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**Environmental context matters: studies of variation in developmental life-history traits in insects**

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# Environmental context matters: studies of variation in developmental life-history traits in insects

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M.E.M., Duke University, 2006

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An abstract of
a dissertation submitted to the Faculty of the
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**Abstract**

Life history traits influence the survival and reproduction of organisms. They can vary across organisms based on previous response to selection and based on plastic responses to abiotic and biotic factors. Here, I examine how life history traits of insects vary in response to environmental conditions and in response to association with microbial symbionts. I introduce life-history theory and traits, how traits vary in response to environment, and the biological systems used in this dissertation. I then use a meta-analytic statistical approach to estimate the variation in life-history traits of the species within the *Culex pipiens* complex. I demonstrate that the temperature of the developmental habitat is a more significant explanatory factor for development rate variation than sub-species identity. The effect of temperature, however, is heterogeneous and the residual variation in development rate is best explained by two factors, density and study methodology. I repeat this approach in another species, *Aedes aegypti*, where environmental context of temperature alone is sufficient to explain development rate variation. To test the results, I assess the impact of three environmental factors over a wide range of values on development in *Ae. aegypti*. Using this empirical approach, I demonstrate that temperature, diet, and density interact to explain developmental life-history traits. I also found that the effect of temperature is mediated by the context of diet and density. Finally, I experimentally consider the impact of an obligate microbial symbiont on development and survival in a hemimetabolous stinkbug reared on two alternative plants. I show that host plant context mediates the impact of symbiosis on its stinkbug host's development. These studies illustrate that environmental context matters for life-history trait variation. The phenotypic expression of these traits is contingent on multiple environmental factors both abiotic and biotic.

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# Chapter 1: Introduction

The environment in which an organism develops impacts its phenotype and, consequently, its fitness (Wolterek, 1909; Schmalhausen, 1949; Van Valen, 1965; Roughgarden, 1975; Lynch & Gabriel, 1987). In this dissertation, I explore how environmental conditions during development alter life-history traits of organisms. I assess the relative importance of multiple environmental factors to explain variation in these traits. In addition, I examine how environments alter the ecological interactions of organisms, specifically symbiotic associations between a host and its obligate symbiont.

I focus on the developmental traits of insects of medical and agricultural importance. Chapters 2 and 3 identify the most important factors impacting development rate variation in *Culex pipiens s.l.* Say and *Aedes aegypti* Linnaeus. Based on these results, I focus on temperature, diet, and density as the critical environmental factors and explore these in another mosquito species amenable to laboratory experimentation, described in Chapter 4. In Chapter 5, I examine how environment can alter the impact of symbiosis on the life-history of *Megacopta cribraria* Fabricius. In my concluding chapter, I briefly summarize key findings of my work and propose future avenues of research.

## 1.1 Variation in life-history traits

"Life-history theory" in evolutionary biology (Roff, 1992) broadly examines how organisms live, die, and reproduce and how this has been shaped by natural selection. Life history-traits are phenotypes that are directly tied to reproduction and survival, such as age at maturation, body size, and fecundity (Stearns, 1989). They are considered defining characteristics of a species, vary within species (Nylin & Gotthard, 1998), and vary in

response to environment (Arnqvist & Johansson, 1998; Bonduriansky *et al.* 2011). Reznick and Endler (1982) found differences in life-history traits such as size at maturation, frequency of reproduction, and size of litter based on the locality and the presence or absence of predators.

Phenotypic variation within a species can be loosely divided as polymorphism (i.e. genetic) or polyphenism[1] (i.e. plasticity), or a combination. Both can underlie differences in life history traits. Variation in ovulation frequency, litter size, lamb growth and development in merino sheep, for example, is a genetic polymorphism associated with variation at a single locus, the FecB gene (Guan *et al*., 2006). Polyphenism in life-history traits is classically demonstrated in aphids in response to the alarm pheromone (E)-β-farnesene (Bowers et al. 1972). In response to environmental triggers, aphids secrete (E)-β-farnesene, which can induce the production of winged offspring (Hatano *et al*. 2010). The morphological differences between winged and wingless phenotypes have been correlated with differences in life history including longer nymphal development (Noda, 1960) and lower offspring production (Campbell & Mackauer, 1977). Polymorphism and polyphenism, may also interact to produce phenotypic variation (Via & Lande, 1995). Braendle *et al*. (2005) examined the environmentally insensitive sex-linked gene, *api*, responsible for the production of winged male aphids. Clones of females with different genotypes of *api* differed in their propensity to produce winged asexual female offspring, indicating a genetic linkage between the factors that control female wing polyphenism and male wing polymorphism.

Plasticity of a phenotype is measured by the extent of change in phenotype over a range of an environmental parameter is a measure of the plasticity of the trait. Phenotypic

[1] Although polyphenism is sometimes specifically used to define traits that show discrete as opposed to continuous variation. Here this term is used in its most general sense to describe phenotypic plasticity, or environmentally contingent phenotypic expression.

plasticity is defined by the profile of phenotypes of a single genotype across the environment, known as the norm of reaction (Wolterek, 1909; Schmalhausen, 1949). For example, the traits of body size and growth show phenotypic plasticity in response to the environmental parameter of temperature, a relationship that has been studied in a wide array of taxa over the last century (Magoon & Culpepper, 1932; Stier & Newton, 1939; Madariaga & Knott, 1951; Lana & Haber, 1952; Kitchen, 1956; Ahlgren, 1987; Gillooly, 2001; Gillooly, 2002; Thomas & Blanford, 2003).

Norms of reaction have traditionally been studied at the static end of ontogeny, the adult form in animals (Arnqvist and Johannson 1998). Examining how phenotypes unfold during development can deepen our understanding of phenotypic plasticity (Pigliucci & Schlichting, 1995), yet there remains a paucity of empirical studies of ontogenetic plasticity of traits in natural populations (Schlichting & Pigliucci 1995). In this work, I compare studies that have considered the plasticity of both morphology and rate of progression throughout the developmental period of organisms. The life-history traits considered here include the duration or rate of development to each juvenile stage, juvenile survival to maturation, and body size at maturation.

### 1.2 Environment and life-history trait variation

Environmental conditions can alter phenotypes of even genetically identical individuals (Wolterek, 1909; Stearns *et al*.,1986; Berrigan, 1994; Gunay *et al*., 2010). From an adaptationist perspective, plasticity of life-history traits may be advantageous when organisms live in heterogenous and changing environments (Stearns *et al*., 1986). Evidence suggests organisms adaptively respond to environmental conditions at different life-stages (Carriere & Roff, 1995, Messina & Fox, 2001, Gillooly *et al*., 2002, Schwander & Leimar,

2011). For example, the tradeoff between clutch size and offspring size has been shown to differ in grasshoppers due to environmental selection based on temperature and diet conditions. Under favorable thermal and feeding conditions maximum fecundity was shown to occur when individuals produced the largest clutches of the smallest eggs, but under poor conditions maternal fitness was optimal when individuals produced small clutches of very large offspring (Hassall *et al*., 2006, Walters & Hassall, 2006).

Due to finite resources, life-history theory assumes that traits have costs (in fitness units) that are traded off against some other fitness components (Sheldon & Verhulst, 1996; Stearns 1989). The tradeoff between fecundity and somatic growth has been empirically measured in many diverse taxa (Honěk, 1993; reviewed in Zera & Harshman, 2001). The trade-off between fecundity and immune defense is evident in big-horn ewes in which parasite loads are higher in lactating versus non-lactating females (Festa-Blanchet, 1996). This suggests that during lactation resources are allocated toward reproduction rather than pathogen defense.

Life-history tradeoffs are not always apparent (Lazzaro *et al*., 2008), and may only become evident in certain environmental contexts (Schmid-Hempel, 2003). In free-flying bumble bees, *Bombus terrestrus*, the energetically costly activity of foraging effort has been shown to reduce resistance to parasitic attack (Konig & Schmid-Hempel, 1995), an effect not observed in captive bees under nutritional stress (Schmid-Hempel & Schmid-Hempel, 1998). Thus, depending on the environmental context, life-history trade-offs may be dampened (Gonzalez *et al*., 1999) or exacerbated (Hasselquist *et al*., 2001), or we may not know the proper environmental condition to identify its impact on a particular trade-off (Schmid-Hempel & Schmid-Hempel, 1998).

Among the environmental conditions considered that impact life-history traits and trade-offs, the factor of temperature has been heavily emphasized (Wolinska & King, 2009). This emphasis may be justified, especially in ectothermic organisms for whom metabolism is directly influenced by temperature (Kingsolver & Huey, 2008). However, there are a variety of other environmental factors, both biotic and abiotic, that influence life-history traits, generally grouped in life-history theory based on density-dependence or independence (Stearns, 1989). In Chapters 2 and 3, I explore the many factors other than temperature that may influence developmental life-history traits. There may always be environmental factors as yet unidentified shaping phenotypic variation and evolution. Famously, periodical cicadas have long and perfectly synchronous life-cycles occurring in prime numbers (i.e. 13 and 17 year cycles) that have not been fully explained by hypotheses of predator satiation, larval competition, and escape of predator life-cycles (reviewed in Williams & Simon, 1995; Yoshimura, 1997).

### 1.3 Environment and symbiosis

Environmental factors can go beyond influencing the life-history of organisms to mediating the outcome of their biotic interactions (Agrawal *et al.,* 2007). Identifying the mechanisms by which this occurs is central to our understanding of community ecology. For example, many studies have examined how environmental context mediates competitive interactions between species in the same guild (Chesson & Warner, 1981; Wiens, 1977; Hutchinson, 1961; Park, 1954). Fewer studies focus on mutualistic interactions, with notable exceptions (Piculell *et al*., 2008). It is hypothesized that the environment alters the costs and benefits to the host of harboring bacterial symbionts. In the legume-rhizobia mutualism, before the root nodule formation is initiated, soil temperature within the root zone influences

rhizobial bacteria survival in the soil as well as the exchange of molecular signals between the two symbiotic partners (Sadowsky, 2005). Further, rhizobia-plant associations are altered by nitrogen levels in soil due to added fertilizers, as these environments select for mycorrhizal fungi that are less beneficial or even parasitic on their host plants (Johnson, 1993; Johnson *et al*. 1997). Similarly, endophytic fungi, a highly diverse group of microorganisms ubiquitous in plant tissues, have interactions with hosts that range from mutualistic to antagonistic (Faeth & Bultman, 2002). Faeth and Fagen (2002) modeled the costs and benefits of harboring endophytic fungi to hosts, suggesting that benefits change along soil nitrogen gradients. Experimental results provide some evidence to support that plant growth and volume was mediated by endophytic fungal infection in conjunction with plant genotype (Faeth & Fagen, 2002).

Just as environment can alter host-microbe interactions, microbial partners, in turn, can dramatically impact life-history traits of their hosts. Studies comparing insects with and without their symbionts show that symbionts can alter host development rate, final body size and survival (de Vries *et al*., 2004; Prado & Almeida, 2009; Prado *et al*., 2009; Brownlie & Johnson, 2009). Changes in microbial symbionts can also alter other important traits such as heat tolerance (Dunbar *et al*., 2007), immunity (Oliver *et al*., 2005), and mating behavior (Miller *et al*., 2010). These symbionts can form lasting evolutionary relationships with their hosts, as with *Buchnera aphidicola*, a bacterial symbiont of aphids, which produces essential amino acids and other nutrients that are in short supply in plant phloem sap (Douglas, 1993; Sandström & Pettersson, 1994; Baumann *et al*., 1995; Sandström & Moran, 1999). Symbionts can also be dynamic partners, entering and leaving populations (Schlulenberg *et al*., 2002; Turelli & Hoffman, 1991). The stability and fate of these interactions may be

mediated by the benefits to host life-history traits. For example, in a study of the sweet potato whitefly, *Bemisia abaci,* Himler *et al*. (2011) found a sweep of symbiont infection through the host population within six years. Symbiont-infected whiteflies were shown to develop faster, survive at higher rates to adulthood, produce more offspring, and produce more daughters. Thus, it may be important to consider both symbionts and environment when examining life-history trait variation.

### 1.4 Mosquito study systems

This research spans three insect study systems that are of medical or agricultural importance. Among these, I chose two mosquito species of public health importance with wide geographical ranges, *Cx. pipiens s.l.* and *Ae. aegypti*. Because of the public health impact of these insects, rearing experiments have been conducted over many decades and throughout their ranges. This provided the opportunity to consider many factors over a wider range of each factor (described in Chapters 2 and 3).

In addition to being major vectors of pathogens, mosquitoes are model insects to explore plasticity of fitness related traits in the laboratory where the aquatic environment can be contained and manipulated. Their developmental period of 1-2 weeks allows for multiple developmental habitat scenarios to be explored in a reasonable time period. In mosquitoes, life history traits are especially responsive to changes in the environment (Nylin & Gotthard 1998). Mosquitoes undergo complex metamorphosis as well as a shift from aquatic to terrestrial habitats, resulting in stage-specific environmental impacts. This allows ontogenetic phenotypic variation to be considered as well as the consequent impacts on adult life-history trait variation.

Natural aquatic environments may be heterogeneous and stressful to mosquito larvae, which are subject to competition for resources and to habitat desiccation (Costanzo *et al*., 2005a; Juliano & Stoffregen, 1994). Ecological differences between species may influence these dynamics. For example, small water volume breeders such as *Ae. albopictus* have been shown to be more tolerant of higher larval density than large water volume breeders like *Cx. pipiens s.l.* (Carrieri *et al*., 2003) and *Cx.* species generally inhabit polluted water rich in organic matter (Chaves et al., 2011).

Adaptive phenotypic plasticity may be highly favored across the heterogeneous developmental environments of these insects. Recent evidence suggests that mosquitoes developing in highly unpredictable environments may adjust developmental patterns based on environmental cues (Costanzo *et al*., 2005a; Aubin-Horth & Renn, 2009). For example, *Ae.* spp. larvae have been shown to actively monitor water volume and accelerate development in response to habitat deterioration (Constanzo *et al*., 2005b) and predation (Kesavaraju & Juliano, 2004). Through experimental manipulation of the environmental cues of the aquatic environment during development, it may be possible to determine the extent to which each environmental factor can moderate certain life-history tradeoffs. For example, Padmanabha *et al*. (2011) have shown a trade-off between development rate and juvenile survival in response to starvation is dependent on temperature in *Ae. aegypti*.

In Chapter 2, I examine impact of temperature and other environmental conditions, including resource availability and intraspecific competition, on larval development of *Cx. pipiens s.l.* through a meta-analysis. I find that while temperature is the most important factor, other factors such as density, sex, and study methodology are also critical in explaining variation in development rate and juvenile survival of these insects. I also test the

hypothesis that the development curve is a basic feature of the species, and show that variability in development rate of these insects appears to be primarily driven by response to certain environmental conditions rather than differences between populations.

Based on the results in Chapter 2, I considered the impact of temperature, intraspecific density, and diet in a second mosquito vector, *Ae. aegypti*. Whereas a lack of reporting of methods limited the analysis in *Cx. pipiens s.l.*, I was able to explicitly consider the environmental context of resource availability both in diet amount and type for *Ae. aegypti*. I found that temperature was sufficient to explain development rate variability. As with *Cx. pipiens*, the effect of temperature on development rate was not found to be homogenous or constant. The sources of heterogeneity of the effect of temperature were difficult to analyze due to lack of consistent reporting of larval rearing methods.

In Chapter 4 I tested the hypothesis that multiple environmental factors interact to predict developmental life history trait variation in a rearing experiment of *Ae. aegypti*. I found that the factors of temperature, amount of food, intraspecific rearing density, and their interactions were significant predictors of development rate variation, whereas temperature alone was sufficient to predict juvenile mortality.

## 1.5 Megacopta cribraria study system

The outcomes of many parasitic interactions are known to be altered by both abiotic and biotic factors (Blanford *et al*. 2003; Mitchell *et al*. 2005; Vale *et al*. 2011; Bryner & Rigling 2011). In contrast, for obligate, mutualistic symbioses, benefits to the host are assumed to be universal. Due to a distinct feature of their biology, *Megacopta cribraria* ('kudzu bug'), stink bugs of the family Plataspididae, are becoming a model system for examining the impact of obligate symbionts (Hosokawa *et al*., 2006). The transfer of symbiotic γ-proteobacteria,

*Candidatus* Ishikawaella capsulata, from one generation to the next is vertical through the use of a protein 'symbiont capsule' (Fukatsu & Hosokawa, 2002), manufactured by the mother, which is loaded with symbionts and deposited with the egg mass. This allows for convenient study of the developmental life-history traits of *M. cribraria* with and without the symbiont.

In the *M. cribraria*-Ishikawaella system, I examine whether environment alters the benefits of the symbiosis for insect hosts. I experimentally consider alternative host plants (kudzu and soybeans) as an ecological context. This factor is of particular interest for several reasons. First, this species recently invaded North America in 2009. Since then it has spread throughout the southeastern United States. The impact of ecological conditions is of particular interest when a host and its associated microbes invade a new habitat. In such cases, symbiotic partners (e.g. fungi, nematodes, bacteria or viruses) can facilitate the establishment and population increase of their insect vectors in novel habitats (Jiu *et al*. 2007; Himler *et al*. 2011; Lu *et al*. 2011; Zhao *et al*. 2013). This system provides the opportunity to consider the role of the novel habitat in shaping the host-microbe interaction.

Second, in their native range, a closely related sister species, *M. punctatissima,* is a major agricultural pest of soybean. Experimental inoculation of *M. cribraria* with the symbionts of *M. punctatissima* confers the ability of offspring to develop on soybean (Fukatsu & Hosokawa, 2002). Although it is not the main emphasis of this work, the results may offer insight into the management of *M. cribraria* as a potential agricultural pest.

In Chapter 5, I examine the impact of the symbiont on the life-history trait variation of *M. cribraria* during development, and observed this effect across alternative leguminous host plants in an outdoor field experiment and under environmentally controlled laboratory

conditions. I find that differences in critical life-history traits of development time, body size, and survival in hosts reared with and without their microbial symbiont are mediated by the ecological context of the plant on which the insects develop, supporting the hypothesis that the symbiosis may be environmentally context dependent.

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## Chapter 2: Variation in development rate and survival in *Culex pipiens s.l.*

**Modified from**: Couret, J. 2013. Meta-Analysis of Factors Affecting Ontogenetic Development Rate in the *Culex pipiens* (Diptera: Culicidae) Complex. *Environmental Entomology*. 42 (4), 614-626 (http://www.bioone.org/doi/full/10.1603/EN12248)

### Abstract

Meta-analysis of 33 studies of developmental timing of *Cx. pipiens s.l.* Linnaeus demonstrates that development rate, or the rate of progression through immature life stadia, is primarily driven by temperature, whereas immature survival is driven by temperature, density, and variability in the environmental conditions. As expected, the linear relationship of temperature and development rate is positive for the larval period as well as development to adult emergence. However, the strength of this association varies significantly. Variation in development rate can be explained using additional environmental factors of intraspecific rearing density, sex, and study methodology. Heterogeneity in development rates even once temperature has been considered emphasizes the need for further research of multiple environmental factors and in changing environments. Immature survival is also significantly impacted by variability in environmental conditions. Development rates vary between subspecies of *Cx. pipiens*, but these population differences are no longer significant once an environmental factor of temperature is considered. Thus, variability in development rate of these insects appears to be primarily driven by response to certain environmental conditions rather than differences between populations. Broad patterns of phenotypic variation across latitude and 96 years of empirical estimates were not significant once environmental rearing conditions had been considered.

## 2.1 Introduction

Environmental variation influences the biotic world at every scale. At the global scale, anthropogenic environment change influences critical aspects of life including community composition (Lavergne *et al*. 2010), organismal phenology, and species ranges (McMahon *et al*. 2011). Shifting temperatures at the regional scale have been associated with changing species abundance of invertebrates (Southward *et al*. 1995), and growth and metabolic rate in ectotherms (Portner *et al*. 2008). Fine-scale fluctuations in environmental conditions such as temperature can impact the development and survival of organisms, a pattern in insects that has drawn attention for decades (Janisch 1932, Krafka 1921, Messenger 1959, Howe 1967, Rueda *et al*. 1990; Kingsolver & Huey 2008). With current predictions of rising temperatures (IPCC 2007), there is a need to understand the inevitable ecological and evolutionary consequences of environmental variation at several scales for the development and survival of organisms (West-Eberhard, 1989; Caswell 1983; Stearns & Koella 1986; Schwander & Leimar 2011; Schneider *et al*. 2011).

Response to environment during development can be highly plastic in many insects (Nylin & Gottard 1983), and this plasticity is a potential mechanism by which organisms can maximize fitness in an inconstant world. In particular, the adjustment of developmental timing can directly impact survival, especially in organisms subject to high intraspecific competition during development (Peters & Barbosa 1977). Historically, few studies have empirically estimated plasticity of developmental timing in response to multiple environmental conditions (Schwander & Leimar 2011). Instead, the focus of both empirical study (Hopp & Foley 2001; Portner *et al*. 2008, Fusco & Minelli 2010; Dell, Pawar & Savage 2011) and modeling (Damos & Savopoulou-Soultani 2012; Worner 1993) has been

primarily on temperature. While the general relationship of temperature with developmental timing is well established in ectotherms (Kingsolver & Huey 2008, Gilooly *et al*. 2001), there is a paucity of empirical data on specific responses of organisms to multiple environmental conditions and across gradients of these conditions (Flenner *et al*. 2010), with notable exceptions (Olejnicek & Gelbic 2000; Yoshioka *et al*. 2012). In the natural environment, other conditions such as resources quality and quantity and density also have biological relevance and consequences for development rate and survival and yet are rarely considered in modeling. Temperature alone may be insufficient to understand the variation in development rates of organisms in the context of heterogeneous and variable environments (Tanigoshi & Logan 1979; Chavez & Kitron 2011).

Empirical data are needed that consider development rates 1) in response to multiple factors, 2) over a gradient of each environmental factor, and 3) at different scales across space (i.e. latitude) and time (i.e. long term climate change; Uller 2008). A dataset of this broad scope is difficult to produce within one experiment, but data may be compiled through meta-analysis of developmental studies. Such a dataset is compiled here for *Cx. pipiens s.l.*, or the common house mosquito. *Cx. pipiens s.l.* is considered a species complex, consisting of five main sub-species (*Cx. p. molestus* Forskal, *Cx. p. pallens* Coquillett, *Cx. p. pipien*s Linnaeus, *Cx. p. quinquefasciatus* Say, *Cx. p. fatigans* Wiedemann) over many regions. This facilitates the detection of broad patterns of phenotypic variation between subspecies populations over geographic regions, across latitudinal gradients, and spanning a century of research. Further, much of the empirical research of this mosquito vector focuses on the developmental period, allowing multiple conditions of the rearing environment to be

compared and evaluated across studies for their impact on the rate of development and survival.

Mosquito development is a topic for which more empirical studies have been conducted with respect to multiple environmental factors (Mogi, 1992; Tun-Lin *et al*. 2000; Teng & Apperson 2000). This is perhaps because the habitat of the immature stages of *Cx. pipiens s.l*. is discrete pools of water, and this life-history feature facilitates experimental manipulation of the developmental environment. Taken individually, studies of *Cx. pipiens s.l*. development mainly address either one or few environmental conditions, or do not address gradients of these conditions (Table 1). However, in combination these data provide estimates over wide ranges of conditions as well as over wider geographic space. This combined dataset lends itself to meta-analysis of mosquito developmental phenotypes. *Cx. pipiens s.l*. is particularly well studied because it is a vector of several human pathogens such as West Nile virus (Turell *et al*. 2001), St. Louis encephalitis (Bailey *et al*. 1978) and Rift Valley fever (Amraoui *et al* 2012). While many studies estimate developmental phenotypes such as rate and survival for *Cx. pipiens s.l*., the impact of temperature is not consistently significant (Shim *et al*. 1989; Rueda *et al*. 1990). Also, the relative importance of the many factors identified as important by empirical analysis has not been established. The public health significance of this species complex underlies the need to have overall estimates of development rate and rate variation in a wide range of environments. Moreover, the combined information from many studies of developmental response of this species under different environmental conditions can be used to better predict phenotypic responses to a rapidly changing environment.

Here, developmental rate and survival are estimated within and between populations based on meta-analysis of empirical studies for *Cx. pipiens s.l.*. While temperature has a known impact on development rate and survival (Rueda *et al*. 1990), I hypothesize that other environmental conditions are necessary to adequately predict and explain variability in these phenotypes. Using meta-analysis, I provide an overall estimate of development rate across a wide range of environmental conditions, determine the relative importance of each factor, and look for broad patterns of development rate across a wide latitudinal range.

## 2.2 Methods

To compare studies using meta-analysis, the effect measure is the linear relationship of temperature and development rate based on linear regression, using the parameters of slope and intercept weighted by study sample variance. This effect measure provides an overall effect estimate for temperature on development rate for each study as well as by subspecies, and an estimate of its variability. I focus on the development rate from first instar larva to adult emergence, as well as the nested development rate for only the larval stages. Because the immature life-stages of *Cx. pipiens s.l.* are subject to competition for resources in a discrete aquatic habitat that is at risk for habitat desiccation, plasticity in developmental timing during these stages may be particularly advantageous (Nylin & Gotthard 1998; Schneider *et al*. 2011). These rates are compared within and between sub-species to estimate the phenotypic response to several different environmental factors and across broad geographic regions and latitudinal clines.

*Literature Search*

I conducted an online search in April 2011 of several databases ranging in scope from general to specialized on mosquito related topics (e.g., NAOsite; see supplementary Table

S2.1). Keywords included: *Cx*., development, life cycle, bionomics, temperature, mosquito, temperature-dependent, larva, larvae, and larval. This search yielded studies in several languages resulting in the inclusion of studies published in French, Spanish, Portuguese, Japanese, and Korean. A preliminary list was subject to further examination by two specialists on *Cx*. mosquito ecology.

*Inclusion criteria and data extraction*

Inclusion is contingent upon reporting of (a) either the mean developmental time, measured in hours or days, or the rate of developmental change from first instar to the pupa stage or to adult emergence; (b) rearing temperature and density (i.e. the number of larvae per volume of water); (c) the number of independent replicates per estimate, and a measure of estimate variability [S.E., S.D. or Variance]; (d) the subspecies: *Cx. pipiens pipiens, Cx. pipiens pallens , Cx. pipiens molestus,* and *Cx. pipiens quinquefasciatus* (here considered equivalent to *Cx. pipiens fatigans*) or *Cx. pipiens sensu latu*; (e) the latitude of origin and, (f) whether the larvae were reared in a constant or variable temperature. Studies were designated as field or laboratory experiments based on the study design and location. When available, the variables of the sex of the emerging adult mosquitoes and temperature-specific survival estimates were considered as well. Data from tables and text were directly entered into spreadsheets, and data from plots were extracted using the ADS's Dexter Data Extraction Applet and ImageJ Software.

*Statistical Analysis*

Experiments for insects have demonstrated an empirical relationship between developmental time and temperature (Atkinson 1995). This relationship can be described by the following equation for thermal summation:

$$D(T - t) = k \qquad (2.1)$$

The development time (in days) is a function of the accumulated temperature degrees (T - t) above the developmental zero, t, and k, a constant for the cumulative effective temperature (Ikemoto 2000, 2005). The developmental zero, t, is the theoretical temperature at which, development ceases. Equation 2.2 can transform development times into rates (Briére *et al*. 1999, Dixon *et al*. 2009), re-written as:

$$\frac{1}{D} = -\frac{t}{k} + \frac{1}{k}T \qquad (2.2)$$

Where the developmental rate, i.e., the inverse of developmental time (1/D), is a linear function of temperature (T). For this model, the development rate (y = 1/*D*) is regressed on temperature (x = *T):*

$$y = B_1 x + B_0 \qquad (2.3)$$

The regression parameter of $B_1$ is the measure of effect of temperature on development rate and is the basis for statistical comparison between studies that estimated development over three or more temperatures.

Using regression parameter estimates, main effects were tested for heterogeneity using the general linear-mixed effects model on the parameters of B1 and B0 of equation (3) for the larval period up to pupation and for the period to adult emergence using the metafor package in R. This method assumes that a given set of independent effect-size estimates is a function of an unknown true effect, moderators (i.e., the meta-analysis term for covariates that could explain heterogeneity in the effect size estimates), and random variation (Vietchbauer, 2010). Thus, I fitted models of the form:

$$\hat{B}1_i = B1 + Moderator + \sigma \qquad (2.4)$$

The vector $\hat{B}1_i$ includes the regression estimates from the different studies, and B1 is the unknown true parameter. Moderator is any of the covariate(s), and $\sigma^2$ is the variance of the random variability affecting parameter estimation. The best moderator(s) were selected based on a forward process model selection, first fitting a random effects model, i.e., with no moderators. In cases with a Hedges Q statistic (Gurevitch *et al*. 2001) sufficient to reject the null hypothesis of homogeneity in the estimates, each available moderator was individually tested. Moderators included sub-species, study author, latitude, density, and sex (the latter only included in development rate to adult emergence). If more than one of these factors was significant, these were also tested in combination, followed by a backward elimination of non-significant factors (Faraway 2006) and Hedges Q statistics for heterogeneity in the residuals, and in any of the moderators (Vietchbauer 2010).

Instantaneous development rates (i.e. inverse development time) for each temperature, here called temperature specific estimates (TSES), were modeled with a linear mixed effects model (Pinheiro & Bates 2000) to account for both the variability across studies and the lack of independence of TSES data coming from studies with similar experimental conditions (Chaves 2010). A linear mixed effects model of TSES allows an explanation of the patterns in a response variable as a function of covariates, unknown random variation, and variability arising from known sources that condition the independence of the observations (Chaves 2010). For example, observations coming from the same studies could be more similar than those coming from different studies. Thus, models of TSES data were fitted (separately for the full developmental period and for the larval stages only) with the following general form:

$$a_i = \mu + B1\mathrm{T}_i + other + \tau + \sigma_i \qquad (2.5)$$

Here, $a_i$ is a single TSES point, $\mu$ is an intercept, B1 is the parameter relating developmental rate with temperature (Ti), other represents any of the covariate(s) we collected (environmental variability, latitude and density in both pupation and emergence, and also sex for the latter), $\tau^2$ represents the variance for the different sources of non-random variability (Study author and subspecies) and $\sigma^2$ is the identical, independent and normally distributed error variance affecting the observations.

The best linear mixed effects models for the TSES were chosen through backward elimination, first fitting a full model (i.e., a model with all the covariates collected) and using the Akaike and Bayes information criteria (AIC and BIC) minimum values to eliminate covariates (Faraway 2004), repeating this process until values for the selection criteria reached a minimum (Venables & Ripley 2002). Models were fitted by restricted maximum likelihood (REML), and parameter inference for the best model was based on a Markov Chain Monte Carlo (MCMC) where uninformative priors were assigned to model parameters and credible limits were generated by sampling the posterior distribution of the samples generated via MCMC (Gilks, Richardson & Spiegelhalter 1996).

## 2.3 Results

The literature search yielded 33 papers and a book chapter fitting the inclusion criteria (Table 2.1, Table S2.2), providing 287 estimates of developmental timing under different environmental conditions (Table S2.3).

| Study | Temperature | Density | Across a gradient? | Sex | Latitude | Subspecies |
|---|---|---|---|---|---|---|
| Kramer 1915 | ✓ | | ✓ | | | |
| De Boisseson 1930 | ✓ | | ✓ | | | |
| De Boisseson 1933 | ✓ | | ✓ | | | |
| Headlee 1942 | ✓ | | ✓ | | | |
| Tekle 1960 | ✓ | | ✓ | | | ✓ |
| Kurihara 1963 | ✓ | | | | | |
| Ishii 1963 | ✓ | ✓ | ✓ | | | |
| Sasa et al. 1965 | ✓ | | | ✓ | | |
| Nayar & Sauerman 1970 | ✓ | | | | | |
| Rosay 1973 | ✓ | | | | | ✓ |
| Shelton 1973 | ✓ | | ✓ | | | |
| Hayes & Hsi 1975 | ✓ | | ✓ | | | |
| Kurihara & Ichimori 1975 | ✓ | | ✓ | | | |
| Gomez et al. 1977 | | ✓ | | | | ✓ |
| Dadd et al. 1977 | ✓ | | | ✓ | | |
| Lobvoka 1980 | ✓ | | ✓ | | | |
| Suleman 1982 | ✓ | ✓ | ✓ | ✓ | | |
| Madder et al. 1983 | ✓ | ✓ | ✓ | | | |
| Mead & Conner 1987 | ✓ | | ✓ | | | |
| Mori et al. 1988 | ✓ | | ✓ | | ✓ | ✓ |
| Shim et al. 1989 | ✓ | | ✓ | | | ✓ |
| Rueda et al. 1990 | ✓ | | ✓ | | | |
| Mogi 1992 | ✓ | | ✓ | ✓ | ✓ | |
| Costa et al. 1994 | ✓ | | ✓ | | | |
| Vianna et al. 1996 | ✓ | | ✓ | | | |
| Olejnicek & Gelbic 2000 | | ✓ | ✓ | ✓ | | |
| Agnew et al. 2000 | ✓ | ✓ | ✓ | ✓ | | ✓ |
| Salazar & Moncada 2004 | ✓ | | | | | |
| Alves et al. 2004 | ✓ | | ✓ | | | |
| Ribeiro et al. 2004 | ✓ | ✓ | | | | |
| Garcia et al. 2010 | ✓ | | ✓ | | | |
| Roberts & Kokkin 2010 | | ✓ | ✓ | | | |
| Chaves et al. 2011 | | ✓ | ✓ | ✓ | | |

**Table 2.1 Empirical studies in the meta-analysis, the study factors considered in each, and whether temperatures or densities were considered across a gradient of rearing conditions**

Of the studies included, 24 of 33 consider a single environmental factor (72.7%). Approximately 88% (n=29) of studies include temperature (alone or with another factor), and 27% of studies consider density (n=4). Seven studies (21%) include sex ratios when reporting development rates or times. Six of 33 (18%) experimental studies estimate development rate in more than one subspecies. Of the eight studies considering two or more factors concurrently, all of these examined gradients of the factors over three or more levels. Only one study considers three factors of temperature, density, and sex (Olejnicek & Gelbic 2000). Although many studies reported some information on diet, generally there was not information on exact amounts or nutritional information of the diet. The larval diet varied in composition and included yeast, beef liver, rabbit food, or dog food. Further, feeding

schedules and regimens varied widely, and all of these issues impeded detailed analysis of this factor. Amount of food was generally reported to be *ad libitum*.

Across all studies the average development time in days for *Cx. pipiens s.l.* to mature to the pupal stage is 16.82 ± 0.73 SE and average time to adult emergence is 19.94 ± 1.99 SE days. Average development times range widely (Table S2.4) and differ significantly between subspecies (ANOVA, F=12.22, p < 8.82e-09). However, sub-species is no longer a significant explanatory factor once the other factors of temperature, intraspecific rearing density, and latitude have been considered (Table 2.2).

| Model | Factor | Estimate | 95% HPD[a] |
|---|---|---|---|
| Pupation[b] | Intercept | −0.070 | −0.0826 to −0.0460 |
| | Temperature | 0.0074 | 0.0064–0.0078 |
| Emergence[c] | Intercept | −0.031 | −0.041 to −0.016 |
| | Temperature | 0.0048 | 0.0042–0.0051 |
| | Density | −0.007 | −0.013 to −0.005 |

**Table 2.2 Parameter estimates for linear mixed effects models considering the impacts of Temperature and other factors on *Cx. pipiens s.l.* developmental period up to pupation and up to emergence.**

[1]HPD: high posterior density, which can be considered the Bayesian equivalent to Maximum Likelihood confidence limits. [2]This model considered interaction between species and study as random factor. Error variance was $2.55 \times 10^{-4}$ (77 d.f.), study variance was $17.3 \times 10^{-4}$ (24 d.f.) and species variance conditioned on the studies was $4.67 \times 10^{-4}$ (30 d.f.). [3]This model considered study as random factor. Error variance was$1.6 \times 10^{-4}$ (186 d.f.), study variance was $7.4 \times 10^{-4}$ (21 d.f.)

No broad patterns were observed in either of the two phenotypes considered across space or time. Latitude of mosquito strain origin was considered over a range from -36.91° to 63.75° based on coordinates reported in the literature when available and otherwise study location. Neither the development rate during the larval stage up to pupation was significant over latitude ($F_{1,133} = 0.0008507$ , $p = 0.9768$; Fig. 2.1, panel A), nor the development rate from embryo to adult emergence ($F_{1,209} = 0.4429$, $p = 0.5065$; Fig. 2.1, panel B). Further there is no discernable difference between ontogenetic development rates in the northern versus southern hemispheres (Fig. 2.1). Finally, there is no detectable temporal trend in

development rates when comparing estimates across a time span of 96 years of published research ($F_{1,206} = 0.07986$, $p = 0.7778$).

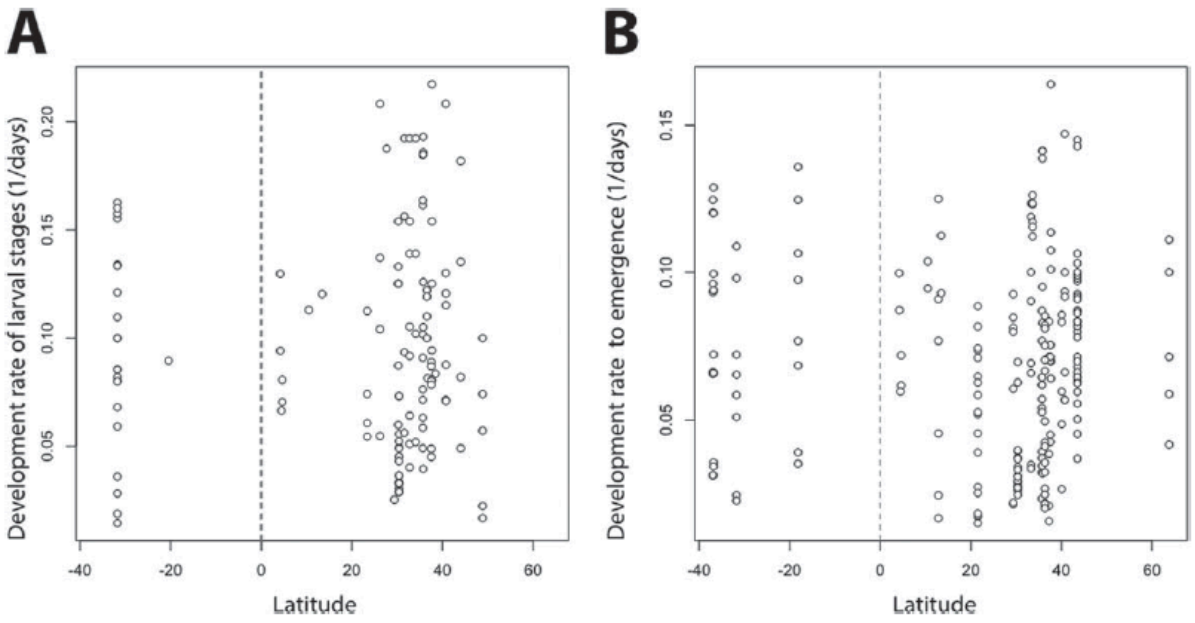


**Figure 1.1 Developmental rate data over latitudinal degrees**
(A) Developmental rate up to the pupal stage and (B) up to adult emergence as a function of latitude.

The first step of the meta-analysis considered the slope and intercept of the relationship between temperature and development rate as the effect measure. Six papers contained effect measures estimates for development during the larval period up to pupation, resulting in 11 independent estimates for comparison (Table S2.5; for parameter estimates see Table S2.6). The cumulative effect measure as estimated across studies for the larval period is positive overall, indicating decreased development time with increased rearing temperatures (Fig. 2.2, panels A through D). For the full developmental period to adult emergence, 18 papers provided effect measure estimates (Table S2.7; for parameter estimates see Table S2.8).

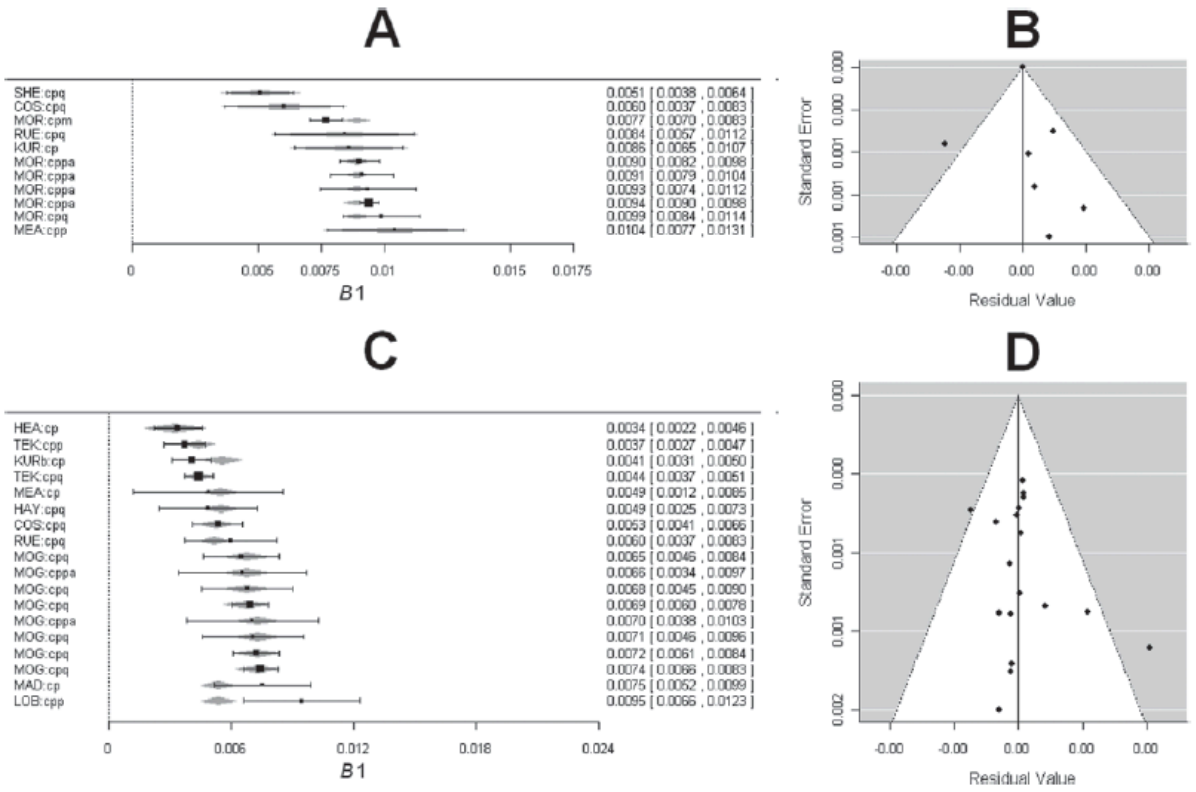


**Figure 2.2 Meta-analysis the effect measure, i.e. the slope of the regression of temperature and development rate.**
(A) Forest plot for rate of development of the larval stages up to pupation. (B) Corresponding funnel plot for (A). (C) Forest plot for rate up to adult emergence. (D) Corresponding funnel plot for (B). A three-letter author code for each study is shown (see Table S2) on the left of the forest plots, followed by a code for the subspecies: *Cx. pipiens s.l.* (cp), *Cx. pipiens quinquefasciatus* (cpq), *Cx. pipiens pallens* (cppa), *Cx. pipiens pipiens* (cpp), *Cx. pipiens molestus* (cpm). Squares represent effect estimates of individual studies. Square size represents the weight given to the study in the meta-analysis, and the horizontal lines represent 95% confidence intervals. The diamond represents the estimate and its 95% confidence intervals according to the model, with the estimated values written to the right of the plot. In the funnel plots, dots represent the residuals of the model presented in the corresponding forest plot and their associated standard error. When the residuals fit within the light cone, it implies that heterogeneity in the main effect is successfully accounted by the model.

The cumulative effect measure for the developmental period from to adult emergence is positive (Fig. 2.3, panel C). Although the cumulative effect measure for both periods of development are positive, there remains residual heterogeneity as values fall outside of the cone in the funnel plots (Fig. 2.2, panels B and D;). This heterogeneity is significant for the larval period up to pupation ($Q_{df = 11} = 242.4396$, $p < .0001$). It is also significant for the period up to adult emergence ($Q_{df=40} = 315.3548$, $p < 0.0001$).

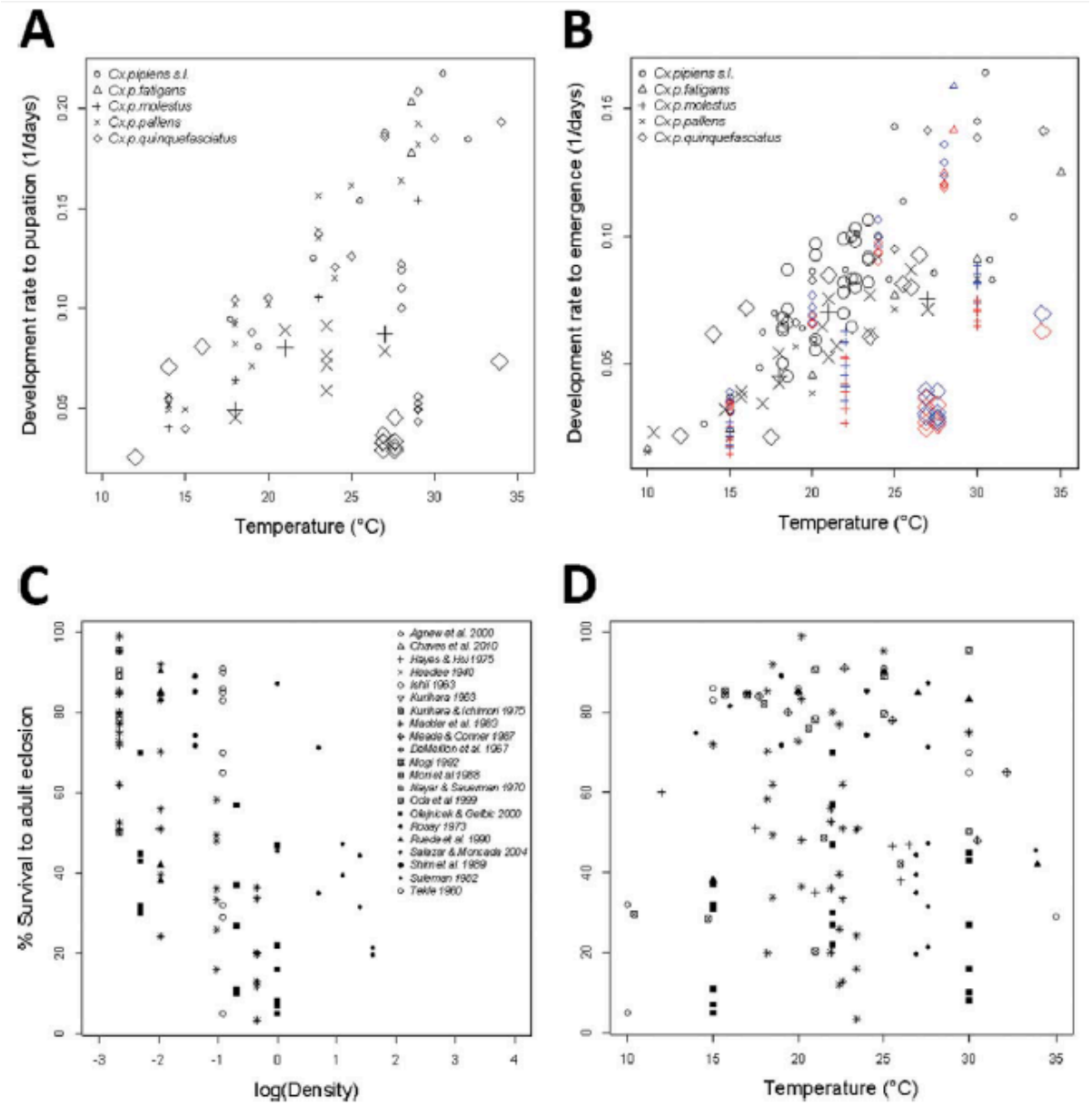


**Figure 2.3 Developmental rates and survival data**
(A) Development rate of larval stages up to pupation as function of temperature. Character shapes represent the *Cx. pipiens* complex sub-species [see legend in panel A] Small and large character sizes represent constant and variable temperature regimes respectively. (B) Development rate from hatch to emergence as function of temperature with character shapes that represent sex [see legend in panel B]. Colors represent sex with females in red, males in blue and both in black. (C) Percent survival as function of natural logarithm transformed density calculated as number of individual larvae/ml. (D) Percent survival as function of temperature. Character shapes represent study author for plots (C) and (D) [see legend in panel C].

Linear mixed effects models of the effect estimates showed several environmental factors could significantly explain this residual heterogeneity in the development rates up to

the pupal stage (Table 2.3) and up to adult emergence (Table 2.4). Based on model selection (Table S2.9), study author as a fixed and random factor best accounts for residual heterogeneity in the effect for development rate up to the pupal stage. Model selection yields two models that best explain the residual heterogeneity in the period to adult emergence: i) study author and ii) density and sex. With the inclusion of these factors, the overall effect estimates for each period no longer exhibit significant residual heterogeneity ($QE_{df = 11}$ = 21.81, p = 0.0826; $QE_{df=40}$ = 19.0320, p = 0.9661).

| Model | Effect | Q | df | *P* |
|---|---|---|---|---|
| Random effects (null) | Random | 61.51 | 10 | <0.0001* |
| Species | Random | 24.18 | 6 | <0.0001* |
| | Fixed | 4.63 | 4 | 0.3450 |
| Study | Random | 22.20 | 5 | 0.0005* |
| | Fixed | 26.68 | 5 | <0.001* |
| Density | Random | 61.51 | 9 | <0.001* |
| | Fixed | 0.0070 | 1 | 0.9335 |
| Latitude | Random | 60.48 | 10 | <0.001* |
| | Fixed | 0.468 | 1 | 0.4939 |

**Table 2.3. Meta-analysis model selection for explanation of development rate to of larval stages up to pupation of *Cx. pipiens s.l.***
In the first row a random effects model is a model that assumes a constant effect size across all studies with all variation being random. In the next rows a moderator (i.e., a covariate in meta-analysis jargon) is included to explain the heterogeneity in the estimators as a fixed factor (i.e., non-random like in ANOVA). Q is an abbreviation for Hedges' Q, a test for heterogeneity among the true effects, with the null hypothesis of no heterogeneity in a random effects model or no residual heterogeneity in the random effects of a mixed model (i.e., one also including fixed effects). For the fixed effects of a mixed model Q is an omnibus test for significant effects of any of the moderators, when there are more than one variable, or the moderator itself when only one moderator is considered (Viechtbauer, 2010). D.f. are model degrees of freedom and P the statistical significance. **Best model is bolded.**

| Model | Effect | Q | df | P |
|---|---|---|---|---|
| Random effects (null) | Random | 97.37 | 17 | <0.0001* |
| Species | Random | 65.41 | 14 | <0.0001* |
| | Fixed | 3.58 | 3 | 0.3106 |
| Study | Random | 2.70 | 8 | 0.9518 |
| | Fixed | 94.67 | 9 | <0.0001* |
| Density | Random | 73.80 | 16 | <0.0001* |
| | Fixed | 6.15 | 1 | 0.0132 |
| Latitude | Random | 54.88 | 16 | <0.0001* |
| | Fixed | 2.51 | 1 | 0.1132 |
| Sex | Random | 28.57 | 15 | 0.0183* |
| | Fixed | 11.48 | 2 | 0.0032* |
| Density and sex[a] | Random | 21.81 | 14 | 0.0826 |
| | Fixed | 24.38 | 3 | <0.0001* |

**Table 2.4 Meta-analysis model selection for the development rate to adult emergence of *Cx. pipiens s.l.***
Refer to Table 1.3 caption for column header explanations. **Best models are bolded**. *Statistically significant ($P<0.05$) [l]There were singularities in the predictor matrix for a model considering Density, Sex and Study and for a model considering Study and Sex at the same time, thus preventing the fitting of models containing all those predictors simultaneously.

Because the first meta-analytic method could only consider studies that estimated development rate at three or more temperatures, many studies were excluded from this portion. Using TSES for the second meta-analytic method allowed the inclusion of studies that considered three or fewer temperatures in experiments. In addition, the first meta-analytic method implicitly includes the impact of temperature and identifies what secondary factors explain heterogeneity in the temperature-development rate linear relationship. The second step of the meta-analysis considers TSES of development rate (an instantaneous rate of 1/development time) and the environmental conditions for each estimate. By this method, temperature is not assumed to be the primary driver of development rate, and all environmental factors are modeled to determine their relative importance in explaining the variance of development rate.

As before, two periods were considered: the larval stages up to pupation and the full ontogenetic period from to adult emergence. Based on the LMEM, temperature is the most significant environmental factor explaining variability in development rates. Other significant explanatory factors considered included sub-species, density, latitude, sex, and environmental variability. Further, temperature is the only fixed factor necessary to explain variability for the development rate up to pupation (Table 2.2) with study author, and sub-species conditioned on study author as random factors (Table S2.8). The model predicts a linear increase in development rate to pupation between 10°C and 35°C (Fig. 2.4, panel A). For the full ontogenetic period from embryo to emergence, the best LMEM model includes

both temperature and intraspecific larval density as fixed factors with study author as a random factor (Table S2.9). The model prediction for development rate values with respect to temperature and intraspecific density are shown in Fig. 2.4, panel B.

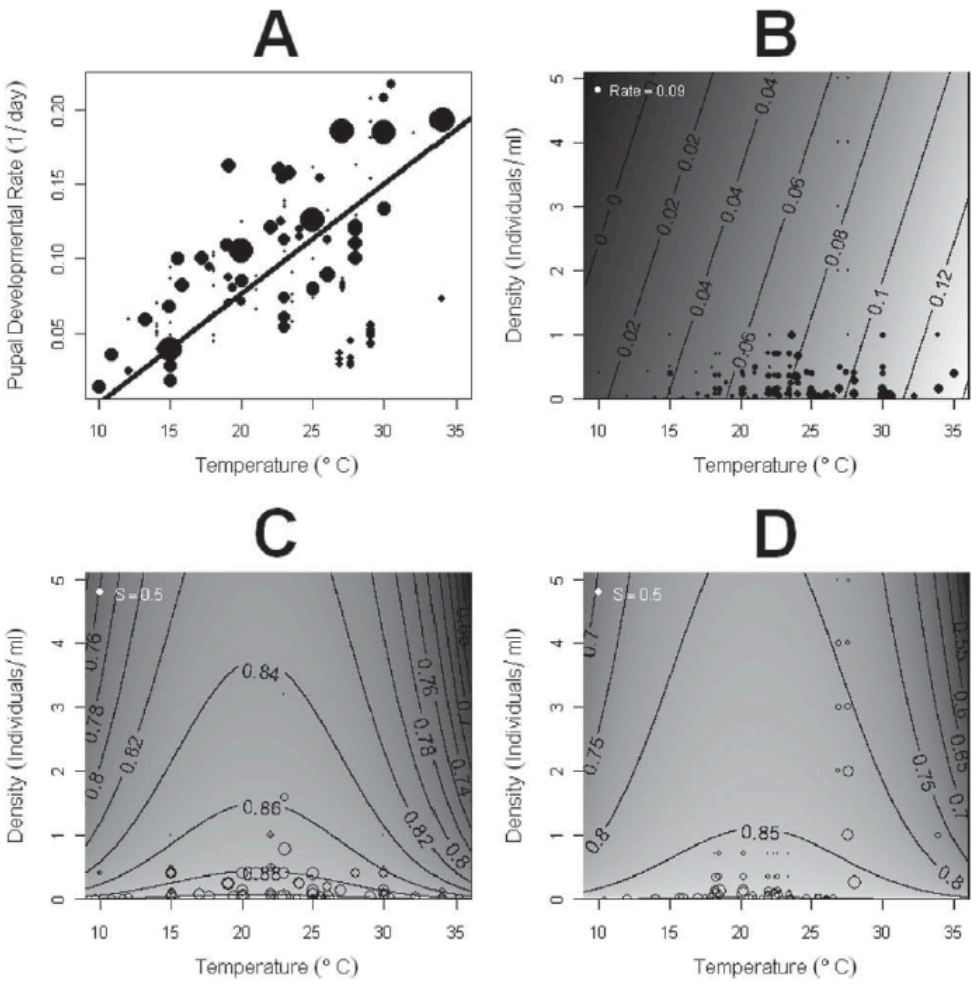


**Figure 2.4 Models for developmental rate and survival as function of temperature and density** (A) Model prediction for the development rate up to pupation. (B) Model prediction for the development rate to adult emergence. Parameters estimates for (A) and (B) are presented in Table 2.3 (C) Model predictions for percent survival in constant environments. (D) Model prediction for percent survival in variable environments. Parameters estimates for (C) and (D) are presented in Table 2.6. In all plots, character shapes represent study, and character size is proportional to the estimate/measure. Contour lines represent developmental rate prediction in panels (A) and (B) and survival predictions in panels (C) and (D). Shading is presented to ease surface visualization, and lighter shades indicate higher predicted values.

The second step of the meta-analysis also allowed for the evaluation of survival to adulthood in *Cx. pipiens s.l.* in the 21 studies that reported this outcome (Fig. 2.3, panels C,

and D). Survival is considered using a generalized binomial LMEM including the factors of temperature, density, and environmental variability as fixed factors and study author and sub-species as random factors. The model that minimized selection criteria included temperature, density, and environmental variability as fixed factors and study author as a random factor (Table 2.5 and Table 2.6). The model prediction of percent survival with respect to temperature and intraspecific rearing density is presented for an environment with constant rearing conditions (Fig. 2.4, panel C) and an environment with variable rearing conditions (Fig. 2.4, panel D).

| Factor | Odds ratio (exp(estimate)) | Estimate | SE | Z | *P* |
|---|---|---|---|---|---|
| Intercept (constant environment) | — | −7.674 | 0.775 | −9.897 | <2e-16[a] |
| Temperature | 1.898 | 0.640 | 0.050 | 12.846 | <2e-16[a] |
| (Temperature)[b] | 0.985 | −0.015 | 0.001 | −13.350 | <2e-16[a] |
| Log(density) | 0.363 | −1.0126 | 0.046 | −21.825 | <2e-16[a] |
| Variable environment | 0.477 | −0.740 | 0.125 | −5.927 | 3.09E-09[a] |

**Table 2.5 Parameter estimates for a generalized binomial linear mixed effects models considering the impacts of temperature, density and environmental variability on *Cx. pipiens s.l.* percent survival to adulthood.**
This model considered the study author as random factor. Deviance was 678.9 (119 d.f.); study variance was 2.92 (9 d.f.). [1]Statistically significant (P<0.005)

| Model (all data) | AIC | ΔAIC | BIC | ΔBIC |
|---|---|---|---|---|
| FF: Temperature (T), density (D), environmental variability (EV)<br>RF: Study, species conditioned on Study | 1,005 | 3 | 1,026 | 5 |
| FF: T, D, EV<br>RF: Species, study conditioned on Species | 1,013 | 11 | 1,034 | 13 |
| FF: T, D, EV<br>RF: Study, species | 1,005 | 3 | 1,026 | 5 |
| FF: T, D, EV<br>RF: Species | 1,650 | 148 | 1,669 | 148 |
| FF: T, D, EV<br>RF: Study*species | 1,011 | 9 | 1,029 | 8 |
| **FF: T, D, EV<br>RF: Study** | **1,002** | **0** | **1,021** | **0** |

**Table 2.6. *Cx. pipiens s.l.* survival binomial generalized linear mixed effects model selection.**
FF and RF stand for fixed and random factors respectively, AIC and BIC for Akaike and Bayes Information criterion respectively. Δ represents the difference with respect to the minimum value**.** **Minimum values for each selection criterion are bolded**.

## 2.4 Discussion

Meta-analyses of both development rate and survival demonstrate that while temperature is a driving environmental factor for development rate of *Cx. pipiens* s.l., this relationship is not constant across study populations or experiments. Further, while many environmental factors have been tested to determine their impact on survival or development rate, this analysis shows that apart from temperature, density and perhaps study methodology are the most significant variables in understanding the variation of these outcomes.

The phenotypic response of development rate to temperature is especially well studied in insects (Wagner, Olson & Willers 1991; Worner 1993; Jarosik *et al*. 2002). Based on first principles, phenotypes such as body size and development rate are often modeled using temperature as the main factor (Gilooly *et al*. 2001; Damos & Savopoulou-Soultani 2012). In particular, the linearized degree-day model (Briére *et al*. 1999) and other thermal summation models are common methods for predicting vector abundance of mosquitoes based on the linear relationship of temperature and development rate. A critical input parameter into such population models is the developmental zero (i.e. the low temperature at which development cannot occur), which is an extrapolated estimate based on the linear relationship between temperature and development rate. For development rate, the positive, linear association of temperature with development rate is confirmed in these results, but cross study comparison shows significant heterogeneity in the slope and intercept of this relationship, requiring other environmental variables to explain this variability. These results caution against the use of a single reference to obtain a parameter estimate of the impact of temperature on *Cx. pipiens* development for application to population or disease transmission models.

In addition to the heterogeneity observed in development rates, a clear shift in the slope of development rate is observed based on the inclusion of the pupal period in *Cx. pipiens s.l.*. There is a difference in development rates between the periods of development from hatch to emergence versus the larval period alone. Variation in development rate decreases during the pupal period, perhaps because this is a non-feeding life stage. The elimination of one source of variability, namely diet, could account for the decrease in heterogeneity of development rates of this stage. Similar differences in the response to temperature of development rate of larval versus pupal life stages have been observed in other holometabolous insects (Folguera, Mensch *et al*. 2010). The impact of temperature on development rate may be stage-specific, and the differences observed during pupation have been attributed to the process of complex metamorphosis (Bentz, Logan, & Amman 1991; Moehrlin & Juliano 1998; De Jong 2001; Petavy *et al*. 2001; Folguera, Ceballos *et al*. 2008; Nylin 2008; Folguera, Mensch *et al*. 2010). While a mechanistic understanding of the shift in development rate is beyond the scope of this analysis, it underscores the need for stage-specific input parameters for mosquitoes in population dynamics or vector-borne disease models. Models of insect population dynamics and vector-borne disease often assume that the slope of the relationship between temperature and development rate is constant across developmental stages, using this estimate as single input parameter (Arnold 1959; Dye 1984; Depinay *et al*. 2004; Kunkel *et al*. 2006).

In contrast to the empirical literature, in insect phonological modeling and prediction, temperature is often the only environmental factor considered (Wagner, Olson & Willers 1991; Worner 1992; Worner 1993). While the majority of studies estimating development rate in *Cx. pipiens s.l.* did so with respect to temperature alone, 8 of 33 studies examined two

or more factors (Table 2.1). Meta-analysis capitalizes on these studies but also allows comparison with studies that only address one factor. It also allows for wider ranges of each factor to be evaluated for outcomes such as survival (Fig. 2.3, panels C and D). Overall, the relative importance of more environmental factors over a wider range can be considered using meta-analysis than have been included in a single study. The results of the meta-analysis indicate that a single-factor approach, namely examining only temperature, is not sufficient to understand the variation in developmental rates or survival to adulthood across populations. However, it only takes the inclusion of one additional factor (intraspecific rearing density) to sufficiently to remove residual heterogeneity of the slope parameter estimates. Considering density provides a critical step toward recreating the naturally changing developmental environment for mosquitoes. Intraspecific competition is inherent in the developmental habitat of *Cx. pipiens* due to the oviposition of eggs in masses that can have over 100 eggs in each mass (Chaves *et al*. 2011).

Survival to adulthood is variable as well and factors including temperature, intraspecific density, and environmental variability are necessary to adequately explain this variability. Perhaps due to the challenges of conducting studies in the field, there is a paucity of information on the impact of changing environments on development rates, with notable exceptions (Chavez & Kitron 2011). These results show that environmental variability is as essential as temperature and density to explain survival estimates. In this analysis, environmental variability encompasses specifically the difference between constant and variable temperatures in rearing conditions but also generally the factors that differ between laboratory versus field experimental conditions. Field studies introduce many sources of

variability in exogenous factors including temperature, light, precipitation, wind, and day length; all aspects that are difficult but not impossible to consider in a laboratory setting.

A distinction in this analysis is the development rate of larval stages only versus the rate embryo to adult emergence. Temperature is consistently significant in both rates. However, study author is the only other significant actor aside from temperature for the larval period. Study author is a variable that indirectly represents many methodological factors, including diet quantity and quality in the rearing environment. These and other methodological factors would ideally be considered explicitly, but were not included in this analysis due to lack of reporting. Recent efforts have been made to standardize mosquito larval diets (Damiens *et al*. 2012). For some diets, such as yeast and beef liver, development rates differences are not evident in *Cx. pipiens quinquefasciatus* when administered at the same concentration (Couret, unpublished data). However, the amount of food provided is known to impact both development rate and survival in *Cx*. species and in other mosquito genera (Teng & Apperson 2000; Chaves *et al*. 2010). Further, the context of diet quality in natural environments may impact development rate and species interactions of mosquito populations (Juliano 2009).

Temperature, density, and sex were all significant factors explaining the variability in development rate from embryo to emergence. Thus, for both rates considered, there is evidence to support the hypothesis that factors other than temperature influence developmental rate. Sex differences in development rates have been shown in previous studies (Hilbert 1995; Tun-Lin & Burkott 2000; Bedhomme *et al*. 2004; Stillwell *et al*. 2010) and this pattern is observed across studies in the meta-analysis. This may be attributed to sexual dimorphism in size (Chambers & Klowden, 1990; Timmermann & Briegel, 1999), or

suggest that developmental timing is not equally important to each sex in holometabolous insects (Nylin & Gotthard 1998; Stillwell *et al*. 2010). Sex was only included as a factor in the analysis of the full developmental period because it was not consistently reported for earlier life stages. Future studies are needed to determine the timing of sexual dimorphism of development rates across early life stages.

Beyond the conditions of the rearing environment, I evaluated development rates across latitude. Variation in development rate across latitude has been hypothesized for the *Cx. pipiens s.l.* with some experimental support in Japan, though without a formal statistical analysis (Mori *et al*. 1988). The foundation for this expectation is the temperature-development relationship combined with temperature gradient from the equator to the poles. One hypothesis is that development will slow as temperatures cool with higher latitudes at the poles (Atkinson 1995; Atkinson 1997). Another, contrasting hypothesis predicts that populations at higher latitudes will demonstrate faster development to compensate for shorter season length (Blanckenhorn *et al*. 2004). There is evidence for both of these patterns of development rate in other insects with respect to latitude (Flenner *et al*. 2010, Hassall 2006, Walters & Hassall 2006). One limitation of studies searching for broad patterns of phenotypic response is in the spatial constraint of species ranges across latitude, and limited number of studies conducted across the range. Meta-analysis in part addresses this issue by expanding the geographic range of data, but is limited in other aspects such as inconsistent methodology and reporting across studies. Here, I looked for any linear pattern in development rate of *Cx. pipiens s.l.* with distance from the equator. The studies afforded a latitudinal range from -36.91° to 63.75°, spanning just over 100 latitudinal degrees, and within this range there is no evidence of a positive or negative association between distance

from the equator and development rate. Meta-analysis of development rates also confirms that in *Cx. pipiens s.l.* there is not empirical support for latitudinal variation once other environmental factors have been considered.

While temperature significantly explains variability in the phenotypic traits of development rate and survival among populations of the *Cx. pipiens* complex, other factors including density, sex, environmental variability, and study author are necessary to adequately address variability in these phenotypes. Many other environmental factors may play an important role in moderating the effect of temperature on development rate. Lack of reporting limited the inclusion of several other potential explanatory factors previously examined in dipteran insects, including disease (DeAngelis *et al*. 1993), nutrient quantity and quality (Loader & Danman 1991, Ali & Gaylor 1992, Legaspi & O'Neil 1994), thermoperiodism (Beck 1983), and presence of predators (Arnqvist & Johannson 1988). Each of these factors is a potential developmental driver and warrants further, especially in the context of additional factors of temperature and conspecific density. These results underscore the need for more research on gradients of multiple environmental factors and their interactions to better understand the drivers of development and survival. Plasticity in developmental phenotypes in response to environmental variation may play a major role in maintaining phenotypic diversity and adapting to heterogeneous and unpredictable habitats.

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# Chapter 3: Variation in development rate and survival in *Aedes aegypti*

**Modified from**: Couret, J, Benedict, MQ. 2014. Meta-analysis of environmental factors affecting development rate and survival in *Ae. aegypti*. BMC Ecology. 14(3):1-15.

**Abstract**

Development rates of *Ae. aegypti* are known to vary with respect to many abiotic and biotic factors including temperature, resource availability, and intraspecific competition. The relative importance of these factors and their interactions are not well established across populations. We performed meta-analysis on a dataset of development rate estimates from 49 studies. Meta-analytic results indicated that the environmental factor of temperature is sufficient to explain development rate variability in *Ae. aegypti*. While diet and density may greatly impact other developmental phenotypes, these results suggest that for development rate these factors should never be considered to the exclusion of temperature. The effect of temperature on development rate is not homogenous or constant. The sources of heterogeneity of the effect of temperature are difficult to analyze due to lack of consistent reporting of larval rearing methods. Temperature is the most important ecological determinant of development rate in *Ae. aegypti,* but its effect is heterogeneous. Ignoring this heterogeneity is problematic for models of vector population and vector-borne disease transmission.

## 3.1 Introduction

The effect of temperature on growth has been studied across a wide diversity of organisms (Davidson 1944a, Ahlgren 1987, Gillooly 2001, Baras *et al*. 2012, Golizadeh and Zalucki 2012, Vitolo *et al*. 2012). Like all poikilotherms, the biochemical and physiological processes of insects depend on body temperature, and ambient environmental temperature

has a profound effect on the metabolic rate and growth of insects. With short generation times and high fecundity, insects are convenient model species both in the laboratory and the field, as over a century of research establishes that temperature influences the duration and rate of development (Cook 1927; Urarov 1931; Janisch 1932; Powsner 1935; Andrewartha & Birch 1954; Messenger 1959; Watt 1968; Wigglesworth 1972; Laudien 1973; Schwander & Leimar 2011a). A main feature of this body of research is the emphasis on prediction of the timing of maturation (Kingsolver & Huey 2008; Yoshioka *et al*. 2012), body size (Gillooly *et al*. 2002; Evans *et al*. 2011), and population dynamics (Juliano 2009; Pesko *et al*. 2009a; Weaver & Reisen 2010). However, with the benefits of simplicity and practicality of considering only temperature for predicting developmental timing come the costs of ignoring other environmental and ecological factors of known importance such as resource availability, competition, and predation.

Particularly in insects of medical importance, such as mosquitoes that vector human pathogens, estimates of developmental characteristics and models of developmental timing are used to guide vector population control efforts (Gilles *et al*. 2010). In particular, controlling the population of the mosquito vector *Aedes* aegypti (Linnaeus) is critical to preventing dengue infection (Vazquez-Prokopec 2011), as there is no vaccine or chemotherapeutic treatment (Ghosh 2008). In *Ae*. *aegypti*, insecticide resistance (McAllister *et al*. 2012; Bisset *et al*. 2013) and continued progress with transgenic strains and their release (Legros *et al*. 2012) underscore the need to understand developmental phenotypes. Increasingly unpredictable climate patterns (Team 2007) motivate the study of development rate in response to varied environmental conditions (Folguera *et al*. 2009; Parkash *et al*. 2013).

Few studies have sought to determine importance of other conditions of the developmental environment relative to temperature to explain individual variation in development rate (Schwander & Leimar 2011a; Kollberg *et al*. 2013). Plasticity of development rate has been demonstrated in many diverse taxa. In mosquitoes, developmental traits vary in response to gradients of abiotic and biotic factors such as diet (Farnesi *et al*. 2009; Chown & Gaston 2010; de Jong 2010; Flenner *et al*. 2010; Yang & Rudolf 2010; Dell *et al*. 2011; Padmanabha *et al*. 2011; Farjana *et al*. 2012), larval rearing density (Agnew *et al*. 2002; Stav *et al*. 2005; Gilles *et al*. 2010), fungal infection (Blanford *et al*. 2012), nutrient quality (Walker *et al*. 1997; Chaves *et al*. 2009), thermoperiodism (Beck 1983), and presence of predators (Arnqvist & Johansson 1998). Inclusion of the variability in development rate with respect to factors other than temperature might improve the realism of models. However, temperature is often considered the main driver of development (Davidson 1944b), and it is unclear whether other factors are necessary to adequately explain variation in development rate. We hypothesize that development rate is significantly influenced by several environmental factors apart from temperature and that the interaction of these factors is an important predictor of development rate variation.

To test these hypotheses in diverse environmental conditions, empirical data is needed that considers development rate 1) in response to multiple factors (Schwander & Leimar 2011b), 2) over a gradient, (i.e. 2 or more levels) of each environmental factor (Yoshioka *et al*. 2012), and 3) across heterogeneous space (Evans *et al*. 2011). Data of such a broad scope may be difficult to produce within one experiment or study. However, we may approach such a dataset by meta-analysis of a compilation of published estimates of development rate with respect to different environmental factors. In this manner, the

phenotype of development rate in response to multiple environmental conditions can be assessed over a wider range of conditions and broader geographical bounds.

*Ae. aegypti* has been well-studied, as it vectors several human pathogens including yellow fever, dengue, and chikungunya, (Pesko *et al*. 2009b, Weaver & Reisen 2009). We conducted a meta-analysis of data from studies of the development of *Ae. aegypti* with an aim toward summarizing the impact of multiple environmental conditions on developmental duration, determining the relative importance these factors, and evaluating their interactions. The conditions evaluated here include temperature, food concentration, food type, larval rearing density, geographic location, and latitude. The linear relationship between development rate and temperature was also evaluated across studies to test the hypothesis that it is a fixed characteristic of the species.

## 3.2 Methods

*Literature Search*

For the literature search and meta-analysis we adhered to PRISMA guidelines. We searched online databases for peer-reviewed research papers in December of 2011 pertaining to *Ae. aegypti* development. Of the two forms of *Ae. aegypti*, *Ae. a. formosus*, was not included because of known differences in ecology (MacClelland 1974), behavior (Tabachnick and Powell 1979), and spatial distribution (Failloux *et al*. 2002) with limited gene flow between forms (Mousson *et al*. 2005). The list of databases searched along with keywords and the number of papers included from each source is summarized in supplementary materials (Table S3.1). The inclusion criteria were as follows. Studies had to report i) the larval rearing temperature, ii) the development time of mosquitoes from hatch to pupation or hatch to emergence in hours or days (data could be in either tabular or graphical

format and graphical data were digitally extracted with PlotDigitizer; copyright 2000-20011, Joseph A. Huwaldt), iii) the number of replicates, and iv) the number of larvae included for each estimate. In order to accomplish a meta-analysis, datasets must have similar experimental designs (Gurevitch *et al*. 2001), and we focused on studies that estimated development time with respect to temperature. We made every effort to include as many environmental factors as possible. Whenever reported we also included other methodological information of diet level (in milligrams of food per larva per day), diet type (main ingredient), larval rearing density (number of larvae per milliliter of water), photoperiod, and global position coordinates of the study or, when available and specified, strain origin (Table 1). Studies with transgenic strains were also included with "transgenic" added as another factor. For studies of laboratory strains of mosquitoes, we used the coordinates of the strain's location of origin. These data were compiled into a Microsoft Excel (Redmond, Washington: Microsoft 2011) spreadsheet and are available in supplementary materials and from the corresponding author.

| Temp | Temp gradient | Density | Density gradient | Diet (amt) | Diet gradient | Diet (type) | Photo-period | Latitude | Author & Year | Journal |
|---|---|---|---|---|---|---|---|---|---|---|
| ✓ | | ✓ | ✓ | | | ✓ | ✓ | Est. | Bargielowski et al. 2011 [58] | PLoS ONE |
| ✓ | ✓ | ✓ | | ✓ | | ✓ | ✓ | ✓ | Farjana et al. 2012 [34] | Med. Vet. Entomol. |
| ✓ | ✓ | ✓ | | ✓ | | ✓ | | ✓ | Mohammed and Chadee 2011 [59] | Acta Trop. |
| ✓ | ✓ | | | | | ✓ | | ✓ | Padmanabha et al. 2011 [60] | Med. Vet. Entomol. |
| ✓ | | ✓ | ✓ | | | ✓ | ✓ | Est. | Maciá 2009 [61] | Rev. Soc. Entomol. Argent. |
| ✓ | | ✓ | ✓ | | | ✓ | ✓ | Est. | Reiskind and Lounibus 2009 [62] | Med. Vet. Entomol. |
| ✓ | | ✓ | | ✓ | | ✓ | ✓ | ✓ | Tejerina et al. 2009 [63] | Acta Trop. |
| ✓ | | ✓ | | ✓ | | ✓ | ✓ | ✓ | Beserra and Castro 2008 [64] | Neotrop. Entomol. |
| ✓ | | ✓ | | | | ✓ | ✓ | ✓ | Chang et al. 2007 [65] | J. Med. Entomol. |
| ✓ | ✓ | ✓ | | ✓ | | ✓ | ✓ | ✓ | Beserra et al. 2006 [66] | Neotrop. Entomol. |
| ✓ | | ✓ | | ✓ | ✓ | ✓ | ✓ | ✓ | Arrivillaga and Barrera 2004 [67] | J. Vector. Ecol. |
| ✓ | | ✓ | | ✓ | ✓ | ✓ | ✓ | Est. | Bedhomme et al. 2004 [68] | Proc. R. Soc. Lond. B. |
| ✓ | | ✓ | | ✓ | | ✓ | ✓ | Est. | Irvin et al. 2004 [69] | PNAS |
| ✓ | | ✓ | ✓ | ✓ | ✓ | ✓ | | Est. | Agnew et al. 2002 [43] | Ecol. Entomol. |
| ✓ | ✓ | ✓ | | ✓ | | ✓ | ✓ | ✓ | Kamimura et al. 2002 [70] | Med. Entomol. Zool. |
| ✓ | | ✓ | | ✓ | | ✓ | ✓ | Est. | Lounibus et al. 2002 [71] | J. Vector. Ecol. |

| Temp | Temp gradient | Density | Density gradient | Diet (amt) | Diet gradient | Diet (type) | Photo-period | Latitude | Author & Year | Journal |
|---|---|---|---|---|---|---|---|---|---|---|
| ✓ | ✓ | ✓ | | | | ✓ | | Est. | Tsuda and Takagi 2001 [72] | Environ. Entomol. |
| ✓ | ✓ | ✓ | | ✓ | | ✓ | ✓ | ✓ | Tun-Lin et al. 2000 [73] | Med. Vet. Entomol. |
| ✓ | | | | | | | | Est. | Costero et al. 1999 [74] | J. Med. Entomol. |
| ✓ | | ✓ | ✓ | | | ✓ | ✓ | Est. | Silva and Silva 1999 [75] | Rev. Soc. Bras. Med. Trop. |
| ✓ | ✓ | | | | | | | Est. | Thu et al. 1998 [76] | SE Asian Trop. Med. |
| ✓ | ✓ | ✓ | | ✓ | | ✓ | ✓ | Est. | Becnel and Undeen 1992 [77] | J. Invertebr. Pathol. |
| ✓ | ✓ | ✓ | | | | ✓ | ✓ | Est. | Rueda et al. 1990 [78] | J. Med. Entomol. |
| ✓ | | ✓ | | ✓ | | ✓ | ✓ | Est. | Ho et al. 1989 [79] | J. Med. Entomol. |
| ✓ | | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | Est. | Russell 1986 [80] | Aust. J. Zool. |
| ✓ | | | | | | ✓ | ✓ | Est. | Soekiman et al. 1984 [81] | ICMR Ann. |
| ✓ | | ✓ | ✓ | ✓ | | ✓ | ✓ | | Dye 1982 [82] | Ecol. Entomol. |
| ✓ | | ✓ | | ✓ | | ✓ | | Est. | Saul et al. 1980 [83] | Am. Midl. Nat. |
| ✓ | ✓ | | | | | ✓ | | Est. | Gilpin and McClelland 1979 [84] | Fortschr. Zool. |
| ✓ | | ✓ | | | | ✓ | | Est. | Dadd et al. 1977 [85] | Mosq. News |
| ✓ | ✓ | ✓ | | | | | | Est. | Lachmajer and Hien 1975 [86] | Inst.t Med. Morskiej I Trop. |
| ✓ | | ✓ | | | | ✓ | | Est. | Ameen and Moizuddin 1973 [87] | Dacca Univ. Stud. |
| ✓ | | ✓ | | ✓ | | ✓ | | Est. | Moore and Whitacre 1972 [88] | Ann. Entomol. Soc. Am. |
| ✓ | | | | | | | | Est. | Southwood et al. 1972 [89] | Bull. World Health Organ. |
| ✓ | | ✓ | | | | | | Est. | Rosay 1972 [90] | Mosq. News |
| ✓ | | | | | | | | | Nayar 1970 [91] | J. Med. Entomol. |
| ✓ | ✓ | | | | | ✓ | | Est. | McCray et al. 1970 [92] | J. Invertebr. Pathol. |
| ✓ | | ✓ | | | | ✓ | | Est. | Keirans 1969 [93] | Mosq. News |
| ✓ | | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | Est. | Moore and Fisher 1969 [94] | Ann. Entomol. Soc. Am. |
| ✓ | | ✓ | | ✓ | ✓ | ✓ | | Est. | Peters et al. 1969 [95] | Mosq. News |
| ✓ | | ✓ | | | | ✓ | | Est. | Brust 1968 [96] | J. Econ. Entomol. |
| ✓ | ✓ | ✓ | | | | ✓ | | Est. | Keirans and Fay 1968 [97] | Mosq. News |
| ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | | Est. | Wada 1965 [98] | Quaestiones entomologicae |
| ✓ | | ✓ | | ✓ | | ✓ | | Est. | Lea 1963 [99] | J. Insect. Physiol. |
| ✓ | ✓ | ✓ | | ✓ | | | | ✓ | Ofuji 1963 [100] | B. Res Inst. Endem. Nagasaki Univ. |
| | | | | | | | | | Christophers 1960 [101] | Cambridge University Press |
| ✓ | ✓ | ✓ | | | | ✓ | | Est. | Bar-Zeev 1958 [102] | B. Entomol. Res. |
| ✓ | ✓ | | | | | | | | Headlee 1940 [103] | J. Econ. Entomol. |
| ✓ | ✓ | | | | | ✓ | | | Headlee 1941 [104] | J. Econ. Entomol. |

**Table 3.1 Studies included in the meta-analysis of *Ae. aegypti* development**
Check marks indicate studies that have reported at least one value of the environmental conditions listed including temperature, diet (mg/larva/day), density (larvae/mL), or photoperiod. Gradient columns indicate whether the study considered three or more levels of the environmental condition. Latitude of origin was either reported (check mark) or estimated (Est.) based on the city of origin of the mosquito strain. Studies that considered transgenic strains are indicated in bold. Development rate estimates for transgenic strains were not included in the meta-analysis. A full bibliography is available in Table S3.2.

*Meta-analysis*

We used two meta-analytic approaches for these data. In our first approach we evaluated estimates of development time from hatch to pupation and development time from hatch to emergence using a mixed linear regression model (Faraway 2006) "nlme" (Pinheiro *et al*. 2013) implemented in the R package (Team 2013). These two dependent variables were analyzed separately. Factors included temperature, larval rearing density, diet level (mg/larvae/day), latitude of strain origin, photoperiod, and publication. For a study to be included in the mixed linear regression model at least one environmental factor had to be reported along with the estimate of development time (i.e. at least one temperature, larval rearing density, or diet level). The variable of sex was not considered for hatch to emergence in this portion of the analysis as many studies reported values for only females or did not report sex at all. Publication author was considered a random factor in our analysis as our primary interest was the in the effects of other variables across studies. Parameters were eliminated using backward model selection and a minimization of the Akaike Information Criterion (AIC) and BIC (Bayesian Information Criterion). Both criteria impose a penalty for increasing the number of parameters in a model. A model with $\Delta$AIC = 2 and $\Delta$BIC = 2 or more units lower than any other model was considered the best (Faraway 2006).

In our second approach, we focused analysis on a temperature range for which development rate (1/development time) can be well approximated with a linear model. Development rates in *Ae*. *aegypti* are well approximated by a linear model within the temperature range from 14 - 31°C (Gilpin & McClelland 1979). The linear model is described by the following equation

$$y = B_1 x + B_0 \quad , \qquad (3.1)$$

where y = 1/development rate, and y is regressed on temperature, x. The parameter $B_0$ represents the developmental zero and $B_1$ is a constant for the cumulative effective of temperature, generally reported as *K* (Ikemoto 2000; 2005). When parameter estimates were not directly reported, linear models were run in the open source package R version 2.14.0 (R Development Core Team 2012). Linear models in this second meta-analytic approach were only conducted on data from studies that estimated development rate over three or more temperatures in order to allow for a regression analysis. For meta-analysis, parameter estimates of $B_1$ and $B_0$ were each used as effect measures, and were weighted by the number of replicates per experiment in a study. We tested the hypotheses that cumulative effect of temperature (*K*) and developmental zero (*t*) are constant properties of a mosquito strain using a test of total heterogeneity, $Q_T$, with Hedge's estimator, a standardized difference method for comparing effect measures (Hedges & Olkin 1985; Gurevitch *et al*. 2001). Next, we used a linear mixed effects model to determine the variables that best explained this heterogeneity including publication, diet, larval rearing density, and latitude of strain origin. We then tested for residual heterogeneity, $Q_E$ (Hedges & Olkin 1985; Gurevitch *et al*. 2001). For this portion of the analysis we were able to include the variable of sex due to greater reporting in this subset of studies. Sex was considered with three categories: male, female, and both.

### 3.3 Results

Based on a literature search of 11 online databases using search terms including *Ae. aegypti*, temperature, diet, larval rearing density, and development rate, we found 27,559 articles, from which 48 journal publications and one book chapter fit the inclusion criteria (Table 3.1). From these, data on development rate were compiled for 66 populations of *Ae. aegypti* (references in Table S3.2) spanning approximately 87° of latitude (Figure 3.1).

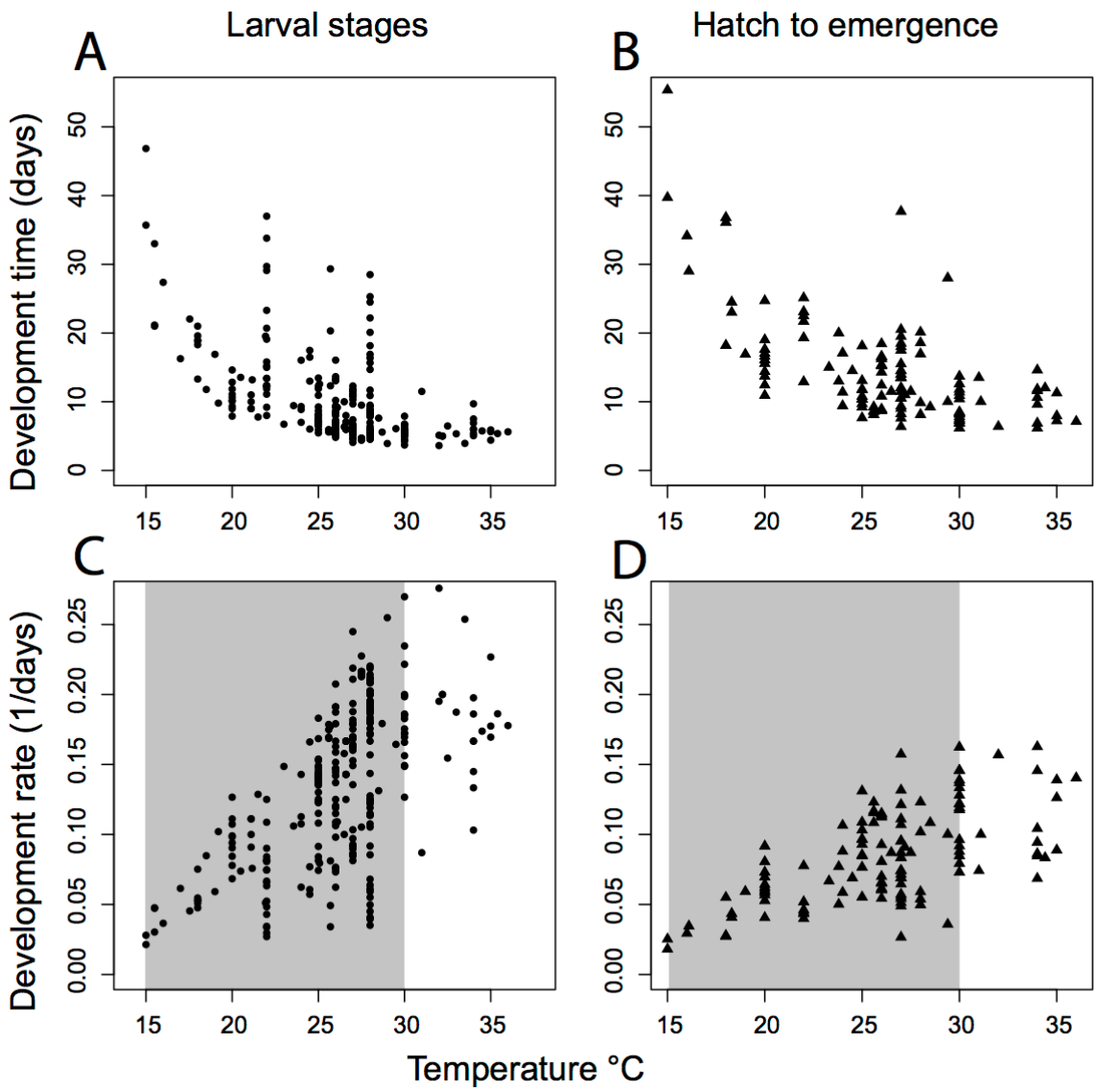


**Figure 3.1 Compiled dataset of development time (days) and development rate (1/days) plotted against temperature for hatch to pupation, i.e. larval stages (A and C, respectively), and hatch to emergence (B and D respectively)**
Shaded gray bars show the subset of data used for linear models of development rate.

Among these studies, 39% evaluated temperature across a gradient of 2 or more levels, and 77% of all reported one intraspecific rearing density whereas 18% considered larval rearing density gradients. Many studies reported food added *ad libitum*, but among the subset of studies that reported diet values, 25% examined diet gradients. Photoperiod was reported in 45% of studies. Some studies were laboratory based and others were field-based

or under semi-natural conditions. This facilitated the comparison of constant versus variable temperatures on development rate (Figure 3.2).

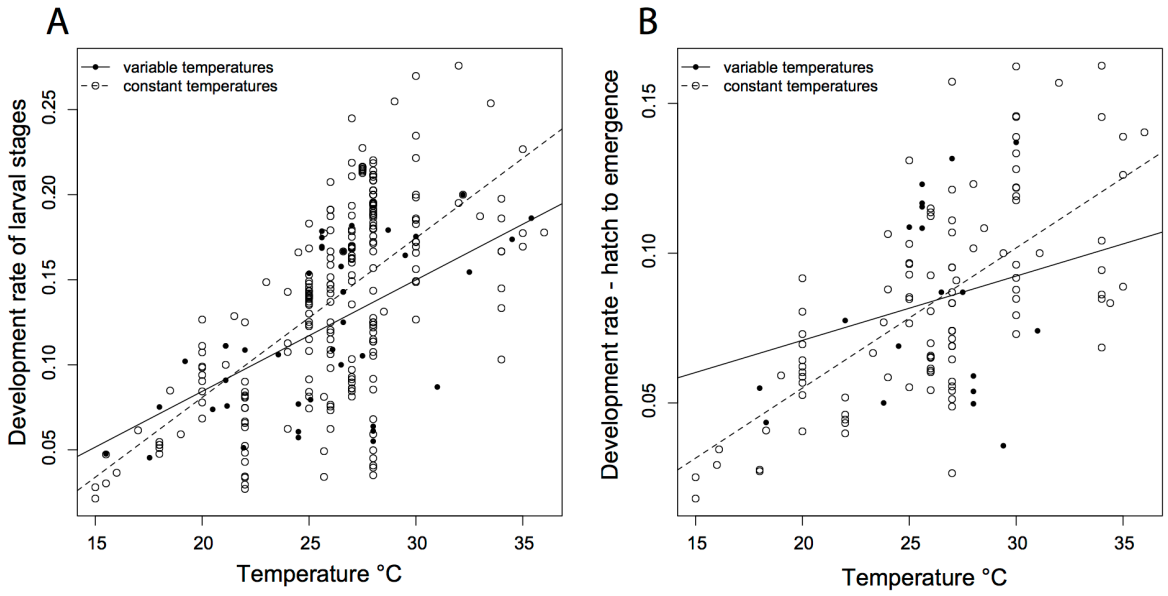


**Figure 3.2 Development rate (inverse development time) estimates for (A) hatch to pupation, i.e. larval stages, and (B) hatch to emergence plotted against temperature.**
Character shape represents whether larvae were reared in constant or variable temperatures. Line type corresponds with character shape and lines indicate linear regression of development rate and temperature for constant and variable temperatures.

The type of diet was reported for 42 of 49 experiments, and of these studies 32 had a unique diet composition. Diets shared across multiple studies included brewer's yeast and Tetramin® Fish Food. Unique diets were combinations of these and various other sources including, but not limited to, rabbit food, dog food, pig chow, pig liver powder, beef liver powder, bacterial infusions, detritus, and unspecified larval broth. Inclusion of diet type led to over-parameterization of models and was dropped from the analysis as a factor due to the number of unique types.

Development time of larval stages, development time from hatch to emergence, and percent survival were compiled into a dataset for the first meta-analytic approach (Table

S3.2). Inclusion required an estimate of development rate of *Ae. aegypti* under at least one value of temperature, larval rearing density, or diet. The compiled dataset had 283 estimates of development time from hatch to pupation and 127 from hatch to emergence (Figure 3.1, panels A and B). Temperatures ranged from 14–37.8°C. Development times were not normally distributed for larval stages (Shapiro-Wilk test, $W = 0.727$, $p < 0.0001$) or from hatch to emergence ($W = 0.7942$, $p < 0.0001$), and therefore estimates were transformed into development rate in the form of the inverse of development time. Development rates were normally distributed for larval stages ($W = 0.9797$, $p > 0.08$) and hatch to emergence ($W = 0.9532$, $p > 0.1$). Development rate showed a significant positive association with rearing temperature across all studies for larval stages ($B_1 = 0.008913$, $t_{281} = 13.50$, $p < 0.0001$, $R^2 = 0.3782$; Figure 3.1, panel C). Similarly, the development rate from hatch to emergence is significantly associated with temperature ($B_1 = 0.0045222$, $t_{125} = 8.725$, $p < 0.0001$, $R^2 = 0.3862$; Figure 3.1, panel D).

For better approximation with a linear model we used a subset of the compiled data over the temperature range of 14 - 31°C resulting in 262 estimates for larval stages and 110 for hatch to emergence. This data subset restricted only the upper boundary of development rate estimates, above which a linear model is no longer a good approximation (Figure 3.1; Gilpin & McClelland 1979). The full GLMM model for development rates included fixed factors of temperature, photoperiod, diet, larval rearing density, and a dummy variable of temperature variability (constant or variable temperature). Estimates under constant temperatures came from laboratory studies. Estimates under variable temperatures came from both field studies in natural or semi-natural conditions and laboratory studies with fluctuating temperature schemes accomplished using environmental chambers. Temperature fluctuations

imposed in laboratory studies differed in magnitude, duration, and the life stage at which mosquitoes were exposed. To broadly assess the difference between constant and variable temperatures we created the dummy variable of temperature variability. Random factors included latitude and publication. Based on the minimum AIC and BIC, the best model for development rate from hatch to pupation included the fixed factor of temperature and the random factor of publication (Table 3.2). Similarly the best model for the development rate from hatch to emergence included only temperature as a fixed factor and the random factor of publication (Table 3.3).

| Fixed Factor | Random Factor | AIC | Δ AIC | BIC | Δ BIC |
|---|---|---|---|---|---|
| T, Ph, D, Dt, EV | Author, Lat | –36.74 | 436.16 | –15.53 | 443.07 |
| T, Ph, D, Dt, EV | Lat | –35.65 | 437.25 | –16.79 | 441.81 |
| T, Ph, D, Dt, EV | Author | –118.3 | 354.6 | –96.28 | 362.32 |
| T, Ph, D, Dt | Author | –124.9 | 348 | –105.6 | 353 |
| T, Ph, Dt, EV | Author | –127.4 | 345.5 | –108.2 | 350.4 |
| T, Ph, D, EV | Author | –196.5 | 276.4 | –175.2 | 283.4 |
| T, D, Dt, EV | Author | –215.4 | 257.5 | –193.9 | 264.7 |
| Ph, D, Dt, EV | Author | 39.35 | 512.25 | 58.62 | 517.22 |
| T, D, Dt | Author | –222.8 | 250.1 | –204.5 | 254.1 |
| T, Dt, EV | Author | –224 | 248.9 | –205.6 | 253 |
| T, D, EV | Author | –395.2 | 77.7 | –374.5 | 84.1 |
| D, Dt, EV | Author | 1.712 | 474.612 | 20.09 | 478.69 |
| T, D | Author | –403.5 | 69.4 | –386.2 | 72.4 |
| T, EV | Author | –466.8 | 6.1 | –448.9 | 9.7 |
| D, EV | Author | –96.4 | 376.5 | –79.1 | 379.5 |
| EV | Author | –131.6 | 341.3 | –117.3 | 341.3 |
| **T** | **Author** | **–472.9** | **0** | **–458.6** | **0** |

**Table 3.2 Linear mixed effects model selection *Ae. aegypti* development rate from hatch to pupation**

Fixed factors considered were temperature (T), photoperiod (Ph), density in larvae/mL (D), diet in mg/larva/day (Dt), and environmental variability (EV). Environmental variability represents constant versus variable temperatures. Random factors included study author (Author) and latitude of origin for the *Ae. aegypti* study strain. AIC and BIC stand for Akaike and Bayes Information Criterion respectively. Δ represents the difference with respect to the minimum value. The best model with minimum values for each selection criterion is bolded. The AIC and BIC have negative values because the models had positive log-likelihoods, which occur because the probability densities evaluated at the observations are below 1, which produces a negative logarithm. ΔAIC and ΔBIC show differences with respect to the model that minimized each information criterion.

| Fixed Factor | Random Factor | AIC | Δ AIC | BIC | Δ BIC |
|---|---|---|---|---|---|
| T, Ph, D, Dt, EV | Author, Lat | –71.58 | 131.32 | –51.24 | 140.86 |
| T, Ph, D, Dt, EV | Lat | –69.23 | 133.67 | –51.25 | 140.85 |
| T, Ph, D, Dt, EV | Author | –73.48 | 129.42 | –55.49 | 136.61 |
| T, Ph, D, Dt | Author | –76.71 | 126.19 | –60.97 | 131.13 |
| T, Ph, Dt, EV | Author | –78.76 | 124.14 | –63.02 | 129.08 |
| T, Ph, D, EV | Author | –89.58 | 113.32 | –73.65 | 118.45 |
| T, D, Dt, EV | Author | –98.46 | 104.44 | –82.06 | 110.04 |
| Ph, D, Dt, EV | Author | 24.1 | 227 | 39.84 | 231.94 |
| T, D, Dt | Author | –105.3 | 97.6 | –91.2 | 100.9 |
| T, Dt, EV | Author | –106.5 | 96.4 | –92.4 | 99.7 |
| T, D, EV | Author | –140.2 | 62.7 | –125 | 67.1 |
| D, Dt, EV | Author | 9.06 | 211.96 | 23.13 | 215.23 |
| T, D | Author | –147 | 55.9 | –134.3 | 57.8 |
| T, EV | Author | –195 | 7.9 | –181.5 | 10.6 |
| D, EV | Author | –12.11 | 190.79 | 0.55 | 192.65 |
| EV | Author | –43.19 | 159.71 | –32.39 | 159.71 |
| **T** | **Author** | **–202.9** | **0** | **–192.1** | **0** |

**Table 3.3 Linear mixed effects model selection *Ae. aegypti* development rate from hatch to emergence**

Fixed factors considered were temperature (T), photoperiod (Ph), density in larvae/mL (D), diet in mg/larva/day (Dt), and environmental variability (EV). Environmental variability represents constant versus variable temperatures. Random factors included study author (Author) and latitude of origin for the *Ae. aegypti* study strain. The best model with minimum values for each selection criterion is bolded.

For the second meta-analytic approach, inclusion required estimation of development rate for at least three temperatures in one experiment. The regression parameters for development rate on temperature are reported in supplementary tables (Tables S3.3 and S3.4). The estimates of the developmental zero (t) and degree-day model constant (K) are calculated and listed for each study for both hatch to emergence (Table 3.4) and hatch to pupation (Table 3.5). The literature search yielded 23 experiments meeting the criteria with the dependent variable development rate from hatch to emergence. The literature search yielded 20 experiments meeting the criteria for development rate from hatch to pupation. Results of experiments conflicted regarding the significance of the relationship between temperature and development rate. For example, considered separately, many of the studies

did not show a significant, positive linear relationship between temperature and development rate (Tables 3.4 and 3.5). Of the 23 studies measuring hatch to emergence, 10 did not find a significant linear association. Similarly, 7 of 20 studies did not show a significant relationship for development rate from hatch to pupation and temperature. However, these data combined demonstrated an overall significantly positive association (Figure 3.2).

| Study | Latitude | Sex | t (°C) | K | n | $r^2$ | p-value | |
|---|---|---|---|---|---|---|---|---|
| Bar-Zeev 1958 [155] | 31.0461 | F | 12.83 | 121.86 | 100 | 0.9959 | 6.21E-06 | *** |
| Bessera et al. 2006 [121] | –7.4908 | C | 13.35 | 186.74 | 120 | 0.9874 | 0.00634 | * |
| Bessera et al. 2006 [121] | –6.38 | C | 9.40 | 280.23 | 120 | 0.9962 | 0.03915 | * |
| Bessera et al. 2006 [121] | –7.2256 | C | 8.42 | 243.21 | 120 | 0.8418 | 0.2604 | |
| Bessera et al. 2006 [121] | –7.3 | C | 13.63 | 173.32 | 120 | 0.9949 | 0.002563 | ** |
| Bessera et al. 2006 [121] | –6.9669 | C | 18.35 | 102.82 | 120 | 0.9644 | 0.1209 | |
| Farjana et al. 2011 [116] | –3.3439 | F | 9.95 | 257.90 | 100 | 0.981 | 0.08805 | |
| Farjana et al. 2011 [116] | –3.3439 | F | 11.44 | 158.13 | 100 | 0.9403 | 0.1572 | |
| Farjana et al. 2011 [116] | –3.3439 | M | 9.95 | 209.14 | 100 | 0.9882 | 0.06917 | |
| Farjana et al. 2011 [116] | –3.3439 | M | 11.69 | 137.59 | 100 | 0.9318 | 0.1682 | |
| Headlee 1940 [157] | 40.486217 | C | 10.21 | 187.68 | 200 | 0.9828 | 0.0838 | |
| Headlee 1941 [156] | 40.486217 | C | 8.38 | 219.88 | 200 | 0.9858 | 0.0007197 | *** |
| Kamimura et al. 2002 [124] | 24.8934 | F | 9.93 | 162.44 | 50 | 0.9902 | 0.06328 | |
| Kamimura et al. 2002 [124] | –7.2653 | F | 10.68 | 151.77 | 50 | 0.9985 | 0.02504 | * |
| Kamimura et al. 2002 [124] | –9.2628 | F | 11.38 | 144.78 | 50 | 0.9472 | 0.1476 | |
| Kamimura et al. 2002 [124] | 24.8934 | M | 8.19 | 176.84 | 50 | 0.9931 | 0.05285 | * |
| Kamimura et al. 2002 [124] | –7.2653 | M | 10.10 | 148.90 | 50 | 0.9977 | 0.03039 | * |
| Kamimura et al. 2002 [124] | –9.2628 | M | 9.09 | 163.45 | 50 | 0.9142 | 0.1893 | |
| Lachmajer & Hien 1975 [139] | 14.0583 | C | 6.85 | 141.43 | 6300 | 0.9958 | 0.04125 | * |
| Ofuji 1963 [153] | 32.2 | F | 10.76 | 133.80 | 20 | 0.96 | 0.00344 | ** |
| Ofuji 1963 [153] | 32.2 | M | 10.45 | 129.82 | 20 | 0.9514 | 0.004608 | ** |
| Rueda et al. 1990 [132] | 35.7721 | C | 11.17 | 129.35 | 20 | 0.8669 | 0.006966 | * |
| Tun-Lin et al. 2000 [127] | –10.58 | C | 46.31 | 332.82 | 200 | 0.8497 | 0.02594 | * |

**Table 3.4 Studies that estimated development rate to adult emergence over three or more temperatures**

Developmental zero (t) and linearized degree day model constant (K) are listed along with the correlation coefficient and p-value of the linear regression between temperature and development rate. Level of significance is indicated by the number of asterisks (* < 0.01; ** < 0.001; *** < 0.0001). Sex is listed as C if values represent a combination of males and females.

| Study | Latitude | t (°C) | K | n | r | p-value | |
|---|---|---|---|---|---|---|---|
| Bar-Zeev 1958 [155] | 31.0461 | –14.21 | 86.22 | 100 | 0.9975 | 0.001269 | ** |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Becnel & Undeen 1992 [131] | 15.87 | 1.13 | 185.46 | 250 | 0.883 | 0.2222 | |
| Bessera et al. 2006 [121] | –7.4908 | –9.91 | 148.46 | 120 | 0.8963 | 0.01464 | * |
| Bessera et al. 2006 [121] | –6.38 | –7.75 | 187.97 | 120 | 0.9758 | 0.001609 | ** |
| Bessera et al. 2006 [121] | –7.2256 | –9.41 | 130.57 | 120 | 0.8663 | 0.02164 | * |
| Bessera et al. 2006 [121] | –7.3 | –4.37 | 200.88 | 120 | 0.4404 | 0.222 | |
| Bessera et al. 2006 [121] | –6.9669 | –12.56 | 114.48 | 120 | 0.8652 | 0.02193 | * |
| Gilpin & McClelland 1979 [61] | –10.9491 | –10.81 | 82.27 | 300 | 0.8875 | 4.80E-07 | *** |
| Kamimura et al. 2002 [124] | 24.8934 | –1.62 | 28.64 | 50 | 0.9634 | 0.1225 | |
| Kamimura et al. 2002 [124] | –7.2653 | –9.70 | 122.78 | 50 | 0.9995 | 0.01357 | * |
| Kamimura et al. 2002 [124] | –9.2628 | –9.89 | 122.34 | 50 | 0.9035 | 0.2011 | |
| Keirans & Fay 1968 [150] | 18.2208 | –10.79 | 102.18 | 50 | 0.9729 | 6.26E-06 | *** |
| Lachmajer & Hien 1975 [139] | 14.0583 | –10.00 | 112.68 | 6300 | 0.9598 | 0.1286 | |
| Mohammed & Chadee 2011 [117] | 10.6389 | 69.92 | 365.94 | 600 | 0.002094 | 0.9069 | |
| Ofuji 1963 [153] | 32.2 | –9.70 | 105.79 | 20 | 0.9095 | 0.01189 | * |
| Padmanabha et al. 2011 [36] | 10.9861 | –9.09 | 100.97 | 160 | 0.9644 | 0.0004806 | *** |
| Rueda et al. 1990 [132] | 35.7721 | –10.65 | 101.43 | 20 | 0.7966 | 0.01671 | * |
| Thu et al. 1998 [130] | 21.914 | 76.45 | 1124.99 | 100 | 0.0356 | 0.8113 | |
| Tsuda & Takagi 2001 [126] | 19.5177 | –10.40 | 153.68 | 50 | 0.6096 | 0.03826 | * |
| Tun-Lin et al. 2000 [127] | –10.58 | –36.15 | 727.80 | 200 | 0.887 | 0.01671 | * |

**Table 3.5 Studies that estimated development rate to pupation over three or more temperatures** Developmental zero (t) and linearized degree day model constant (K) are listed along with the correlation coefficient and p-value of the linear regression between temperature and development rate. Level of significance is indicated by the number of asterisks (* < 0.01; ** < 0.001; *** < 0.0001).

The linear association between development rate and temperature had significant heterogeneity for both hatch to pupation ($Q_T = 242.4396$, $p < 0.0001$) and hatch to emergence ($Q_T = 403.5$, $p < 0.0001$). A linear mixed effects model was used to determine what other environmental factors might explain the heterogeneity in this relationship. Additional factors considered were initial larval rearing density, diet level (mg/larva/day), strain origin, latitude, and publication author. The model including only temperature as a fixed factor and the random factor of publication author best explained the heterogeneity in slope estimates for both the pupation group and emergence group. Once publication was included in the model, the test of residual heterogeneity was no longer significant for hatch to pupation ($Q_E = 4.8582$, $p < 0.3022$) or hatch to emergence ($Q_E = 2.23$, $p < 0.8971$). Similarly, the developmental zero was significantly heterogeneous for both the hatch to pupation

development rate ($Q_T = 92.3908$, $p < 0.0001$) and hatch to emergence ($Q_T = 675.6708$, $p < 0.0001$). Once temperature had been considered, the residual heterogeneity in the developmental zero was explained by publication author such that the test for residual heterogeneity was no longer significant (hatch to pupation: $Q_E = 2.2802$, $p < 0.6844$; hatch to emergence: $Q_E = 1.0234$, $p < 0.9847$).

Asymmetry was apparent when plotting effect measures against study size in funnel plots (Figure 3.3). In the absence of systematic heterogeneity, points should fall within the range indicated by the inverted cone in funnel plots. Asymmetry may be a result of publication bias or systematic heterogeneity. With the inclusion of publication author as a random effect in the model, the asymmetry was no longer evident and the funnel plots no longer indicated heterogeneity for hatch to emergence or hatch to pupation (Figure S3.1 and Figure S3.2).

The range of diets considered across all studies was 0.01 mg/larva/day to 435.2 mg/larva/day. However, 96.6% of studies used values within the range of 0.01 mg/larva/day to 6.8 mg/larva/day. Comparisons of diet level with development rate are shown in Figure 3.4, panels A and C. The larval rearing density considered across the studies ranged from 0.01 larvae/mL to 8 larvae/mL, and comparisons with development rate are shown in Figure 3.4, panels B and D. Approximately 69% of larval rearing density levels used by studies in the meta-analysis fell between 0.1 larva/mL and 1 larva/mL.

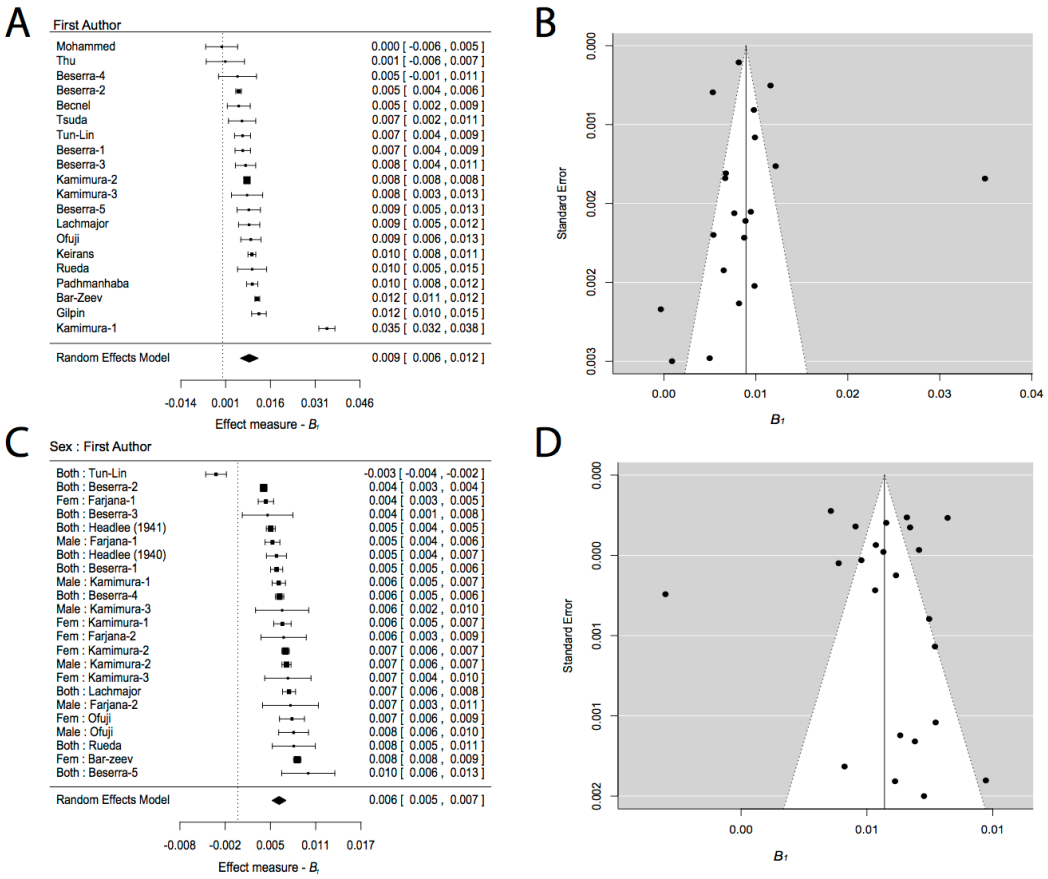


**Figure 3.3 Meta-analysis of the effect of temperature, i.e. $B_1$ - the slope of the regression of temperature and development rate**

**(A)** Forest plot for development rates of hatch to pupation, i.e. larval stages. **(B)** Funnel plot corresponding to plot **(A). (C)** Forest plot for development rates from hatch to emergence. **(D)** Funnel plot corresponding to plot **(C)**. The weight of the study is indicated by the size of the square and the diamond indicates the overall effect estimate from the random effects model. First authors are listed on the left of the forest plots and, when applicable, the strain identifier is listed by number (for full references see Additional file 1: Table S2). Squares represent effect estimates of individual studies. Square size represents the weight given to the study in the meta-analysis, and the horizontal lines represent 95% confidence intervals. Estimated values and confidence intervals are written to the right of the plot. In the funnel plots, points represent the residuals of the model presented in the corresponding forest plot and their associated standard error. When the residuals fit within the light cone, it implies that heterogeneity in the main effect is successfully accounted by the model.

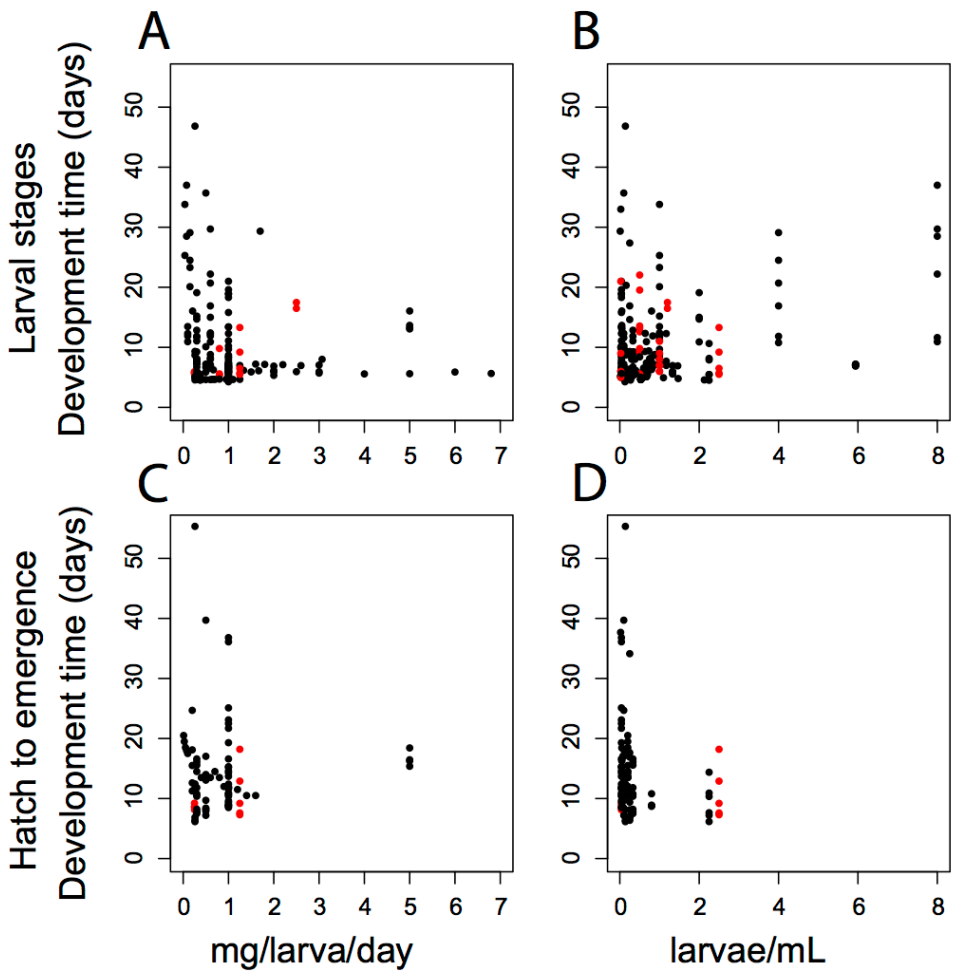


**Figure 3.4 Development time of hatch to pupation, i.e. larval stages, compared to diet (A) and density (B), and development time from hatch to emergence compared to diet (C) and density (D).**
Character color indicates laboratory (black) and field studies (red).

## 3.4 Discussion

We hypothesized, first, that development rate is significantly influenced by several environmental factors and that the interaction of these factors is an important predictor of development rate variation. The results of both meta-analytic approaches suggest that temperature is the main fixed factor driving development rate, to the exclusion of other factors of known importance such as diet and density. This bolsters the contention that temperature is the most important ecological determinant and, when modeling development,

sufficient to predict development rate (Damos 2012). When larvae experience nutritional deprivation or high densities, this can dampen (Couret *et al*. 2014) or exacerbate (Padmanabha *et al*. 2011) the impacts of temperature. Thus, while research suggests that diet (Tun-Lin *et al*. 2000; Barrera *et al*. 2006) and larval rearing density (Gilpin & McClelland 1979; Dye 1984) do matter, these results underscore that they should not be considered to the exclusion of temperature. Based on model selection, the relative importance of these factors can be ranked as temperature followed by temperature variability, larval rearing density, then diet, and lastly photoperiod (Table 3.2, Table 3.3). The relative importance of factors is consistent between the periods of hatch to pupation and hatch to emergence. While this analysis shows other variables such as latitude were not significant in explaining development rate variation, they may impact other important life history traits including survival, body size, fecundity (Kollberg *et al*. 2013), and morphology (Fitzgerald & Tipping 2013).

The relationship between temperature and development rate is linear within a median temperature range (Roltsch *et al*. 1990; David *et al*. 1997; Kontodimas *et al*. 2004; Logan 2011), and features of this linear relationship, such as slope and intercept, have biological interpretations. The slope of this relationship is considered the cumulative effect of temperature on the rate of development, and the intercept can be interpreted as the theoretical temperature at which development can no longer occur (Ahlgren 1987; Ikemoto 2000), also called the developmental zero. Although at extremes of low temperature the development curve is non-linear, the linear portion is extrapolated to the intersection with the temperature axis to estimate the developmental zero (Arnold 1959). This extrapolation based on slope may, in part, explain the large variation in the estimates reported in Table 4 and Table 5. This

may also explain estimates that were less than zero, which is biologically implausible. Meta-analysis these parameters across many studies allows for outliers to be more easily identified.

Despite these limitations, the developmental zero is often considered a fixed characteristic of a species for the purposes of modeling and predicting population abundance (Briere *et al*. 1999; Ikemoto 2000; Jarosík *et al*. 2002; de Jong 2010). Thus, we also sought to test the hypothesis that the effect of temperature and the developmental zero are fixed characteristics of *Ae. aegypti* strains. While the meta-analytic results are consistent with a positive, linear relationship between temperature and development rate, tests for heterogeneity suggest a significant amount of variation in response to temperatures within this range. These data do not support the hypothesis that the developmental zero and the effect of temperature are fixed constants. Both the effect of temperature and the developmental zero are heterogeneous across studies considered in the meta-analysis. These results have implications for the modeling of development rate as well as population abundance, which often relies on development times of larval populations(Juliano 2009; Richardson *et al*. 2011). These compiled data may be used as the basis for modeling these parameters as a distribution rather than choosing one value from a single study. Variation in development time (i.e. the inverse of development rate) has been modeled as a continuous random variable with a distribution of frequencies, such as the normal distribution (Pradhan 1945) or with a heterogeneity factor (Yang *et al*. 2009). Other modeling approaches to incorporate development rate variation stochastically by treating development rate as a random variable dependent on the variability in the level of catalytic enzymes (Sharpe *et al*. 1977; Curry 1978; Sharpe & Hu 1980), positing a biophysical basis for variability.

There are several hypotheses to address why the response to temperature may be

heterogeneous. Our results indicate that factors of larval rearing density, diet, latitude, and photoperiod were not factors that could explain heterogeneity of the effect of temperature. A limitation of this analysis was the narrow range of reported values of diet and initial larval rearing density. While many studies reported at least one level of different factors such as temperature, diet, and larval rearing density, few studies in *Ae. aegypti* examined development across gradients of multiple environmental conditions. Such experiments are needed in order to establish the relative importance of environmental factors in the variation of development rates. Assessing the impact of varied environmental conditions on the developmental phenotypes of mosquito larvae can be complex with interactive effects (Hagstrum & Workman 1971; Kingsolver & Huey 2008; Gilles *et al*. 2010). For example, Padhmanhaba *et al* (2011) show that increased the rearing temperature for starved *Ae. aegypti* larvae impacts development rate, and this impact changes depending on the larval stage and the temperature.

Publication author was adequate to explain heterogeneity in the effect of temperature on development rates. It is difficult to identify the aspects of this factor to describe its significance in explaining development rate variation. We evaluated the dichotomy of laboratory versus field experiments, which generally corresponded to constant versus variable temperatures. Mosquito response to variable rather than constant temperatures has been a recent focus both for life history traits and vectorial capacity (Paaijmans *et al*. 2010; Lambrechts *et al*. 2011; Richardson *et al*. 2011; Carrington *et al*. 2013a; Carrington *et al*. 2013b; Carrington *et al*. 2013c). Variable temperatures have been shown to increase (Huffaker 1944), decrease (Roltsch *et al*. 1990), and have no impact (Joshi 1996) on development rates of mosquitoes and other insects. Inconsistency in the relationship between

temperature and development rate has been attributed to field conditions versus laboratory conditions (Padmanabha *et al*. 2011). To test this, we compared development rates estimated under constant versus variable temperature conditions, which corresponded to laboratory versus field conditions. This comparison showed no significant difference overall in the relationship between development rate and temperature based on temperature variability for either larval stages or to hatch to emergence (Fig. 3.2). This finding is consistent with recent reports that *Ae*. *aegypti* life-history traits depend not only on variability but also the magnitude of temperature fluctuations (Carrington *et al*. 2013a).

The factor of publication may be a proxy for methodological differences such as diet composition (i.e. ingredients of diet). Of the 49 studies, almost all reported information on diet composition. However, few used the same diet preparations, and this prevented this factor from being included in meta-analysis. Some diets were created from detritus of the larval habitat in order to mimic natural conditions(Lounibos *et al*. 2002; Maciá 2009; Padmanabha *et al*. 2011) or incorporated detritus(Reiskind & Lounibus 2009). The majority however provided no explanation for the choice of diets. Diet choice can influence development rate as well as interspecific larval competition (Murrell & Juliano 2008; Murrell *et al*. 2011) and adult wing length (Padmanabha *et al*. 2011b). To facilitate comparison of larval performance across populations, these findings support a need for standardization of diet composition for laboratory colonies. This is especially important in the context of transgenics. Our literature search yielded only two studies with estimating development rate of *Ae*. *aegypti* transgenic strains. The low sample size impeded statistical comparison of transgenic versus wild-type development rate estimates, leading to singularity errors in the linear mixed effects modeling. Future comparisons of transgenic and wild strains in other

important life-history traits such as body size, fecundity, and longevity may also be informative. Estimating and evaluating life-history traits across different environmental conditions is critical to provide a basis for comparison between wild and transgenic strains and may guide future transgenic release programs(Irvin 2004; Bargielowski *et al*. 2011; Legros *et al*. 2012).

Other factors not considered in this analysis may also impact the effect of temperature, and perhaps contribute to heterogeneity. Examples include genetic variation, microbial symbiotic partners, and maternal effects. Population differences in larval survival and body size in response to different temperatures have been demonstrated in other insects (Bochdanovits 2003) but such differences have also been attributed to adaptive phenotypic plasticity through a hormonal cascade that stops growth (Ghosh *et al*. 2013). Inclusion of latitude as a variable was one proxy for comparing populations broadly. Latitude has been suggested as a potential gradient for local adaptation to thermal stress in mosquitoes (Mori *et al*. 1988). However, our results suggest latitude does not explain heterogeneity of the effect of temperature. The strain origin/study location was included as a random effect as another indirect proxy for genetic differences in population, but we found no associations with strain origin. There is evidence of genetic structure across geographic space (Olanratmanee *et al*. 2013) and seasons (Endersby *et al*. 2011), but examples of strong local adaptation in development rate is lacking in *Ae*. *aegypti* populations (Richardson *et al*. 2011). Richardson et al. (2011) suggested that the lack of strong local adaptation may be evidence of a limited capacity to evolve in response to thermal stress. More studies are needed to evaluate the potential for adaptive phenotypic plasticity in response to temperature in *Ae*. *aegpyti* that could explain the heterogeneity of responses characterized here. Further, in natural

conditions other ecological factors not considered here such as interspecific competition, such as between *Ae. aegypti and Ae. albopictus* (Lounibos *et al*. 2002; Farjana *et al*. 2012), and predation (Kontodimas *et al*. 2004) may impact development rate and warrant further investigation.

Life-history traits such as body size and fecundity have been experimentally considered across multiple environmental conditions in few studies (Smith & Fretwell 1974; Fox & Czesak 2000). However, the importance is gaining recognition and more empirical estimates of these traits across environments have been made available since the preparation of this work (Muturi & Alto 2011; Richardson *et al*. 2011; Muturi *et al*. 2012; Carrington *et al*. 2013a). This is a limitation of conducting a meta-analysis in a rapidly developing field of research. Recent advances suggest variation in these traits has been attributed to responses to environmental conditions during development (Carriere & Roff 1995; Gillooly *et al*. 2002; Carrington *et al*. 2013c) as well as adaptive genetic responses due to selection at different temperatures (Messina & Fox 2001; Schwander & Leimar 2011a). Developmental life-history traits are of particular epidemiological importance for arboviral disease dynamics as they have been associated with critical aspects of vectorial capacity such as changes in bite rate, dispersal (Maciel-De-Freitas *et al*. 2007) and virus infection and dissemination (Alto *et al*. 2008).

Beyond utility for vector population control, development rate estimates may be useful for modeling and understanding disease transmission. There is evidence that larval environment impacts adult dispersion of *Ae. aegpyti* (Schneider *et al*. 2004) as well as arbovirus infection (Alto *et al*. 2005). Depinay *et al* (2004) have demonstrated improved predictive power for malaria transmission dynamics when using vector population

parameters including life-history traits of anopheline mosquitoes. Meta-analysis confirms that temperature is the most important ecological determinant of development rate in *Ae. aegypti* but that the effect is heterogeneous. Ignoring the heterogeneity in response to temperature may be problematic for using development rate estimates to model vector populations and predicting the impact of temperature on vector-borne disease transmission.

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## Chapter 4: Study of the interactive effects of the developmental environment on *Ae. aegypti* life-history traits

**Modified from**: Couret J, Benedict MQ, Doston EM. 2014. Temperature, density, and diet interact to influence larval development of *Ae. aegpyti*. *PLoS ONE*. 9(2), e87468.

### Abstract

Many environmental factors, biotic and abiotic interact to influence organismal development. Given the importance of *Ae. aegypti* as a vector of human pathogens including dengue and yellow fever, understanding the impact of environmental factors such as

temperature, resource availability, and intraspecific competition during development is critical for population control purposes. Despite known associations between developmental traits and factors of diet and density, temperature has been considered the primary driver of development rate and survival. To determine the relative importance of these critical factors, wide gradients of conditions must be considered. We hypothesize that 1) diet and density, as well as temperature influence the variation in development rate and survival, 2) that these factors interact, and this interaction is also necessary to understand variation in developmental traits. Temperature, diet, density, and their two-way interactions are significant factors in explaining development rate variation of the larval stages of *Ae. aegypti* mosquitoes. These factors as well as two and three-way interactions are significantly associated with the development rate from hatch to emergence. Temperature, but not diet or density, significantly impacted juvenile mortality. Development time was heteroskedastic with the highest variation occurring at the extremes of diet and density conditions. All three factors significantly impacted survival curves of experimental larvae that died during development. Complex interactions may contribute to variation in development rate. To better predict variation in development rate and survival in *Ae. aegypti*, factors of resource availability and intraspecific density must be considered in addition, but never to the exclusion of temperature.

## 4.1 Introduction

The rate of development and survival of organisms can vary greatly in response to many biotic and abiotic factors of the environment. Higher temperatures are often associated with faster development rate and have variable impacts on immature survival in insects (Wigglesworth, 1972; Laudien, 1973; Rueda *et al*., 1990; Kingsolver & Huey, 2008a; Kirby

& Lindsay, 2009; Arrese & Soulages, 2010). Density-dependent competition in insects is also associated with delayed maturity (Dijkstra, 1986; Merritt *et al*., 1992; Gimnig *et al*., 2002; Stav *et al*., 2005; Legros *et al*., 2009) and increased juvenile mortality (Southwood *et al*., 1972; Arnqvist & Johansson, 1998; Agnew *et al*., 2002; Gilles *et al*., 2010; Roberts & Kokkinn, 2010). Similarly, food availability and nutrient quality have known associations with development rates and mortality (Farnesi *et al*., 2009; Yang *et al*., 2009; Flenner *et al*., 2010; Yang & Rudolf, 2010; Dell *et al*., 2011; Padmanabha *et al*., 2011; Farjana *et al*., 2012). Despite the demonstrated associations of diet and density with developmental life-history traits, temperature remains a primary focus to explain development rate variation in insects (Howe, 1967; Mead & Conner, 1987; Robinson & Partridge, 2001; Kingsolver & Huey, 2008a; Damos, 2012). Selection for shorter development times is strong relative to other life history traits (Kingsolver & Pfennig, 2004; Kingsolver & Huey, 2008a). Thus, development time is an important candidate for understanding how a phenotype varies under different environmental conditions.

Differences observed in development times of the yellow fever mosquito *Ae. aegypti* Linnaeus from different geographical locations have been attributed to climatic differences (Hopp & Foley, 2001). Tun-Lin *et al*. (2000) suggest local adaptation to temperature and other climatic variables is occurring as the mean development for *Ae. aegypti* populations from Raleigh, NC (Rueda *et al*., 1990) and Israel (Bar-Zeev, 1958a) reared at the same temperatures differ by five days. Yet differences of up to 25 days are reported for a single population of *Ae. aegypti* in New South Wales at similar temperatures through experimental manipulation of diet and density during development(Russell, 1986). Rather than being locally adapted, we expect that *Ae. aegypti* developmental phenotypes are highly plastic in

response to several environmental factors. Empirical estimates of larval performance in response to gradients of three environmental factors in *Ae. aegypti* are rare with notable exceptions (Wada, 1965a; Moore & Fisher, 1969; Russell, 1986). We seek to determine the relative importance of temperature, diet, and density on developmental performance as well as evaluate their interactive effects. Studying the relationship between environmental variables that impact mosquito biology is critical to guide public health controls and improve understanding of the epidemiology of vector-borne disease (Waldock *et al*., 2013).

We estimate juvenile mortality and development rate of *Ae. aegypti* across four-level gradients of three factors: temperature, diet concentration, and intraspecific density. Determining the relative importance of multiple factors and evaluating potential interactions during development is foundational to understanding phenotypic responses of organisms to complex and changing environments (Stearns & Koella, 1986; Callahan *et al*., 1997; Garland & Kelly, 2008). Estimating the effects of environmental conditions on mosquito larvae is also critical information in the controlling of natural larval populations (Juliano, 2009). *Ae. aegypti,* once mature, vectors dengue virus, the most commonly transmitted arthropod-borne virus in the world (World Health Organization 2012), and yellow fever, one of the most lethal (World Health Organization, 2013).

## 4.2 Methods

*Ae. aegypti colony*

All experimental *Ae. aegypti* were reared from dried F2 eggs originating from wild caught eggs collected in Iquitos, Peru (Apperson, C., personal communication). Adults were supplied with 2% sugar solution at all times and offered a blood meal (human) five days after emergence. For maintenance of the population, one of the authors provided the blood meal

with full consent (JC). In consultation with the Institutional Review Board (IRB) of CDC, the IRB process is aimed toward protecting research subjects and this action is not subject to review. Mosquito colonies were maintained at 28°C and 80% relative humidity with a 12L:12D schedule and 30 minutes of gradual transition of light levels to simulate sunrise and sunset.

*Experiment*

Larval development rate and survival were quantified in artificial containers over gradients of temperature, food concentration (mg/ml/day), and conspecific density. Performance across conditions was evaluated by measuring mortality rates during development (dead individuals/cup), time to pupation (days since hatching), and time to emergence (days since hatching). Dead larvae were removed daily to determine mortality and estimate survival curves. We estimated development time through the daily counting and removal of molts for each life stage from II-instar to adult emergence. The diet mixture used was comprised of beef-liver powder, tuna meal, and vitamin mix in water (Damiens *et al*., 2012) and was tested at 1%, 2%, 4%, and 8% concentrations (10 mg/ml, 20 mg/ml, 40 mg/ml, and 80 mg/ml of diet mixture in deionized water respectively). Four initial density levels (10, 20, 40, and 80 larvae/cup) were tested. At initial densities, 500 µl of diet mixture was added to experimental cups. Each day the volume of the diet mixtures administered to containers was adjusted according to the daily number of larvae in that container in order to maintain a constant ratio of mg/larva/day (Table 4.1). This four by four array was tested across four temperatures (21°C, 24°C, 27°C, 30°C) resulting in 64 unique combinations. This three-factor and four level experimental design was replicated twice.

| Diet mixture | 5mg/500µL | 10mg/500µL | 20mg/500µL | 80mg/500µL |
|---|---|---|---|---|
| Initial density | | | | |

| | | | | |
|---|---|---|---|---|
| 10 larvae | 0.5 | 1 | 2 | 4 |
| 20 larvae | 0.25 | 0.5 | 1 | 2 |
| 40 larvae | 0.125 | 0.25 | 0.5 | 1 |
| 80 larvae | 0.0625 | 0.125 | 0.25 | 0.5 |

**Table 4.1 Experimental design of diet, density, and resultant ratios of mg/larva/day**

Synchronous hatching was induced using a barometric chamber at 85 mmHg for 15 minutes. First-instar larvae were placed in water previously brought to the experimental treatment temperature. Larvae were left to mature for 12 h with 5 ml of a 2% w/v mixture of the larval diet in order to allow enough growth to facilitate the pipette transfer of the correct number of first-instar larvae into experimental containers. Following this period, first instar larvae were transferred to artificial rearing cups with 250 ml of filtered rainwater. Rearing containers were 473 ml white, plastic, cylindrical food containers (Bauman Paper Co., Lexington, KY). The volume of water in each cup was maintained at 250 ml throughout the experiment by adding water as needed to a fill-line marked in permanent marker. Temperature and relative humidity were logged each hour throughout the duration of the experiment and remained constant, maintained by environmental chambers at the Centers for Disease Control and Prevention insectary facilities (Atlanta, GA).

*Statistical Analysis*

All tests were computed using R v3.0.1 statistical programming language (R Development Core 2013). A general linear mixed effects model (GLMM) with Poisson error and log link with the nlme package v3.1-109 (Pinheiro *et al*., 2013) was used to compare the dependent variables of mean duration of larval stages as well as the mean duration of the entire juvenile period from hatch to emergence ($\log_{10}$-transformed) to fixed variables of temperature, mg/ml/day of diet, initial density, and random factors of replicate, generation, and individual. Individual was included as a random factor due to the repeated measures of

development time recorded daily for experimental containers. Development times were also averaged for each of the 64 treatments. These computed means represented independent observations characterizing each container allowing the use of a completely randomized (CR) ANOVA rather than a repeated/related measures ANOVA. The inverse of mean development time (1/hours of development) was used to both normalize (Shapiro-Wilk test) and linearize development with respect to temperature (Ikemoto & Takai, 2000; Jarosík *et al*., 2002). Development rates of the larval stages as well as the development rate from hatch to emergence were computed for each treatment and analyzed using CR ANOVA. Mortality rate estimates were non-parametric and heteroskedastic and so were analyzed using the Kruskal-Wallis test. Survival functions were analyzed using Kaplan-Meier analysis with the survival package v2.37-4 (Therneau, 2013) and the Weibull function. Mantel-Cox Log-Rank tests were performed to determine whether increases in temperature, diet, or density significantly affected survival.

## 4.3 Results

*Development rate*

Mean development time was not normal for larval stages (Shapiro-Wilk test, $W = 0.7981$, $p < 0.0001$) or for the period from hatch to emergence (Shapiro-Wilk test, $W = 0.8471$, $p < 0.0001$). The inverse of mean development time (1/hours of development) was used to both normalize (Shapiro-Wilk test, $W = 0.977$, $p < 0.2747$; Shapiro-Wilk test, $W = 0.9755$, $p < 0.2312$) and linearize development with respect to temperature (Ikemoto & Takai, 2000; Jarosík *et al*., 2002).

Mean development times from hatch to pupation (i.e. larval stages; Table 4.2) and from hatch to emergence (Table 4.3) were estimated across gradients of temperature, diet,

and density. Development time of larval stages decreased at higher temperature across all diet and density treatments. Development time increased with increasing initial density level as well as decreasing diet level. The greatest variation in development time of larval stages occurred when the lowest diet level was paired with the higher initial density levels. The impact of diet level on development time from hatch to emergence was most evident at the highest initial density level.

| | | Temperature °C | | | |
|---|---|---|---|---|---|
| | | 21 | 24 | 27 | 30 |
| All diets and densities | | 12.71 (0.17) | 10.68 (0.18) | 9.36 (0.16) | 8.62 (0.14) |
| Diet | 1% | 18.95 (0.47) | 16.57 (0.53) | 14.71 (0.4) | 13.82 (0.42) |
| | 2% | 11.81 (0.19) | 10.7 (0.2) | 9.92 (0.20) | 9.27 (0.16) |
| | 4% | 10.23 (0.11) | 8.11 (0.11) | 6.58 (0.94) | 6.39 (0.08) |
| | 8% | 9.88 (0.09) | 7.49 (0.05) | 6.24 (0.12) | 5.74 (0.06) |
| Density | 80 | 14.35 (0.28) | 12.61 (0.30) | 11.32 (0.25) | 10.26 (0.24) |
| | 40 | 11.11 (0.17) | 9.05 (0.17) | 7.8 (0.17) | 7.27 (0.12) |
| | 20 | 10.2 (0.14) | 7.88 (0.10) | 6.54 (0.16) | 6.00 (0.09) |
| | 10 | 10.57 (0.14) | 7.73 (0.09) | 6.18 (0.12) | 6.18 (0.11) |

**Table 4.2. Mean development time of larval stages for all treatments with standard error in parentheses.**
For each diet, values are averaged across density treatments. For each density, values are averaged across diet.

| | | Temperature °C | | | |
|---|---|---|---|---|---|
| | | 21 | 24 | 27 | 30 |
| All diets and densities | | 16.23 (0.18) | 13.16 (0.18) | 11.51 (0.15) | 9.92 (0.12) |
| Diet level | 1% | 22.62 (0.50) | 19.19 (0.56) | 16.58 (0.39) | 14.51 (0.37) |
| | 2% | 15.34 (0.20) | 13.26 (0.20) | 11.88 (0.19) | 10.87 (0.16) |
| | 4% | 13.63 (0.11) | 10.55 (0.10) | 9.14 (0.07) | 8.07 (0.08) |
| | 8% | 13.37 (0.09) | 9.98 (0.05) | 8.45 (0.07) | 7.18 (0.05) |
| Density | 80 | 17.99 (0.30) | 15.10 (0.31) | 13.23 (0.25) | 11.30 (0.22) |
| | 40 | 14.48 (0.18) | 11.62 (0.17) | 10.21 (0.17) | 8.95 (0.12) |
| | 20 | 13.63 (0.13) | 10.36 (0.13) | 9.03 (0.10) | 7.74 (0.10) |
| | 10 | 14.14 (0.15) | 10.18 (0.13) | 8.70 (0.09) | 7.82 (0.11) |

**Table 4.3. Mean development time from hatch to emergence for all treatments with standard error in parentheses.**

For each diet, values are averaged across density treatments. For each density, values are averaged across diet.

Mean development rate (1/days of development time) of the larval stages was significantly impacted by all of the fixed independent factors and their two-way interactions (Table 4.3). Random factors of replicate, generation, and individual were not significant (Table 4.3). Initial densities of 40 and 80 larvae per cup had the greatest impact on reducing development rate (Fig. 4.1, panel A). At the highest initial density (80 larvae/cup) the impact of mg/ml/day of diet was more evident with an average difference of 14.2 days between the lowest and highest diet levels across all four temperatures. In contrast, at the lowest initial density (10 larvae/cup) the average difference between the lowest and highest diets was 0.8 days across all temperatures. Similar results were seen for development time from hatch to emergence (Fig. 4.1, panel B).

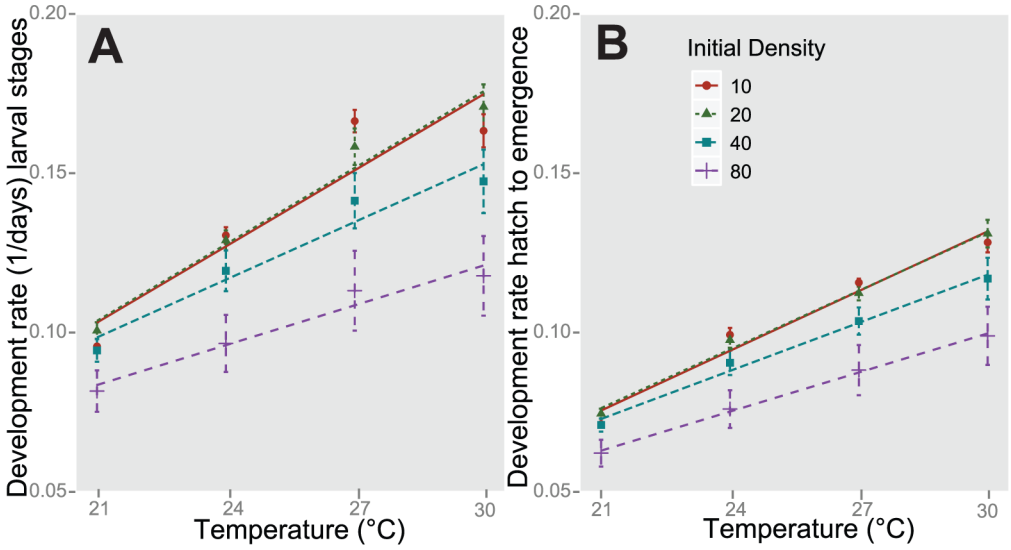


**Figure 4.1 Mean development rate for larval stages (A) and from hatch to emergence (B)** Bars indicate standard error. Character shape, color, and line type indicate initial density level. Lines indicate simple linear regression for density treatments.

Development rate of both the larval stages and the period from hatch to emergence was also significantly impacted by mg/ml/day of diet (Fig. 4.2). At the lowest diet level (0.02 mg/ml/day), the average difference between the lowest and highest density treatments was

14.4 days. At the highest diet level (0.16 mg/ml/day) the average difference is 0.4 days. The development rate for the larval stages was significantly associated at three of four temperature treatments (21°C, 24°C, and 27°C; $F_{2419,3} = 295.392$, $p < 2.2e\text{-}1$).

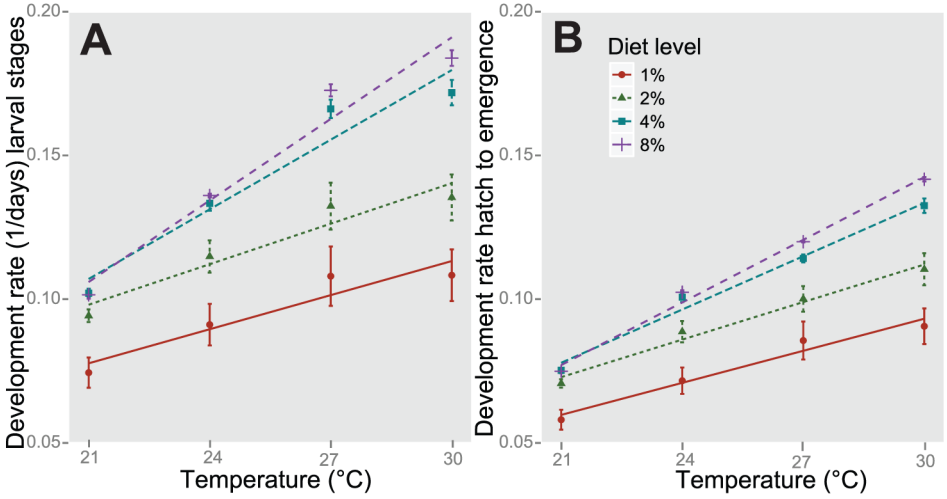


**Figure 4.2 Mean development rate for larval stages (A) and from hatch to emergence across diet treatments**
The amounts of each diet (mg/ml) were added to experimental cups daily. Bars indicate standard error. Character shape, color, and line type indicate diet treatment. Lines indicate simple linear regression for diet treatments.

All three factors of temperature, diet, and density significantly explain the variation in mean development rate of larval stages (Table 4.4). These factors, as well as the two and three-way interactions, were significant for the development rate from hatch to emergence (Table 4.5).

| | Df | SS | MS | F | p | |
|---|---|---|---|---|---|---|
| Temp | 3 | 0.029 | 0.029 | 143.9 | < 0.000 | *** |
| Diet | 3 | 0.021 | 0.021 | 104.4 | < 0.000 | *** |
| Density | 3 | 0.018 | 0.018 | 88.19 | < 0.000 | *** |
| Temp x Diet | 9 | 0.003 | 0.003 | 14.34 | < 0.000 | *** |
| Temp x Density | 9 | 0.002 | 0.002 | 8.587 | <0.001 | ** |
| Diet x Density | 9 | 0.005 | 0.005 | 24.50 | < 0.000 | *** |
| Temp:Diet:Density | 27 | 0.000025 | 0.000025 | 0.122 | < 0.8 | |

**Table 4.4 CR ANOVA of temperature, diet (mg/ml/day), and initial density for development rate of larval stages**

| | Df | SS | MS | F | p | |
|---|---|---|---|---|---|---|
| Temp | 3 | 1.2152 | 0.4051 | 2424.8 | < 0.000 | *** |
| Diet | 1 | 1.2481 | 1.2481 | 7471.4 | < 0.000 | *** |
| Density | 1 | 0.3255 | 0.3255 | 1948.4 | < 0.000 | *** |
| Temp x Diet | 3 | 0.1318 | 0.0439 | 263 | < 0.000 | *** |

| | | | | | | |
|---|---|---|---|---|---|---|
| Temp x Density | 3 | 0.0306 | 0.0102 | 61.1 | < 0.000 | *** |
| Diet x Density | 1 | 0.1676 | 0.1676 | 1003.3 | < 0.000 | *** |
| Temp:Diet:Density | 3 | 0.0115 | 0.0038 | 22.91 | < 0.000 | *** |

**Table 4.5 CR ANOVA of temperature, diet, and initial density for development rate from hatch to emergence**

We examined the changes in development rate with respect to the ratio of diet to density in order to assess the importance of the unit of mg/larva/day. By statistically comparing mg/larva/day within and between levels, we determined that the development rate of larval stages was not affected by higher initial numbers of larvae as long as the amount of mg/larva/day remained constant (Table 4.6).

| | Initial density | | | | $\chi^2$ | p |
|---|---|---|---|---|---|---|
| Ratio | 10 | 20 | 40 | 80 | | |
| 0.0625 | * | * | * | 20.89 | | |
| 0.125 | * | * | 12.12 | 12.26 | 0.0008 | 0.98 |
| 0.25 | * | 9.22 | 8.34 | 8.5 | 0.0392 | 0.98 |
| 0.5 | 8.45 | 7.66 | 7.15 | 7.54 | 0.1162 | 0.99 |
| 1 | 7.82 | 6.92 | 7.15 | * | 0.063 | 0.97 |
| 2 | 7.21 | 6.84 | * | * | 0.0095 | 0.92 |
| 4 | 7.41 | * | * | * | | |

**Table 4.6 Mean duration of larval stages for mg/larva/day across density levels and $\chi^2$ tests.** Duration of larval stages reported in days. Ratios only possible at certain combinations of diet and density levels considered (see Table 1) otherwise indicated as *. Within ratio comparisons (Wald chi-square) were tested when two or more containers shared the same ratio of mg/larva.

In comparing development of larval stages to mg/larva/day it was evident that as the amount of food per larva decreased, the differences between temperature treatments were smaller (Fig. 4.3, panel A). In addition, as the amount of food per larva increased up to 1 mg/larva/day, the temperature treatment difference became apparent, but remained relatively constant at higher food doses (Fig. 4.3, panel A). The same pattern was observed for the development rate from hatch to emergence (Fig. 4.3, panel B). The relationship between temperature and larval development rate was significantly and positively linearly associated at each level of food/larva/day as determined by simple linear regression (Fig. 4.4, panel A; Table S4.1). At 21°C, the lowest temperature treatment, there were smaller differences in development rate and differences increased with temperature such that the widest differences

in development rate occurred at the 30°C (Fig. 4.4, panel A). The same pattern was found with the development rate from hatch to emergence (Fig. 4.4, panel B).

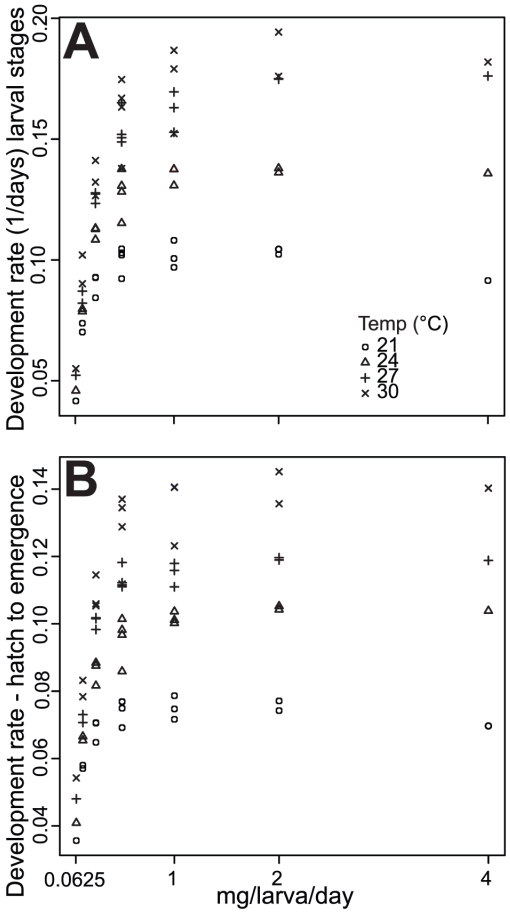


**Figure 4.3 Development rate of larval stages (A) and from hatch to emergence (B) across mg/larva/day levels.**
Character shape represents temperature in which larvae were reared.

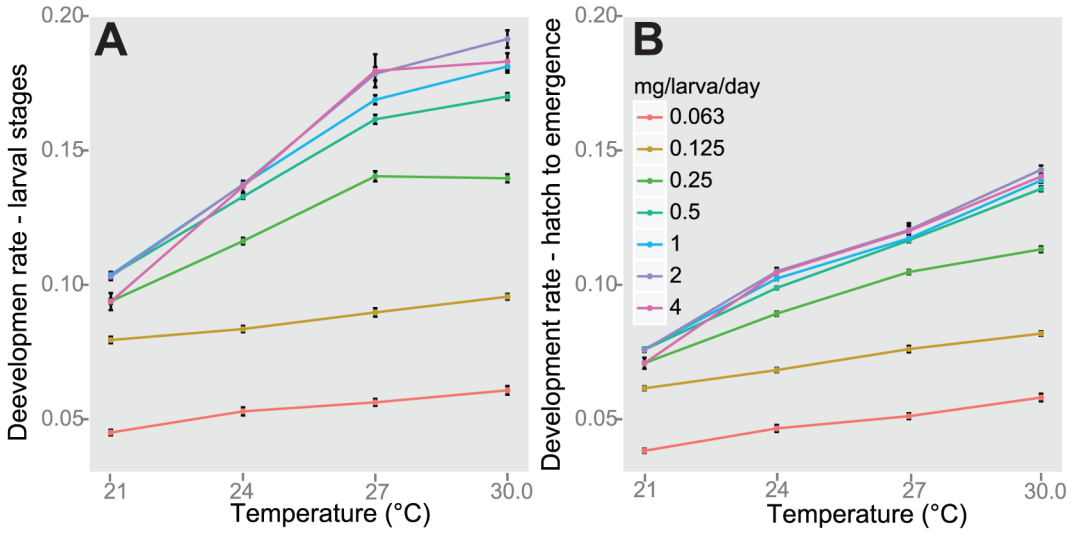


**Figure 4.4 Development rate of larval stages (A) and hatch to emergence (B) across temperature and mg/larva/day.**
Line color indicates levels of mg/larva/day. Black bars indicate standard error.

*Survival*

Mortality was defined as death during immature stages or during molting to the adult form. Of the 4,800 experimental larvae, 429 died for an overall mortality rate of 9% across all treatments (Fig. 4.5, panels A through C). Mortality rate differed significantly across temperature treatments (Kruskal-Wallis, $X^2_{df=3} = 10.79$, $p < 0.05$), but not diet (Kruskal-Wallis, $X^2_{df=3} = 4.66$, $p > 0.1$) or initial density level (Kruskal-Wallis, $X^2_{df=3} = 0.56$, $p > 0.9$).

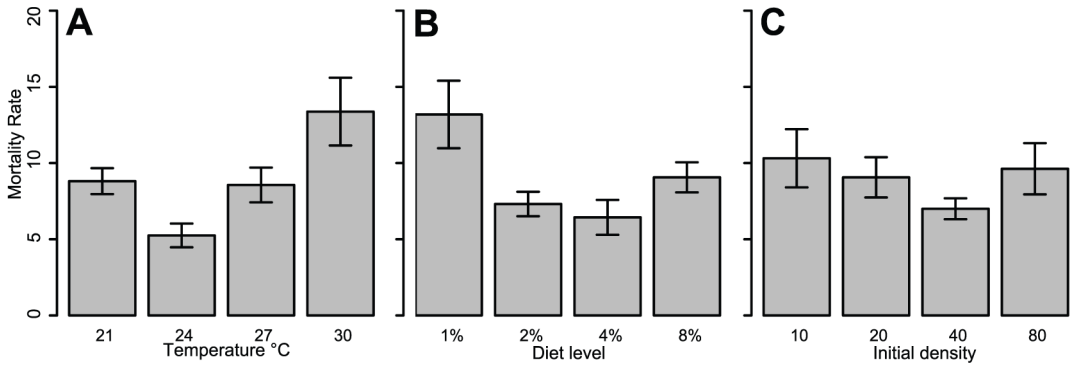


**Figure 5. Mortality rate across temperature (A), diet concentration (B), and initial density (C).**

Survival curves were estimated based on the subset of larvae that died before or during emergence (n = 429; Fig. 4.6, panel A). The larvae that survived to adulthood (n=4,371) were excluded from analysis because their inclusion flattened the survival

functions such that differences could not be visualized. Instead we examine the patterns of survival among those larvae that died before maturation. Temperature groups had significantly different survival functions (logrank, Mantel-Cox $X^2_{df=3} = 8.39$, $p < 0.05$), and this impact appeared to be mainly driven by the 21ºC treatment (Fig. 4.6, panel B). Both diet (logrank, Mantel-Cox $X^2_{df=3} = 105.4$, $p < 0.0001$) and initial density (logrank, Mantel-Cox $X^2_{df=3} = 93.99$, $p < 0.0001$) demonstrated highly significant differences in survival functions, and these differences were evident at every level of each factor (Fig. 4.6, panel C; Fig. 4.6, panel D). While percent mortality was highest at the lowest diet level, larvae in these treatments also survived longer than those from higher diets. Larvae in higher density treatments survived longer than those in lower density treatments, and with similar mortality across densities (Fig. 4.6, panel C).

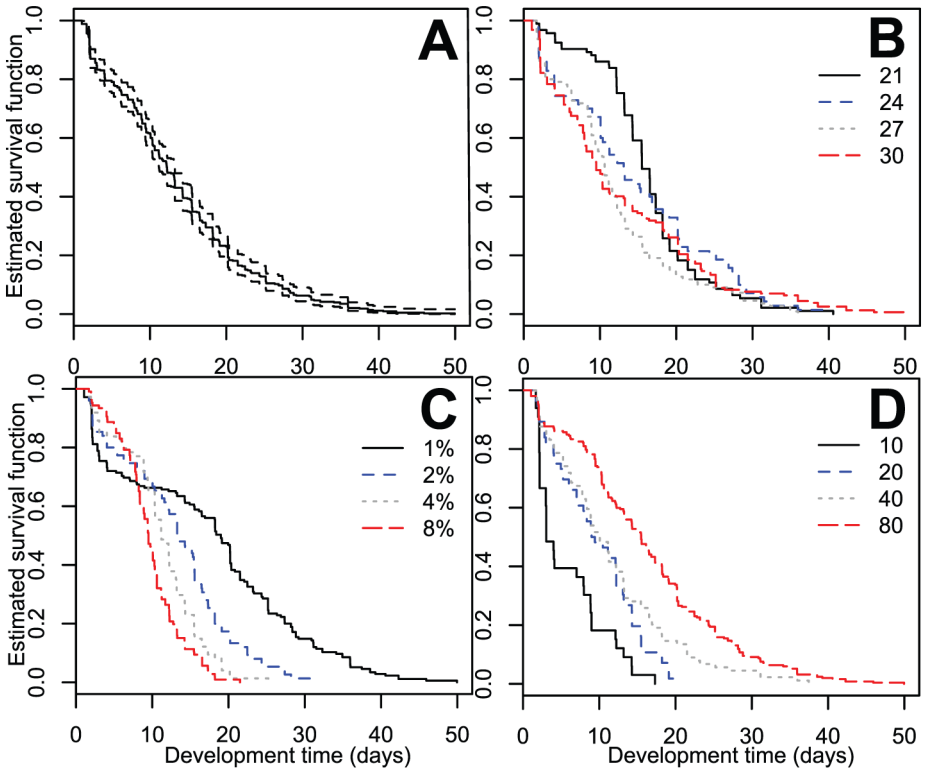


**Figure 6. Survival curves over all treatments (A), by temperature (B), diet (C), and initial density (D).**
Treatments are distinguished by line type and color.

## 4.4 Discussion

We sought to estimate larval development and survival of *Ae. aegypti* across wide gradients of environmental conditions of temperature, diet, and density, and hypothesized all three factors and their interactions would influence variation in developmental timing and survival. During larval stages of mosquitoes, the aquatic environment can be experimentally manipulated to facilitate the study of environmental impacts on phenotypic variation both in the laboratory and under natural conditions (Huffaker, 1944; Lake & Friend, 1968; Clay & Venard, 1972; Tun-Lin *et al*., 2000; Kontodimas *et al*., 2004; Gilles *et al*., 2010; Farjana *et al*., 2012; Yoshioka *et al*., 2012).

A GLMM model including factors of temperature, diet, and initial density and their two-way interactions best explains development rate variation for the larval stages (Table 4.3). For the entire developmental period from hatch to emergence, these factors as well as their two-way and three-way interactions are significant (Table 4.4). It is unclear why the developmental period from hatch to emergence would include a three-way interaction whereas the larval stages alone would not. The difference between these dependent variables is the exclusion or inclusion of metamorphosis, a complex developmental process to which plasticity in both juvenile and adult phenotypes has often been attributed (Bentz *et al*., 1991; Moehrlin & Juliano, 1998; Petavy *et al*., 2001; Folguera *et al*., 2008; de Jong, 2010; Folguera *et al*., 2010). Development rate differences in response to temperature have been observed between larval and pupal stages in the fruitfly *Drosophila buzzatii* (Folguera *et al*., 2010). Thus the impact and interaction of environmental factors may be stage-specific.

To progress to the next life stage, mosquito larvae require a minimum amount of nutrition in order to trigger hormonal developmental cascades (Nijhout *et al*., 2010). The

interactions observed here suggest that these thresholds of resource requirements are both temperature-dependent and density-dependent. Mechanisms for temperature-dependence of development rate include the impact of temperature on the use of fat body energy reserves(Arrese & Soulages, 2010) as well as the temperature-dependent growth of food resources of periphyton populations on which larvae feed (Merritt *et al*., 1992). The interactive effect of diet and density can be difficult to distinguish experimentally, and we chose an experimental design well-suited to this purpose (Mori, 1979; Mori *et al*., 1988; Gilles *et al*., 2010).

Several hypotheses address the impact of crowding on mosquito larvae development including tactile interference (Wada, 1965b; 1965a; Dye, 1984; Roberts & Kokkinn, 2010), chemical waste toxins, chemical signals exhibited by larvae, and stress from food partitioning (lower food per capita) at higher numbers. Ammonia accumulates in aquatic larval habitats due to larval waste and may retard (Moore & Fisher, 1969) or accelerate(Walker *et al*., 1991; Chaves & Kitron, 2011) growth. This may occur because ammonia influences the microbe populations on which mosquito larvae feed, or it may be a stressor to developing larvae. Growth retardant factor is also produced by overcrowded larvae, an effect that is not species-specific (Moore & Fisher, 1969). Growth retardant factor has been shown to lengthen development and prevent pupation in some Culicine mosquito species(Roberts, 1998) but not others(Roberts & Kokkinn, 2010). In a partitioned container designed to test the impact of shared water without the mechanical interference of crowding, Yoshioka et al. 2012(Yoshioka *et al*., 2012) do not find conspecific density to impact development time from hatch to emergence. These mechanisms are not mutually exclusive (Roberts & Kokkinn, 2010). Our design does not distinguish between these factors, but rather focuses on

distinguishing the impact of density from those of diet level, especially in the context of a limited resource environment. Our results indicate that larvae receiving the same amount of food per capita exhibit lower development rates at higher densities, an effect that is consistent across temperature.

At the lowest temperature, there were narrower differences between diet and density treatments than at intermediate and high temperatures (Fig. 4.3). The interaction between temperature, diet, and density may provide an alternative explanation of long-standing puzzle in insect physiology that insects are bigger when reared at colder temperatures. Many hypotheses focus on the impact of temperature to explain variation of life-history traits such as body size (Atkinson, 1995; Karl & Fischer, 2007; Kingsolver & Huey, 2008b) and development rate (Gillooly, 2001; Gillooly *et al*., 2002; Knies & Kingsolver, 2010).

Our results confirm a high degree of plasticity in development times in *Ae. aegypti* of a single brood that have as broad a range as distinct populations across continents and latitudes (Bar-Zeev, 1958b; Ofuji, 1963; Nayar & Sauerman, 1970; Ameen & Moizuddin, 1973; Russell, 1986; Silva & Silva, 1999; Tsuda & Takagi, 2001; Kamimura *et al*., 2002; Lounibos *et al*., 2002; Chang *et al*., 2007; Tejerina *et al*., 2009; Padmanabha *et al*., 2011). We cannot establish based on these data whether this plasticity is adaptive or non-adaptive. There is limited evidence suggesting that developmental timing and body size in mosquito larvae adaptively respond to changes in water volume (Juliano & Stoffregen, 1994). Another potential mechanism for plasticity of developmental timing is adaptive plasticity of behaviors in mosquitoes. Behavioral changes in foraging in response to controphic competition (Stav *et al*., 2010) and oviposition (Yoshioka *et al*., 2012) in response to conspecific larval density have been observed in mosquitoes.

While reduced larval survival with higher larval density has been observed in mosquito rearing studies (Tun-Lin *et al*., 2000), we did not find initial density to impact percent mortality of *Ae. aegypti*. This may be some indication that the initial density conditions here considered did not span a wide enough gradient to have a significant effect. In contrast, the schedule of survivorship among those larvae that died before emergence was significantly impacted by density as well as diet. Those larvae reared in lower diet concentrations survived longer than higher diet concentrations. This shift may be either a direct result of starvation on critical hormonal signals necessary for maturation(Nijhout *et al*., 2010) or another mechanism such as an increase in haemolymph lipid concentration that has been observed in insects under nutritional stress (Beenakkers *et al*., 1985; Ziegler, 1991). In other Aedine mosquitoes, temperature has demonstrated effects on larval (Tun-Lin *et al*., 2000; Kirby & Lindsay, 2009) and adult (Bayoh & Lindsay, 2004) survival. Survival analysis in related species, *Ae. albopictus,* found no impact of either diet or density factors on the timing of survival over similar treatment levels as used in this study(Yoshioka *et al*., 2012). There is some evidence in other mosquito genera that temperature and density may interact to influence larval survival (Lyimo *et al*., 1992). It may be that due to the same interactions observed for development rate, it is only when considering temperature, diet, and density that the impacts on survivorship curves become evident.

Examining the plastic responses of *Ae. aegypti* to heterogeneous environmental conditions addresses broad questions in ecology and evolution (Pigliucci, 2005; Reiskind & Lounibus, 2009; Padmanabha *et al*., 2011; Farjana *et al*., 2012) as well as targeted public health questions (Irvin, 2004; Bargielowski *et al*., 2011; Little *et al*., 2012). Mosquitoes are historically important in the field of medical entomology, as vectors of human pathogens. In

*Ae. aegypti,* recent evidence shows that shifting climatic patterns have impacted the timing of developmental stages (Kearney *et al*., 2009) of this mosquito. There is an awareness that variation in environmental conditions and their impact on mosquito physiology (Worner, 1992a; Joshi, 1996; David *et al*., 1997) can influence vectorial capacity for dengue virus transmission (Reiter, 2007; Lambrechts *et al*., 2011). Even recent population dynamics models of *Ae. aegypti* and other mosquito vectors simplify the impact of environmental conditions to include only the influence of temperature (Wagner *et al*., 1991; Worner, 1992b; Damos, 2012). Our results provide empirical estimates of life-history traits critical to modeling mosquito population abundance over wide gradients of these environmental conditions and illustrate the importance of interactive effects in modulating developmental timing. These findings support the need to include more complexity when predicting the population dynamics of this arboviral vector.

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# Chapter 5: The impact of symbiosis on the invasive stink bug *Megacopta cribraria* is mediated by the context of host plant


## Abstract

The outcomes of many parasitic host-microbe interactions are known to be dependent on environmental context. In contrast, the benefits for hosts of obligate, mutualistic microbial symbioses are assumed to be universal across environments. It remains unknown whether the costs and benefits of obligate symbioses are altered by ecological conditions. We sought to test the hypothesis that the impact of a microbial symbiont on its host is contingent on the ecological context of host plant in an herbivorous insect. We focused the sap-feeding insect, *Megacopta cribraria*, which invaded the southeastern United States from Asia in 2009 and has since expanded its range throughout the Southeast. We assessed host life-history traits, including development time, survival, and size, in the presence and absence of the obligatory microbial symbiont *Candidatus* Ishikawaella capsulata on two host plants. We found that differences in development of hosts with and without microbial symbionts were mediated by the host plant context. Our results support the hypothesis that this host-microbe interaction is environmentally contingent, which has important implications for understanding the nature of symbiosis.


## 5.1 Introduction

Many insects associate with microorganisms that support host development (Waller & LaFage 1985; Holldobler & Wilson 1990; Adams & Douglas 1997; Bourtsiz & Miller

2003; Moran et al. 2008; Nikoh 2011) and can mediate their ability to use resources in their environment (Wilkinson *et al*. 2001; Raghu *et al*. 2002; Parker et al. 2006). In obligate insect-microbe associations these symbionts are often found in specialized cells or organs in their hosts and are thought to provision nutrients, or, as in phytophagous insect hosts, detoxify plant allelochemicals (Buchner, 1966; Douglas, 1988; Dowd 1991). In many insects, symbionts can alter host development rate, body size, and survival (de Vries et al. 2004; Prado & Almeida 2009; Prado et al. 2009; Brownlie & Johnson 2009). Maintenance of these symbiotic associations is thought to be regulated by an intimate dialogue between host and microbe, and will be selected for based on benefits conferred to the host (Ruby, 2008; Werner et al. 2014). Particularly in invasive species, these symbionts, by altering growth and survival of hosts, may play an important role in determining colonization success (Pringle et al. 2009; Parker et al. 2006).

Environmental factors have long been shown to mediate the outcome biotic interactions within and between species (Agrawal *et al*. 2007). For example, many studies have examined how environmental context mediates competitive interactions between species in the same guild (Chesson and Warner 1981; Wiens, 1977; Hutchinson 1961; Park 1954). The outcomes of many parasitic interactions are contingent upon abiotic (e.g, temperature; Blanford *et al*. 2003; Bryner & Rigling 2011; Sadd 2011) and biotic (e.g., food availability; Fellous & Koella 2010; Manson *et al*. 2010) factors. This environmental mediation has also been shown in some mutualistic interactions (Picullel *et al*. 1998; Setala *et al*. 1997; Bronstein 1994). In the mutualism between leguminous plants and nitrogen-fixing rhizobial bacteria housed in root nodules, before root nodule formation is initiated, soil temperature within the root zone influences rhizobial survival in the soil as well as the

exchange of molecular signals between the two symbiotic partners (Sadowsky, 2005). Nitrogen levels in the soil can also influence these associations; addition of nitrogenous fertilizers reduces bacteria uptake and nodule formation (reviewed in Zahran 1999). In another plant-microbe association, endophytic fungi in the plant's tissues have been shown to range from mutualistic to antagonistic towards their plant hosts depending on endophyte genotype, plant genotype, and environmental conditions (Faeth & Bultman, 2002; Faeth & Fagen, 2002).

Environmental context may dramatically change when organisms invade a novel environment, and microbial symbionts can play an important role in successful invasion. The ability of the kudzu vine to invade new habitats is due in part to its ability to establish symbioses with native mycorrhizal fungi (Greipsson & DiTommaso 2006; Tytova et al. 2013). Viruses brought by invasive plants can also aid invasions by weakening native species in competition with invaders (Malmstrom et al. 2005; Roossinck 2011). In contrast, the legume *Cytisus scoparius* is limited in its ability to colonize new habitats because it is symbiont limited (Parker et al. 2006). In animals, the colonization success of invasive bark beetles depends on its associations with fungi and gut-associated bacteria (Klepzig & Six 2004; Vasanthakumar et al. 2006; Klepzig et al. 2009). Changes in microbial symbionts can also alter other important traits such as heat tolerance (Dunbar et al. 2007), parasite defense (Oliver et al. 2005), and mating behavior (Miller et al 2010).

The maintenance of mutualistic associations has been described using market theory in which commodity exchanges dictate costs and benefits of the partnership for each 'actor' (Werner et al. 2014). In the study of obligate symbioses, however, costs are generally overlooked and benefits to the host are assumed to be universal rather than environmentally

contingent. In terms of host plants, one of the most important biotic factors influencing insect development, survival and interactions with other species, there is some evidence that benefits provided by a symbiont may be context dependent. Wilkinson et al. (2001) demonstrated that aphids with the obligate symbiont *Buchnera aphidicola* had increased larval mass than aphids without the symbiont when reared on some host plants but not others. However, the intimacy of aphid-*Buchnera* symbiosis required antibiotic clearing of symbionts in mothers, which also impacted facultative symbionts and could introduce other effects. This illustrates a broad challenge in quantifying the fitness benefits of obligate symbioses to hosts and testing whether such benefits are universal or environmentally contingent on factors such as host plant.

Due to the manner of transmission of their obligate symbionts, stink bugs of the genus *Megacopta* (Plataspidae) provide a system to quantitatively measure the impacts of obligate symbionts on host developmental traits. *Megacopta* spp. harbor extracellular gamma-proteobacteria, *Candidatus* Ishikawaella capsulata, in midgut crypts (Fukatsu & Hosokawa 2002; Fukatsu *et al*. 2006; Hosokawa et al. 2007a; Hosokawa et al. 2007b). The bacteria are vertically transmitted via protein capsules, manufactured by the mother, that are loaded with symbionts and deposited alongside each egg mass. Aposymbiotic nymphs hatch and probe these capsules to ingest symbionts (Hosokawa *et al*. 2008). By removal or heat-treatment of capsules Fukatsu & Hosokawa (2002) demonstrated that preventing establishment of a symbiont population within sister species *Megacopta punctatissima* Fabricius can delay development, decrease body weight, and lessen adult coloration (Fukatsu & Hosokawa 2002), suggesting an essential need for symbiosis for the host. This study was conducted with insects feeding on a single host plant (soybean) under controlled laboratory

conditions, and it is unknown how abiotic and biotic factors could alter the benefits conferred by the symbiont.

*M. cribraria* recently invaded North America from Asia. Since being observed in Georgia in 2009 (Eger *et al*. 2010), it has expanded its range to seven southeastern United States (Ruberson et al. 2012, Gardner et al. 2013), and continues to expand north, south, and west (W. Gardner, personal communication). Its expansion is closely associated with the distribution of the host plant kudzu vine (*Pueraria montana*; Eger et al. 2010, Suiter et al. 2010). In Asia, *Megacopta* spp. occur on kudzu (Hibino & Ito, 1983; Tayutivutikul & Yano, 1990; Hosokawa *et al*. 2007a). Reports of the pest-status of *M. cribraria* for soybean (*Glycine max* (L.) Merril) in Asia are conflicting (Guanguan *et al*., 2006; Hosokawa *et a*l. 2007a), but studies have shown *M. cribraria* can feed on soybean when inoculated with the symbionts of *M. punctatissima* (Hosokawa et al. 2007b). *M. punctatissima* has been shown to impact soybean growth by approximately 10-30% (Kikuchi and Kobayashi 2010).

In its expanded North American range, *M. cribraria* is widely reported on soybean (Gardner et al. 2013) and recent evidence suggests soybean may be a suitable host plant for invasive *M. cribraria* (Del Pozo-Valdivia and Reisig 2013, Seiter et al. 2013). It is presumed that the microbial symbiont *Ishikaewlla* is as essential for *M. cribraria* as for its sister species, but this has not been empirically tested. We evaluated the impact of the host-microbe interaction on *M. cribraria* development across different host plants and under different environmental conditions. We sought to test the hypothesis that the impact of a microbial symbiont on its host is contingent on the ecological context of host plant.

## 5.2 Methods

*Overview*

We measured development time, juvenile survival, and adult body size upon emergence in *M. cribraria*. The impact of symbiont acquisition on development was compared on alternative host plants, kudzu vines and soybean plants. The experiment was first conducted in the field. Based on those results we repeated the design in the laboratory to focus on development of early instars (up to third). Thus, we were also able to draw some comparisons between rearing methodologies (i.e. laboratory versus field). For each methodology, we used a two-by-two factorial design of host plant by symbiont status (Fig. 5.1).

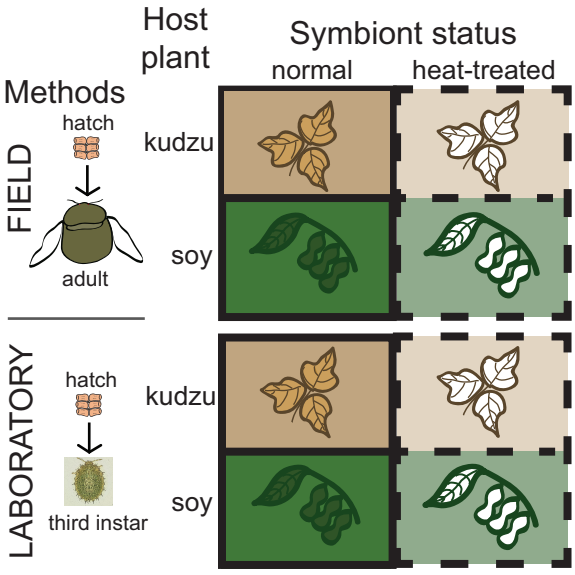


**Figure 5.1. Experimental design for field experiment and laboratory experiment**

*Experiment Set-Up*

Soybean plants were started from seed (Public Variety UA 5612) in a greenhouse. For the field experiment, fourteen day-old seedlings were transplanted into one of four outdoor raised beds (3 x 6 ft) in a community garden in Doraville, Georgia, United States. One soybean plant was planted in each square foot resulting in 18 plants per bed. Each bed was enclosed in a mesh tent (4 x 8 ft) secured to a raised bed frame and wooden posts, and

tethered to the ground. The kudzu treatment was conducted in a patch of wild kudzu adjacent to the community garden. Wild *M. cribraria* and other insects were removed from young kudzu shoots by hand and using compressed air. Shoots were then enclosed in cylindrical mesh tents (1.5 x 2.5 ft) sealed with duct tape. Twenty tents were set up one week prior to the beginning of the experiment and checked daily for infiltration of wild *M. cribraria* or other insects.

For the laboratory experiment, plants were placed in BugDorm mesh tents (36x36x72 inches) with four plants per tent. Soybeans were grown from seed. We collected root nodules of kudzu plants from nearby patches and grew them in two gallon pots until they were established (Frye et al. 2007; Zhang et al. 2012). Tents were housed in environmental chambers maintained at 25C and approximately 70%RH, with an extended-day schedule of 16L:8D.

*Insect sampling and experimental manipulation*

For both the field and laboratory experiments, egg masses were collected from the top stratum of new kudzu growth (Fig. 5.2, panel A) on and near the Emory University (Atlanta, Georgia) campus. Using forceps we removed symbiont capsules and separated egg masses into single eggs (Fig. 5.2, panel B). Washes of 70% ethanol and 4% formalin were used to clean eggs, but not symbiont capsules (as described in Fukatsu *et al*. 2002). Eggs were glued together using diluted, non-toxic Elmer's Glue into experimental egg masses of 20 eggs, positioned to mimic natural egg mass configurations (Fig. 5.2, panel C).

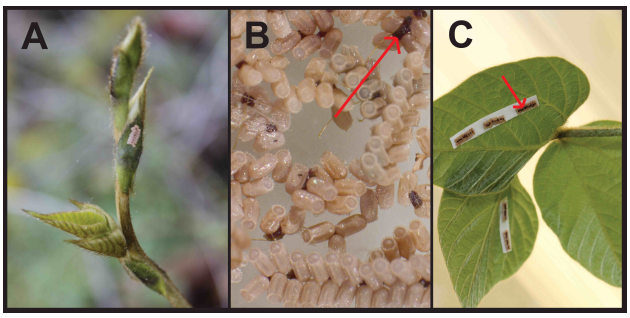


**Figure 5.2 *M. cribraria* egg masses A) in natural environment on kudzu, B) under stereoscopic dissection, and C) reconstructed and glued to soy plant leaf.**

To each experimental egg mass, we added 10 symbiont capsules. For normal symbiont treatments, the previously removed symbiont capsules were glued along the central line of the experimental egg masses. Care was taken that glue was not applied on or near the perculum of eggs so as not to impede hatching. For the symbiont negative treatments, in an effort to reduce juvenile wandering, which is known to increase in first instar nymphs when symbiont titers are low (Hosokawa *et al*. 2008) and which may impact juvenile mortality through increased death by desiccation, we provided autoclaved symbiont capsules so that negative treatment nymphs could probe. In laboratory populations, treatments with heat-treated capsules had reduced wandering behavior and mortality compared to eggs masses with no capsules (Couret, J. unpublished data). Symbiont capsules were heated to 190°C for 15 minutes in an autoclave. Subsequently, heat-treated capsules were glued along the central line of the experimental egg masses in a similar manner to the normal treatments.

For the field experiment, 10 experimental egg masses were glued on the underside of leaves of each plant, resulting in 200 eggs per plant. This number of egg masses was determined based on published estimates of hatch rate (Zhang *et al*. 2012) and survival to adulthood for *M. cribraria* reared with heat-treated symbiont capsules on soy (Fukatsu *et al*. 2002). We sought to have an adult sample size of 30 individuals for each treatment. Due to

contamination of soy plants with spider mites, 18 soy plants were dropped from the analysis. Due to the time constraints of experimental egg mass preparation and delays due to inclement weather, experimental tent set up was staggered over six weeks (April 7th to May 22nd, 2012).

The laboratory experiment included four replicate plants for each treatment with 200 eggs per plant.

*Life-history traits measurement*

We measured several life-history traits including development time to each instar, survival to each instar, and adult body size. Development time and survival were determined by counting the numbers of each instar and adults each week after hatch in the field and each day after hatch in the lab. Data collection in the field continued until all insects emerged as adults or died. In the laboratory, data collection continued until all insects matured to third instar or died. In the laboratory dead nymphs were removed from tents daily; this was not possible in the field. In both experiments, only insects found on the plant were counted. Especially for early instars in the field, it was not possible/feasible to find insects not on the plants.

During the field experiment, each week all adults that had emerged within the last week were collected in mesh bags and brought to the laboratory. A subset was weighed and photographed under a stereo-microscope. Subsets of third instar nymphs from the laboratory experiment were similarly catalogued. Approximately equal numbers were collected from each treatment. Body size was measured and compared based on scutellum width for adults from the field. For third instar nymphs from the laboratory experiment, we used distance

between eyes as a proxy of body size as measured from the dorsal view. JPEG images were analyzed using ImageJ software to obtain body measurements.

*Statistical analysis*

Survival over time was plotted and analyzed as a step function using Kaplan-Meier survival analysis with the 'survival' package (Therneau 2013). Differences in survival between treatments were analyzed with the Log-Rank test and Cox regression analysis (Klein & Moeschberger 2003). We censored both datasets at the end of the field and laboratory experiments, which corresponded to 22 weeks in the field and the time at which insects matured to third instar in the lab.

While one would ideally compare the time between stadia rather than from hatch to each stage, such data were not estimable based on our data as individuals could not be tracked. For the field experiment, weekly count data of the number of insects and life-stage on each plant. Weekly counts coupled with data on initial hatch time were used to determine time (in weeks) for the developmental periods from hatch to first instar, hatch to second instar, hatch to third instar, hatch to fourth instar, hatch to fifth instar, and hatch to adult. These five periods were analyzed separately as dependent variables. We recognize that these variables are overlapping and therefore correlated. Rather than analyzing only the period from hatch to adult, we opted to compare the experimental factors to development at each stage in order to determine whether the associations were consistent throughout development. These six dependent variables were analyzed using a Poisson distributed General Linearized Model (GLM) with log-link. Explanatory factors included host plant species and symbiont status (*i.e.*, normal or heat-treated symbiont capsules). Adult body size was compared across

treatments. Scutellum width was measured as a proxy for body size and analyzed using two-way ANOVA with factors of host plant and symbiont status.

For the laboratory experiment, development was monitored daily and time to develop from hatch to third instar was recorded for each experimental insect. These data were analyzed using a two-way analysis of variance (ANOVA) and multiple comparisons were tested using Tukey's HSD (honest significant difference). Factors included host plant and symbiont status. All statistical analyses were conducted in R v3.0.2 (R Core Development Team).

## 5.3 Results

*Impacts of Symbiosis and Host Plant Species on Development and Survival to Adulthood in a Field Setting*

Hatch rates were similar across treatments in the field and ranged from 40-50%. Percent survival in each treatment is summarized in Table 5.1.

| | | **Symbiont status** | |
|---|---|---|---|
| | | normal | heat-treated |
| **Host plant** | kudzu | 9.6 | 1.1 |
| | soy | 41.8 | 0.6 |

**Table 5.1. Percent of *M. cribraria* eggs to survive to adult emergence under field experimental conditions**

In the field experiment, a log-rank test indicated significant differences in survival curves between treatments ($X^2_{df=3} = 1425, p < 2e\text{-}16$). Survival was lower in treatments with heat-treated symbiont capsules than normal capsules. Survival was lower on kudzu than soy regardless of symbiont status (Fig. 5.3A). Host plant, symbiont status, and their interaction

were significant predictors of survival curves for *M. cribraria* in the field experiment (Wald test, $X^2_{df=3}$= 1442, $p$ < - $2^{-16}$; Table 5.2). In the field, the greatest mortality occurred in the earliest life stages, namely the first three weeks.

| Field | Variable | Coefficient (*B*) | S.E. | z | *p* value | Risk ratio | 95% CI |
|---|---|---|---|---|---|---|---|
| | **Plant** | -0.9943 | 0.03256 | -30.537 | < 2e-16 | 0.37 | 0.3472 - 0.3943 |
| | **Symbiont** | -0.6888 | 0.03629 | -18.982 | < 2e-16 | 0.5 | 0.4677 - 0.5392 |
| | **Plant * Symbiont** | 0.4072 | 0.04611 | 8.832 | < 2e-16 | 1.5 | 1.3728 - 1.6448 |
| **Lab** | **Plant** | -0.4219 | 0.1116 | -3.781 | < 0.0002 | 0.65 | 0.5270 - 0.8161 |
| | **Symbiont** | -1.4231 | 0.1409 | -10.1 | < 2e-16 | 0.24 | 0.1828 - 0.3176 |
| | **Plant * Symbiont** | 0.9025 | 0.1932 | 4.672 | < 2e-16 | 2.47 | 1.6886 - 3.6005 |

**Table 5.2. Results of Cox regression analysis showing predictors of juvenile survival of *M. cribraria***

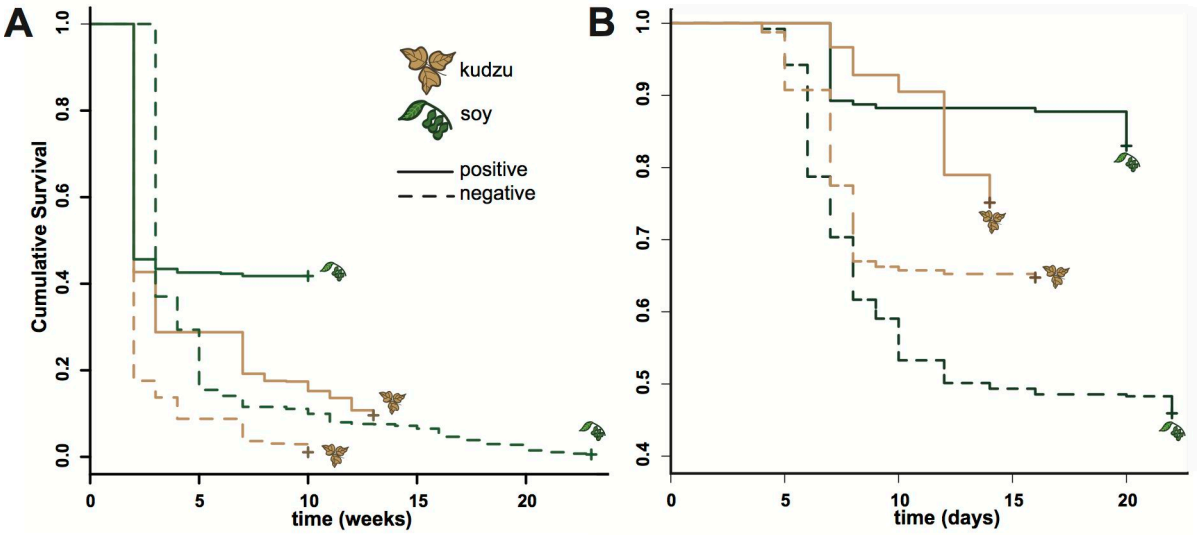


**Figure 5.3. Survival of *M. cribraria* is impacted by both host plant species and symbiont availability.** A) Survival to adult in field experiment and B) Survival to third instar nymph in laboratory experiment. Treatment is indicated by color and line type (see figure legend).

Comparisons of development time to each life stage showed that differences widened with each interval to older life-stages (Fig. 5.4). Development time was longer for kudzu than soybean. Heat-treated symbiont treatments had longer development times than normal symbiont treatments.

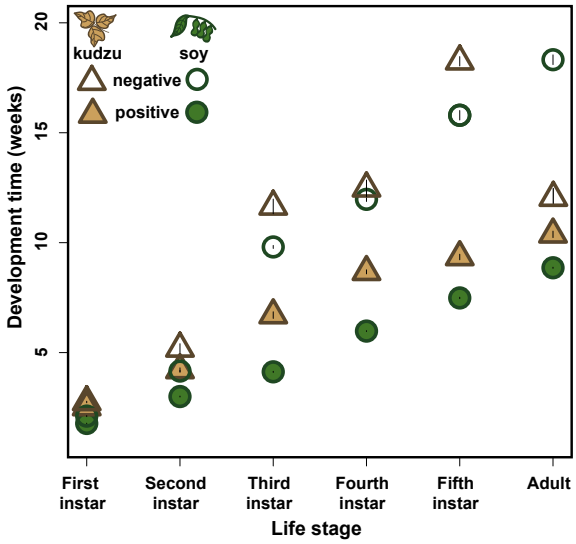


**Figure 5.4 Development time (in weeks) of *M. cribraria* in the field experiment**
Treatment is indicated by color and line type (see figure legend).

Results of GLMs of developmental periods from hatch to each life stage indicated that host plant and symbiont status were significant predictors of development time for all periods (Table 5.3). The interaction between host plant and symbiont status was significant for all development periods except for hatch to third instar and for hatch to fifth instar.

| Interval | Variable | Coefficient (*B*) | S.E. | z | *p* value |
|---|---|---|---|---|---|
| **Hatch to first** | **Plant** | -0.20 | 0.05 | -4.09 | 4.37e-05 |
| | **Symbiont** | 0.48 | 0.05 | 9.06 | < 2e-16 |
| | **Plant * Symbiont** | 0.26 | 0.06 | 4.50 | 6.88e-06 |
| **Hatch to second** | **Plant** | 1.39 | 0.10 | 14.12 | < 2e-16 |
| | **Symbiont** | 0.86 | 0.11 | 7.75 | 8.94e-15 |
| | **Plant * Symbiont** | -0.50 | 0.11 | -4.43 | 9.60e-06 |
| **Hatch to third** | **Plant** | 1.21 | 0.06 | 21.98 | < 2e-16 |
| | **Symbiont** | 0.80 | 0.03 | 30.68 | < 2e-16 |
| **Hatch to fourth** | **Plant** | 0.74 | 0.12 | 5.97 | 3.09e-09 |
| | **Symbiont** | 0.79 | 0.13 | 5.91 | 3.50e-09 |
| | **Plant * Symbiont** | 0.78 | 0.14 | 5.70 | 1.18e-08 |
| **Hatch to fifth** | **Plant** | 1.28 | 0.06 | 21.54 | < 2e-16 |
| | **Symbiont** | 1.94 | 0.06 | 31.64 | < 2e-16 |
| **Hatch to adult** | **Plant** | -0.57 | 0.20 | -2.80 | 0.00504 |
| | **Symbiont** | 1.19 | 0.15 | 8.10 | 5.44e-16 |
| | **Plant * Symbiont** | 1.09 | 0.21 | 5.22 | 1.81e-07 |

**Table 5.3. Results of the GLMs of development time of *M. cribraria*.** Plant is the factor of host plant (kudzu or soybean), symbiont is the factor of symbiont status (normal or heat-treated), and interval is the development time period from egg hatch to each instar stage and to adult.

We found significant differences in adult body size comparisons between treatments (Fig. 5.5). In normal symbiont treatments, both sexes of *M. cribraria* reared on kudzu were larger than their soybean counterparts. Similarly in heat-treated symbiont treatments, insects reared on kudzu were larger than those reared on soybean. Further, the apparent magnitude of difference between normal and heat-treated symbiont treatments differed for each host plant. On kudzu this difference was 0.1.8 (0.03) mm for females, and not significant, 0.005 mm (0.09), for males. On soybean, this difference was 0.38 (0.07) mm for females and 0.28 (0.13) mm for males. These differences between normal and heat-treated symbiont individuals were significant for all groups except for kudzu males.

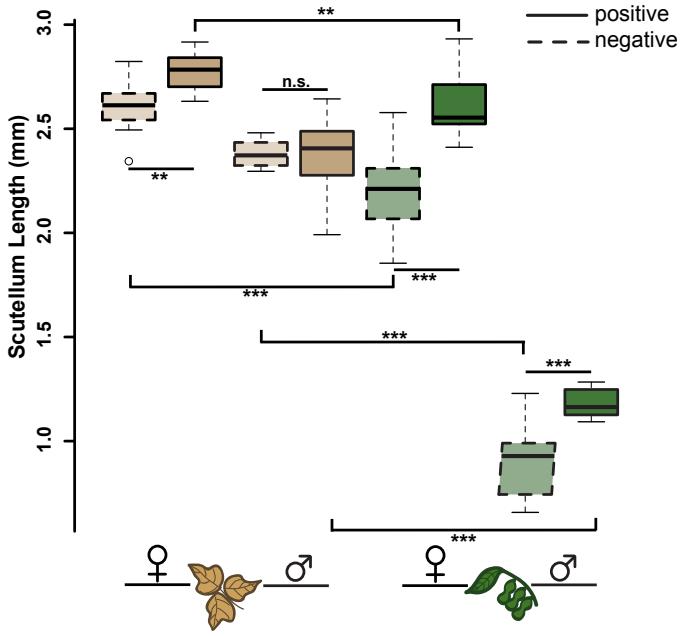


**Figure 5.5 Scutellum length (in mm) of adult *M. cribraria*.** Significance level is indicated asterisks (*** < 0.0001; ** < 0.001; * < 0.01).

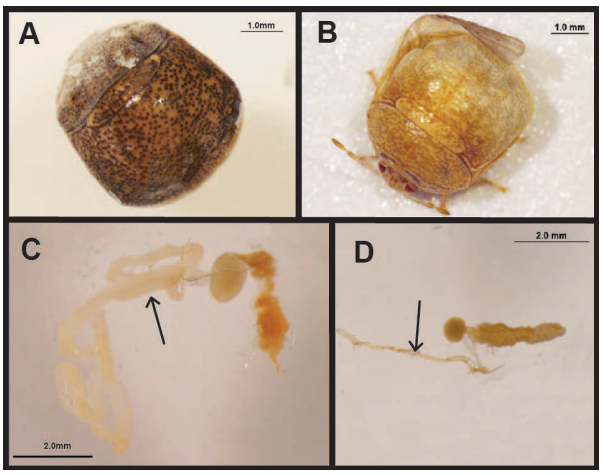


**Figure 5.6. Adult female *M. cribraria* and mid-guts from field experiment on soy plants.** Whole body images (panels A and B) and dissected mid-gut images (panels C and D) from individuals reared with normal (panels A and C) and heat-treated symbiont capsules (panels B and D). Scale bar of 1mm is shown in black. Crypt bearing mid-gut sections are indicated by arrows.

Upon adult emergence we observed morphological differences between normal and heat-treated symbiont treatments in soybean reared *M. cribraria*. Differences in cuticle color and wing formation were visibly evident (Fig. 5.6, panels A and B). Microscopic dissection of these samples revealed stunted midgut development (Fig. 5.6, panels C and D). Such differences were not observed between symbiont treatments for *M. cribraria* reared on kudzu.

*Impacts of Symbiosis and Host Plant Species on Survival and Development in a Laboratory Setting*

Hatch rates in the laboratory ranged from 70-90% and were not affected by treatment. There were significant differences in survival to third instar between treatments ($X^2_{df=3}$ = 142, $p < - 2^{-16}$). In congruence with the field experiment, host plant, symbiont status, and their interaction were significant predictors of survival during early developmental stages (Wald test, $X^2_{df=3}$ = 126.5, $p < - 2^{-16}$; Table 2). Heat-deactivated symbiont treatments showed

decreased cumulative survival compared to normal symbiont treatments (Fig. 5.3B). For treatments with normal symbiont capsules, significant differences between host plants in survival became evident at day 12, with a reduction in survival on kudzu below that of soybean. In heat-deactivated symbiont treatments, cumulative survival decreased for both host plants in a similar manner. After day 7, survival on kudzu was constant, whereas survival on soy continued to decrease.

In a pattern consistent with the field results, development time from hatch to third instar was longer for kudzu than soybean. In the laboratory, *M. cribraria* reared on kudzu took an average of 1.9 days longer to mature to third instar than on soybean (Tukey difference of means, adj. $p < 0.0005$). Also consistent with the field results, across both host plants development time was longer in heat-treated symbiont treatments than normal symbiont treatments (Fig. 5.7), with a difference of approximately 14.1 days (Tukey difference of means, adj. $p < 0.000$). For the heat-treated symbiont treatment, development time was approximately 8.65 days longer on kudzu than soybean (adj. $p < -2^{-16}$). The results of ANOVA of development time from hatch to third instar indicated that host plant, symbiont status, and the interaction of these factors were significant predictors (Table 5.4).

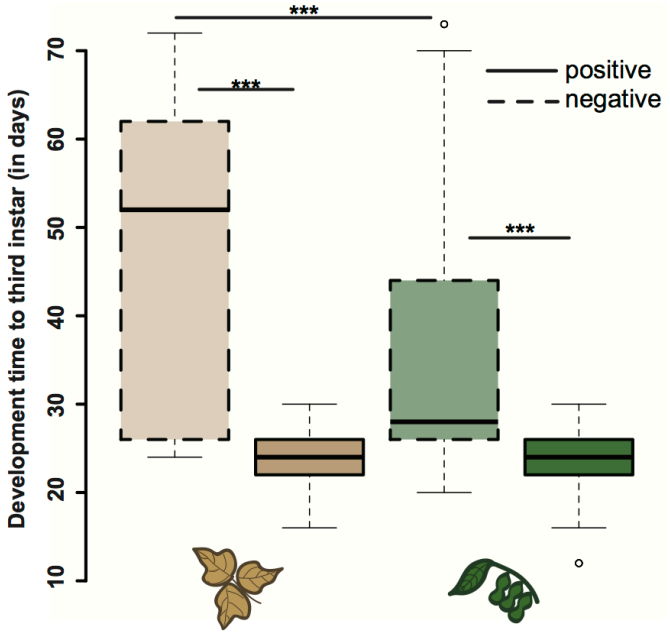


**Figure 5.7. Development time of *M. cribraria* under laboratory conditions from hatch to third instar.** Significance level is indicated asterisks (*** < 0.0001; ** < 0.001; * < 0.01).

| Variable | Sum of Squares | D.f. | Mean Square | F value | Prob( <F) |
|---|---|---|---|---|---|
| **Plant** | 995 | 1 | 995 | 12.18 | < 0.0005 |
| **Symbiont** | 62471 | 1 | 62471 | 764.53 | < 0.000 |
| **Plant * Symbiont** | 5138 | 1 | 5138 | 62.88 | < 0.000 |
| **Error** | 113334 | 1387 | | | |

**Table 5.4 Two-way ANOVA of development time to third instar for *M. cribraria* reared in the laboratory.** Plant is the factor of host plant (kudzu or soybean), and symbiont is the factor of symbiont status (normal or heat-treated)

## 5.4 Discussion

We sought to estimate the impact of host-plant and the presence of symbionts on the development of *M. cribraria*. As expected based on previous investigations in its sister species, *M. puntatissima* (Hosokawa *et al*., 2007), we found a significant impact of normal symbiont capsules versus heat-treated capsules on the survival, development time, and body size of *M. cribraria*. We hypothesized that the environmental context would interact with symbionts to influence host development. Our results confirmed there is a significant

interaction between the impact of symbionts and host plant on host life-history traits, including both survival and development rate.

Studies in *Megacopta spp*. have reported life-history trait estimates in the context of a single host plant, namely soybean (Fukatsu & Hosokawa 2002; Zhang *et al*. 2012; Del Pozo-Valdivia & Reisig 2013), in the presence of symbionts. We explored *M. cribraria* life-history trait variation in the context of two experimental environments, field and laboratory, finding that development time, survival curves, and body size were significantly impacted by symbiont status, by host plant, and by the interaction between these factors. To our knowledge this is the first study to estimate the impact of symbiont status on *M. cribraria* developmental traits in alternate host plants. While several factors might contribute to these differences, the interaction of plant and symbiont status suggests the overall importance of ecological context in mediating the effects of symbiosis on host fitness.

Comparing our estimates of survival on soybean to previous work, survival in the normal symbiont treatment in the laboratory experiment was lower than experiments conducted with *M. punctatissima* from its native range under similar environmental conditions (Fukatsu & Hosokawa 2002; Hosokawa *et al*. 2007b). Differences may reflect the species differences between *M. punctatissima* and *M. cribraria*, or may reflect the changes in the host and symbiont populations that occurred during and after invasion. Brown *et al*. (2013) found that in the first two years of the invasion, the microbial symbiont *Ishikawaella* showed evidence of positive and negative selection as well as differential selection for genes involved in nutrient provisioning for two host plants, kudzu (*Pueraria montana* Lour. [Merr.] variety *lobata* [Willd.]) and soybean (*Glycine max* (L.) Merril). A common garden

experiment to compare native to invasive *M. cribraria* would provide better insight into these differences.

Zhang et al. (2012) examined juvenile survival of *M. cribraria* on soybean under similar field conditions, resulting in estimates ten times lower than reported here. One methodological difference was that we controlled intraspecific rearing density across replicates, whereas Zhang *et al*. allowed insects from neighboring plants to freely oviposit on soy plants before relocating plants for nymphal development. It has also been suggested that these insects are sensitive to conditions limiting their movement or shading plants (Ruberson *et al*. 2012). Zhang et al. used fine mesh cloth bags around plants, whereas we surrounded raised beds of soy plants in a large mesh tent. Such differences in the rearing methodology may account for differences in survival observed across these studies.

In *M. punctatissima*, nymphs deprived of symbionts showed delayed development, arrested growth, and abnormal body coloration (Fukatsu & Hosokawa 2002). We observed similar impacts of delayed development in invasive *M. cribraria* on soybean as a result of symbiont deprivation. Development time of *M. cribraria* reared with normal symbiont capsules on soybean is consistent with other recent estimates from its expanded range (Del Pozo-Valdivia & Reisig 2013). These results indicate that soybean is a potentially suitable host plant for *M. cribraria*, providing sufficient nutrition to support development and emergence. However, we also observed differences in development time symbiont-harboring insects when reared on kudzu and soybean. In field trials, *M. cribraria* reared on soybean developed faster than those on kudzu. This suggests soybean is a more suitable host plant for development of *M. cribraria*, and further research is needed to determine the impact of host plant on other critical traits such as fecundity. We also found development time differences

in symbiont-deprived treatments, and here the interaction between symbiont status and host plant becomes evident. The impact of lacking symbionts appeared more severe on soy than kudzu in the field experiment. This disproportionately negative effect of delayed development on soy suggests that manipulation of the symbiont is one potential avenue for biological control of this invasive pest species.

We found differences in adult body size between host plants in both symbiont present and absent treatments that provide further evidence of an interaction. Moreover, we observed morphological differences among soy-reared insects between symbiont treatments that were consistent with previous work in *M. punctatissima* (Fukatsu & Hosokawa 2002; Hosokawa *et al*. 2007b). However, we did not see these differences in kudzu-reared insects. It is possible that the ecological context of host plant impacts the expression of genes controlling pigmentation. With partial interference of Laccase2 through RNAi in *M. punctatissima,* there is some lightening of cuticular pigment (Futahashi *et al*. 2011). *M. punctatissima* is morphologically indistinguishable from *M. cribraria*. While it may not be taxonomically distinct (Eger *et al*. 2010; Jenkins *et al*. 2010; Ruberson *et al*. 2012), it is considered a separate species in China and known to cause the loss of high volumes of soy crop in these regions (Guanguan *et al*. 2006). The lightened color of aposymbiotic insects in our results appears to be more extensive. These results are suggestive that the symbiont may play a role in the expression of this and potentially other pigmentation and cuticle development genes. These phenotypic differences provide an opportunity for further study of the role of the bacterial symbiont in host development.

The significant impacts of symbiont deprivation on *M. cribraria* life-history traits was expected based on studies of symbiosis in *M. punctatissima*. The Ishikawaella symbiont is

thought to provide essential amino acids and vitamin synthesis for *M. punctatissima*, and is more closely related to *Buchnera*, the intracellular nutritional symbiont of aphids, than to other extracellular obligate symbionts (Nikoh *et al*. 2011). This similarity reflects in its reduced genome size, high AT content, and few mobile genetic elements (Nikoh *et a*l. 2011). Despite the reduced genome size, many genes involved in metabolism of amino acids were conserved, and *Ishikawaella* can synthesize all essential amino acids as well as some vitamins and co-factors. Just as Buchnera compensates for lack of nutrients in the aphid diet (Baumann *et al*. 1997), so *Ishikawaella* may aid *Megacopta spp*. in subsisting on the low nutrient plant-sap diet (Nikoh *et al*. 2011).

Experimentally replacing bacterial symbiont strains between *M. punctatissima* and *M. cribraria* has been tested by switching symbiont capsules on egg masses, and alters the insect host's ability to survive on soybean (Hosokawa *et al*. 2007b). It is therefore possible that ecological differences such as plant utilization that have been attributed to species differences in these insects are actually due to differences in bacterial symbiont strains. *M. cribraria* in its expanded range is most genetically similar to the *M. cribraria* populations in Asia that are not considered soybean pests (Jenkins *et al*. 2010), but comparisons of the genotype of symbionts across the host range may be help clarify the apparent changes in plant use in its expanded range. Brown *et al*. (2013) have shown that invasive *M. cribraria* likely arrived with the pest-phenotype of *Ishikawaella,* conferring the ability to utilize soybean. The evolution of the symbiosis after its invasion has been influenced both by population bottlenecks that occur during invasion (Jenkins & Eaton 2011) and by differential selection based on host plant (Brown *et al*. 2013). Further comparisons between the symbionts of

native and expanded ranges could potentially provide insight into the differences for host-microbe evolution based on plant utilization (Jenkins & Eaton 2011; Brown *et al*. 2013).

Although many aspects of the insect habitat shifted during an invasion, the two main host plant species associated with *M. cribraria,* kudzu and soybean, are widespread in the southeastern U.S. (Medic *et al*. 2014). Although soybean is widely distributed as an agricultural crop, the invasion and expansion of *M. cribraria* is closely associated with the distribution of kudzu, and kudzu is presumed to be the primary host plant (Eger *et al*. 2010; Suiter *et al*. 2010). *M. cribraria* initially invaded central Georgia in 2009 (Eger *et al*. 2010), expanding to seven southeastern states in subsequent years (Ruberson *et al*. 2012; Gardner *et al*. 2013). In the southeast on kudzu, *M. cribraria* appears to be bivoltine, and that it is the second generation of the season that uses soybean (Ruberson *et al*. 2012). Our results support reports that *M. cribraria* can feed on soybean (Seiter *et al*. 2013a), and that *M. cribraria* can become a pest of soybean (Ruberson *et al*. 2012). Recent genomic evidence suggests that soybean can provide nutrition to adult *M. cribraria* as well as support development (Brown *et al*. 2013; Del Pozo-Valdivia & Reisig 2013; Seiter *et al*. 2013b).

The differential ability of *M. cribraria* to different host plants has major agricultural implications and will influence the boundaries of its range in the United States. These results contribute to the novel finding that environmental context mediates an obligate host-microbe interaction in an insect-bacteria system. Here, the ecological context of host plant can mediate the impact of obligate symbionts on the life history of its insect host. This finding has implications for understanding the ecology and evolution of *M. cribraria* in its expanded North American range. Microbial symbionts may be key drivers not only of host development and physiology but host ecology as well. Ecological cues may change the costs

and benefits of maintaining symbiotic relationships, and may impact the evolutionary stability of these partnerships.

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## Chapter 6: Conclusion

Variation in life-history traits during development in response to environmental conditions can have important consequences for many ecological and evolutionary processes, including interactions within and between species (Chesson & Warner, 1981; Wiens, 1977; Hutchinson, 1961; Park, 1954), coevolution (Piculell *et al*., 1998; Setala *et al*., 1997; Bronstein, 1994), and invasion (Klepzig & Six, 2004; Vasanthakumar *et al*., 2006; Klepzig *et al*., 2009). Especially in study of insects that transmit pathogens, the effects of environment on insect performance have been and remain topical due to the profound impact of their population dynamics on infectious disease epidemiology (Vazquez-Prokopec, 2011). Furthermore, there is growing appreciation for the role microbial partners have in shaping host life-history phenotypic variation and ecological interactions. Additional work is needed that addresses host-microbe interactions across different environmental conditions (Wilkinson *et a*l., 2001) and examines the molecular drivers of these species interactions (Whitham *et al*., 2008).

My dissertation has focused on the impacts of temperature, intraspecific density, diet and symbiosis on the life-history traits of insects, with particular reference to the interaction of these factors. By using techniques from field ecology, laboratory rearing experiments, and linear mixed effects statistical modeling, I was able to explore the complex effects of multiple environmental factors on the development and growth of these insects and for a host-microbe interaction.

In Chapter 2, although the law of effective temperature predicted that temperature alone was sufficient to explain the variation in development rate of ectotherms, I found that

temperature dependence was heterogeneous. Across the studies considered in the meta-analysis of *Cx. pipiens s.l.* development rate, this heterogeneity was significant and could be explained using secondary factors of density, sex, and study methodology. Further, the effect of temperature differed based on the life-stages considered in the analysis, particularly the pupal phase. This suggests the process of metamorphosis, during which resources are no longer taken in, may change the impact of temperature. It would be interesting to expand the analysis to consider the impact of temperature on larval versus pupal development in all holometabolous insects. My results illustrate that the effect of temperature on development is context dependent, and should not be considered a constant feature of the species.

Although in Chapter 2, I found that other factors were necessary to explain development this result was not consistent with the meta-analysis of studies of development for another mosquito species, *Ae. aegypti* in Chapter 3. Temperature was sufficient to explain the variation of development rate in *Ae. aegypti*. I attribute this in part to the limited ranges of diet concentration and intraspecific density that were used in the literature available for meta-analysis. Most of the rearing studies provided food *ad libitum*, and used low-competition environments to ensure high juvenile survival. In this light, the meta-analysis of juvenile survival makes more sense, and indicates that temperature is the only important environmental factor for survival. Based on these results, I aimed to consider wider ranges of diet and levels of intra-specific competition in Chapter 4.

> "The success of a species, its numbers, sometimes its size, etc., are often determined largely by the degree of deviation of a single factor (or factors) from the range of optimum of the species." V.E. Shelford, 1913

The idea that environmental factors constrain fitness is not new, thought it remains a relevant and fundamental aspect of understanding the evolution of life-history traits. Schmalhausen (1949) formalizes the premise presented by Shelford (1913) in a principle ('Schmalhausen's law') stating that biological systems at the boundary of their tolerance along any dimension of existence become more vulnerable to small changes along the other dimensions (Schmalhausen, 1949; Awerbuch *et al.,* 2002; Chaves, 2010a; Chaves, 2010b; Chaves & Kitron, 2011). According to this principle the variance of data is not simply stochasticity, or noise, interfering with the detection of so-called "main effects", but rather an indicator of stressful conditions leading to greater vulnerability (Lewontin & Levins, 2000). Another relevant hypothesis is Leibig's law of the minimum, stating that it is not the total amount of resources, but the scarcest resource that limits and organism's growth rate. The results of the rearing experiment of *Ae. aegpyti* (Chapter 4) demonstrate these principles. Indeed, at the lower extremes of diet conditions and the upper extremes of density, the effect of temperature diminished and the relative importance of diet or density factors increased. This may explain the significant three-way interaction of temperature, diet, and density in the model of development rate from hatch to emergence for *Ae. aegypti*.

Much of the work on mosquitoes has immediate application toward modeling mosquito population dynamics. The parameters of the developmental zero and the cumulative effect of temperature are used to predict mosquito populations in a wide variety of models. These models are often used in public and environmental health arenas to make decisions on mosquito abatement programs. The parameters determined through meta-analysis provide a better estimate of the effect of temperature, and provide a variance around this estimate that reflects phenotypic variation in response to secondary factors of diet and

study methodology. It remains to be seen if these estimates will improve our prediction of mosquito population dynamics in natural systems, and this is an important next step to validate the statistical models developed here.

One limitation of the statistical modeling that I used to explore development rate variation in these mosquitoes was in the employment of linear models. Because the development curve for insects contains linear and non-linear portions, temperature dependent development models are often organized by whether they are linear or non-linear (Harcourt & Yee, 1982; Wagner *et al.,* 1984a; Worner, 1992). This distinction relates to whether insects are assumed to well adapted to local climatic conditions such that they are not expected to be exposed to temperature extremes during development (Campbell *et al*., 1974; Gilbert *et al*., 1976). This assumption may be violated in natural populations, and the importance of extreme temperature events may increase with global climatic change (Day & Shaman, 2009). The goal of the statistical modeling here was to estimate parameters, describe their variation, and determine the environmental factors that best explained this variation. The models used here, as with many linear models, are based on using the mid-range portions empirical curves (Belehradek, 1926).

Overall, I used the mosquito systems to explore the relative importance of exogenous environmental factors on phenotypic variation of life-history traits. However, there is growing evidence that environmental factors can play an important role in mediating even intimate mutualistic interactions (Piculell *et al*. 2008; Bronstein, 1994). I explore this possibility in an obligate symbiosis. The *M. cribraria* system allowed me to consider whether symbiont benefits to hosts were ecologically contingent. I found that the ecological context of host plant interacts with symbiont presence to influence development time, juvenile

survival, and body size. My result contributes the novel finding that environmental context mediates an obligate host-microbe interaction. These findings have broad implications for our understanding of the nature of symbiosis. Ecological cues may change the costs and benefits of maintaining symbiotic relationships, and may impact the evolutionary stability of these partnerships.

Here I have explored the plasticity of critical organismal phenotypes in response to abiotic and biotic factors including temperature, resources and resource availability, intraspecific density, and microbial partners. As with most scientific research, I have uncovered more questions than answers, and there are many potential avenues for further inquiry. A natural extension of this work is to examine the role of environmental context as mediating communities of interacting species. The *M. cribraria* system may be further studied in this regard as I recently co-discovered that a parasitoid wasp of *M. cribraria* has invaded North America (Gardner *et al*., 2013). This presents an opportunity to explore the coevolutionary dynamics among hosts, symbionts, and parasitoids in different ecological contexts (*i.e*. plants). Understanding how the host-microbe partnership changes within the broader context of community composition will further our understanding of ecological processes and trophic interactions.

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## Appendix I: Supplemental tables

See supplementary excel files.

**Table S2.1 Databases included in the literature search and websites**

**Table S2.2 Publication information for the considered in the meta-analysis**

**Table S2.3 Compiled dataset employed in the analyses with linear mixed effect models**

**Table S2.4. Mean developmental time in days estimated based on weighted average of studies included in meta-analysis reported from egg to pupation and from egg to adult emergence**

**Table S2.5 Effect estimates employed in the pupation rate regression parameter estimate meta-analyses**

**Table S2.6. Parameter estimates for the pupation rate of *Cx. pipiens s.l.* regression parameter (*B*1) best meta-analysis**

The intercept is the estimate for the COS study, other estimates are additive in relation to this one. For details about the studies see Table S1.

| Parameter | Estimate | SE | Z | P |
|---|---|---|---|---|
| Intercept (COS) | 0.006 | 0.0013 | 4.7693 | <.0001* |
| KUR | 0.0026 | 0.0017 | 1.5014 | 0.1333 |
| MEA | 0.0044 | 0.0019 | 2.3104 | 0.0209* |
| MOR | 0.0029 | 0.0013 | 2.2565 | 0.0240* |
| RUE | 0.0024 | 0.0019 | 1.2374 | 0.2159 |
| SHE | -0.0009 | 0.0015 | -0.6226 | 0.5335 |

*Statistically significant (P<0.05)

**Table S2.7 Effect estimates employed in the emergence rate regression parameter estimate meta-analyses**

**Table S2.8. Parameter estimates for the adult emergence rate of *Cx. pipiens s.l.* regression parameter (*B*1) best meta-analyses**

The intercept for the model considering Study as source of heterogeneity is the estimated rate for the COS study and other estimates are additive in relation to this one. For details about the studies see Table S1. The intercept for the model considering Sex and Density as source of heterogeneity is the estimated rate for both sexes (Males and Females), other estimates are additive in relation to this one.

| Model | Parameter | Estimate | SE | Z | P |
|---|---|---|---|---|---|
| Study | Intercept (COS) | 0.0053 | 0.0006 | 8.6182 | <.0001* |
| | HAY | -0.0005 | 0.0014 | -0.33 | 0.7414 |
| | HEA | -0.0019 | 0.0009 | -2.2561 | 0.0241* |
| | KURb | -0.0013 | 0.0008 | -1.6242 | 0.1043 |
| | LOB | 0.0041 | 0.0016 | 2.6086 | 0.0091* |
| | MAD | 0.0022 | 0.0014 | 1.6015 | 0.1093 |
| | MEA | -0.0005 | 0.002 | -0.2373 | 0.8124 |

| | | | | | |
|---|---|---|---|---|---|
| | MOG | 0.0018 | 0.0007 | 2.6504 | 0.008* |
| | RUE | 0.0007 | 0.0013 | 0.4959 | 0.62 |
| | TEK | -0.0012 | 0.0007 | -1.698 | 0.0895 |
| Sex and Density | Intercept (Both Sexes) | 0.0056 | 0.0005 | 11.4677 | <.0001* |
| | Density | -0.0027 | 0.0012 | -2.226 | 0.026* |
| | Females | 0.0017 | 0.0007 | 2.5848 | 0.0097* |
| | Males | 0.0023 | 0.0006 | 3.5308 | 0.0004* |

*Statistically significant (P<0.05)

**Table S2.9. *Cx. pipiens s.l.* development rate to the pupal stage linear mixed effects model selection**

FF and RF stand for fixed and random factors respectively , AIC and BIC for Akaike and Bayes Information criterion respectively. Δ represents the difference with respect to the minimum value. **Minimum values for each selection criterion are bolded.** The AIC and BIC have negative values because the models had positive log-likelihoods, which occurs because the probability densities evaluated at the observations are below 1, which produces a negative logarithm. ΔAIC and ΔBIC show differences with respect to the model that minimized each information criterion.

| Model | AIC | ΔAIC | BIC | ΔBIC |
|---|---|---|---|---|
| FF: Temperature (T), Density (D),Latitude (L),Sex (S), Environmental Variability (EV) | -670 | 51 | -641 | |
| RF: Study, Species conditioned on Study | | | | 65 |
| FF: T, D, L, S, EV | -667 | 54 | -638 | |
| RF: Species, Study conditioned on Species | | | | 68 |
| FF: T, D, L, S, EV | -669 | 52 | -643 | |
| RF: Study, Species | | | | 63 |
| FF: T, D, L, S, EV | -598 | 123 | -572 | |
| RF: Species | | | | 134 |
| FF: T, D, L, S,EV | -666 | 55 | -637 | |
| RF: Study*Species | | | | 69 |
| FF: T, D, L, S, EV | -657 | 64 | -631 | |
| RF: Study | | | | 75 |
| FF: D, L, S, EV | -482 | 239 | -456 | |
| RF: Study, Species conditioned on Study | | | | 250 |
| FF: T, L, S, EV | -682 | 39 | -655 | |
| RF: Study, Species conditioned on Study | | | | 51 |
| FF: T, D, S, EV | -684 | 37 | -657 | |
| RF: Study, Species conditioned on Study | | | | 49 |
| FF: T, D, L, S | -679 | 42 | -653 | |
| RF: Study, Species conditioned on Study | | | | 53 |
| FF: T, D, L, EV | -688 | 33 | -665 | |
| RF: Study, Species conditioned on Study | | | | 41 |
| FF: D, L, EV | -497 | 224 | -476 | |
| RF: Study, Species conditioned on Study | | | | 230 |
| FF: T, L, EV | -699 | 22 | -679 | |
| RF: Study, Species conditioned on Study | | | | 27 |
| FF: T, D, L | -697 | 24 | -677 | |
| RF: Study, Species conditioned on Study | | | | 29 |
| FF: T, D, EV | -701 | 20 | -681 | |
| RF: Study, Species conditioned on Study | | | | 25 |
| FF: D, EV | -510 | 211 | -493 | |
| RF: Study, Species conditioned on Study | | | | 213 |
| FF: T, EV | -712 | 9 | -695 | |
| RF: Study, Species conditioned on Study | | | | 11 |

| | | | | |
|---|---|---|---|---|
| FF: T, D | -710 | 11 | -693 | |
| RF: Study, Species conditioned on Study | | | | 13 |
| FF: EV | -520 | 201 | -506 | |
| RF: Study, Species conditioned on Study | | | | 200 |
| **FF: T** | **-721** | **0** | **-706** | |
| **RF: Study, Species conditioned on Study** | | | | **0** |

**Table S2.10. *Cx. pipiens s.l.* development rate to adult emergence linear mixed effects model selection**

FF and RF stand for fixed and random factors respectively, AIC and BIC for Akaike and Bayes Information criterion respectively. Δ represents the difference with respect to the minimum value. **Minimum values for each selection criterion are bolded.** The AIC and BIC have negative values because the models had positive log-likelihoods, which occurs because the probability densities evaluated at the observations are below 1, which produces a negative logarithm. ΔAIC and ΔBIC show differences with respect to the model that minimized each information criterion.

| Model | AIC | ΔAIC | BIC | ΔBIC |
|---|---|---|---|---|
| FF: Temperature (T), Density (D),Latitude (L),Sex (S), Environmental Variability (EV) | -1262 | | -1225 | |
| RF: Study, Species conditioned on Study | | 56 | | 76 |
| FF: T, D, L, S, EV | -1275 | 43 | -1242 | 59 |
| RF: Species, Species conditioned on Study | | | | |
| FF: T, D, L, S, EV | -1277 | 41 | -1247 | 54 |
| RF: Study, Species | | | | |
| FF: T, D, L, S, EV | -1163 | 155 | -1133 | 168 |
| RF: Species | | | | |
| FF: T, D, L, S,EV | -1278 | 40 | -1244 | 57 |
| RF: Species *Study | | | | |
| FF: T, D, L, S, EV | -1280 | 38 | -1250 | 51 |
| RF: Study | | | | |
| FF: D, L, S, EV | -1062 | 256 | -1035 | 266 |
| RF: Study | | | | |
| FF: T, L, S, EV | -1285 | 33 | -1258 | 43 |
| RF: Study | | | | |
| FF: T, D, L, EV | -1297 | 21 | -1273 | 28 |
| RF: Study | | | | |
| FF: T, D, L, S | -1284 | 34 | -1258 | 43 |
| RF: Study | | | | |
| FF: T, D, S, EV | -1296 | 22 | -1269 | 32 |
| RF: Study | | | | |
| FF: D, L, EV | -1078 | 240 | -1058 | 243 |
| RF: Study | | | | |
| FF: T, L, EV | -1301 | 17 | -1281 | 20 |
| RF: Study | | | | |
| FF: T, D, EV | -1313 | 5 | -1293 | 8 |
| RF: Study | | | | |
| FF: T, D, L | -1302 | 16 | -1282 | 19 |
| RF: Study | | | | |
| FF: D, EV | -1094 | 224 | -1077 | 224 |
| RF: Study | | | | |
| FF: T, EV | -1317 | 1 | -1300 | 1 |
| RF: Study | | | | |

| | | | | |
|---|---|---|---|---|
| **FF: T, D<br>RF: Study** | **-1318** | **0** | **-1301** | **0** |

**Table S3.1 Online databases searched in December 2011 for research papers pertaining to *Ae. aegypti* development rate**

**Table S3.2 Full bibliography for the 65 studies included in the factors influencing development rate and survival of *Ae. aegypti***

**Table S3.3 Linear regression parameter estimates for studies that experimentally examined the relationship between development rate and temperature for the life stages from first instar to adult emergence.**

**Table S3.4 Linear regression parameter estimates for studies that experimentally examined the relationship between development rate and temperature for the life stages from first instar to pupation.**

**Table S4.1 Parameter estimates and F tests of larval development rate and temperature, as shown in Figure 4.**

| mg/larva/day | Slope | Intercept | Adj. R | F | p |
|---|---|---|---|---|---|
| 0.0625 | 0.00155 | 0.00917 | 0.97 | 98.36 | 0.01 |
| 0.125 | 0.02593 | 0.01685 | 0.83 | 34.32 | 0.001 |
| 0.25 | 0.00483 | -0.00796 | 0.83 | 34.32 | 0.001 |
| 0.5 | 0.00689 | -0.04007 | 0.81 | 67 | < 0.0001 |
| 1 | 0.00796 | -0.06018 | 0.86 | 66.71 | < 0.0001 |
| 2 | 0.00944 | -0.09058 | 0.93 | 87.8 | < 0.0001 |
| 4 | 0.010387 | -0.118531 | 0.88 | 23.24 | 0.04 |