were further analyzed by all pairwise comparisons of treatment means and adjusted using the Bonferroni correction method (Rice 1989).

Results

To identify parental effects, offspring reared in low nutrient environments from dissimilar nutrient parents were compared (HL v. LL) and offspring reared in high nutrient environments from dissimilar nutrient parents were compared (HH v. LH). It was expected that offspring from low larval nutrient parents will have decreased fitness-related life history traits and greater susceptibility to infection relative to offspring from high larval nutrient parents.

Life History Traits

A total of 4,336 offspring emerged to adulthood (2,436 males and 1,900 females). These adults were used in the life history trait analysis for development time (males and females), and survivorship. Female size was only estimated for females with measurable wings that successfully fed on blood (511 females). See Table 2-2 for descriptive statistics. MANOVA showed significant parental nutrition effects on life history traits for offspring reared in low nutrient environments (HL vs LL) ($p= 0.007$) but not on offspring reared in high nutrient environments (HH vs. LH) ($p=0.173$) (Table 2-3). In low nutrient offspring, female development time contributed the most to the significant parental effect followed by survivorship (Figure 2-2). Female size and male development time contributed little to the treatment effect. Low nutrient female offspring from low nutrient parents (LL) developed more slowly (14.47 ± 0.30 days) than low nutrient female offspring from high nutrient parents (HL) (13.06 ± 0.21 days).

Body Infection with Dengue Virus

The mean dengue viral titer of the infectious blood meals was 7.2±0.3 Log10 PFUe/mL, which is within the range of viremia in humans (Stramer et al. 2012). This titer is expected to result in approximately 75% of *Ae. aegypti* blood-fed with dengue to

have infected bodies (Nguyet et al. 2013). Of the 1900 females, a total of 266 successfully fed on the dengue infectious blood meal and were tested for infection. Of these, 92 were assayed at 24 hours, 95 at 3 days, and 78 at 14 days. Of the 266 females, 195 (73%) were positive for dengue in the body. Descriptive statistics for percent infected, body titer, and percent infected with disseminated infections can be found in Table 2-4. Contingency table analysis showed that body infection did not depend on parental treatment (high nutrient offspring: p= 0.7869, $\chi 2$ = 0.07, d.f.=1; low nutrient offspring: p= 0.514, $\chi 2$ = 0.43, d.f.= 1) or day by parental treatment interaction effect (high nutrient offspring: p= 0.106, $\chi 2$ = 4.49, d.f.=2; low nutrient offspring: p= 0.428, $\chi 2$ = 1.70, d.f.=2) for any of the treatment groups (Table 2-5). Body infection did depend upon day of assay post-infection (high nutrient offspring: p<0.0001, $\chi 2$ = 56.88, d.f.=2; low nutrient offspring: p<0.0001, $\chi 2$ = 36.99, d.f.=2).

Viral Titer and Dissemination

GLIMMIX showed that treatment (PxF1) (p= 0.0455, F= 2.84, d.f.= 3), day (p< 0.0001, F= 44.30, d.f.= 2), and treatment by day interaction (p= 0.0148, F= 2.92, d.f.= 6) significantly affected viral titers. Subsequent contrasts (Table 2-6) found that at 24 hours and 3 days, viral titers were not significantly different for offspring treatment groups. At 14 days when offspring were reared in high nutrients, those from low nutrient parents (LH) had 54% higher viral titers (93,275 PFUe/mL) compared to those with high nutrient parents (HH) (42,312 PFUe/mL) (Figure 2-3). At 14 days offspring reared in high nutrients from low nutrient parents (LH) had 5.7% lower viral titers (93,275 PFUe/mL) compared with offspring reared in low nutrients from high nutrient parents (HL) (98,900 PFUe/mL). Contingency table analysis found that parental larval nutrition did not

significantly affect virus dissemination in low nutrient offspring (p=0.589, $\chi 2$ =0.29, d.f.= 1). Parental larval nutrition but was marginally significant in affecting viral dissemination in high nutrient offspring (p=0.0534, $\chi 2$ =3.73, d.f.= 1), with offspring from high nutrient parents (HH) having fewer disseminated infections (18.2%) than those from low nutrient parents (LH) (45.8%).

Discussion

Life History Traits

We investigated whether parental nutrition influences offspring life history traits (development time, survivorship, and size) and susceptibility to infection with dengue. While other studies have been conducted on parental effects in mosquitoes, we are unaware of any studies on parental larval nutrition as it relates to offspring life histories and arboviral infection. Allocation of resources to life histories is influenced by environmental stress, including parental environmental stress, and underlying plasticity and constraints on these traits (Boggs 2009a). According to our results, offspring reared in high nutrients did not display significant differences in the life history traits measured (male development time, female development time, female size, and survivorship) regardless of parental nutrition. As holometabolous insects, mosquito larvae must allocate maternal- and larval-derived nutrients to maintenance, growth, and storage (Boggs 2009a). When larval-derived nutrients are high, the importance of maternally-derived nutrients is likely lower than when larval-derived nutrients are low. It is possible that high nutrient offspring were able to achieve the maximum growth rate for the population under the given temperature and food sources.

Offspring reared in low nutrients did display significant differences in life history traits attributed to parental nutrition with female development time contributing the most to this effect. Specifically, females reared in low nutrients, from low nutrient parents (LL) extended the juvenile developmental period by 10% compared with females from high nutrient parents (HL). A minimum amount of nutrition is required for mosquito larvae to pupate and fully mature (Chambers and Klowden 1990). The differences in development time of LL females vs. HL females may reflect differences in maternal egg resource allocation as a product of parent larval nutrition. Mothers from greater nutrient larval environments may have been able to allocate more nutrients to eggs, resulting in offspring with more resources that required less from the environment to reach the threshold to develop. *Ae. aegypti* larvae display significant development time plasticity and can develop very quickly or resist starvation and develop very slowly (Barrera and Medialdea 1996). The difference in development time due to parental larval nutrition may reflect some of this underlying plasticity. While survivorship contributed to the significant effect of parental larval nutrition in offspring reared in low nutrients (HL vs. LL), it did so minimally and was not significantly different between these two groups. Males in low nutrients were robust to parental nutrient effects.

Aedes aegypti larval nutrient metabolism varies significantly by sex (Chambers and Klowden 1990) with females requiring greater resources to pupate. This may be the reason significant differences were seen in low nutrient female offspring but not male offspring. The lower nutrient requirements of the male mosquito likely allowed males to develop more quickly regardless of parental nutrition. A study conducted on the collembolan *Orchesella cincta* showed that parental nutrition did not influence male

development time, however, it did influence male weight at maturity and the production of spermatophores (Zizzari et al. 2016). When considering parental effects on *Ae. aegypti* males in the future, it would be worthwhile to measure alternative life history parameters like sperm quality.

While parental nutrition did not lead to significant differences in measured life history traits of high nutrient offspring, survivorship did vary between offspring from low food parents (LH) and those from high food parents (HH). Offspring reared in high food, from parents reared in low food (LH) had the highest survivorship out of all four treatments. When compared to HH offspring, LH offspring had over 11% greater survivorship. The low nutrient status of LH parents may have resulted in fewer, higher quality eggs being produced allowing for greater survivorship. These results are consistent with studies on the grasshopper *Chorthippus biguttulus,* whose high-nutrient parents produced superior offspring which developed faster than those from low-nutrient parents (Franzke and Reinhold 2012).

Infection with Dengue-1 Virus

Body infection with dengue did not depend upon parental nutrition, day, or day by parental nutrition interaction effect for any treatment groups. Offspring were equally likely to be infected with dengue regardless of treatment group. These results suggest that susceptibility to arboviral infection was not influenced by parental larval nutrition and the efficacy of the midgut barrier is predominantly genetically controlled. This is consistent with studies on quantitative trait loci (QTLs) in *Ae. aegypti* that show phenotypic variance in dengue virus infection is associated with midgut infection barrier QTLs (Black et al. 2002, Bennett et al. 2005). Several studies on parental effects in arthropods have shown that parental nutrient deprivation leads to heightened resistance

to pathogens (Ben-Ami et al. 2010, Stjernman and Little 2011, Shikano et al. 2015), but this is inconsistent with our results. Other studies demonstrate no parental effects on offspring disease resistance (Valtonen et al. 2012) or parental stress leading to lower pathogen resistance (Triggs and Knell 2012, Saastamoinen et al. 2013). Variation in resistance to bacterial pathogens associated with parental nutrient deprivation has been shown to vary significantly between different host genotypes in the crustacean *Daphnia magna* (Stjernman and Little 2011).

The mosquitoes used in this study were the F4 generation of a 2-year-old colony and were likely genetically similar contributing to similar infection rates across treatment groups. The proportion of mosquitoes infected was lower than some studies using *Ae. aegypti* and dengue and higher than others (Buckner et al. 2013, Nguyet et al. 2013, Richards et al. 2012, Alto et al. 2014). This was expected given the importance of viral strain within serotypes (Alto et al. 2014, Lambrechts et al. 2009), viral dose (Nguyet et al. 2013), and mosquito population (Bennett et al. 2002) in terms of susceptibility to infection. The same viral strain, viral dose, and mosquito population were used by Buckner et al. (2013), yet the proportion of individuals infected in this study was much lower and may be related to differences in temperature and food between the two studies.

The midgut microflora of *Ae. aegypti* influences dengue virus infection and the presence of certain microbial species can decrease infection rates (Ramirez et al. 2012). In this experiment, while differing amounts of oak leaf infusion and invertebrate carcasses were used for nutrient treatment groups, all experimental containers were provided the same amount of tire-water inoculum containing micro-organisms. Since all

nutrient sources were identical and varied only by amount, the midgut microflora of the treatment groups was likely similar in diversity. This may have contributed to similar infection rates between treatment groups. Follow-up experiments manipulating both parental and offspring midgut microflora would help to elucidate whether microbes played a role in observed results.

Viral Titer and Dissemination

Offspring treatment and offspring by day treatment significantly affected viral titers, but only after 14 days. Infection at 24 hours marks the initial ingestion of the virus and its early entry into the midgut. Differences in susceptibility to infection at this point are typically seen when mosquitoes imbibe different amounts of virus (Gubler et al. 1979). Since our mosquitoes were offered the same amount of virus, little to no difference in titer was expected at 24 hours and this is consistent with our results. At 3 days, new virions are released from the midgut of infected mosquitoes and start infecting additional tissues (Ramirez and Dimopoulos 2010). During this time, Toll pathway defenses against infection are active and can influence dengue titers (Ramirez and Dimopoulos 2010). Consequently, 3 days provides a snapshot of early infection. We did not observe any treatment differences in virus titer at 3 days suggesting that the early immune response was not affected by parental larval nutrition.

The extrinsic incubation period (EIP) for dengue virus in *Ae. aegypti* is generally accepted to be at 7-14 days. However, recent modeling suggests that this period may be between 2-15 days at 30°C (Chan and Johansson 2012). We used 14 days to test viral titers and to test for dissemination since it is close to the longest expected EIP and would increase our ability to detect differences amongst treatment groups. At 14 days, LH offspring had 54% higher viral titers than HH offspring. The low nutrient condition of

parents may have resulted in higher viral titers despite offspring being reared in high nutrients. These results are consistent with studies on insects where offspring of low nutrient parents demonstrate reductions in the expression of innate immune markers (Triggs and Knell 2012, Saastamoinen et al. 2013). Life history theory demonstrates that resources are limited and trade-offs occur in terms of how organisms invest these resources in growth, maintenance, storage, and reproduction. Immune responses carry physiological costs and trade-offs exist when allocating resources to immune function (Rauw 2012). It is possible that when food conditions are limited in the parental environment, offspring have reduced immune investment as an adaptation to have additional resources for other life history traits. It is interesting that differences in viral titers were not seen at 24 hours or 3 days, but were at 14 days for HH vs LH offspring suggesting differences occur in terms of the immune response late in the infection cycle. In *Ae. aegypti,* infection with dengue virus is modulated by the Toll and JAK-STAT pathways (Xi et al. 2008, Ramirez and Dimopolous 2010) and results may be related to differences in these pathways or other barriers to infection.

Dissemination of the virus into leg tissues indicates the virus has escaped the midgut barrier in the mosquito. Of offspring infected at 14 days, dissemination rates were between 40% and 50% in LH, HL, and LL offspring. HH offspring, demonstrated dissemination rates of 18.2% of those infected. This is marginally significantly different than rates seen in LH offspring. It is possible that when offspring were reared in high nutrients, those from high nutrient parents (HH) were in better condition than those from low nutrient parents (LH). The efficacy of the midgut barrier may have been influenced by both parental and offspring nutrition leading to fewer disseminated infections when

nutrition is high for both generations. Larval nutrient deprivation has been shown to increase viral susceptibility and dissemination in *Ae. aegypti* (Muturi et al. 2011) as well as other vector mosquitoes (Grimstad and Walker 1991). Our observations marginally support the hypothesis that high quality larval nutrition for parents and offspring led to reductions in dissemination. Viral dissemination is not a reliable indicator of transmission potential due to additional barriers to infection. However, dissemination to secondary tissue does demonstrate that the virus has bypassed the midgut infection barrier (Chamberlain and Sudia 1961). Future studies should consider parental effects on transmission.

Conclusion

In summary, nutritional quality behaves as predicted for a stressor in which it is expected that good condition parents will produce high quality offspring and poor condition parents will produce lower quality offspring (Mousseau and Fox 1998b, Knyeb and Toft 2006, Jones and Widemo 2005). These results suggest that parental larval nutrition is important to *Ae. aegypti* female offspring. Parental diet effects were seen on development time, infection with dengue after the extrinsic incubation period, and dissemination. Carry-over effects of parental nutrition are evident in this system, and the ones measured do not appear to confer any advantages to offspring in low nutrient environments for the traits considered. Future studies should consider manipulating the types of nutrients provided, measuring offspring immune response to pathogens at earlier time points, and considering additional male traits.

Table 2-1. Dengue-1 serotype specific primers and probes designed by Callahan et al. 2011.

Primer/Probe	Sequence (5'—3')	Genomic Region	GenBank #
Forward Primer	GAC ACC ACA CCC TTT GGA CAA	NS5 (8586-8606)	
Reverse Primer	CAC CTG GCT GTC ACC TCC AT	NS5 (8692-8673)	M87512
Probe	AGA GGG TGT TTA AAG AGA AAG TTG ACA CGC G	NS5 (8606-8638)	

Table 2-2. Mean descriptive statistics (± S.E.) per container for offspring life history traits, by treatment group.

Treatment	n (containers)	Female development (days)	Male development (days)	Survivorship (%)	Female wing length (mm)
HH	8	8.32 ± 0.15	7.76 ± 0.16	60.86 ± 5.45	2.64 ± 0.02
LH	8	8.32 ± 0.09	7.54 ± 0.06	72.72 ± 3.71	2.61 ± 0.01
HL	9	13.06 ± 0.21	10.47 ± 0.17	63.31 ± 1.14	2.32 ± 0.03
LL	9	14.47 ± 0.30	11.34 ± 0.24	64.06 ± 2.19	2.29 ± 0.05

Table 2-3. MANOVA for effects of parental nutrition on offspring development (dev) time (females, males), survivorship to adulthood, and female wing length.

					Standardized Canonical Coefficients			
F1 Food	Comparison	Num d.f. / Den d.f.	Pillai's trace	*p*	Female dev	Male dev	Survivorship	Female wing length
High	HH v. LH	4 / 11	0.415	0.173	-1.09	0.56	-0.89	0.61
Low	HL v. LL	4 / 12	0.664	0.007	1.94	-0.05	0.99	0.11

Table 2-4. Dengue virus infection and titer by treatment and day.

Day	Trt	% Viral RNA	Titer (PFUe/mL)	% disseminated
1	HH	100 (N= 25)	8,862 ± 1,061	--
	LH	95.5 (N= 22)	5,855 ± 748	--
	HL	92.6 (N= 27)	6,065 ± 1,060	--
	LL	100 (N= 9)	7,670 ± 2,520	--
3	HH	25.8 (N= 31)	1,964 ± 557	--
	LH	73.9 (N =23)	8,070 ± 3,420	--
	HL	37.5 (N= 24)	5,143 ± 2,194	--
	LL	50.0 (N =12)	1,735 ± 780	--
14	HH	22.7 (N= 22)	42,312 ± 12,369	18.2
	LH	33.3 (N= 23)	93,275 ± 17,704	45.8
	HL	45.5 (N= 22)	102,850 ± 37,138	50.0
	LL	30.0 (N= 10)	98,900 ± 39,448	40.0

Table 2-5. Body infection by parental nutrition, parental by day treatment effects, and day effect.

Effect	Offspring Comparison	$\chi 2$	p	d.f.
High Food F1				
Parental Treatment	HH v. LH	0.07	0.7869	1
Day x Parental Treatment	HH v. LH	4.49	0.106	2
Day	HH v. LH	56.88	<0.0001	2
Low Food F1				
Parental Treatment	HL v. LL	0.43	0.514	1
Day x Parental Treatment	HL v. LL	1.70	0.428	2
Day	HL v. LL	36.99	<0.0001	2

Table 2-6. Pairwise contrasts for Log10 virus titer in offspring by treatment (trt) and day adjusted using the Bonferroni method.

Trt 1	Trt 2	Day	Estimate	S.E.	d.f.	T Value	Pr>|t|	Critical *p*
HH	HL	1	0.2003	0.5636	58	0.36	0.7236	0.0125
HH	LH	1	0.4800	0.5636	58	0.85	0.3979	0.0062
HH	LL	1	0.3708	0.5866	58	0.63	0.5298	0.0071
HL	LH	1	0.2797	0.5636	58	0.50	0.6216	0.01
HL	LL	1	0.1705	0.5866	58	0.29	0.7724	0.0166
LH	LL	1	-0.1092	0.5866	58	-0.19	0.8529	0.025
HH	HL	3	0.6741	0.6384	58	1.06	0.2954	0.0045
HH	LH	3	-0.08353	0.6011	58	-0.14	0.8900	0.05
HH	LL	3	1.2698	0.6668	58	1.90	0.0618	0.0033
HL	LH	3	-0.7576	0.5694	58	-1.33	0.1886	0.0041
HL	LL	3	0.5957	0.6384	58	0.93	0.3547	0.0055
LH	LL	3	1.3533	0.6011	58	2.25	0.0282	0.0031
HH	HL	14	-0.3770	0.7073	58	-0.53	0.5960	0.0083
HH	LH	14	-2.4464	0.6174	58	-3.96	0.0002*	0.0027
HH	LL	14	-1.1468	0.7700	58	-1.49	0.1418	0.0038
HL	LH	14	-2.0694	0.6609	58	-3.13	0.0027*	0.0029
HL	LL	14	-0.7697	0.8053	58	-0.96	0.3431	0.005
LH	LL	14	1.2996	0.7276	58	1.79	0.0793	0.0035

*Denotes significant p-value after Bonferroni correction

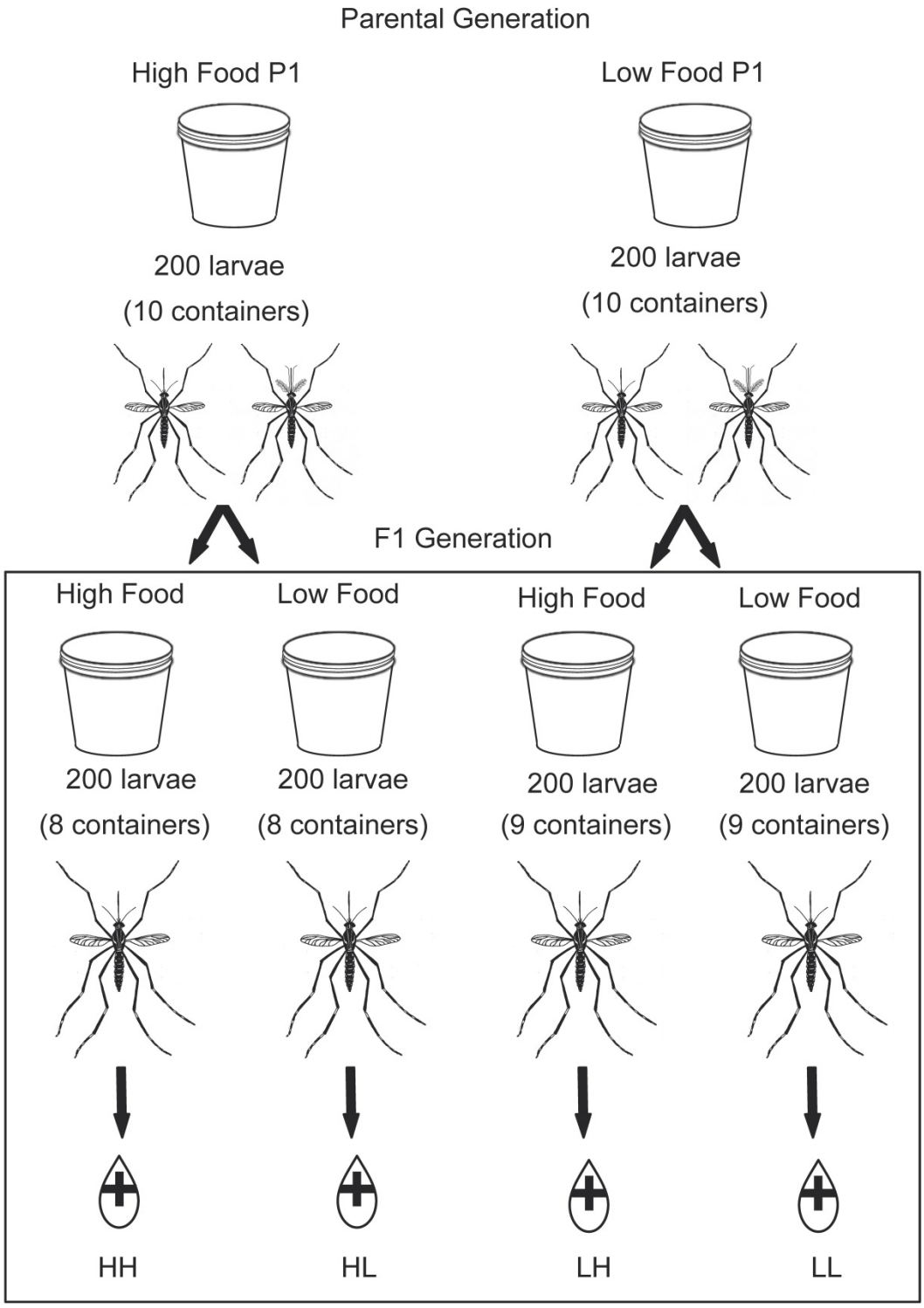

Figure 2-1. Experimental design for parental and offspring nutrition treatments.

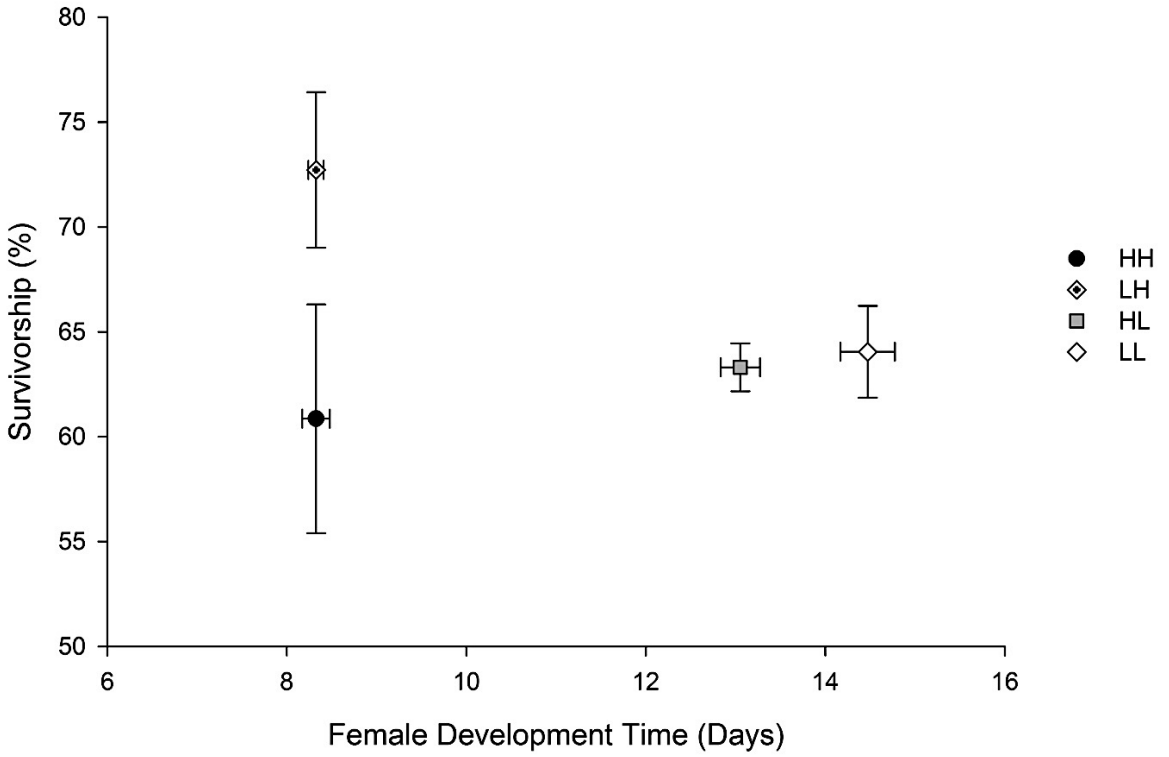

Figure 2-2. Bivariate plot of least square (LS) means (±SE) for offspring female development time and survivorship by treatment.

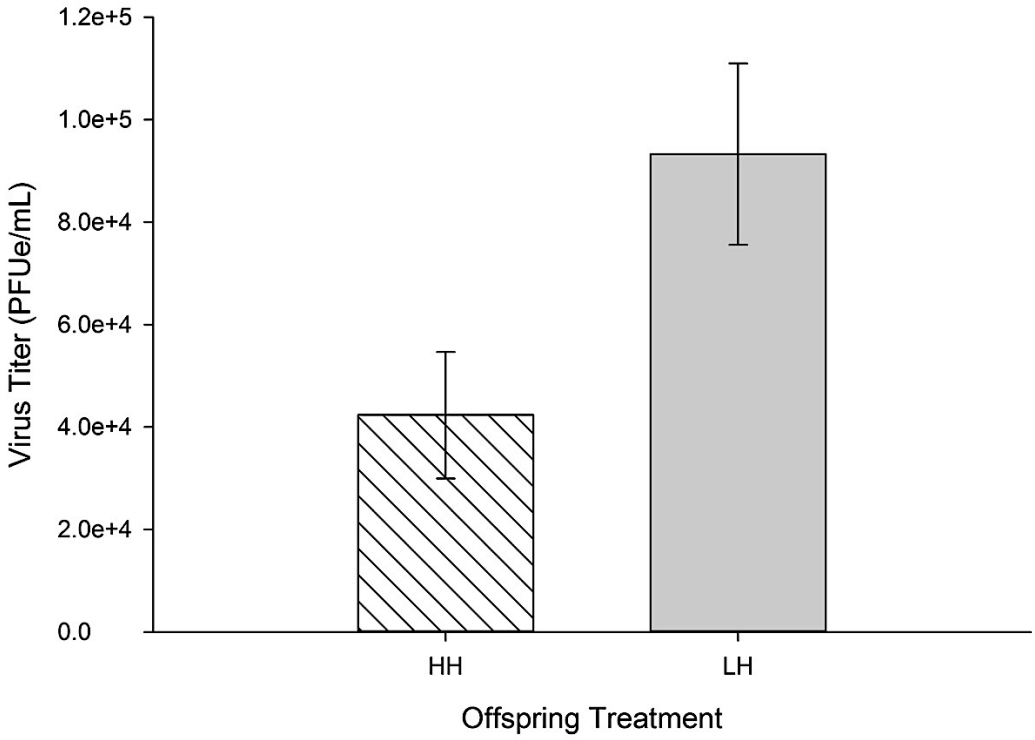

Figure 2-3. Means (±SE) of body dengue-1 virus titer (plaque forming unit equivalents/mL) of infected offspring (14 days post infectious blood meal) reared in high food, which varied by parental larval nutrition.

CHAPTER 3
MATERNAL AND PATERNAL NUTRITION IN A MOSQUITO INFLUENCES OFFSPRING LIFE HISTORIES BUT NOT INFECTION WITH AN ARBOVIRUS

Introduction

The environmental conditions experienced by parents can influence offspring phenotype generating parental effects. Parental effects are increasingly being recognized as a source of phenotypic variation in offspring (Kirkpatrick and Lande 1989, Mousseau and Dingle 1991a) potentially influencing several generations of descendants (Plaistow et al. 2006). Parental effects can be derived from the mother or the father. Given the larger gamete size and greater energy expenditure by females for reproduction, there has been more research on maternal effects. However, there has been increasing recognition of the importance of fathers in contributing to parental effects. The contribution of each parent can influence offspring in different ways (Bonduriansky and Head 2007, Valtonen et al. 2012, Bonduriansky et al. 2016) and given the expectation that parents will maximize their own fitness, these effects are largely context dependent (Qvarnström and Price 2001, Plaistow et al. 2006). In some circumstances, parental effects generate adaptive phenotypic responses in offspring to environmental stimuli (Bernardo 1996a).

In natural ecosystems, resources are limited, reproductive effort is costly, and life history theory predicts that trade-offs exist intergenerationally (Stearns 1989). Nutrient acquisition leads to context and sex-specific trade-offs between the allocation of resources to growth and to reproduction (Boggs 2009b). The nutritional ecology of insects, and underlying phenotypic plasticity, influences responses to environmental stimuli influencing hatching, feeding strategies, diapause, and allocation of resources to growth, maintenance, and development (Slansky 1982). In holometabolous insects,

both larval and adult nutrients, acquired through feeding or transferred during mating, can influence reproduction (Rivero et al. 2001, Boggs 2009a). Nutrition has also been linked to parental effects on a variety of different offspring traits in insects including fecundity (Futuyma et al. 1993, Grech et al. 2007, Frago and Bauce 2014), feeding strategy (Grech et al. 2007), growth rate (Rotem et al. 2003, Franzke and Reinhold 2012, Valtonen et al. 2012, Frago and Bauce 2014), size (Franzke and Reinhold 2012, Cahenzli and Erhardt 2013), and immunity (Boots and Roberts 2012).

Aquatic stages of the yellow fever mosquito, *Aedes aegypti,* occur in both natural and man-made containers and produce adults which are primary vectors of emergent mosquito-borne pathogens including dengue, chikungunya, yellow fever, and Zika viruses. Nutrient availability in container systems comes from degradation of allochthonous inputs of plant and animal detritus by microorganisms which form the basis of the mosquito diet and influence growth, development, and survival of larvae (Murrell et al. 2011). The quality and quantity of the larval and adult diet of container mosquitoes influence life history trajectories (Fish and Carpenter 1982, Carpenter 1983, Lounibos et al. 1993, Telang and Wells 2004, Yee and Juliano 2006, Muturi et al. 2011, Alto et al. 2012) and vector competence (Grimstad and Walker 1991, Zhang et al. 1993, Nasci and Mitchell 1994, Muturi et al. 2011, Telang et al. 2012). Vector competence refers to susceptibility to infection and transmission of pathogens. Larval nutrition has also been linked to parental effects on offspring female development time and dengue virus load in *Ae. aegypti* (Chapter 2), however, only combined parental effects were considered. Given the expectation that maternal and paternal contributions to parental

effects likely vary given different strategies for maximizing fitness between females and males, further investigation is warranted.

Here, the hypothesis that both the maternal and paternal larval nutrient environments in *Ae. aegypti* contribute to transgenerational effects on offspring life history traits (growth, development, survival to adulthood) is tested. Four different mating crosses were considered: high food females x high food males, high food females x low food males, low food females x high food males, and low food females x low food males. Previous research in this system, which considered high food offspring from high food parents versus low food parents, found that two high food parents produced female offspring that developed more quickly, had lower viral titers, and fewer disseminated infections, than those from low nutrient parents (Chapter 2). As a result, it is expected that two high food parents will result in offspring with more rapid development, increased survivorship, and larger sizes (cumulative growth) than offspring from both mixed food parents and low food parents. Additionally, we expected two high food parents to have offspring that are more resistant to infection with dengue virus with lower virus loads than offspring of mixed food parents and low food parents. We expect to find both maternal and paternal effects, but the life history traits affected may differ. Though this experiment focuses on an important mosquito vector and arbovirus system, this research will help improve our general understanding of how the maternal and paternal environment can shape offspring phenotypes in organisms lacking parental care.

Materials and Methods

Mosquito Population

Ae. aegypti larvae were collected from containers (buckets, plastic cups, planter trivets) in the field in Key West, FL in 2012 and used to establish a laboratory colony. These mosquitoes were reared in an insectary set at 28±0.5°C with a 12:12 (light:dark) hour photoperiod. Larvae were reared in enamel pans (24 x 36 x 5 cm) holding 1.5 L of water in cohorts of ~200 mosquitoes. Larvae were fed an equal mixture of lactalbumin:*Saccharomyces cerevisiae* yeast provided in 200 mg increments two to three times per week. Pupae were transferred to water-filled cups and placed in cages (45.7 x 45.7 x 45.7 cm, BugDorm, MegaView Science Co. Ltd. Taichung, Taiwan) to emerge as adults. Adults were sustained on a 10% sucrose solution provided using cotton wicks and fed on live chickens weekly. All chicken care was provided under the animal use and care policies of the University of Florida's Institute of Animal Care and Use Committee (IACUC Protocol 201003892). Experimental design for both parental and offspring treatment is described below and summarized in Figure 3-1.

Parental Treatment

The F_{10} colony generation was used for this study and will be referred to as the parental (P) generation. P generation larvae were hatched in enamel pans (24 x 36 x 5 cm) containing 1.5 L of water and 200 mg of larval food in cohorts of ~200 mosquitoes. P larvae were then removed from the nutrients and individually placed in cylindrical vials (26 mm diameter x 82 mm height) containing 10 mL of water containing either low (0.75 mg) or high larval food (3.0 mg). Due to development time asymmetry attributed to differential food quantities, low food P larvae were hatched 1 week prior to high food P larvae to ensure mating potential between P food treatment crosses. Upon emergence,

adults were sexed and placed in nutrient and sex-specific cylindrical cardboard cages (9.7 x 9.7 cm ht. x dia.), containing cohorts of ≤ 50 mosquitoes, sustained on a 10% sucrose solution. Once all adults emerged, they were assigned to one of the following mating cross treatment groups:

- High food ♀ x High food ♂: H♀H♂
- High food ♀ x Low food ♂: H♀L♂
- Low food ♀ x High food ♂: L♀H♂
- Low food ♀ x Low food ♂: L♀L♂

Mosquitoes were cold anesthetized, and placed into mating cross treatment specific cages H♀H♂, H♀L♂, L♀H♂, and L♀L♂ for 7 days for mating. After 7 days, females were allowed to feed on live chickens. Females were cold anesthetized, and blood-fed females were placed individually in cylindrical tubes (82 x 26 mm ht. x dia.) containing a piece of seed germination paper as an oviposition substrate. Females that did not feed were discarded. After two days, enough water was added to each tube to fully dampen the paper without pooling in the tube. Females were given one week to oviposit after which they were frozen and stored at -80°C. Oviposition papers were removed from the tubes and allowed to dry until slightly damp (about 24 hours). After 24 hours, eggs were stored in a plastic container for one week to allow for embryonation and preparation of F_1 rearing cups.

Offspring Treatment

F_1 generation eggs were hatched by placing the egg paper from one female into a cylindrical plastic cup (7.6 cm by 2.5 cm depth) containing 20 mL of water and 20 mg of larval food. A total of 213 different families successfully had offspring. After 24 hours, larvae were rinsed to remove nutrients and were then counted. After larvae were counted, half the number from each female were placed into high food cups and half

into low food cups containing approximately 20 mL of water. Larval densities ranged from 1 to 52 larvae. Larvae in the high food treatment were provided 3.0 mg of larval food per larva. Larvae in the low food treatment were provided 0.75 mg of larval food per larva. Providing food on a per larva basis and rearing offspring between 1-52 larvae reduced potential effects of intraspecific competition for food and/or space (Moore and Whitacre 1972, Macia 2009, Legros et al. 2009, Couret et al. 2014). Supplemental food was provided weekly and consisted of half the initial amount of food per larva. F_1 larvae were checked daily for pupation. Pupae were transferred to cylindrical vials (8.2 x 2.6 cm ht. x dia.) for emergence. After emergence, adults were sexed and transferred to family and treatment specific cylindrical cardboard cages (9.7 x 9.7 cm ht. x dia.) at a density ≤ 50 mosquitoes per cage. Cages contained females within a 3-day age span. Adults were sustained on a 10% sucrose solution for 7-9 days post emergence. Twenty-four hours prior to blood feeding, sugar was removed and replaced with water to increase propensity to blood feed. Development time, in days from hatch until emergence, and survivorship to adulthood were measured for offspring.

Dengue Virus Infection

F_1 females were offered blood infected with dengue-1 virus, originally isolated from a human infected in Key West, FL in 2010 (GenBank: JQ675358.1, Shin et al. 2013). Dengue-1 virus was propagated using monolayers of African Green monkey kidney (Vero) cells in 175 cm^2 tissue culture flasks using previously established methods (Alto and Bettinardi 2013, Alto et al. 2008). Each monolayer of Vero cells was inoculated with 250 µL of DENV-1 at a multiplicity of infection of 1.2 followed by an hour incubation period at 37°C in a 5% CO_2 chamber. After the incubation period, 25 mL of media (199 media, 10% fetal bovine serum, 0.2% antimycotic, and 0.2% penicillin-

streptomycin) were added to each flask. To ensure similar viral titers for each blood meal, the number of host cells available for infection was carefully controlled by seeding flasks with a known number of cells. Additionally, studies on comparative growth of dengue viruses in cell culture (Shin et al. 2013) were completed under similar conditions enabling the time to harvest the virus during the stationary phase to be determined. Infected cell cultures were held for 7 days after which the media (containing dengue virus) was added to defibrinated bovine blood (Hemostat, Dixon, CA) in a 1:2 ratio of media:blood. A sample of each blood meal was taken to determine dengue titers using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR).

F_1 females were offered the infectious blood meal for one hour using the Hemotek® feeding system with membranes consisting of hog intestines. This methodology was used to allow for oral ingestion of virus particles, which mimics how mosquitoes would become infected in nature. After one hour, females were cold anesthetized, and fully engorged females were placed in cages and sustained on a 10% sucrose solution, those that did not feed were discarded. Female mosquitoes were housed for 14 days, the approximate length of the extrinsic incubation period for dengue virus at 28°C in *Ae. aegypti* (Watts et al. 1987, Rohani et al. 2009, Chan and Johansson 2012). After 14 days, females were frozen, dissected, and stored at -80°C in 2.0 mL microcentrifuge tubes until assayed to determine dengue infection status, body viral titer, and viral dissemination into leg tissues.

Infection Status

F_1 blood-fed females were dissected using sterile techniques so that the body, legs, and wings were separated prior to storage. Wings were mounted on slides and

lengths, in millimeters from alula to wing tip, were measured using computer imaging software (i-Solution lit©, AIC, Princeton, NJ) to approximate body size (reviewed in Armbruster and Hutchinson 2002). Bodies and legs were stored separately in 2.0 mL centrifuge tubes containing 1.0 mL of BA-1 diluent (M-199, 0.05M Tris pH 7.6, 1% bovine serum albumin, 0.35 g/L sodium bicarbonate, 100 U/mL streptomycin, 1 ug/mL Fungizone) until processed. Body and leg tissues were homogenized directly in the BA-1 diluent with two metal beads at 25 Hz for 3 minutes in a Qiagen ® Tissue Lyser. A 140 µL aliquot of homogenate was used to extract RNA using the QIAamp Viral RNA Mini Kit (Qiagen Inc., Valencia, CA) following the manufacturer's protocol. Quantitative RT-PCR was performed using the SuperScript® III Platinum® one step qRT-PCR kit (Invitrogen Life Technologies, Carlsbad, CA) and fluorogenic probes (TaqMan®, Applied Biosystems, Foster City, CA). Mosquito bodies were tested for the presence of virus using the dengue-1 specific primers and probes designed in Callahan et al. (2001) and described in Table 2-1. The reagent quantities and run protocol for PCR used in this experiment are described in Buckner et al. (2013). The titer of each sample was calculated in plaque forming unit equivalents per milliliter (PFUe/mL) using a standard curve method (Bustin 2000). The standard curve was generated from serial dilutions of dengue virus of known titer. Stock viral titer was established using the plaque assay method as described in Alto et al. (2008). RNA was subsequently extracted from leg tissues for samples indicating a viral infection in the body tissue through qRT-PCR. Leg RNA was tested using the sample protocol as body tissues. Dissemination of virus into the hemocele indicates the virus has escaped the midgut defense barrier which is an advanced infection and a precursor to transmission potential.

Statistical Analyses

Analysis of variance (ANOVA) was used to determine parental mating cross treatment effects on the number of offspring produced (PROC GLM, SAS v. 9.3). Multivariate analysis of variance (MANOVA) was used to determine the contribution of maternal and paternal larval nutrient effects on high and low food offspring 1) life history traits; and 2) susceptibility to infection with dengue virus and dissemination into secondary tissues (PROC GLM, SAS v. 9.3). Not all raw data met the assumptions of univariate normality and homogeneity of variances. In order to meet the requirements for parametric testing, survivorship was transformed by the square root of the proportion dead. Additionally, viral titer was transformed using a Log10 (viral titer +1) transformation to normalize the residuals. To further analyze these data, pairwise multivariate contrasts of means were evaluated using the sequential Bonferroni method ($\alpha = 0.05$). The contribution of each life history trait to significant treatment effects and their relationships to each other (positive or negative) was measured using standardized canonical coefficients (Scheiner 2001). Similarly, standardized canonical coefficients were used to determine the contribution of infection and dissemination parameters measured to significant treatment effects and their relationships to each other (Scheiner 2001).

Results

ANOVA showed significant differences in the number of offspring per female between treatment groups (see Figure 3-1). Subsequent pairwise contrasts showed that females from H♀H♂ produced significantly more offspring than H♀L♂ ($p<0.0001$), L♀H♂ ($p<0.0001$), and L♀L♂ ($p<0.0001$). Additionally, H♀L♂ females produced

marginally significantly more offspring than L♀H♂ females (p= 0.05) and significantly more offspring than L♀L♂ females (p= 0.02). Females from L♀H♂ and L♀L♂ did not produce different amounts of offspring (p= 0.997). To identify maternal effects, offspring reared in high nutrients from dissimilar treatment mothers (H♀H♂-high vs. L♀H♂-high; H♀L♂-high vs. L♀L♂-high) were compared; and offspring reared in low nutrients from dissimilar treatment mothers were compared (H♀H♂-low vs. L♀H♂-low; H♀L♂-low vs. L♀L♂-low). To identify paternal effects, offspring reared in high nutrients from dissimilar treatment fathers (H♀H♂-high vs. H♀L♂-high; L♀H♂-high vs. L♀L♂-high) were compared and offspring reared in low nutrients from dissimilar treatment fathers were compared (H♀H♂-low vs. H♀L♂-low; L♀H♂-low vs. L♀L♂-low). It was expected that both mothers and fathers would contribute significantly to offspring life history traits and infection with dengue virus.

A total of 2,143 high food offspring emerged to adulthood (1199 males and 944 females) from 214 different families. Of the low food offspring 2,040 emerged to adulthood (1118 males and 922 females) from 213 different families. These adults were used to measure male development time, female development time, and survivorship to adulthood. Female wing lengths, used as an estimate of size, were measured only for females that successfully blood-fed with measurable wings (657 females). In some instances wings were not measureable due to damage incurred during the dissection process. See Table 3-1 for descriptive statistics for high food and low food offspring.

MANOVA showed significant parental nutrient mating cross effects on both high food (Pillai's trace=0.28, p<0.0001) and low food (Pillai's trace=0.22, p<0.005) offspring life history traits. Subsequent multivariate contrasts showed significant maternal

nutrition effects on both high food offspring ($p < 0.001$) and low food offspring ($p=0.015$) when offspring were sired by high food fathers (Table 3-2, H♀H♂-high vs L♀H♂-high and H♀H♂-low vs. L♀H♂-low). In high food offspring, female development time contributed the most to this effect followed by male development time. Daughters from two high food parents (H♀H♂-high) took on average 2.5 days longer to develop than those from high food fathers and low food mothers (L♀H♂-high). Sons from two high food parents (H♀H♂-high) took a little over 1.25 days longer to develop than those from high food fathers and low food mothers (L♀H♂-high). In low food offspring, the percent dead contributed the most to this effect followed by female development time (Table 3-2). In low food offspring, those from two high food parents (H♀H♂-low) had 4% fewer survive than those from high food fathers and low food mothers (L♀H♂-low). Low food daughters, from two high food parents took two days longer to develop than those from a high food father and low food mother. There was not a significant maternal nutrition effect on either high food offspring ($p=0.54$) or low food offspring ($p=0.43$) when offspring were sired by low food fathers (H♀L♂-high vs. L♀L♂-high and H♀L♂-low vs. L♀L♂-low).

Multivariate contrasts showed significant paternal nutrition effects on high food offspring ($p=0.008$) and low food offspring ($p=0.018$) when the offspring were from high food mothers (Table 3-2, H♀H♂-high vs. H♀L♂-high and H♀H♂-low vs. H♀L♂-low). In high food offspring, female development time contributed the most to this effect with those from two high food parents (H♀H♂-high) taking 18% longer to develop than those from low food fathers and high food mothers (L♀H♂-high). Male development time contributed the second most to this effect with males from two high food parents

(H♀H♂-high) taking 7.7% longer to develop relative to those from low food fathers and high food mothers (L♀H♂-high). In low food offspring, female development time contributed the most to this effect followed by female wing length and male development time which contributed similarly. There was not a significant paternal nutrition effect on either high food offspring or low food offspring when generated by low food mothers (L♀H♂-high vs. L♀L♂-high and L♀H♂-low vs. L♀L♂-low).

The mean viral titer of the dengue infectious blood meals was 5.73±0.03 Log_{10} PFUe/mL. This is slightly lower than the 50% mosquito infectious dose (MID_{50}) expected for dengue-1 virus (Nguyet et al. 2013) which enabled us to identify both treatment enhancement and mitigation effects on mosquito infection. Of the 944 high food female offspring, 365 successfully blood-fed and survived the extrinsic incubation period for dengue of 14 days. Of the 922 low food female offspring, 304 successfully blood-fed and survived the 14 days. Descriptive statistics on percent infected, body titer, and percent of those infected with disseminated infections for high food and low food offspring in Table 3-3.

MANOVA showed that parental nutrient mating cross did not affect proportion infected with dengue, body titer, or dissemination of the virus to leg tissues for high (Pillai's trace=0.0.03, p=0.92) or low (Pillai's trace=0.09, p=0.37) food offspring. Multivariate contrasts showed that maternal larval environment did not affect proportion infected with dengue, body titer, or dissemination of the virus to leg tissues in either high food (H♀H♂-high vs. L♀H♂-high, p= 0.85) or low food offspring (H♀H♂-low vs. L♀H♂-low, p= 0.36) sired by high food fathers. Multivariate contrasts showed the same was true for high food (H♀L♂-high vs. L♀L♂-high, p=0.34) and low food offspring (H♀L♂-

low vs. L♀L♂-low, p= 0.76) sired by low food fathers (Table 3-4). Similarly, multivariate contrasts showed that paternal larval environment did not affect proportion infected with dengue, body titer, or dissemination of the virus to leg tissues in either high food (H♀H♂-high vs. H♀L♂-high, p= 0.79) or low food offspring (H♀H♂-low vs. H♀L♂-low, p= 0.10) from high food mothers. Multivariate contrasts showed the same was true for paternal effects on high food (L♀H♂-high vs. L♀L♂-high, p= 0.82) and low food offspring from low food mothers (L♀H♂-low vs. L♀L♂-low, p= 0.82) (Table 3-4).

Discussion

We investigated whether maternal and paternal larval nutrition influences offspring life history traits (development time, survivorship, and size) and susceptibility to infection with dengue virus. To our knowledge, this is the first study conducted on mosquitoes that considers both maternal and paternal nutrient effects as they relate to offspring life histories and interactions with arboviruses. We show that parental effects on mosquito offspring can be maternally and paternally derived.

We found evidence that the maternal environment contributed to parental effects when mothers mated with high food fathers but not when mothers mated with low food fathers, regardless of offspring nutrition. The effects were primarily mediated through female offspring development in high food offspring and survivorship in low food offspring. Daughters required between 13% (low food) and 23% (high food) longer, and sons required between 8.6% (low food) and 12.8% (high food) longer to develop when they had two high food parents (H♀H♂-high and H♀H♂-low) than those from low food mothers mated with high food fathers (L♀H♂-high and L♀H♂-low). Offspring from two high food parents (H♀H♂-high and H♀H♂-low) had reduced survivorship (8.6% lower in high food offspring, and 4.1% lower in low food offspring) compared to those individuals

from low food mothers and high food fathers (L♀H♂-high and L♀H♂-low). Our results were inconsistent with studies on malaria vector *Anopheles stephensi* suggesting that parental food does not influence offspring life history traits other than fecundity (Grech et al. 2007). However, our results were consistent with studies on *Drosophila melanogaster* fruit flies, which demonstrated that offspring of one standard food parent and one low food parent developed the quickest compared with other parental crosses (Valtonen et al. 2012).

The mechanism underlying faster development time of female offspring from the H♀L♂ treatment (low food mothers x high food fathers) compared with the H♀H♂ treatment (two high food parents) is unknown. Studies on insects suggest the mechanisms behind parental effects include differences in resource allocation and nutrient provisioning to offspring (Bonduriansky and Day 2009, Lyko et al. 2010, Newcombe et al. 2013, Newcombe et al. 2015), transfer of symbionts or hormones (Fox et al. 1997, Koehler and Kaltenpoth 2013, Liu et al. 2014), and changes in the epigenome (Chittka and Chittka 2010, Oppold et al. 2015). Differential nutrient provisioning by parents influences offspring phenotype (Bonduriansky and Day 2009). In *Ae. aegypti*, larval nutrition influences female teneral reserves affecting the number of eggs laid during the first ovarian cycle (Telang et al. 2006). In our study, high food females that mated with high food males (treatment H♀H♂) produced 45% more offspring than low food females that mated with high food males (treatment H♀L♂). It is likely that H♀H♂ females had greater teneral reserves increasing the quantity of eggs produced. Additionally, high food mothers, when mated with high food fathers, may have produced more eggs, with fewer resources per egg, in response to the benign

environment both parents experienced. Varying environmental conditions likely shift allocation efforts between immunity and reproduction (Schwenke et al. 2016). Other studies on the dung beetle *Onthophagus taurus* found that mothers provide more resources to offspring sired by larger fathers (Kotiaho et al. 2003), corroborating our observations. With benign environmental conditions, producing many small offspring is likely the best bet-hedging strategy to maximize long-term fitness (Olofsson et al. 2009). Low food mothers, when mated with high food fathers, may have allocated more resources to a fewer number of eggs in response to the higher risk environment. In the Cabbage White Butterfly, *Pieris rapae,* low food mothers produced higher mass eggs, which developed into larger offspring suggesting potential adaptive maternal effects (Rotem et al. 2003). Given that these trends were not seen when high and low food mothers mated with low food fathers, the effects may reflect physiological changes associated with the transfer of accessory gland products to females during mating. Effects may also be associated with differential allocation of maternal and larval-derived nutrients (Boggs 2009b). Alternatively, nutritionally-stressed H♀L♂ females, may have laid eggs with fewer resources, and offspring developed more quickly to compensate for lack of nutrients (Metcalfe and Monaghan 2001).

We also found evidence of paternal effects on high and low food offspring life history traits, but only when offspring were from high food mothers similar to the pattern observed with maternal effects. Daughters required between 15.2% (high food) and 9.2% (low food) longer to develop when they had two high food parents (H♀H♂-high and H♀H♂-low) than when they had a high food mother and low food father (H♀L♂-high and H♀L♂-low). Similarly, high food sons required 7.7% longer to develop when

they had two high food parents (H♀H♂-high) than those from high food mothers and low food fathers (H♀L♂-high). When offspring originated from low food mothers, we did not identify paternal effects on offspring, suggesting condition-specific transgenerational effects in this system. Male insects produce substances within their accessory glands that are transferred to females during mating and influence female physiology and behavior (Fuchs et al. 1969, Klowden 1999). Additionally, seminal fluid seems to mediate paternal larval diet effects on offspring in the fly, *Telostylinus angusticollis* (Crean et al. 2014). There is evidence that male-derived substances from mating influence female life spans, feeding behavior, and egg production in *Ae. aegypti* (Fuchs et al. 1969, Helinski and Harrington 2011). Additionally, male accessory gland products facilitate fertilization in *Ae. aegypti*, potentially by triggering female physiological responses required in order for fertilization to occur (Degner and Harrington 2016). The quality or quantity of accessory gland products in semen produced by high food compared to low food males may be one reason that H♀H♂ mothers produced significantly more offspring than H♀L♂ mothers. Our results were consistent with other studies in insects demonstrating paternal effects on offspring phenotype (Valtonen et al. 2012), which may be generated by seminal fluid products (Crean et al. 2014) and maternal response to paternal phenotype (Sheldon 2000).

Our results suggest that both maternal and paternal food intake influence offspring and that these effects primarily influence female offspring. This is consistent with previous research on *Ae. aegypti* which demonstrated that sex-specific transgenerational effects of parental nutrition on offspring life history traits (Chapter 2). Results from Chapter 2 found that females from two high food parents developed more

quickly than those from two low food parents, and results here in Chapter 3 suggest the opposite. While there are many similarities between the two studies, the food sources and rearing densities varied which may explain the differences in offspring response to parental larval food.

We did not find any evidence of maternal or paternal effects on offspring susceptibility to infection with dengue virus, viral titer, or disseminated infection regardless of parental treatment group or offspring nutrition. Trade-offs often exist between different life history traits and immunity (Schmid-Hempel 2005) and there are physiological costs associated with allocating resources to immune function (Rauw 2012). In the moth, *Trichoplusia ni* there is evidence of transgenerational trade-offs between the transfer of nutritional stress tolerance and immune priming (Shikano et al. 2015). Additionally, other studies on *Drosophila melanogaster* have shown similar results to ours in that life history traits but not susceptibility to bacterial infection were influenced by parental nutrients (Valtonen et al. 2012). Our results indicate that offspring were equally susceptible to infection, however, we did see trends in that low food offspring generally sustained higher body titers and disseminated infection rates than high food offspring. It is likely that offspring larval nutrition is more important than parental larval nutrition in influencing infection with virus. Additionally, larval nutrient deprivation has been shown to influence susceptibility to infection with dengue virus and dissemination of the virus outside of the midgut in *Ae. aegypti* (Muturi et al. 2011). Our results for susceptibility to infection are consistent with previous results in Chapter 2, which suggest that parental nutrients do not affect this trait.

We did not find any effects on offspring interactions with dengue virus. Our results are inconsistent with other studies on insects demonstrating adaptive transgenerational immune priming to viruses in response to maternal nutrient deprivation (Boots and Roberts 2012) and studies demonstrating reductions in immunity associated with parental food (Triggs and Knell 2012). Studies in mosquitoes have shown complex interactions between maternal effects and offspring immune traits (Voordouw et al. 2008, Lorenz and Koella 2011). There have been many studies demonstrating immune priming in insect offspring associated with infection in the parental generation (Moret and Schmid-Hempel 2001, Sadd et al. 2005, Moret 2006, Freitak et al. 2009, Roth et al. 2009, Zanchi et al. 2011, Eggert et al. 2014, and Dubuffet et al. 2015). As a result, it would be beneficial to consider how both maternal and paternal infection with pathogens influences offspring life histories and immune traits in *Ae. aegypti.* Additionally, traits linked to immunity, other than susceptibility to infection, in *Ae. aegypti* should be considered.

In summary, our study demonstrates the importance of considering both the maternal and paternal environment when considering offspring life histories. Our results show that early parental nutrition can influence offspring, similar to results found by Valtonen et al. (2012). We found evidence of maternal effects on development time of both daughters and sons, however, these effects were only evident when mothers mated with high food fathers. Additionally, we found evidence of paternal effects on development time of daughters and sons, and similarly these effects were only seen when fathers mated with high food mothers. High condition partners may have had increased ability to influence mates thus generating these nutrient effects. In the traits

measured, sons were not affected as much as daughters. This may be related to the greater nutrient requirements of female *Ae. aegypti* for development (Chambers and Klowden 1990). Further investigation into maternal and paternal effects on additional life history traits of sons is warranted. Given the longer development times of offspring from two high food parents, compared with those from two dissimilar parents, our results may indicate potential bet hedging by parents in which two high nutrient parents resulted in significantly more offspring (Olofsson et al. 2009). However, longer development times, coupled with higher fecundity may not be maladaptive given that these two factors offset one another mathematically when considering per capita growth rates of populations (Livdahl and Sugihara 1984). Future studies should consider the intrinsic rate of increase to obtain further clarity on how parental larval nutrition influences population performance. It is possible that intraspecific competition influenced our results, although with the feeding regimen and densities used we believe such effects to be minimal. We did not find any evidence of transgenerational effects derived from either parent on offspring susceptibility to dengue virus infection, body titer, and viral dissemination. Further study should consider how introducing pathogens to the parental generation, may influence offspring immune traits and the mechanisms behind the findings.

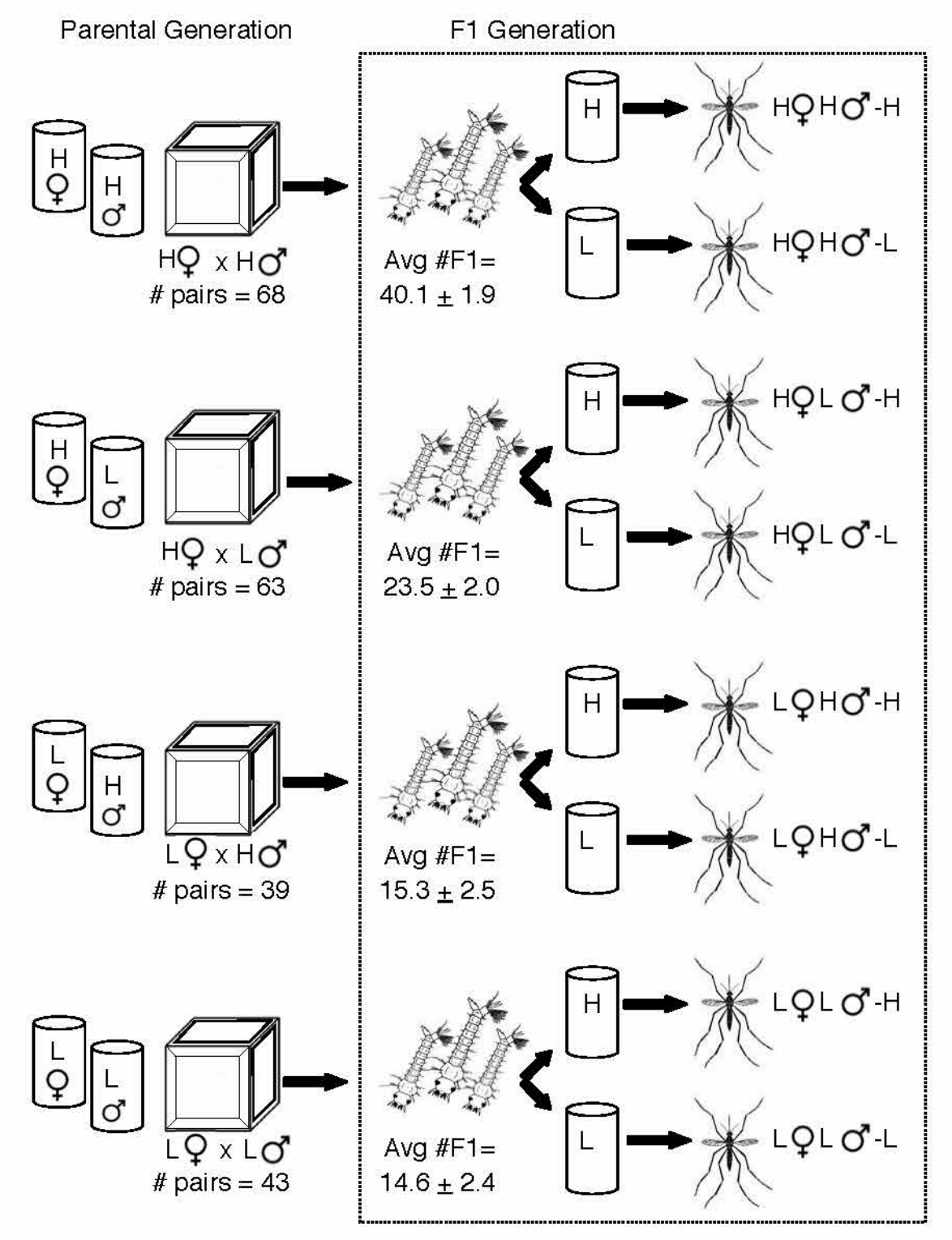

Figure 3-1. Experimental design and the average number of offspring produced by parental mating cross.

Table 3-1. Mean descriptive statistics (± S.E.) for high food and low food offspring by treatment (trt) group.

F1 Food	Parental Trt	Female development (days)	Male development (days)	Survivorship (%)	Female wing length (mm)
High	H♀H♂	11.23 ± 0.34	10.02 ± 0.23	75.79 ± 2.67	2.76 ± 0.02
	H♀L♂	9.52 ± 0.25	9.30 ± 0.24	81.73 ± 2.95	2.76 ± 0.03
	L♀H♂	8.70 ± 0.12	8.74 ± 0.13	84.41 ± 4.03	2.78 ± 0.02
	L♀L♂	9.32 ± 0.23	9.25 ± 0.26	87.04 ± 2.93	2.79 ± 0.04
Low	H♀H♂	15.40 ± 0.39	13.42 ± 0.36	72.81 ± 2.58	2.45 ± 0.02
	H♀L♂	13.98 ± 0.38	12.39 ± 0.28	73.88 ± 3.50	2.40 ± 0.03
	L♀H♂	13.40 ± 0.41	12.27 ± 0.42	76.94 ± 4.03	2.40 ± 0.03
	L♀L♂	12.95 ± 0.32	11.65 ± 0.38	80.36 ± 3.24	2.49 ± 0.06

Table 3-2. MANOVA for main effects and multivariate pairwise contrasts of maternal nutrition and paternal nutrition effects on offspring development (dev) time (females, males), survivorship to adulthood, and female wing length.

					Standardized Canonical Coefficients			
Comparison		Num d.f. / Den d.f.	Pillai's trace	*p*	Female dev	Male dev	Sqrt % dead	♀ wing length
High Food F1								
Parental larval nutrition		12/441	0.28	<0.0001	1.36	-0.58	0.53	-0.11
Maternal	H♀H♂-High vs L♀H♂-High	4/79	0.31	<0.0001*	1.38	-0.33	0.24	0.24
	H♀L♂-High vs L♀L♂-High	4/63	0.05	0.54	0.23	0.07	0.78	-0.58
Paternal	H♀H♂-High vs H♀L♂-High	4/88	0.14	0.008*	1.51	-0.99	0.30	0.15
	L♀H♂-High vs L♀L♂-High	4/54	0.12	0.14	1.22	-0.29	-0.21	0.23
Low Food F1								
Parental larval nutrition		12/372	0.22	0.005	0.81	0.53	0.47	0.06
Maternal	H♀H♂-Low vs L♀H♂-Low	4/64	0.17	0.015*	0.64	0.37	0.69	0.34
	H♀L♂-Low vs L♀L♂-Low	4/55	0.07	0.43	0.24	-0.01	0.71	-0.78
Paternal	H♀H♂-Low vs H♀L♂-Low	4/76	0.14	0.018*	0.78	0.38	0.23	0.39
	L♀H♂-Low vs L♀L♂-Low	4/43	0.06	0.64	0.38	0.33	0.16	-0.75

*Denotes significant p-value after Bonferroni correction

Table 3-3. Mean descriptive statistics (± S.E.) for high and low food offspring infection with dengue-1 by treatment (trt) group.

F1 Nutrition	Trt	n (families)	% Infected	Log 10 (Titer) (PFUeq/mL)	% Disseminated Infection
High Food	H♀H♂	55	65.4 ± 5.3	3.99 ± 0.21	65.3 ± 6.1
	H♀L♂	42	63.0 ± 6.1	4.09 ± 0.22	74.7 ± 6.9
	L♀H♂	30	52.5 ± 8.2	4.10 ± 0.31	71.9 ± 8.7
	L♀L♂	29	66.1 ± 7.9	3.81 ± 0.34	62.9 ± 8.9
Low Food	H♀H♂	45	89.0 ± 3.7	4.21 ± 0.19	91.7 ± 3.2
	H♀L♂	37	71.2 ± 6.1	3.79 ± 0.29	87.0 ± 5.0
	L♀H♂	25	71.3 ± 7.5	3.64 ± 0.36	93.2 ± 3.9
	L♀L♂	24	76.3 ± 8.9	3.74 ± 0.36	90.4 ± 2.3

Table 3-4. MANOVA for main effects and multivariate pairwise contrasts of maternal and paternal nutrition effects on offspring dengue-1 infection, virus titer, and percent of infected with disseminated infections.

					Standardized Canonical Coefficients		
Effect	Comparison	Num d.f. / Den d.f.	Pillai's trace	*p*	% Infected	Log10 (Titer) (PFUeq/mL)	% Disseminated Infection
High Food F1							
Parental larval nutrition		9/351	0.03	0.92	-0.08	0.08	0.60
Maternal	H♀H♂-High vs L♀H♂-High	3/61	0.01	0.85	0.74	-0.10	0.77
	H♀L♂-High vs L♀L♂-High	3/52	0.06	0.34	-0.87	0.05	0.79
Paternal	H♀H♂-High vs H♀L♂-High	3/76	0.01	0.79	0.01	-0.28	1.12
	L♀H♂-High vs L♀L♂-High	3/37	0.02	0.82	-0.63	0.27	0.63
Low Food F1							
Parental larval nutrition		9/333	0.09	0.37	1.00	-0.28	0.11
Maternal	H♀H♂-Low vs L♀H♂-Low	3/60	0.05	0.36	1.02	0.02	-0.35
	H♀L♂-Low vs L♀L♂-Low	3/47	0.02	0.76	0.91	0.41	-0.47
Paternal	H♀H♂-Low vs H♀L♂-Low	3/70	0.09	0.10	0.89	-0.64	0.59
	L♀H♂-Low vs L♀L♂-Low	3/37	0.02	0.82	0.79	-0.59	0.17

*Denotes significant p-value after Bonferroni correction

CHAPTER 4
MATERNAL AND PATERNAL LARVAL NUTRIENT CONDITION INFLUENCE ON EGG QUANTITY AND QUALITY IN A MOSQUITO

Introduction

Transgenerational effects occur when the conditions experienced by a mother and/or father contribute to offspring phenotype. These effects are important factors contributing to the ecology of organisms and can influence evolutionary responses to selection (Kirkpatrick and Lande 1989). The environmental conditions experienced by parents can enhance offspring fitness (Mousseau and Dingle 1991a, Agrawal et al. 1999, Marshall and Uller 2007) or reduce offspring fitness (Vijendravarma et al. 2010). These effects can be maternally-derived and/or paternally derived, and are often context dependent (Qvarnström and Price 2001, Plaistow et al. 2006). While significantly more attention has been paid to transgenerational effects in organisms displaying parental care, these effects have been identified in a wide range of taxa including invertebrates (Mousseau and Fox 1998a). In insects, mothers and fathers have been shown to influence different offspring traits, often in a sex-specific manner (e.g. Bonduriansky and Head 2007, Roth et al. 2009, Garcia-Gonzalez and Simmons 2011, Dew-Budd et al. 2016, Chapter 2, Chapter 3), possibly due to the evolution of sex-specific reproductive investment strategies (Zanchi et al. 2011).

The mosquito, *Aedes aegypti,* is one of the most important vectors of arthropod-borne (arbo) viruses including Zika, dengue, chikungunya, and yellow fever viruses. Previous studies on *Ae. aegypti* have characterized the influence of parental larval nutrition on offspring life history traits and mosquito-virus interactions (Chapter 2, Chapter 3). These effects were maternally- and paternally-derived, as determined by experimental manipulations of parental mating crosses (Chapter 3). Despite the

importance of these observations, no mechanisms were considered. While transgenerational effects have been widely documented for a variety of insects, the mechanisms behind these effects are largely speculative. Proposed mechanisms involved in transgenerational effects in insects include egg resource provisioning (Rossiter 1991, Sinervo 1991, Fox et al. 1999, Rolff 1999, McIntyre and Gooding 2000, Freitak et al. 2009, Gibbs et al. 2010, Urbanski et al. 2010b), epigenetic marking (Bongiorni et al. 1999, Bonduriansky and Head 2007, Freitak et al. 2009, MacDonald 2012), sex-specific genomic imprinting (Bongiorni et al. 1999, MacDonald 2012), transfer of symbionts (Moran et al. 2008), transfer of immunodefense factors (Tidbury et al. 2010, Boots and Roberts 2012, Triggs and Knell 2012, Pölkki et al. 2012), and elevation of germline mutation (Bonduriansky and Head 2007). Of these, egg size is one of the more tractable ways to identify transgenerational effects and has not been investigated in this context for mosquitoes (Uller 2008).

Egg size is typically used as an indicator of reproductive investment (Winkler and Wallin 1987, Sinervo 1990, Sinervo and Licht 1991, Bernardo 1996b, Fox and Mousseau 1996, Fox et al. 1999, Giron and Casas 2003). Egg size variation has been documented in *Ae. aegypti* (Steinwascher 1984) and may be a proximate mechanism by which mothers can influence offspring phenotype. In a laboratory study, females from higher larval nutrient environments acquired larger amounts of lipid, glycogen, and proteins allowing them to provision a higher number of eggs compared to females in low food environments (Telang et al. 2006). How females allocate resources to eggs may influence offspring development (Perez and Noriega 2012). Given that *Ae. aegypti* egg yolks are primarily composed of protein, lipids, glycogen, and water (Briegel et al.

2003), the quantities of these macronutrients may vary depending upon the nutritional status of the mother (Briegel 1990, Ziegler 1997) and possibly the nutritional status of the father.

During and after mating, female *Ae. aegypti* undergo many different behavioral and physiological changes from exposure to male ejaculate and seminal fluid proteins (sfps) produced by male accessory glands (Klowden and Chambers 1991, Sirot et al. 2011). These sfps may modulate use of proteins, hormone levels, and how sperm is utilized (Sirot et al. 2011) all of which may ultimately influence egg development generating paternal effects. Adult body size is associated with larval nutrition in *Ae. aegypti* (Schneider et al. 2004). Small males likely experience seminal depletion more quickly than large males and semen depletion can influence female longevity and fecundity (Helinski and Harrington 2011). When mated with males depleted in sfps and or sperm, females produce fewer eggs and had reduced longevity relative to those that mated with non-depleted males (Helinski and Harrington 2011) and this may lead to paternal effects.

While experiments have considered the influence of female body size on egg size (Steinwascher 1984) and larval nutrition on the endocrinology of mosquito egg development (Telang et al. 2006), the influence of combined parental nutrition on egg size and nutrient composition has not been characterized. Previous research suggests that both maternal and paternal larval nutrition influences offspring life histories in *Ae. aegypti* (Chapter 2, Chapter 3). Egg size is considered a tractable way to test for plasticity and parental effects (Uller 2008). Here we consider how parental larval

nutrition and mating cross influences the number produced, average size, lipid content, and protein content of *Ae. aegypti* eggs.

Materials and Methods

Study Organism and Laboratory Colony

Ae. aegypti larvae were collected in 2012 from field containers in Key West, FL and used to establish a laboratory colony. Colony mosquitoes were reared in an insectary at 28±0.5°C with a 12:12 (light:dark) hour photoperiod. Mosquitoes were hatched in enamel pans (24 x 36 x 5 cm) containing 1.5 L of water and 200 mg of larval food in cohorts of approximately 200. Larval food consisted of a 1:1 mixture of lactalbumin:*Saccharomyces cerevisiae* yeast which was provided twice a week in 200 mg aliquots. Upon pupation, mosquitoes were transferred to water-filled cups and placed in plastic cages (45.7 x 45.7 x 45.7 cm, BugDorm, MegaView Science Co. Ltd. Taichung, Taiwan) for emergence. Adults were sustained on a 10% sucrose solution provided in cotton wicks and females were provided blood meals weekly from live chickens. Chicken care followed the animal use and care policies of the University of Florida's Institutional Animal Care and Use Committee (IACUC Protocol 201003892).

Parental Treatment

For this study, the F_{12} colony generation was used and will herein be referred to as the parental (P) generation. P generation larvae were hatched in enamel pans (24 x 36 x 5 cm) containing 1.5 L of water and 200 mg of larval food. After 24 hours, P larvae were removed from nutrients and placed individually into cylindrical vials (82 x 26 mm ht. x dia.) containing 10 mL of water with either low (0.75 mg) or high larval food (3.0 mg). Low food larvae were hatched 1 week prior to high food larvae to ensure mating between P larvae treatment groups given that low food larvae take longer to develop. A

total of 1400 high food and 1400 low food larvae were hatched and reared. Once mosquitoes emerged, adults were sexed and placed in treatment and sex specific cages in numbers no greater than 50 mosquitoes per cage. Adults were sustained on 10% sucrose solution. After all adults emerged, they were used in the following parental mating cross treatments:

- High food ♀ x High food ♂: H♀H♂
- High food ♀ x Low food ♂: H♀L♂
- Low food ♀ x High food ♂: L♀H♂
- Low food ♀ x Low food ♂: L♀L♂

Mosquitoes were cold anesthetized and placed into treatment specific cages at a 1:1 male to female ratio for 7 days to allow for mating. In order to maintain the 1:1 male:female ratio, subsets of males were haphazardly selected from the high and low food treatments. After one week, females were allowed to blood feed on live chickens for one hour. After the feeding period, females were cold anesthetized and fully engorged blood-fed females were placed individually into cylindrical tubes (82 x 26 mm ht. x dia.) containing a small piece of damp cotton and seed germination paper as an oviposition substrate. Females were given one week to oviposit after which egg papers and adult females were removed and stored at -80°C until processed. Wings were removed from females and mounted onto slides for wing length determination using computer imaging software (i-Solution lit©, AIC, Princeton, NJ). Wings were used to estimate body size (reviewed in Armbruster and Hutchinson 2002) and were measured in millimeters from alula to wing tip. See Figure 4-1 for experimental design and parental nutrient mating crosses.

Egg papers were photographed using an Olympus SZ61 stereomicroscope fitted with an Infinity 1 digital camera. Total egg number per female was recorded. Length and

width measurements were taken for a subset of 10 eggs per female to approximate average egg size using the ImageJ 1.50i software. For females laying fewer than 10 eggs, all were measured. Under the assumption that egg shape is a prolate spheroid, egg volume was estimated using the methods described by Hawley (1985). Specifically, the following equation was used where V= volume, L= length, and W= width:

$$V = \pi L W^2/6$$

Egg Nutrient Composition Quantification Assays

Triacylglycerides were measured through colorimetric determination using the BioVision Triglyceride Quantification Kit (BioVision, Mountain View, CA; cat #K622-100). Ten eggs per assay were manually homogenized using sterile pestles in centrifuge tubes with 1 mL of 5% NP-40 detergent (lysis buffer). Ten eggs were used to ensure measurements were within the range of sensitivity of the kit based upon previous studies on first instar larvae (Perez and Noriega 2012). We used the manufacturers protocol (BioVision) to perform the assay. Briefly, 30 μL of homogenized egg sample were added to wells individually on a 96-well clear microplate, along with 46 μL of triglyceride assay buffer, 2 μL of triglyceride probe, and 2 μL of triglyceride assay mix. Due to potential interference from endogenous compounds in the sample interfering with the assay, each sample well was intentionally spiked with 2 nmol of triglyceride standard. Plates were mixed, covered with foil to protect from the light, and allowed to incubate at room temperature for 60 minutes. Plates were read on a spectrophotometer (Bio-Tek, Synergy HT) with absorbance set to 570 nm. The assay kit contained serial dilutions of standards containing known quantities of lipids which were ran on a

microplate in triplicate. Data produced from these standards was used to generate a standard curve which was used to quantify lipid content in the eggs.

Proteins were quantified using the Thermo Scientific Pierce 660 nm Protein Assay Kit (Pierce Biotechnology, Rockford, IL; cat #22662). For protein quantification, 10 eggs were pooled and prepared for each assay to try and remain within the sensitivities of the kit as determined by studies on 1st instar larvae (Perez and Noriega 2012). Eggs were prepared for protein analysis using the methods described by Giron et al. (2002). Specifically, 10 eggs were crushed using a sterile pestle into 800 µL of physiological water containing 0.001% Triton X-100 (Pierce Biotechnology, Rockford, IL; cat #85111) and placed at 4°C for five days to allow proteins to dissolve into the detergent for analysis. After five days, the protocol provided by Pierce Biotechnology for protein quantification was followed. Briefly, 8 µL of sample and 150 µL of protein assay reagent were added to each well of a 96-well clear microplate. Each sample was intentionally spiked with 2 µL of 17% BSA standard to ensure that the assay would register within the BSA standard curve. Plates were mixed on a plate shaker set to medium speed for one minute and incubated at room temperature for 5 minutes. After incubation, plates were read at an absorbance of 660nm. Samples were quantified based upon a standard curve generated from standards.

Statistical Analyses

Analysis of variance (ANOVA) was used to compare female size (wing length) among treatment groups (PROC GLM, SAS v. 9.3). Multivariate analysis of variance (MANOVA) was used to determine the contribution of parental larval nutrient mating cross effects on the quantity of eggs produced during the first gonotrophic cycle, egg

volume, egg lipid content, and egg protein content (PROC GLM, SAS v. 9.3). Significant effects were further analyzed with multivariate pairwise contrasts of means adjusted using the sequential Bonferroni method (α=0.05). The contribution of each egg parameter to significant treatment effects and their relationship to each other (positive/negative) was measured using standardized canonical coefficients (Scheiner 2001). Specifically, to consider maternal contribution to egg number, volume, and composition eggs from H♀H♂ vs. L♀H♂ and H♀L♂ vs. L♀L♂ were compared. To consider paternal contribution to aforementioned effects H♀H♂ vs. H♀L♂ and L♀H♂ vs. L♀L♂ were compared. Lastly, to consider combined parental effects H♀H♂ vs. L♀L♂ and H♀L♂ vs. L♀H♂ were considered.

Results

Of the 2,800 larvae, a total of 2,030 survived to adulthood (72.5%). In the low food treatment, 689 males and 496 females emerged and in the high food treatment, 431 males and 414 females emerged. Of these, 207 males and 207 females were used for the H♀H♂ treatment, 207 males and 207 females for the H♀L♂ treatment, 248 males and 248 females for the L♀H♂ treatment and 248 males and 248 females for the L♀L♂ treatment. See Table 4-1 for summary statistics on parental treatment numbers, percent blood feeding, percent of blood fed females successfully laying eggs, and blood fed female wing length. When comparing high food females, ANOVA results showed that those that mated with high food males (H♀H♂) did not have different mean wing lengths than those that mated with low food males (H♀L♂) (p=0.9988). When comparing low food females, ANOVA results showed that those that mated with high food males (L♀H♂) did not have different mean wing lengths than those that mated with

low food males (L♀L♂) (p=0.7501). ANOVA also showed that high food blood fed females had significantly longer mean wing lengths than low food blood fed females (H♀H♂ vs. L♀H♂, p<0.0001; H♀H♂ vs. L♀L♂, p<0.0001; H♀L♂ vs. L♀L♂, p<0.0001; H♀L♂ vs. L♀H♂, p<0.0001).

See Figure 4-2 for descriptive statistics on egg parameters measured. MANVOA showed significant parental nutrient mating cross effects on egg production (Pillai's trace=0.444, p<0.0001). Subsequent analyses demonstrated significant maternal nutrient effects on egg parameters measured (Table 4-2, H♀H♂ vs. L♀H♂ and H♀L♂ vs. L♀L♂). In females that mated with high food males, egg count followed by lipid content contributed the most to this effect. Specifically, high food females that mated with high food males (H♀H♂) laid nearly 2 times more eggs than low food females that mated with high food males (L♀H♂). Low food females that mated with high food males laid eggs with 1.4 times higher lipid content than high food females that mated with high food males. In females that mated with low food males, egg count contributed the most to the effect. Specifically, high food females that mated with low food males (H♀L♂) laid 1.9 times more eggs than low food females that mated with low food males (L♀L♂).

Multivariate contrasts demonstrated significant paternal effects on egg production when males mated with low food females (L♀H♂ vs. L♀L♂, p=0.0113) but not when males mated with high food females (H♀H♂ vs. H♀L♂, p=0.4591). In males that mated with low food females, lipid content contributed most to the effect. Specifically, high food males that mated with low food females (L♀H♂) generated eggs with 1.4 times higher lipid content than those from low food males that mated with low food females (L♀L♂).

Low food females that mated with high food males (L♀H♂) laid eggs with the highest lipid content compared to all other treatment groups (Figure 4-2).

When comparing two high food parents (H♀H♂) and two low food parents (L♀L♂), Multivariate contrasts demonstrated significant parental effects on egg production ($p<0.0001$). The number of eggs laid contributed the most to this effect followed by egg volume. Two high food parents (H♀H♂) laid 1.9 times the number of eggs that two low food parents laid (L♀L♂). Additionally, the average volume of eggs produced by two high food parents (H♀H♂) was about 4% larger in volume than eggs laid by two low food parents (L♀L♂). When comparing two completely dissimilar parental nutrient mating crosses (H♀L♂ vs. L♀H♂), MANOVA demonstrated significant parental effects on egg parameters measured with egg count contributing most to the effect followed by egg volume and lipid content which contributed similarly. High food females that mated with low food males (H♀L♂) laid on average 25.6 more eggs which were 6.9 percent greater in volume than low food females that mated with high food males (L♀H♂). However, low food females that mated with high food males (L♀H♂) laid eggs with 1.4 times greater lipid content than high food females that mated with low food males (H♀L♂) (Figure 4-2).

Discussion

Previous research on *Ae. aegypti*, found significant parental larval nutrient effects on offspring life history traits (Chapter 2, Chapter 3). Results were largely context dependent and sex-specific (Chapter 2, Chapter 3) and the potential mechanisms behind these effects were not investigated. Egg size is used as an indicator of parental investment in offspring, and as a measure of parental effects (Uller 2008). Despite this,

egg size is not always a reliable indicator of egg quality or composition (Fox and Czesak 2000, McIntyre and Gooding 2000, Giron and Casas 2003). Here we investigated whether maternal and paternal larval nutrition influences in the first gonotrophic cycle the number produced, size, lipid content, and protein content in *Ae. aegypti* eggs.

Maternal Effects

We found evidence that maternal larval nutrition influences the number of eggs produced by females as well as their lipid investment. In Diptera, numbers of eggs produced are highly correlated with female body size (Berrigan 1991). It is also argued that a tradeoff exists between egg size and egg number (Smith and Fretwell 1974, Berrigan 1991). In *Ae. aegypti* larval nutrition strongly influences adult female body size (Schneider et al. 2004) and body size influences the number of eggs laid by females (Steinwascher 1984, Briegel 1990, Dieng et al. 2016). Additionally, *Ae. aegypti* larval nutrition influences female metabolic reserves, which ultimately influence fecundity for the first ovarian cycle, with high-reserve females producing more eggs than low-reserve females (Briegel 1990, Telang et al. 2006). Previous studies have found that larger females can lay 4-5 times more eggs than smaller females (Briegel 1990). Consistent with these studies, we found that high nutrient, larger females laid twice the number eggs during the first ovarian cycle than low nutrient, smaller females regardless of paternal nutrition. The difference between eggs laid by our larger vs. smaller females was less than Briegel (1990). The discrepancy between our observations and others in the number of eggs produced may be attributable to the range of sizes of adult mosquitoes and/or the source of host blood. It would be worth considering alternative host blood meal sources in subsequent studies, given that host species can influence fecundity (Bennett 1970, Gunathilaka et al. 2017). While we found that high food

females laid significantly more eggs than their low food counterparts, we did not assess the viability of the eggs, hatching rates, or subsequent offspring phenotypes and these should be considered in future studies.

We also found that when mated with high nutrient males, low nutrient females laid eggs with significantly higher lipid content than those laid by high food females. This is surprising given that high larval nutrient females have been found to be lipid rich relative to low larval food females (Telang et al. 2006) and mating does not appear to alter nutritional state of females in terms of whole body glycogen, lipid, and protein content (Klowden and Chambers 1991). Additionally, small females and large females have been found to deposit similar proportions of lipids in ovaries post-blood meal (Naksathit et al. 1999) giving rise to the expectation that large females would lay eggs with more lipids. While our results were not expected, other studies have verified that lipid content in *Ae. aegypti* eggs is highly variable (Briegel 1990). Underlying plasticity in lipid resource allocation to eggs may be one source of maternal effects in this system. A previous study also showed that when low nutrient females mated with high nutrient males, subsequent female and male offspring reared in high food, developed the most quickly compared to other mating treatment groups (Chapter 3). It is possible that additional lipid, allocated by mothers, may explain the faster development time demonstrated in the previous study.

The availability of maternally-derived lipid reserves is critical during pharate first-instar quiescence, which allows the unhatched larva to survive during unfavorable environmental conditions (Clements 1992, Vinogradova et al. 2007). In unpredictable and variable environments, females may use alternative provisioning strategies to

maximize fitness (Olofsson et al. 2009), and increased lipid reserves may improve the ability of eggs to survive quiescence. Interestingly, a study conducted by Dieng et al. (2016) found that small females that mated with large males produced eggs with low hatching rates at 24-hours, perhaps this is indicative of quiescence. The ability of *Ae. aegypti* eggs to undergo prolonged quiescence has contributed significantly to the range expansion of this species (Vinogradova 1965, Clements 1992). Also, a laboratory study found that females reared from eggs that have undergone extended quiescence, were more tolerant to starvation and had longer lifespans than those from short quiescence eggs (Perez and Noriega 2013). Increasing lipid content in eggs, may allow for extended pharate larval quiescence, and ultimately greater survival of female offspring. In stressful environments, maternal survival is likely reduced, and females may allocate additional resources during the first gonotrophic cycle to compensate for reduced life span and increase fecundity.

Paternal Effects

We found paternal effects on egg production when males mated with low food females but not high food females. Specifically, when high food males mated with low food females, resulting eggs had higher lipid content than when low food males mated with low food females. The transfer of juvenile hormone III in male accessory gland fluid to females during mating has been shown to reduce the likelihood of follicular resorption and increase storage lipids in females (Clifton et al. 2014). This may explain the higher lipid content in eggs from low food females that mated with high food males. Previous study on this system, demonstrated significant paternal effects on offspring life history parameters when males mated with high food females but not low food females (Chapter 3) which is different than what we found. Given that environmental stressors

can be life-stage specific (Loeschcke and Krebs 1996, De Block and Stoks 2005), it is possible that additional lipid content in eggs buffers against environmental stressors experienced in the egg stage and do not translate to the larval and adult stages. A study conducted by Fernandez and Klowden (1995) found that starved males transferred less protein during mating. Male accessory gland proteins in *Ae. aegypti* modulate host seeking behavior, prevent subsequent mating, and stimulate oviposition (Leahy and Craig 1965, Lee and Klowden 1999). Given that seminal fluid proteins influence female physiology, this may explain why we saw high food males, but not low food males, influencing egg production in low food females. Larger males produce more spermatozoa than smaller males (Ponlawat and Harrington 2007) and this likely applies to seminal fluid proteins. Although previous research suggests that even small quantities of seminal fluid proteins, transferred by small males reared on suboptimal larval nutrients, were enough to render females refractory to subsequent matings (Dickinson and Klowden 1997), it is possible that larger quantities of these proteins may have higher impact on female reproduction than smaller quantities.

Additional Comparisons

When considering two high larval food parents compared to two low larval food parents, we found significant effects on egg production with two high food parents laying a higher number of larger eggs than two low food parents. Diet quality often determines size and is correlated with good condition (Blanckenhorn 2000), as such it is expected that two high quality parents will have increased reproductive output. Given the importance of larval nutrition on life history traits in mosquitoes (Teng and Apperson 2000, Arrivillaga and Barrera 2004, Farjana et al. 2012, Couret et al. 2014), it is unsurprising that we would see these effects on reproductive output, especially for the

first gonotrophic cycle. Previous studies have shown *Ae. aegypti* females that are nutritionally-stressed as larvae tend to be smaller as adults and lay fewer eggs per clutch (Reyes-Villaneuva 2004, Farjana and Tuno 2013). Results from Chapter 2, found that offspring from two low food parents had greater survivorship than offspring from two high food parents.

Lastly, when considering egg production by high food mothers mated with low food fathers (H♀L♂) vs. low food mothers mated with high food fathers (L♀H♂), we also found significant effects. Low food mothers that mated with high food fathers laid fewer and smaller eggs with higher lipid content than high food mothers that mated with low food fathers. Differences in egg quantity between high and low food mothers was expected given that egg number is correlated with female size in this system (Steinwascher 1984, Briegel 1990, Dieng et al. 2016). However, the increased lipid content was unexpected and as discussed previously may indicate increased resource provisioning in eggs in response to nutrient deprivation.

Conclusions

Life-history theory predicts that reproductive effort is likely to vary, and that investment in reproduction will be high when expected returns in fitness are high (Brommer 2000). Additionally, mate quality is likely to influence reproductive investment, with higher quality mates leading to greater investment in reproduction (Sheldon 2000). Here, female and male larval nutrition influenced egg quantity and composition in *Ae. aegypti.* High nutrient females laid the greatest number of eggs, regardless of male nutrition, and eggs produced had similar quantities of proteins and lipids. These results suggest that male quality did not influence female reproductive effort for high nutrient females. It is possible that male quality may influence transfer of other materials to

eggs, that were not measured in this study, such as vitamins, minerals, and hormones. Future studies should consider how male quality affects the transfer of additional substances to eggs as well as the viability of eggs produced.

The quantity of lipids in eggs was similar in treatment groups with the exception of eggs laid by low nutrient females that mated with high nutrient males. When low nutrient females mated with high nutrient males, they laid eggs with the highest lipid content relative to all other treatment groups. Lipid content is associated with ability to undergo quiescence and this ability may confer an advantage for stressful larval conditions encountered by offspring, which may be indicative of an adaptive parental effect. Regardless, additional reproductive effort in eggs produced by low nutrient females that mated with high nutrient males, indicates support for the differential allocation hypothesis. Differential allocation in this system may only be evident when females experience nutritional deprivation. Females produce larger gametes than males, and consequently the nutritional status of the female may be more important than that of the male. Results of this study support differential reproductive investment in offspring but only when females have limited food resources. Results do not support the reproductive compensation hypothesis which predicts that females increase reproductive effort when mating with low quality mates to counteract negative effects on offspring fitness (Gowaty et al. 2007). Larval nutrition influences both female and male quality, and likely that of subsequent offspring.

Table 4-1. Summary statistics for parental treatment (trt) groups

Trt	# ♂	# ♀	% Blood fed (n)	% Laid eggs (n)	Blood fed ♀ Wing Length (mm) ± SE
H♀H♂	207	207	59.9 (124)	81.5 (101)	2.45 ± 0.02
H♀L♂	207	207	77.8 (161)	81.4 (131)	2.45 ± 0.01
L♀H♂	248	248	38.7 (96)	78.1 (75)	2.07 ± 0.02
L♀L♂	248	248	51.2 (127)	70.9 (90)	2.09 ± 0.01

Table 4-2. MANOVA for main effects and multivariate pairwise contracts of parental larval nutrient mating cross on egg count, egg volume (mm^3), lipid content (pmol/egg), and protein content (μg/egg).

Effect	Comparison	Num d.f. /Den d.f.	Pillai's trace	P	Standardized Canonical Coefficients			
					Egg Count	Egg Volume	Lipid Content	Protein Content
Mating Cross Effects		12/645	0.444	<0.0001	0.970	0.410	-0.469	0.121
Maternal	H♀H♂ vs L♀H♂	4/93	0.505	<0.0001*	0.992	0.344	-0.632	0.418
	H♀L♂ vs L♀L♂	4/117	0.264	<0.0001*	1.164	0.312	0.071	-0.049
Paternal	H♀H♂ vs H♀L♂	4/147	0.024	0.4591	-0.096	1.012	-0.174	-0.332
	L♀H♂ vs L♀L♂	4/63	0.184	0.0113*	-0.006	-0.375	0.949	-0.291
Combined	H♀H♂ vs L♀L♂	4/91	0.323	<0.0001*	1.204	0.142	-0.185	0.146
	H♀L♂ vs L♀H♂	4/119	0.390	<0.0001*	0.890	0.500	-0.506	0.169

*Denotes significant p-value after Bonferroni correction

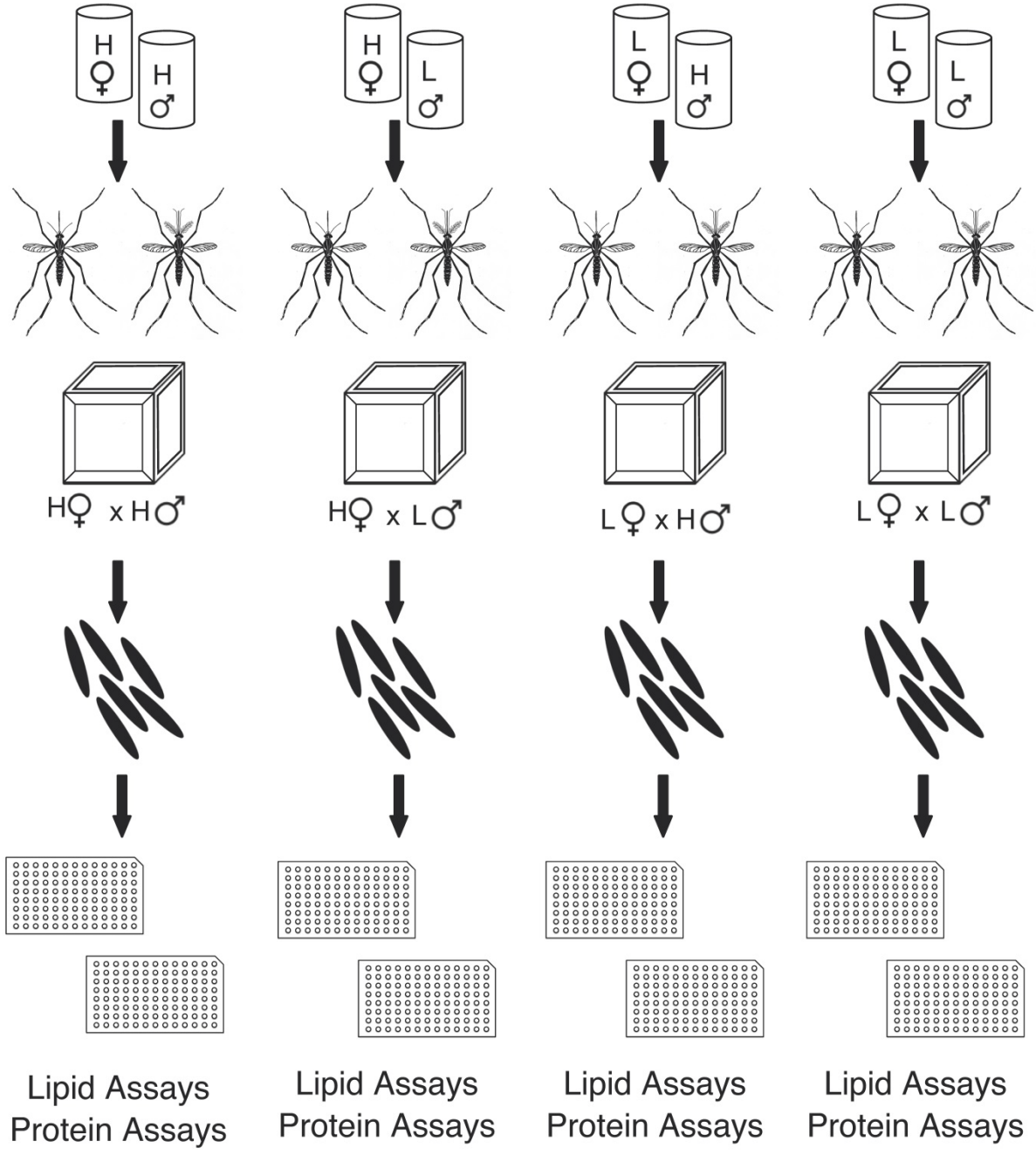

Figure 4-1. Experimental design describing parental mating crosses and assays.

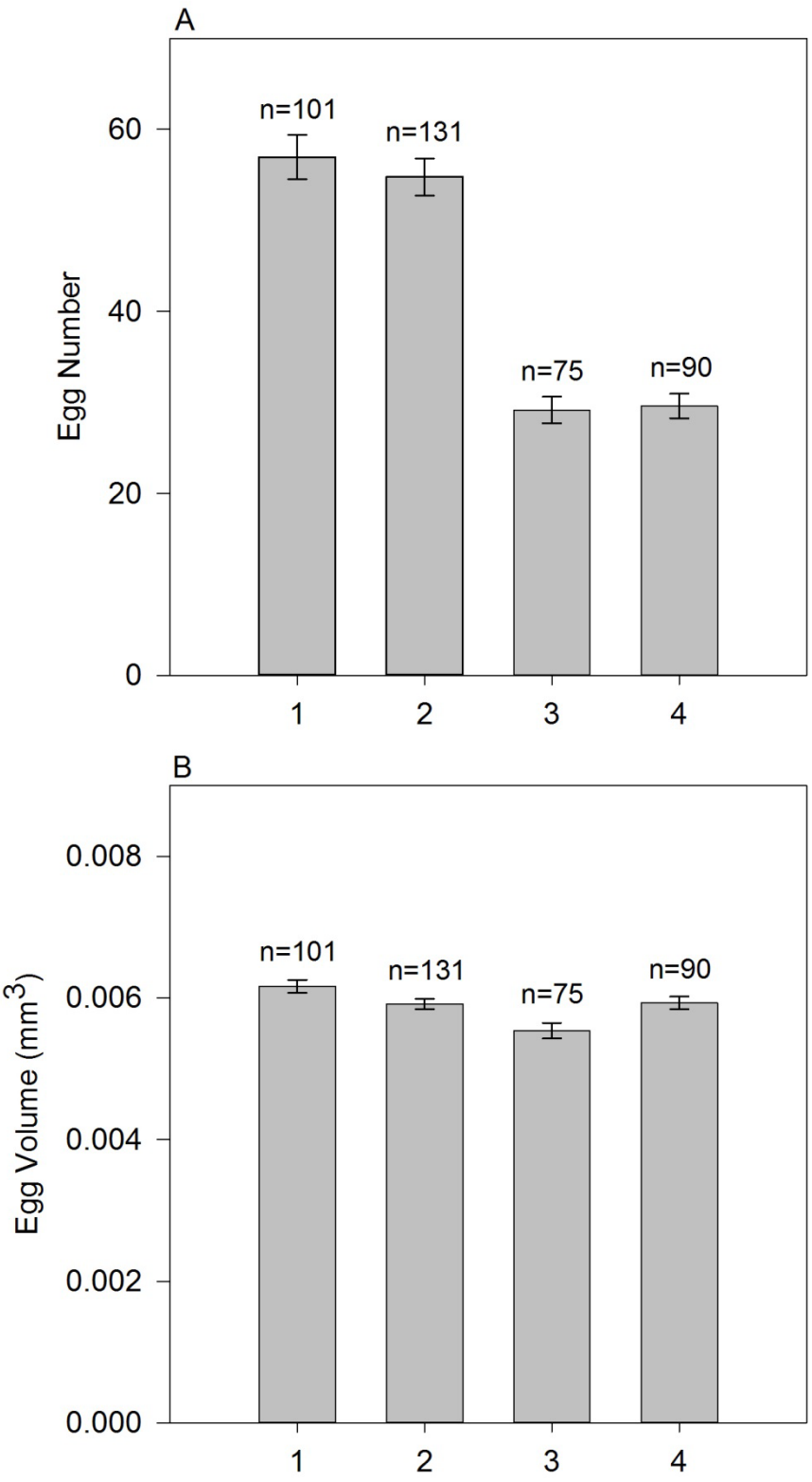

Figure 4-2. Mean descriptive statistics (± S.E.) by parental treatment group with 1= H♀H♂, 2:=H♀L♂, 3: L♀H♂, and 4: L♀L♂ for A) Egg Count, B) Egg Volume, C) Protein Content and D) Lipid Content.

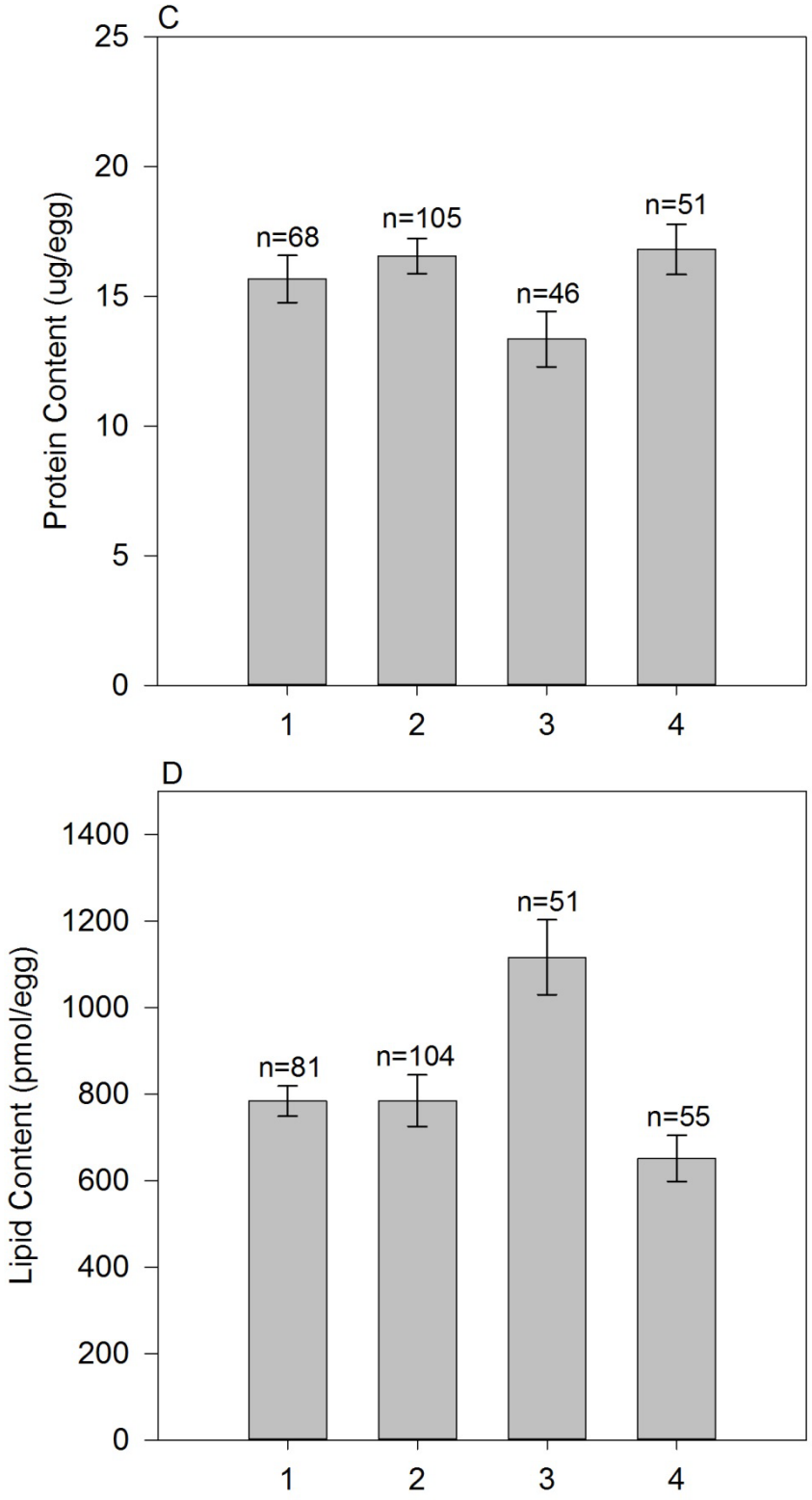

Figure 4-2. Continued

CHAPTER 5
CONCLUSIONS AND FUTURE STUDIES

The phenotype of an organism is influenced not only by its genotype and the environment in which it lives, but oftentimes the environment of its parents as well. The influence of the parental environment on offspring phenotype is generally referred to as parental or transgenerational effects and is considered a form of inherited phenotypic plasticity (Carrière 1994). In circumstances where parental effects confer a fitness advantage to offspring they may be adaptive (Mousseau and Dingle 1991a, Bernardo 1996a, Agrawal et al. 1999, Marshall and Uller 2007) and when they reduce offspring fitness maladaptive (Vijendravarma et al. 2010). As a source of phenotypic plasticity, parental effects can influence both the direction and rate of evolution (Kirkpatrick and Lande 1989). These factors make understanding how parental effects can influence offspring life history trajectories important in understanding the ecology and evolution of the organism.

One of the major limitations in our understanding of parental effects is that they are largely context dependent (Qvarnström and Price 2001, Plaistow et al. 2006) and a majority of research on this topic has focused on organisms that display parental care (Mousseau and Fox 1998a). In organisms that do not display parental care, most attention has been paid specifically to maternal effects. In insects, maternal effects have been identified in over 70 different species, representing a wide range of taxa (Mousseau and Dingle 1991a). In recent years, studies focusing on paternal effects have demonstrated that they can be important even in organisms that lack parental care and can contribute to offspring in unexpected ways (Bonduriansky and Head 2007, Roth et al. 2009, Bonduriansky et al. 2016, Zajitschek et al. 2017).

In mosquitoes, a majority of the research available pertaining to parental effects considers maternal influence on offspring diapause. Additional studies have considered parental food, oviposition site selection, immune response, infection with parasites, vertical transmission of arboviruses, and exposure to parasites. Results of these studies demonstrate that there are many factors parents experience that can influence offspring, and that there are many offspring traits that vary in response to parental effects. Overall it is clear that parental effects can influence host-parasite interactions and subsequently are an important consideration when studying vector mosquitoes (Otti and Sadd 2008). In the present experiments, *Ae. aegypti*, an important arbovirus vector, was used to investigate how parental larval nutrition affects offspring life history traits and interaction with dengue virus, and how parental larval nutrients affect egg production.

Life History Traits

Parental larval nutrition led to significant life history effects on offspring (Table 2-3, Table 3-2) in some treatments and effects appeared to be sex specific. In Chapter 2, effects were only identified when offspring were reared in low nutrients but not high nutrients. This suggests that parental nutrition in this system may be a factor when offspring have fewer available food resources. This may indicate threshold-dependent parental effects (Bonduriansky et al. 2016) in response to parental nutrition. Specifically, low-nutrient females from low nutrient parents took approximately 10.8% longer to develop than those from high nutrient parents, when reared on food sources encountered in the wild. Given that females much achieve a larger body size than males to pupate (Chambers and Klowden 1990) it is possible that potential parental effects on male offspring may appear when food resources are even more limited than females.

Results from Chapter 3, seem to support this hypothesis given that there was a greater difference in female development time than male development time between treatment groups. To test this hypothesis a wider range of nutrient conditions would need to be considered.

Interestingly, the results from Chapter 2 and Chapter 3 were quite different. In Chapter 3, offspring from two high food parents took the longest to develop relative to offspring from other treatment groups. This is in direct opposition to results from Chapter 2, in which offspring from two high food parents developed more quickly than those from two low food parents. These differences may be attributable to different nutrient regimes provided in the two experiments. In Chapter 2, varying amounts of invertebrate carcasses and oak leaves were used as basal nutrients for microorganisms introduced via tire water inoculum. In Chapter 3, nutrients consisted of a 1:1 ratio of yeast:lactalbumin in varying amounts. It can be speculated that these different nutrient regimes resulted in different macronutrient ratios in the studies, although this cannot be confirmed. The quantity and ratio of macronutrients has been linked to generating significantly different maternal and paternal larval nutrient effects on the growth and development of male and female offspring in the neriid fly, *Telostylinus angusticollis* (Bonduriansky et al. 2016). Specifically, Bonduriansky et al. (2016) found that maternal effects on size were mediated by protein and paternal effects by carbohydrate, and male and female offspring responded in different ways. The study conducted by Bonduriansky et al. (2016), and the differences between results in Chapters 2 and 3, demonstrate the complexity of nutrient mediated parental effects. Utilizing nutritional geometry, by varying the ratio and/or quantity of two macronutrients simultaneously in

an array, may be a more powerful way to study how nutrition influences parental effects in *Ae. aegypti.*

In Chapter 3, evidence for a maternal larval nutrient effect on offspring was found when mothers mated with high food fathers not low food fathers (Table 3-2). In Chapter 4, significant maternal larval nutrient effects on egg production were found regardless of paternal nutrition (Table 4-2). When considering offspring traits, those from two high food parents took longer to develop and had reduced survivorship compared to those from low food mothers and high food fathers. When considering egg production, high food mothers produced more eggs than their low food counterparts, regardless of the nutrient condition of the fathers. However, low food mothers that mated with high food fathers also produced eggs with significantly higher lipid content than those that mated with high food fathers. Additionally, mothers that mated with high food fathers produced significantly more offspring than those that mated with low food fathers (Chapter 3) even though they produced the same number of eggs (Chapter 4). When these results are considered together, it can be speculated that the nutrient condition of the mother influences her reproductive strategy and this likely varies depending upon the condition of her mate. Additionally, these results suggest that maternal larval nutrition can influence offspring and that such effects are stage-specific and context dependent. This provides support for a growing body of literature demonstrating maternal nutrition effects in a system lacking parental care (Saunders 1966, Langley et al. 1978, Rossiter 1991, Rossiter 1996, Gould 1988, Bonduriansky and Head 2007, Grech et al. 2007, Freitak et al. 2009, Boots and Roberts 2012, Valtonen et al. 2012, Triggs and Knell 2012).

Life history theory predicts that trade-offs exist between the optimum number of offspring and their size (Smith and Fretwell 1974). Under benign environmental conditions, high food females that mated with high food males, may be able to maximize fitness by producing many eggs and investing less resources per offspring. Low food females that mated with high food males, on the other hand may have increased fitness by increasing the lipid content of the eggs produced given that lipid reserves are associated with pharate larval quiescence (Clements 1992, Vinogradova et al. 2007) and this may confer an advantage in less predictable environments. If additional lipid confers an advantage, this would support an adaptive parental effects hypothesis. Additionally, it is expected that female reproductive effort would increase in the presence of an attractive mate (Sheldon 2000) and that high quality males are able to transfer more nutrients to the female during mating for use in maintenance and egg production (Boggs 1990). Life history theory also would predict that to maximize fitness the best strategy for two low food parents would be to invest more resources into a fewer number of offspring. While eggs from two low food parents had similar lipid and protein content relative to those from high food mothers, it is possible that a greater portion of total available maternal reserves were dedicated to egg production. However, results from Chapter 2 support other parental nutrition studies demonstrating that two low quality parents produce lower quality offspring less tolerant of environmental stressors (Mousseau and Fox 1998b, Jones and Widemo 2005, and Kyneb and Toft 2006). Future studies should consider the relationship between total available maternal reserves, and egg count and content, and maternal survivorship to better understand female provisioning strategy.

In Chapter 2, evidence of paternal nutrition effects were found, with female offspring development time contributing most to these effects, but only when fathers mated with high food mothers. This is similar to the trend seen with maternal effects where they were only identified when mothers mated with high food fathers. The modern view of the differential allocation hypothesis suggests that parents trade-off current and future reproduction and that mate quality affects this trade-off (Sheldon 2000). In *Ae. aegypti,* the total number of sperm produced is highly correlated with body size, with large males producing significantly more sperm than small males (Ponlawat and Harrington 2007) and transferring more sperm during mating (Ponlawat and Harrington 2009). It would be expected, that if paternal effects were solely due to sperm quantity, these effects would be seen when males mated with low food females as well as high food females. Given that these effects are specific to high food females, it is possible that males alter investment during reproduction in response to female quality, through 'cryptic male mate choice' (Bonduriansky 2001). Additionally, female physiology and behavior are influenced by the transfer of seminal fluid proteins during mating (Fuchs et al. 1969, Klowden 1999) which may contribute to paternal effects identified. It is hypothesized that males should invest more sperm in high quality females than low quality females, but only if males are able to recognize female quality (Reinhold et al. 2002, Wedell et al. 2002). Additionally, males that transfer fewer resources to low quality females, are more likely to retain resources that can be transferred to additional females, potentially increasing fitness (Edward and Chapman 2011). An analysis of ejaculate quantity in arthropods found that increased amounts of ejaculate led to increased fecundity but reductions in life span in females (South and Lewis 2010). It

may be worth considering how ejaculate quantity, female survivorship, and fecundity vary in response to larval nutation. It is important to recognize that present experiments did not allow for mate choice. Specifically, all experiments were controlled in that males and females only were able to choose from either high or low nutrient mates. Mate choice may ultimately influence reproductive output and maternal and paternal effects in this system. Overall, experiments generated support for both maternal and paternal effects in this system on life history traits.

Parental Effects on Infection with Dengue

Overall, there was little evidence to support parental nutrient effects on infection with dengue virus. In Chapter 2, parental larval nutrition did not significantly affect susceptibility to infection with dengue virus, however, after the extrinsic incubation period of dengue virus, titers did vary significantly between high food offspring from high and low food parents. Specifically, high food offspring, from two high food parents, had significantly lower viral titers by more than 50% compared to those from two low food parents. Once a mosquito obtains a dengue infectious blood meal, the virus must infect and replicate in the midgut, disseminate and infect secondary tissues, infect and escape the salivary glands, and enter into the saliva in order for a mosquito to become a potential vector. Throughout this entire process, mosquitoes mount an immune response against the arbovirus. In mosquitoes, the immune system is comprised of the humoral and cellular defense components which work together to defend the mosquito against pathogens. Larval nutrition influences both aspects of the immune system and nutrient stress is linked to decreased numbers of haematocytes and changes in immune related gene expression (Telang et al. 2012). The results from Chapter 2 could be explained by differences in immune response. Future research should consider how

parental nutrition influences offspring immune responses including haematocyte counts, phenoloxidase activity, and/or expression of antimicrobial peptides (e.g. defensin and cecropin) to name a few. Research in *Plodia interpunctella* moths found that parental diet influences offspring phenoloxidase activity and haematocyte count (Triggs and Knell 2012), demonstrating that considering these variables may be a good starting point.

In Chapter 3, parental larval nutrition did not influence offspring dengue titer in any of the four parental mating crosses considered. In Chapter 2, dengue titer was significantly lower in high food offspring from two high food parents relative to high food offspring from two low food parents. However, offspring from two high food parents also developed the most rapidly relative to those from two low food parents. Consequently, improved offspring performance is seen in terms of development and infection indicating potentially higher quality offspring. When considering Chapter 3, high food offspring from two high food parents, did not have faster development or survival and did not vary in terms of viral titer in those that became infected demonstrating a consistent pattern with Chapter 2, despite different results.

There is significant evidence supporting transgenerational immune priming in insects; however, these studies typically consider priming in response to parents challenged with a pathogen. When parents are subjected to an immune challenge, offspring may be more responsive to the same or similar challenge supporting evidence of immune priming in invertebrates (Schmid-Hempel 2005). Immune priming in insects affects not only eggs, but also offspring larvae and adults (reviewed in Trauer-Kizilelma and Hilker 2015) indicating that underlying mechanisms influence the transcription of

immune-related genes (Eggert et al. 2014, Freitak et al. 2009, Freitak et al. 2014, Trauer-Kizilelma and Hilker 2015). If transgenerational immune priming does occur in *Ae. aegypti,* challenging parents and subsequent offspring with similar immune challenges may be the most efficient method of identifying these effects. When considering infection with arboviruses like dengue, measuring transcription of genes associated with the Toll, immune deficiency (IMD), and Janus kinase/signal transducers and activators of transcription (JAK_STAT) pathways (reviewed in Sim et al. 2014) would provide a good indicator of immune response and potential priming.

Maternal and Paternal Effects on Male Progeny

In Chapters 2 and 3, parental larval nutrition primarily influenced female offspring development time. Male progeny were robust to these effects in the traits measured. It is important to note that male development time and offspring survivorship were the only two traits considered. Given the importance of females in the transmission of arboviruses this research focused primarily on female offspring. Despite this, there have been quite a few studies in other insects that have found parental effects on male specific traits (e.g. Bonduriansky and Head 2007, Cator and Harrington 2011, Buzatto et al. 2012, Bonduriansky et al. 2016, Zajitschek et al. 2017). In *Onthophagus taurus,* maternal crowding was associated with the production of larger horned offspring across a wider range of body sizes compared with offspring from mothers reared singly (Buzatto et al. 2012). This species displays male dimorphism which influences the reproductive tactic employed by the individual. Males with horns have elaborate weaponry, fight and guard access to females, consequently larger horns are likely associated with more success with this reproductive tactic (Buzatto et al. 2012). In the fly, *Telostylinus angusticollis,* high condition fathers produced larger male offspring with

increased mating success when reared in low nutrient environments (Bonduriansky and Head 2007). In the fly, *Drosophila melanogaster,* sons of high larval protein fathers fared better during sperm competition compared to sons of low larval protein content fathers (Zajitschek et al. 2017). It was speculated that females may have detected these treatment-induced differences in males and allocated more resources towards reproduction with higher quality males (Zajitschek et al. 2017). Lastly, in *Ae. aegypti* harmonic convergence of fathers with mothers, speculated to be one way males can signal quality in this species, increased mating success of sons (Cator and Harrington 2011). These examples demonstrate that both maternal and paternal effects can influence male secondary sexual characteristics and mating success. Future studies should consider how maternal and paternal effects influence additional male traits in *Ae. aegypti.*

Mechanisms Behind Parental Effects

While parental, maternal, and paternal larval nutrient effects on offspring were considered, potential mechanisms behind these effects were not identified. Allocation of resources towards eggs, was the closest proximate mechanism considered. Despite this, it is clear that there are many different mechanisms that generate parental effects. The proposed mechanisms involved in parental effects insects include egg resource provisioning (Rossiter 1991, Sinervo 1991, Fox et al. 1999, Rolff 1999, McIntyre and Gooding 2000, Freitak et al. 2009, Gibbs et al. 2010, Urbansky et al. 2010b), epigenetic marking (Bongiorni et al. 1999, Bonduriansky and Head 2007, Freitak et al. 2009, MacDonald 2012), sex-specific genomic imprinting (Bongiorni et al. 1999, MacDonald 2012), transfer of symbionts (Moran et al. 2008), transfer of immunodefense factors (Tidbury et al. 2010, Boots and Roberts 2012, Triggs and Knell 2012, Pölkki et al.

2012), and elevation of germline mutation (Bonduriansky and Head 2007) to name a few. Investigations into egg resource provisioning demonstrated that lipid content varied depending upon parental nutrition. In the present study, only egg volume, egg number, lipid content, and protein content were considered. However, eggs contain a variety of other components including carbohydrates, vitamins, minerals, and hormones. Consequently, additional considerations of egg content should be considered in concert with offspring life history traits in an effort to further understand the results of the present studies.

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BIOGRAPHICAL SKETCH

Kylie Zirbel grew up in Rochester, New York and spent most of her childhood playing outside which fostered her interest in nature. This led to Kylie obtaining a Bachelor of Science degree in biology from Le Moyne College. Kylie particularly enjoyed the interdisciplinary nature of her bachelor's degree program which encouraged her to learn about the interplay between science and politics. In pursuit of these interests, and to escape harsh Upstate winters, Kylie moved to Virginia to pursue a Master of Science degree in environmental science and policy from George Mason University. While at GMU, Kylie worked for the Disease Carrying Insects Program in Fairfax County, Virginia which introduced her to the field of medical entomology. After this experience, Kylie continued her trek southbound and joined the entomology program at the University of Florida. Kylie received her Doctor of Philosophy degree in entomology from the University of Florida in the fall of 2017. In her spare time, Kylie enjoys exploring all that the great outdoors of Florida has to offer and going on adventures with her pack of mutts.