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Miho Yoshioka April 20, 2011

Fitness consequences of oviposition site selection by the mosquito species Aedes albopictus (Diptera: Culicidae)

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Abstract
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In the naturally heterogeneous environment, mosquito egg-laying or oviposition is intimately related to the search for water habitats where the aquatic immature stages can develop to adulthood. According to the oviposition-preference offspring-performance (P-P) hypothesis for insects, if optimizing offspring performance and fitness ensures high overall reproductive fitness for an individual, the female should accurately assess details of the heterogeneous environment via sensory or perceptive behavior, and then lay her eggs in sites with offspring-suitable conditions. Particularly for a skip-ovipositing female mosquito that disperses her eggs of a single batch in multiple sites, she must select favorable site conditions while "skipping" unfavorable site conditions.

In a laboratory setting, we examined the skip oviposition behavior of the mosquito *Aedes albopictus* by empirically testing the P-P hypothesis and focusing on two habitat conditions: diet and conspecific density (CD) (number of pre-existing larvae of the same species). First, in order to determine which oviposition site conditions were favorable for the aquatic juvenile stages (larvae and pupae), larval development was monitored from the first-instar larval stage through adult emergence over two ascending gradients of diet and CD. Individuals developed significantly faster with each increasing level of diet except from the third (7.2mg) to fourth level (20mg). Regarding, CD, significantly faster development resulted from the first level (zero conspecific larvae) compared to that resulting from the fourth level (80 conspecific larvae). These results are congruent with the hypothesis that higher food and lower conspecific larval density would increase diet availability per capita, thereby reducing density-dependent competition for both food and space. However, the ultimate number of viable adults indicated that even container treatments with suboptimal larval conditions maintained overall high survival and gross mosquito productivity.

Upon concluding which diet and CD treatments significantly increased (and decreased) larval performance in the first experiment, these treatments were used to provide the conditions for the single-female oviposition assays. The laboratory assays are currently being conducted under these treatment conditions. The impressive ecological plasticity of *Aedes albopictus* allows it to thrive in natural and artificial containers commonly found in urban and suburban environments. Therefore, this manipulative study may help us gain a better understanding of the mechanisms underlying the oviposition behavior of *Aedes albopictus* found in nature.

Keywords: habitat selection, oviposition, diet, conspecific density, mosquito, *Aedes albopictus*

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Table of Contents

Introduction

Study species

This study focused on the breeding preferences of the Asian tiger mosquito *Aedes albopictus* under conditions commonly found in urban and suburban environments (Yoshioka 2010). *Aedes albopictus* is an invasive, opportunistic feeder and possesses an impressive ecological plasticity—it rapidly adapts to a variety of habitats, including natural and artificial containers (Braks et al 2004, Paupy et al 2009). Moreover, *Ae. albopictus* is one of two principal mosquito vectors of Chikungunya virus (CHIKV) and Dengue virus (DENV) overseas (Darsie & Ward 2005, Paupy et al 2009). Although its origins are in southeastern Asian forests, *Aedes albopictus* in the United States commonly occurs in urban and suburban environments, where natural and artificial containers provide abundant sources of larval habitat (Hawley 1988, Braks et al 2004). Due to its opportunistic feeding behavior and wide range of hosts, *Ae. albopictus* is generally regarded as a poor vector of human pathogens, including arboviruses (Richards et al 2006, Paupy et al 2009). However, previous host choice experiments and blood meal analyses indicate that *Ae. albopictus* prefer the blood of humans over that of animals (Niebylski et al 1994, Delatte et al 2010). Therefore, its strong prevalence in anthropogenic environments increases the risk for it to become a "bridge vector" for the transmission of pathogens between animals and humans (Mitchell 1995, Juliano & Lounibos 2005, Paupy et al 2009).

Very recently, autochthonous Dengue cases began to arise in the Florida Keys—three identified in August 2009, and the most recent reported case in April 2010 (Trout et al 2010). Being the first Dengue cases acquired in the continental U.S. outside the Texas-Mexico border since 1945 and the first cases acquired in Florida since 1934, these cases were comprised of a total of 57 locally acquired Dengue infections. Although local control measures were

immediately implemented for the recent Florida outbreaks, comparable efforts, especially those used to combat the invasion of *Ae. albopictus,* are largely nonexistent in much larger urban areas in the southeastern United States. *Aedes albopictus* is not as an efficient vector for Dengue virus as *Ae. aegypti*; however, the future regarding viruses carried by mosquito species in the U.S. is not entirely certain.

Aedes albopictus adults can fly only short distances but the fact that most colonization has been by passive transport urges research surrounding the key agents facilitating such passive transport. In this case, we focus on artificial containers. Previous studies have looked at the relationship of artificial containers and *Ae. albopictus* breeding (Braks et al 2003, Simard et al 2005, Richards et al 2006, Yee et al 2010); however, laboratory studies testing the oviposition preference-offspring performance (P-P) hypothesis with *Ae. albopictus* and linking it to manmade habitat conditions have been scarcely conducted (Nayar & Sauerman 1975, Mogi 1982, Soekiman et al 1984, Yap et al 1995). Containers found in residential backyards (e.g. flower pots, plastic buckets and cups) are of particular concern not only because they are common habitats for *Ae. albopictus* larvae (Braks et al 2003, Simard et al 2005, Richards et al 2006, Yee et al 2010), but also because they are highly abundant and located in close proximity to human activity. Nevertheless, specific container conditions that may control the prevalence and/or fitness of *Ae. albopictus* offspring at the aquatic stage are largely unknown.

Oviposition preference-offspring performance hypothesis

Females can influence offspring survival and phenotype through a variety of maternal behaviors. For mosquitoes, one such maternal behavior that can heavily impact offspring survival and phenotype is oviposition site selection (Bernardo 1996). In the natural world, egg-laying or ovipositon involves the process by which the female selects a suitable habitat for her

eggs to hatch and her progeny to develop (Resetarits & Wilbur 1989). Particularly for mosquitoes, such process is intimately related to the search for aquatic habitats where the immature stages can develop to adulthood (Silver 2008). A female's decision about where to oviposit has serious consequences for her reproductive fitness, as oviposition site affects offspring survival, juvenile performance, and ultimate offspring phenotype and fitness (Resetarits 1996). Moreover, oviposition behavior and non-random site selection constitute key life history traits of mosquitoes (Resetarits & Wilbur 1989, Resetarits 1996).

Oviposition site selection could be either an adaptation to optimize offspring development and survival or, alternatively, the response to certain habitat characteristics that may help distinguish them regardless of their potential outcome on mosquito offspring (Yanoviak 2001, Ellis 2008). For instance, oviposition site selection of container-breeding *Aedes sp.* mosquitoes have often been associated with black or dark-colored containers (McDaniel et al 1976, Beehler et al 1992, Yap et al 1995, Yanoviak 2001). This apparent trend may be attributed to a potential predator-defense strategy that reduces visible exposure of *Aedes* larvae or eggs in the water column. However, other researchers argue that it may be nothing more than the species' natural ability to better detect dark-colored containers over light-colored containers (Yanoviak 2001).

Whether a gravid (ready to lay eggs) female mosquito actively searches for oviposition habitats to maximize offspring fitness or as a response to habitat characteristics may have different manifestations for both oviposition behaviors. Single clutch ovipositors, such as *Culex sp.* mosquitoes, take the risk of high offspring mortality upon laying a single cluster ("egg raft") of a few hundred eggs at one site. If the oviposition site interrupts egg hatching and/or development, most likely a substantial portion, if not all, of the other eggs in the clutch will

experience similar detrimental effects. On the other hand, "skip" ovipositors (Mogi and Mokry 1980), such as *Aedes sp.*, distribute individual eggs in several oviposition sites rather than as a single clutch in one site (Chadee & Corbet 1987, Apostol et al 1994). Essentially, successfully developed individual eggs from dispersed containers can behave collectively as a clutch or cluster to indicate efficient allocation of reproductive energy. This study focused on skip ovipositors, since their site selection will be more finely grained and easily quantifiable than for clutch ovipositors.

Ecological theories of optimal foraging and habitat selection can also be extrapolated to oviposition site selection. The ideal free distribution (IFD) theory postulates that habitats differ in their suitability to support an individual organism, and that organisms would preferrentially "select" those habitats that maximize their overall fitness (Fretwell & Lucas 1970). Moreover, the quality of a habitat may change over time due to potential negative effects of crowding and density-dependence, impacting the selection by organisms (Fretwell & Lucas 1970). With regards to oviposition, the IFD theory assumes that a female will allocate the majority of her offspring in the most offspring-favorable habitat available until the conspecific competition within that habitat gets too strong, obligating the female to seek other available sites to lay the rest of her eggs.

A concept related to the IFD theory is the oviposition preference-offspring performance (P-P) hypothesis (Rausher 1983, Valladares & Lawton 1991, Nufio & Papaj 2004, Ellis 2008). This hypothesis proposes that considering that juvenile insects have limited dispersal capabilities, females should oviposit in habitats according to their perceived habitat quality, which is a function of the nutrient levels and the abundance of pre-existing conspecific larvae (larvae of the same species). Essentially, the quality of an oviposition site decreases as larval

density (and intraspecific competition) within the site increases. That competition-driving factors like the presence of conspecific larvae and nutrient abundance can vary in quantity from site to site and thus drive site quality, an ovipositor should have sensory mechanisms or naturally selected behaviors that enable the evaluation of such factors. Without either behavior assumed under the P-P hypothesis, hatching rate, larval performance, and overall offspring fitness may not be optimized.

We approached the life history of *Ae. albopictus* by evaluating whether its oviposition behavior is congruent with the IFD theory—whether females have adapted to principally allocate their eggs in container habitats that have conditions optimal for offspring performance and fitness. Specifically, we empirically tested the P-P hypothesis by performing experiments of *Ae. albopictus* oviposition site selection over gradients of aquatic habitat qualities under controlled laboratory conditions. Given that immature mosquito survival is density-dependent, and also that potential oviposition sites vary with respect to pre-existing conspecific presence (presence of the same species) and nutrient level, a female mosquito must evaluate such conditions via sensory or perceptive behavior to ensure optimization of hatching success, larval performance, and overall offspring fitness.

Aedes albopictus is an invasive species originally from temperate and tropical parts of Southeast Asia and can be found in Europe, North and South America and Africa. In the U.S., it is primarily a nuisance mosquito. The ecological plasticity observed in *Ae. albopictus* allows the species to thrive in natural and artificial containers readily available in both urban and suburban environments (Braks et al 2004, Paupy et al 2009). Therefore, this laboratory study helps us gain a better understanding of the mechanisms underlying *Aedes albopictus* oviposition behavior found in nature.

Materials and Methods

In order to assess whether oviposition site selection maximizes offspring fitness, I conducted two interrelated laboratory experiments. In the first experiment, *Ae. albopictus* performance and, ultimately, fitness in response to two gradients of habitat conditions, diet level (diet) and density of pre-existing conspecific larvae (conspecific density referred to as CD hereafter), were monitored daily. By recreating those two-factor treatments with the most contrasting performance and fitness outcomes (from experiment 1), experiment 2 assessed whether *Ae. albopictus* females follow the predictions of the IFD theory for oviposition. Both experiments were conducted in the indoor controlled insectary chambers located at the Centers for Disease Control and Prevention (CDC) Division of Parasitic Diseases/Entomology Branch (1600 Clifton Rd. Atlanta, GA 30333). The insectary chambers were accessed via a 12-month Guest Researcher pass (approved October 20, 2010, expires October 20, 2011).

Experiment 1:

Larval Development: assessing mosquito performance and fitness over habitat condition gradients

We identified and analyzed *Ae. albopictus* immature performance and ultimate fitness in container habitats by measuring life history traits across two water condition gradients: diet and conspecific density (CD). Four diet levels and four CD levels were used to provide a four-by-four design of container treatments, yielding a total of 16 different treatments (Table 1). A treatment type is hereafter referred by its designated "container identification code" indicated in Table 1. Ten experimental larvae were exposed to each container treatment. Only the experimental larvae were monitored for the quantification of life history traits. The conspecific larvae were used solely to provide the CD conditions within the container treatments and did not contribute towards the experimental data.

The diet used in this study was a mixture of solid tuna meal (M. Q. Benedict, personal communication), lowheat desiccated, non-defatted solid Argentinian beef liver powder (Now Foods, Bloomingdale, IL), and solid vitamin mix (BioServ, www.bio-serv.com) mixed at the ratio 2:2:1 respectively. The solid mixture was thoroughly mixed in pure water to make a 2% diet aqueous solution for efficient diet-transfer purposes. The diet and CD levels were combined in all possible ways (four diet levels X four CD levels) to make 16 distinct treatments in total. Each treatment was replicated three times (total of 48 containers) under constant temperature (28˚C), relative humidity (80%), and Diel regime 12:12 light: dark with 2 crepuscular hours (one hour for sunrise and one hour for sunset).

Each larval rearing container was made out of a 16-ounce white, plastic, cylindrical food container (Bauman Paper Company, Lexington, KY) divided by a white mesh screen positioned perpendicularly to the bottom of the container (Figure 1A). The mesh screen was permeable enough to allow the diet solution to flow homogeneously throughout the container but impermeable enough to keep conspecific larvae separate from the monitored experimental larvae. The mesh screen was inserted in the container by first cutting the container in half, gluing the mesh cloth to one of the halves, then gluing the other half along the perimeter of the mesh cloth where it was glued to the first half. The mesh-divided container was placed in another undivided container of the same size to catch minimal amounts of water dripping from the divided container (Figure 1A). Fourty-eight hours prior to the addition of any larvae, each container was filled with 300 mL of pure water, along with two doses of the appropriate food concentration (Table 1), to allow the start of bacterial development (a diet source for *Ae. albopictus* [Merritt et al 1992]) and settling of food particles. Hereafter, a food dose entails a 200μL solution with the appropriate food concentration (every food dose was added by using a

Eppendorf 5mL micropipette [VWR International, Radnor, PA]). During the 48-hour food-settling period, all containers were placed in the insectary chamber under the temperature, humidity, and Diel conditions stated above.

Following the 48-hour food-settling period, one food dose of the appropriate diet level (referred to as diet) was added to each of the 48 containers. Then, a total of 10 F3 generation recently (24-hours post hatching) emerged *Ae. albopictus* I-instar larvae were added to each container, into one side of the mesh division. These 10 larvae were the experimental larvae monitored throughout the experiment and represented technical replicates within each container. Into the other side of the mesh division, the appropriate number of conspecific larvae were added (Table 1), with half of the conspecific larvae added in each container as I-instar larvae and the other half added as III- or IV-instar larvae. Conspecific eggs to be reared to III- and IV-instar stages were hatched 2 days before the hatching of experimental and conspecific eggs reared to I-instar stage. These proportions of conspecific life stages were used to simulate natural conditions (Yoshioka 2010) as well as to maintain conspecific presence for the entire experimental period. The additions of experimental and conspecific larvae to the containers marked day_0 of the experiment. Therefore, the number of days to each life stage was measured from the date of egg-hatch (day_0).

The development of the experimental larvae (10 technical replicates per container) for all 48 containers (three biological replicates per container treatment type) was monitored daily. Days to reach each life stage were obtained by counting the number of molts to each life stage starting from II-instar larva all the way to pupa (mosquito larvae molt before reaching each life stage). Each time a molt was found in a container, it was recorded for the next life stage and then removed from the container. When conspecific larvae had reached the pupal stage inside a

container, the pupae were removed from the container in order to prevent the escape of subsequently emerged conspecific adults. Mosquito pupae do not feed in the water column; therefore, the removal of the conspecific pupae did not interfere with the integrity of potential density-dependent effects. However, when experimental larvae had reached the pupal stage, the pupae were kept inside the container and the molts to pupa removed.

When larvae in the experimental side of the container division (Exp-side hereafter) reached pupation, the container was covered with a mesh screen. A small ~1cm opening was cut in the screen above the Exp-side, which was covered with a cotton ball (Figure 1B). The removal of the cotton ball allowed extraction of emerged experimental adults via a mouth aspirator (John W. Hock Company, Gainesville, FL) (Figure 1C). Each emerged adult from the Exp-side was sexed and up to five females per container were placed individually into 2mL microvials (Sarstedt Incorporated, Newton, NC) for wing-length measurements. Body size is a good proxy for mosquito fitness, and wing length is closely correlated with body mass (Nasci 1986, Clements 1992). The fitness variable was measured from adult females because the sex ultimately determines production of offspring and selective pressures influencing oviposition behavior were of particular interest in the study. Female wing-length measurements were conducted for two replicates of each container treatment. Each time an adult was extracted, the remaining pupal casing was removed from the container, and the developmental days to adult emergence recorded. When a dead individual was found in a container, the life stage of the individual was recorded and the body removed.

Every day following the larval development monitoring process described above, a food dose of the appropriate concentration was added to each container. Each container received one dose of food daily. Finally, the water level of each container was maintained daily at 300 mL by

adding pure water when needed. Wings were separated from the body of the adult females under a Leica MS5 stereozoom microscope (Leica Microsystems Incorporated, Bannockburn, IL). Following dissection, photographs of the wings were taken using a MagnaFire 2.1C CCD camera (Optronics, Goleta, CA) under an Olympus BX60F-3 microscope (Olympus Optical Co., Ltd., Japan) at 4X magnification. Wing length measurements were conducted using the image processing program ImageJ (Rasband, W. S., U. S. National Institutes of Health, Bethesda, Maryland).

For each container, experimental larval performance was characterized by measuring survival rate (individuals that died/total 10 experimental larvae), time to pupation (days since egg-hatch), percent pupation (number of individuals that pupated/10 total individuals), pupation success rate (number of individuals that pupated/number of individuals that reached IV-instar-stage), time to adult emergence (days since egg-hatch), percent emergence (number of individuals that emerged as adult/10 total individuals), and emergence success rate (number of individuals that emerged/total number of individuals that pupated). Time to pupation and time to emergence were measured to determine which treatments promoted relatively high larval development rate. Percent pupation and percent emergence essentially quantified the mosquito productivity resulting from the 10 total experimental larvae within each treatment; whereas the success rate of pupation and emergence measured the probability of later aquatic stages (i.e. IV-instar and pupal stage) to produce pupae and fully developed adults, respectively.

Experiment 1: Data analysis

Daily survival of mosquitoes under the different diet and conspecific density (CD) levels was analyzed using Kaplan-Meier Survival Analysis (KMSA) tests. KMSA is an exploratory method of generating survival plots for event history data (e.g. time to death) (Kaplan & Meier

1958). Mantel-Cox Log-Rank tests (Mantel 1966) compared the observed number of deaths with the calculated expected number across both gradients of diet and CD and determined whether increasing diet and/or CD affected mosquito survival. Two-way Completely Randomized (CR) Analysis of Variance (ANOVA) tests allowed comparing mean differences in time to pupation and time to adult emergence across the different diet and CD levels. A log transformation was used to transform the non-normal data for time to adult emergence, allowing utilization of the Two-Way CR ANOVA parametric test. Although the study had a blocked design (10 monitored larvae within each of the three biological replicates [of container treatment type]), the mean of the 10 experimental larvae, for both time to pupation and time to emergence, was computed for each monitored container before the CR ANOVA. Essentially, these computed means represented independent observations provided by each container and thus validated the use of a CR ANOVA as opposed to a repeated/related measures ANOVA. For percent pupation, pupation success rate, percent emergence, and emergence success rate, due to the skewed distribution of values and high frequency of tied events for the variables, all values were rank-ordered within each variable and analyzed using the Kruskal-Wallis (K-W) non-parametric analysis of variance (analyzing potential diet and CD effects independently). Regression and correlation analyses allowed estimating the association between fitness and the different diet and CD levels. All statistical tests were performed in SPSS 17.0.0 (IBM SPSS Statistics 2008).

Experiment 2: Empirical estimation of oviposition site preference

Twelve different container treatment types were used in the oviposition assays, which encompassed experiment 2 of the study. Treatment conditions were chosen based on the data analyses resulting from the time to pupation and emergence data—since the observed values of these variables showed clear differences. Diet and CD affected development time across diets 1,

2, and 3 and between CD levels A and D. Therefore, these specific diet and CD levels were used to create the oviposition site conditions. In order to maintain the integrity of site condition gradients, a CD level of 10 conspecific larvae was added as a condition. A diet level of no food (diet ID "0" [Table 2]) was also added as a treatment condition. The nomenclature for each treatment used in the oviposition assays is described in Table 2.

With the exception of treatments with no food (i.e. diet 0 [Table2]), all container treatments were made with six 200µL doses of the food solution at the appropriate concentrations. After pure water was added to fill each container up to the 300mL mark, the diet dose additions were made. Then, appropriate numbers of conspecific larvae were added. As in experiment 1, half of the conspecific larvae were added as I- and II-instars and the other half as III- and IV-instars. These proportions were used to simulate conditions found in nature (Yoshioka 2010). Containers used in experiment 2 did not have the mesh divisions that were necessary in experiment 1, in which the experimental larvae had to be kept separated from the conspecific larvae. Instead for experiment 2, the conspecific larvae were allowed to swim freely throughout the whole 16-ounce container (Bauman Paper Company, Lexington, KY). Coffee filter paper was lined along the walls of each of the 12 containers, eventually soaking completely once touching the solution present inside the container (egg dish protocol as described in Clemons et al 2010).

For each assay, the 12 containers were positioned randomly in an enclosed insect tent with the dimensions 75 by 75 by 115 cm (MegaView Science Co., Ltd., Taichung, Taiwan). Upon adult emergence, the adult female to be used in an assay was allowed five days for mating before bloodfeeding on a sedated rabbit (CDC mosquito bloodfeeding procedures were followed). Immediately after being bloodfed, the gravid (bloodfed and ready to lay eggs) female

was released into the enclosed tent housing the 12 containers and allowed to oviposit for 3 days. The enclosed tent was placed in an environmental chamber controlled under constant temperature (28˚C), relative humidity (80%), and Diel (12:12 light:dark) conditions (same conditions as those used in experiment 1). The female was released at sunset and removed at dusk to mimic natural *Ae. albopictus* oviposition conditions. Upon completion of each 3-day assay, filter papers were removed from the containers and the number of eggs laid by the female was recorded for each container. The fate (dead or alive) of the gravid female was also noted at the end of each assay. A total of eight females were used to conduct eight oviposition assays.

Experiment 2: Data analysis

Resulting data were analyzed using frequency tables (showing presence/absence of eggs for each container). Correlation analyses were also used to relate larval performance resulting in experiment 1 with frequency of eggs laid across container treatments.

Results

Experiment 1: Larval Development

Total larval development time:

The larval development experiment lasted a total of 18 days post egg-hatch of the experimental larvae (the last experimental pupae to emerge as adults across all 48 containers emerged on day 18). The shortest time for an experimental larva to complete development (reach adult stage) was 13.3 days, and the associated container was one of the three 1B replicates (refer to Table 1). Across all replicates, experimental larvae of 4A took the shortest mean time to complete development (7.7 days [SD±0.81 days]), and those of 1D took the longest mean time to complete development (13.6 days [SD±2.47 days]) (Figure 2).

Survival:

Of the total 480 experimental larvae monitored, 39 individuals died throughout the larval development experiment. These 39 deaths included larvae of all four instar stages, as well as pupae and adults that died upon emergence. The mean lifespan for a larva through adult emergence was 17.0 days (SD±0.19 days) across all replicates. Looking at the different levels of diet alone, diet 4 resulted in the longest mean lifespan per larva at 17.3 days (SD±0.19 days), and diet 2 resulted in the shortest mean lifespan per larva at 16.7 days (SD±0.46 days). However, diet did not significantly affect lifespan (log rank Mantel-Cox, $X^2=0.49, p>0.5$) (Figure 3). Looking at the different levels of conspecific density (CD) alone, CD level B resulted in the longest mean lifespan per larva at 17.3 days (SD±0.41 days), and CD level A resulted in the shortest mean lifespan per larva at 16.8 days (SD± 0.49days). However, CD alone also did not significantly affect lifespan per larva (log rank, Mantel-Cox, $X^2=2.86, p>0.4$) (Figure 4).

Time to pupation:

Of the total 480 larvae that were monitored throughout the 18-day experiment, 448 larvae (93.3%) pupated. This number that reached pupation was also 96.8% of the total number of larvae that successfully reached the IV-instar stage (the life stage before pupation). The mean time to pupation (post egg-hatch) was 7.4 days (SD±1.93 days). Across all container treatments and replicates, the amount of time the experimental larvae took to reach pupation stage per container ranged from an average of 5.3 days (SD±0.50 days) from a replicate of treatment 4A to 12.2 days (SD±2.74 days) from a replicate of treatment 1D. Overall, the container treatments resulting in experimental larvae with the longest time to pupation was 1D, pupating at 11.4 days (SD±0.88 days) followed by 1C pupating at 10.6 days (SD±1.19 days) (Figure 5). The container treatments resulting in experimental larvae with the shortest time to pupation was 4A, pupating at 5.5 days (SD±0.14 days) followed by 4B pupating at 5.80 days (SD±0.17 days). In terms of

diet, the shortest and longest mean time to pupation was observed as expected in the highest and lowest diet levels (i.e. diet 4 and diet 1 respectively). Also, the shortest and longest mean time to pupation was observed as expected in the lowest and highest CD levels (i.e. level A and level D respectively).

Independently, diet and CD were both significant predictors of time to pupation (Two-Way CR ANOVA, diet: $F\ (3, 32)=57.6, p<0.001$, CD: $F\ (3, 32)=3.6, p<0.05$) (Table 5). Post-Hoc tests indicated that time to pupation was significantly affected with each increasing diet level except from diet 3 to 4 (Two-Way CR ANOVA Post-Hoc test, $p>0.5$) (Table 5). In addition, time to pupation only significantly increased from CD level A to D (Two-Way ANOVA Post-Hoc test, $p<0.05$); CD levels A, B, and C did not statistically differ in time to pupation.

Percent pupation and pupation success rate:

The following pupation rate variables (per container) were analyzed for the effect of diet and CD separately: 1) percentage of the 10 experimental larvae per container that pupated (referred as "percent pupation") and 2) pupation success rate (number of larvae that reach IV-instar larvae stage that then successfully reach pupation stage). According to the Kruskal-Wallis test, percent pupation was significantly different across both diet and CD (Kruskal-Wallis analysis of variance, diet: $X^2=28.6, df=3, p<0.001$, CD: $X^2=21.3, df=3, p<0.001$). However, as there was no directionality (increase or decrease in percent pupation with ascending diet and/or CD) indicated by the means of both groups (Table 6), no biological interpretations could be made. Pupation success rate mirrored the same statistical pattern (Kruskal-Wallis analysis of variance, diet: $X^2=28.8, df=3, p<0.001$, CD: $X^2=32.1, df=3, p<0.001$), and because the means of both the diet and CD groups lacked directionality (Table 6), no biological interpretations

regarding pupation success could be made with confidence. The heterogeneity found in both variables of pupation rate across the container treatments was perhaps not related to diet availability nor CD. Moreover, in nature, development time may be a better indicator of larval performance compared to ultimate productivity (absolute number of larvae that pupate and emerge as adults). Particularly with breeding sites found in urban and suburban settings, the presence of water inside artificial containers is often short-lived. Essentially, after rainfall and consequent flooding of containers, the containers must continue to hold water for about a week to allow hatched eggs sufficient time to develop and reach the adult stage.

Time between pupal and adult stage:

Across all container types, the mean time for a larva to become adult post-pupation was 2.2 days (SD±0.28 days), but this measurement variable did not vary statistically across diet or CD conditions (Two-Way CR ANOVA, diet: $F(3, 32)=2.7, p>0.05$, CD: $F=0.844, p>0.4$). Since pupae do not feed in the water column and usually adhere to the water surface to breathe, values of this variable did not differ across treatments. Direct density-dependence effects (i.e. limited swimming space and food availability) may have been absent with regards to pupae.

Time to emergence:

Of the total 480 larvae that were monitored for larval development, 445 larvae (92.7%) emerged as adults. This number was also 98.0% of the total number of larvae that successfully reached the pupation stage (life stage before adult emergence). The mean time to emergence (post egg-hatch) was 9.6 days (SD±1.93 days). Across all container types and replicates, the amount of time experimental larvae took to reach adult stage within a container ranged from 7.6 days (SD±0.97 days) from a replicate of treatment 4A (Table 1) to 13.8 days (±2.82 days) from a replicate of treatment 1C. Overall, the treatment types resulting in larvae that took the longest

time to adult emergence were 1D, emerging at 13.5 days (SD±0.97 days) followed by 1C emerging at 12.4 days (SD±1.22 days) (Figure 6). The container types resulting in the shortest time to emergence were treatment 4A, emerging at 7.7 days (SD±0.10 days) followed by 4B emerging at 8.0 days (SD±0.31 days). Because time to emergence largely consists of time to pupation, these trends, as well as the following results logically resemble those of the expected and observed results for time to pupation.

Independently, diet and conspecific density (CD) were both significant predictors of time to emergence (Two-Way CR ANOVA, diet: $F\ (3, 32)=49.0,\ p=<0.001$, CD: $F\ (3, 32)=3.0,\ p<0.05$) (Table 7). Mirroring the statistical pattern seen with time to pupation, Post-Hoc tests indicated that time to emergence was significantly affected with each increasing diet level except from diet 3 to 4 (Two-Way CR ANOVA Post-Hoc test, $p>0.2$) (Table 7). In addition, time to emergence only significantly increased from CD level A to D (Two-Way ANOVA Post-Hoc test, $p<0.05$); CD levels A, B, and C did not statistically differ in time to emergence. Increasing the CD levels from A to B to C did not significantly affect larval development time, suggesting that a CD magnitude of at least 80 conspecific larvae (represented by CD level D) is needed to induce intraspecific crowding and resource competition intense enough to significantly impact larval performance.

Percent emergence and emergence success rate:

The following emergence rate variables (per container) were analyzed for the effect of diet and CD separately: 1) percentage of the 10 experimental larvae that emerged as adults (referred as "percent emergence" hereafter) and 2) emergence success rate (number of larvae that reach pupae stage that then reach adult stage). Percent emergence was significantly different across both diet and CD (Kruskal-Wallis analysis of variance, diet: $X^2=29.6,\ df=3,\ p<0.001$, CD:

$X^2=23.3, df=3, p<0.001$). However, as there was no directionality (increase or decrease in percent emergence with ascending diet and/or CD) indicated by the means of both groups (Table 8), no biological interpretations could be made. Emergence success rate mirrored the same statistical pattern (Kruskal-Wallis analysis of variance, diet: $X^2=22.3, df=3, p<0.001$, CD: $X^2=27.3, df=3, p<0.001$), and because the means of both the diet and CD groups lacked directionality (Table 8) no biological interpretations regarding emergence success could be made with confidence.

Similar to the values observed for percent pupation and pupation success rate, the raw data for percent emergence and emergence success rate did not signal any strongly apparent differences across container treatments. As mentioned earlier, the treatments do not seem to affect the ultimate number of viable offspring regardless of how much time had elapsed post egg-hatch. Although some individuals took more time to accumulate the necessary amount of food to grow and develop vital bodily functions, once such resources were obtained, individuals showed essentially equal success rates to reach the pupation and emergence life stages. In other words, regardless of the overall time each individual took to develop, after reaching the IV-instar larval stage, all individuals had accumulated essentially the same amount of minimum energy required to pupate and later emerge as adults.

Adult female wing length:

[Female wing-length measurements are currently being quantified and will be analyzed once all replicates have been observed.]

Experiment 2: Empirical estimation of oviposition site preference

[Four of the eight oviposition assays have been conducted, and the resulting data will be analyzed once observations are made for all eight replicates.]

Discussion

The P-P hypothesis proposes that females should oviposit in habitats according to their perceived quality, a function of various factors including nutrient level and abundance of pre-existing conspecific larvae (Rausher 1983, Valladares & Lawton 1991, Nufio & Papaj 2004, Ellis 2008). Moreover, since site quality is manifested in such factors, an ovipositor should have sensory mechanisms or naturally selected behaviors that enable the evaluation of a potential oviposition site's characteristics. Without either behavior congruent with the P-P hypothesis, hatching rate, larval performance, and overall population fitness may not be optimized. This study approached the life history of *Ae. albopictus* by evaluating whether its oviposition behavior was congruent with the P-P hypothesis—whether females have adapted to principally allocate their eggs in container habitats that have conditions optimal for offspring performance and fitness. Simulating temperature, humidity, and Diel conditions typical of a temperate suburban environment where *Ae. albopictus* mosquitoes are prevalent, this laboratory study tested the P-P hypothesis by determining potential associations between within-container conditions (diet and conspecific density levels) and productive life stages of *Ae. albopictus*.

Congruent with the results of this study, shorter development time for *Ae. albopictus* under higher diet conditions has been observed in previous studies (Zahiri et al 1997). Moreover, in an observational study of backyard containers set in suburban Atlanta, Georgia, Yoshioka (2010) found that the presence of organic matter inside the container was a significant predictor for the presence of "productive immatures" (IV-instar larvae and pupae). As expected, larval development time decreased with increasing diet level from diet 1 to diet 3, and such findings were not significantly influenced by the presence of conspecific individuals. That the larvae did not develop significantly faster under diet 4 compared to those under diet 3 suggests that

fundamental development processes and successful adult emergence may not successfully occur without a minimum amount of development time offered by the habitat (in this study, the critical minimum development time was suggested to be 15.7 days, which was observed under diet 3 and without any presence of conspecific larvae). At any rate, the inverse relationship of development time and diet for diets 1 through 3 found in the study are of practical concern. The accumulation of food in the form of leaf litter and bacteria occur fairly frequently in backyard containers, and such event is prolonged if containers continue to be left unmanaged or are not emptied by house owners.

As the duration of limited food availability increases, so does the time required for offspring development to adult stage. Similarly, overcrowding of mosquito larvae generally results in retarded growth and high mortality, small and non-uniform size of adults, and decreased fecundity (Shannon & Putnam 1934, Terzian & Stahler 1949, Ikeshoji 1965, Moore & Fisher 1969). Whether the prolonged development time observed in the larvae reared with 80 other conspecific larvae resulted from visible crowding effects (Dye 1984, Zahiri et al 1997, Zahiri & Rau 1998), increased food partitioning (lower food per capita) (Dye 1984), or toxins induced by such sources of stress (Kuno & Moore 1975) need to be tested. The negative effect of 80 additional conspecific larvae on the experimental larvae most likely encompassed a combination of the possible factors. In terms of crowding effects, simply seeing high amounts of conspecific larvae on the other side of the mesh division could have influenced swimming behavior and therefore foraging success for each experimental larva. Regarding food partitioning, comparing CD level A and D, as the number of individuals within the container increased from 10 larvae (10 experimental larvae and zero conspecific larvae) to 90 larvae (10 experimental larvae and 80 conspecific larvae), food per capita (per larva) throughout the whole

container decreased. Less food per capita also means that the rate at which a single larva locates and feeds on food sources is decreased. Such effects of high conspecific larval density have been shown to lead to toxin production (Kuno & Moore 1975) and/or severe depletion of haemolymph glucose levels (Zahiri et al 1998), thus stunting or prolonging development time to adult stage. By implementing more advanced technology, future studies may be able to further specify the process directly caused by the drastic increase of conspecifics that ultimately results in prolonged development time.

As described above, under low food and high conspecific presence, individual larvae took a longer time to develop compared to those under high food and low (or no) conspecific presence. However, percent pupation and percent emergence, as well as pupation and emergence success rates did not differ across diet or conspecific density levels. Similar to the values observed for percent pupation and pupation success rate, the raw data for percent emergence and emergence success rate did not signal any strongly apparent differences across container treatments. The treatments did not seem to affect the ultimate number of viable offspring regardless of how much time had elapsed post egg-hatch. Although some individuals took more time to accumulate the necessary amount of food to grow and develop vital bodily functions, once such resources were obtained, individuals showed essentially equal success rates to reach the pupation and emergence life stages. In other words, regardless of the overall time each individual took to develop, after reaching the IV-instar larval stage as a time-independent event, all individuals had accumulated essentially the same amount of minimum energy required to pupate and later emerge as adults.

Life stage-specific effects of larval rearing conditions have been previously examined. Regarding diet, under low food availability, IV-instars outcompete younger instars and therefore

suffer relatively less severe effects (Brust 1968, Moore & Fisher 1969). The results from our study are in agreement with this hypothesis; once a larva reached the IV-instar stage, it may have reached adequate development to swim and forage more successfully than it did when it was at a younger life stage.

Although low diet and high presence of conspecific larvae delayed offspring development and thus negatively affected larval performance, such container conditions did not inhibit or interfere with overall production of viable adults. Detailed knowledge of larval and oviposition dynamics over gradients of diet and conspecific larval density can be used to elucidate processes that take place in aquatic habitats dominating real-world suburban neighborhoods. Backyard artificial containers like plastic buckets and flower-pots are often left exposed to various weather events, be it moderate wind or high rainfall, for prolonged periods of time. The containers accumulate organic matter (via leaf litter, dirt deposits, other insect larvae) over time, and through subsequent oviposition events, they recruit conspecific mosquito individuals over time. By creating ascending gradients of diet availability and conspecific larval density, this study essentially took snapshots of such nutrient accumulation and conspecific recruitment over time.

However, as this study provided experimental larvae with water for the whole experimental duration, it eliminated the environmental factor of precipitation, which in nature is a major determinant of successful larval performance. Considering that precipitation fluctuations were absent in the study, perhaps it is not surprising that most of the experimental larvae successfully reached the adult stage. On the other hand, development time significantly varied across some treatments. It may be inferred that if the experimental design were a semi-natural one exposed to natural weather conditions, the likelihood of the larvae that developed

significantly slower compared to others to successfully reach adulthood would highly depend on rainfall frequency and magnitude. In nature, the amount of water present inside artificial containers is dependent on such precipitation factors (Kitron et al 1989). In a suburban context, adequate rainfall facilitates adequate collection of water by container sites; however, under sporadic precipitation or generally low inputs of rain, containers remain relatively dry. Moreover, for containers located in areas of high temperature and low shade, evaporation rates substantially increase. Therefore, it may be disadvantageous for a female ovipositor to lay her eggs in such containers holding limited amounts of water.

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Figures and Tables

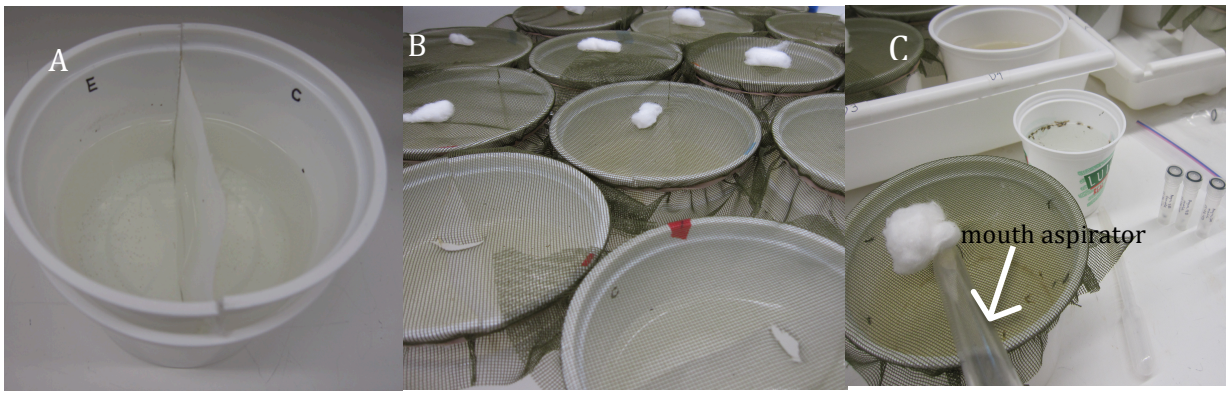


Figure 1A) Plastic 16-ounce food container (Bauman Paper Company, Lexington, KY) divided by a white mesh screen positioned perpendicularly to the bottom of the container. The mesh screen was permeable enough to allow the beef mixture solution to flow homogeneously throughout the container but impermeable enough to keep pre-existing conspecific larvae ("C" side of cup) separate from the experimental larvae ("E" side of cup), B) upon pupation within a container, the top of the container was covered with a mesh screen to prevent emerging adults from escaping. The small opening in the mesh screen covered by a cotton ball allowed easy extraction of adults upon emergence, C) adults were extracted with a mouth aspirator (John W. Hock Company, Gainesville, FL) for vialing or disposing.

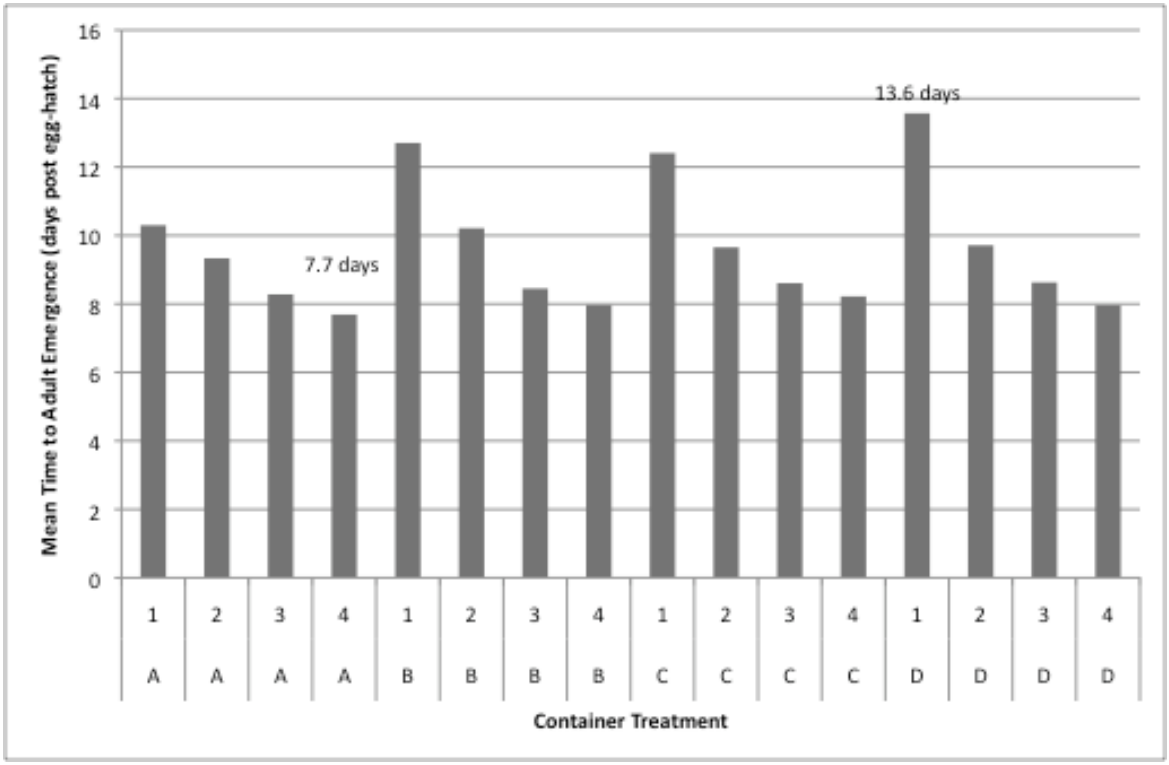
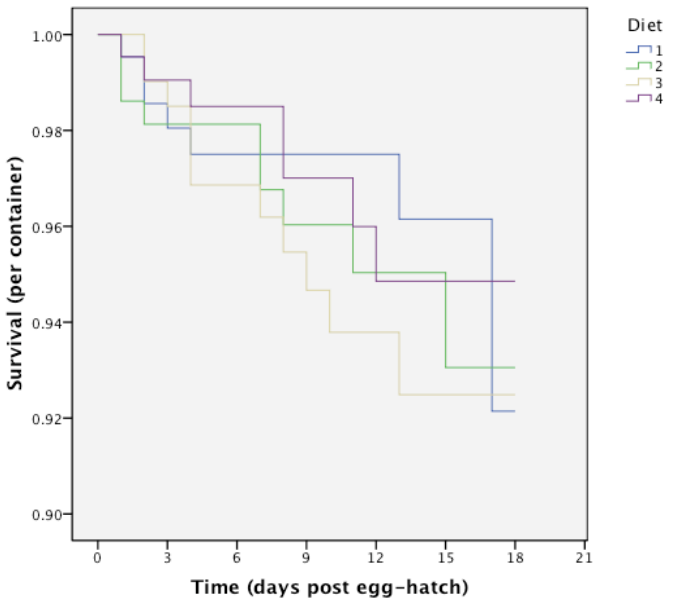
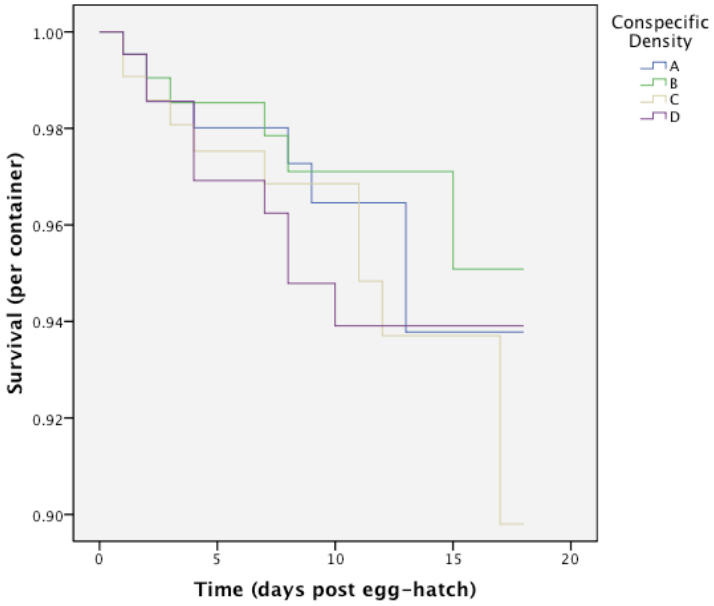


Figure 2 Mean development time to adult emergence across all container treatments. Values for the shortest and longest mean development time are labeled for treatment 4A and 1D respectively. Refer to Table 1 for nomenclature of treatments (numbers 1-4 indicate diet level and letters A-D indicate conspecific density level). Each treatment was replicated three times (N=3).



Diet: 1: 2.0mg, 2: 3.6mg, 3: 7.2mg, 4: 20.0mg

Figure 3 Survival Analysis Curve using diet level as a potential predictor of mean lifespan (per container type) across all replicates (N=3). Survival is representative of the experimental larvae (10 experimental larvae per container) reared under the various diet conditions. Each Diet was added (in constant amounts) to respective containers daily. The graph was obtained by performing a Kaplan Meier Survival Analysis (p=0.921; no difference in survival across diet).



Conspecific Density: A: zero larvae, B: 20 larvae, C: 40 larvae, D: 80 larvae

Figure 4 Survival Analysis Curve using conspecific density (CD) as a potential predictor of mean lifespan (per container type) across all replicates (N=3). Survival is representative of the experimental larvae (10 experimental larvae per container) reared under the various CD conditions. Half of each conspecific density treatment (e.g. 10 of 20 larvae for CD level B) were added as 1st-Instar larvae whereas the other half was added as 3rd or 4th-instar larvae. The graph was obtained by performing a Kaplan Meier Survival Analysis (p=0.414; no difference in survival across CD).

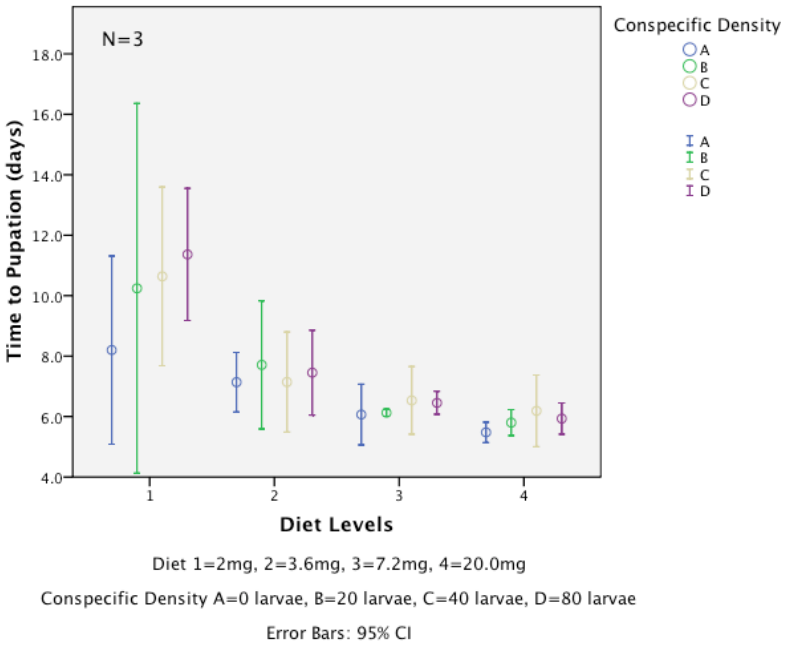


Figure 5 Mean within-container time to pupation (days) across the various diet level and conspecific density (CD) conditions. Data are comprehensive of the whole 18-day time duration of the larval development experiment. Mean points are representative of three biological replicates of each container type with each biological replicate consisting of 10 technical replicates (10 experimental larvae). Each container type has a distinct diet and CD identification, and error bars represent standard deviation from the means.

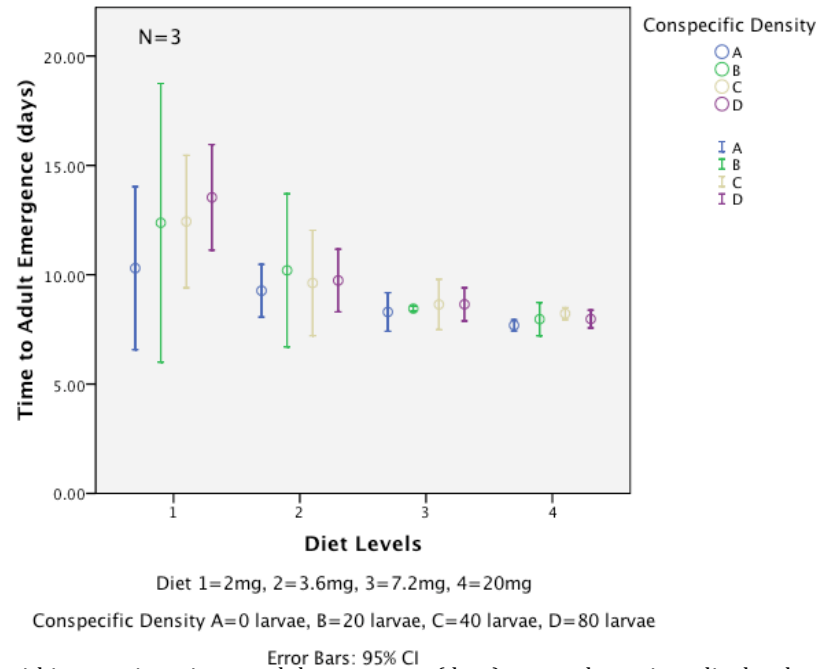


Figure 6 Mean within-container time to adult emergence (days) across the various diet level and conspecific density (CD) conditions. Data are comprehensive of the whole 18-day time duration of the larval development experiment. Mean points are representative of three biological replicates of each container type with each biological replicate consisting of 10 technical replicates (10 experimental larvae). Each container type has a distinct diet and CD identification, and error bars represent standard deviation from the means.

Table 1 Nomenclature for container treatments used in the larval development experiment (experiment 1). Each treatment is identified by diet and conspecific density level.

Conspecific Density / Diet (per daily dose)	ID A: zero larvae	B: 20 larvae	C: 40 larvae	D: 80 larvae
ID 1: 2.0 mg	Container identification code: 1A	1B	1C	1D
2: 3.6 mg	2A	2B	2C	2D
3: 7.2 mg	3A	3B	3C	3D
4: 20.0 mg	4A	4B	4C	4D

Table 2 Nomenclature for container treatments used in oviposition assays (experiment 2). Each treatment is identified by diet and conspecific density level.

Conspecific Density / Diet (per dose)	ID a: zero larvae	b: 10 larvae	c: 80 larvae
ID 1: 2.0 mg	1a	1b	1c
2: 3.6 mg	2a	2b	2c
3: 7.2 mg	3a	3b	3c
0: no food	0a	0b	0c

Table 3 Estimated marginal means of time to pupation (per container type in days) based on diet and conspecific density independently obtained from a Two-Way completely randomized analysis of variance (ANOVA). Means take the average of the three biological replicates of each container type, each replicate consisting of 10 technical replicates (10 monitored larvae).

			95% Confidence Interval	
Diet	Mean Time to Pupation (days)	Standard Deviation	Lower Bound	Upper Bound
1 (2mg)	10.1 [a]	2.85	9.60	10.62
2 (3.6mg)	7.4 [b]	1.67	6.85	7.87
3 (7.2mg)	6.3 [c]	1.15	5.78	6.81
4 (20mg)	5.9 [c]	0.94	5.34	6.36
Conspecific Density				
A (zero larvae)	6.7 [a]	1.74	6.21	7.23
B (20 larvae)	7.5 [a]	2.67	6.96	7.98
C (40 larvae)	7.6 [a]	2.53	7.11	8.14
D (80 larvae)	7.8 [b]	2.72	7.29	8.31

Different letters indicate significant difference ($p < 0.05$).

Table 4 Estimated marginal means of time to adult emergence (per container in days) based on diet and conspecific density independently obtained from a Two-Way completely randomized analysis of variance (ANOVA). Means take the average of the three biological replicates of each container type, each replicate consisting of 10 technical replicates (10 monitored larvae).

			95% Confidence Interval	
Diet	Mean Time to Adult Emergence (days)	Standard Deviation	Lower Bound	Upper Bound
1 (2mg)	12.2 [a]	2.84	11.59	12.74
2 (3.6mg)	9.7 [b]	1.80	9.13	10.28
3 (7.2mg)	8.5 [c]	1.14	7.93	9.09
4 (20mg)	8.0 [c]	0.80	7.39	8.54
Conspecific Density				
A (zero larvae)	8.9 [a]	1.84	8.31	9.47
B (20 larvae)	9.7 [a]	2.67	9.17	10.32
C (40 larvae)	9.7 [a]	2.35	9.15	10.31
D (80 larvae)	10.0 [b]	2.72	9.40	10.55

Different letters indicate significant difference ($p < 0.05$).

Table 5 Two-Way completely randomized analysis of variance (ANOVA) of time to pupation (days post-egg hatch) to determine if diet and/or conspecific density was a significant predictor of time to pupation.

Factors	**df**	**F**	**p-value**
Diet	3	57.640	< 0.001 *
Conspecific Density (CD)	3	3.559	0.025 *
Diet * CD	9	1.512	0.186

*p-values < 0.05 were considered statistically significant.

	Comparisons between Levels		Mean Difference (i-j in days)	p-value		Comparisons between Levels		Mean Difference (i-j in days)	p-value
Diet	1 (i)	2 (j)	2.8	< 0.001*	CD	A (i)	B (j)	-0.7	0.174
		3	3.8	< 0.001*			C	-0.9	0.073
		4	4.3	< 0.001*			D	-1.1	0.024*
	2	3	1.1	0.026*		B	C	-0.2	0.971
		4	1.5	0.001*			D	-0.3	0.790
	3	4	0.4	0.601		C	D	-0.2	0.961

Post-hoc Tukey comparisons between treatment levels of diet and conspecific density (CD) and associated significance values. Multiple comparisons were analyzed for diet and CD independently. p-values < 0.05 were considered statistically significant.

Table 6 Mean values and standard deviations for percent pupation (% pupation) and pupation success rate (in %) across treatment levels of diet and conspecific density (CD) independently. Means take the average of the three biological replicates of each container type, each replicate consisting of 10 technical replicates (10 monitored larvae).

	Level	Mean (% pupation)	Standard Deviation		Level	Mean (% pupation)	Standard Deviation
Diet	1	94.2	9.00	CD	A	92.5	10.55
	2	90.8	9.96		B	95.0	10.00
	3	91.7	11.15		C	93.3	9.85
	4	96.7	6.51		D	92.5	7.54

	Level	Mean (pupation success rate [%])	Standard Deviation		Level	Mean (pupation success rate [%])	Standard Deviation
Diet	1	95.8	0.09	CD	A	95.7	0.07
	2	96.6	0.05		B	95.7	0.09
	3	95.6	0.08		C	96.5	0.07
	4	99.2	0.03		D	99.2	0.03

Table 7 Two-Way completely randomized analysis of variance (ANOVA) of time to emergence (days post-egg hatch) to determine if diet and/or conspecific density was a significant predictor of time to emergence. Non-normal time to emergence values were transformed using the log(x) transformation for the parametric ANOVA test.

Factors	**df**	**F**	**p-value**
Diet	3	49.039	< 0.001 *
Conspecific Density (CD)	3	3.029	0.044 *
Diet * CD	9	0.996	0.463

*p-values < 0.05 were considered statistically significant.

	Comparisons between Levels		Mean Difference (i-j in days)	p-value		Comparisons between Levels		Mean Difference (i-j in days)	p-value
Diet	1 (i)	2 (j)	0.1	< 0.001*	CD	A (i)	B (j)	-0.0	0.152
		3	0.2	< 0.001*			C	-0.0	0.124
		4	0.2	< 0.001*			D	-0.4	0.044*
	2	3	0.1	0.007*		B	C	-0.0	0.998
		4	0.1	< 0.001*			D	-0.0	0.935
	3	4	0.0	0.290		C	D	-0.0	0.962

Post-hoc Tukey comparisons between treatment levels of diet and conspecific density (CD) and associated significance values. Multiple comparisons were analyzed for diet and CD independently. p-values < 0.05 were considered statistically significant.

Table 8 Mean values and standard deviations for percent emergence (% emergence) and emergence success rate (in %) across treatment levels of diet and conspecific density (CD) independently. Means take the average of the three biological replicates of each container type, each replicate consisting of 10 technical replicates (10 monitored larvae).

	Level	Mean (% emergence)	Standard Deviation		Level	Mean (% emergence)	Standard Deviation
Diet	1	93.3	9.00	CD	A	92.5	10.553
	2	90.8	9.96		B	95.0	10.000
	3	91.7	11.15		C	93.3	9.847
	4	96.7	6.51		D	92.5	7.538

	Level	Mean (emergence success rate [%])	Standard Deviation		Level	Mean (emergence success rate [%])	Standard Deviation
Diet	1	98.2	4.12	CD	A	99.2	2.89
	2	99.2	2.89		B	100.0	0.00
	3	99.2	2.89		C	95.7	5.27
	4	97.5	4.52		D	99.2	2.89

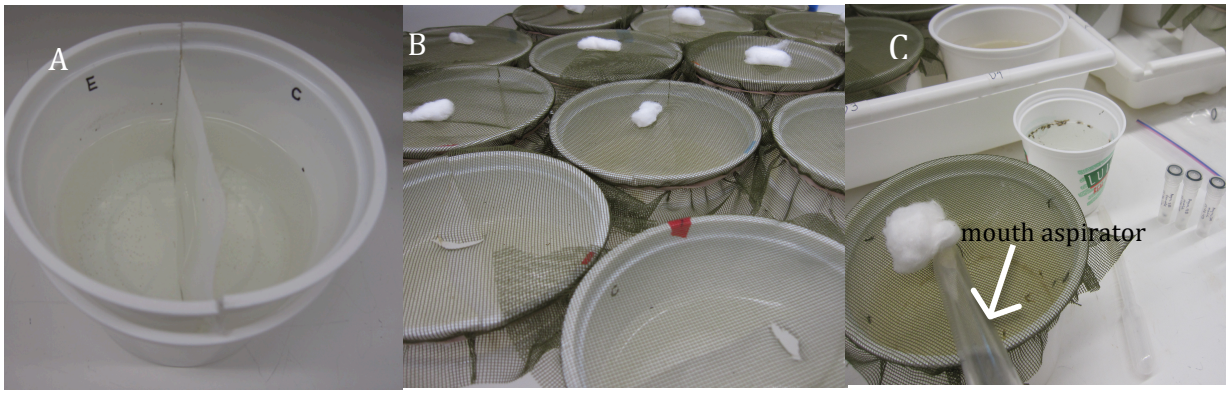


Figure 2A) Plastic 16-ounce food container (Bauman Paper Company, Lexington, KY) divided by a white mesh screen positioned perpendicularly to the bottom of the container. The mesh screen was permeable enough to allow the beef mixture solution to flow homogeneously throughout the container but impermeable enough to keep pre-existing conspecific larvae ("C" side of cup) separate from the experimental larvae ("E" side of cup), B) upon pupation within a container, the top of the container was covered with a mesh screen to prevent emerging adults from escaping. The small opening in the mesh screen covered by a cotton ball allowed easy extraction of adults upon emergence, C) adults were extracted with a mouth aspirator (John W. Hock Company, Gainesville, FL) for vialing or disposing.

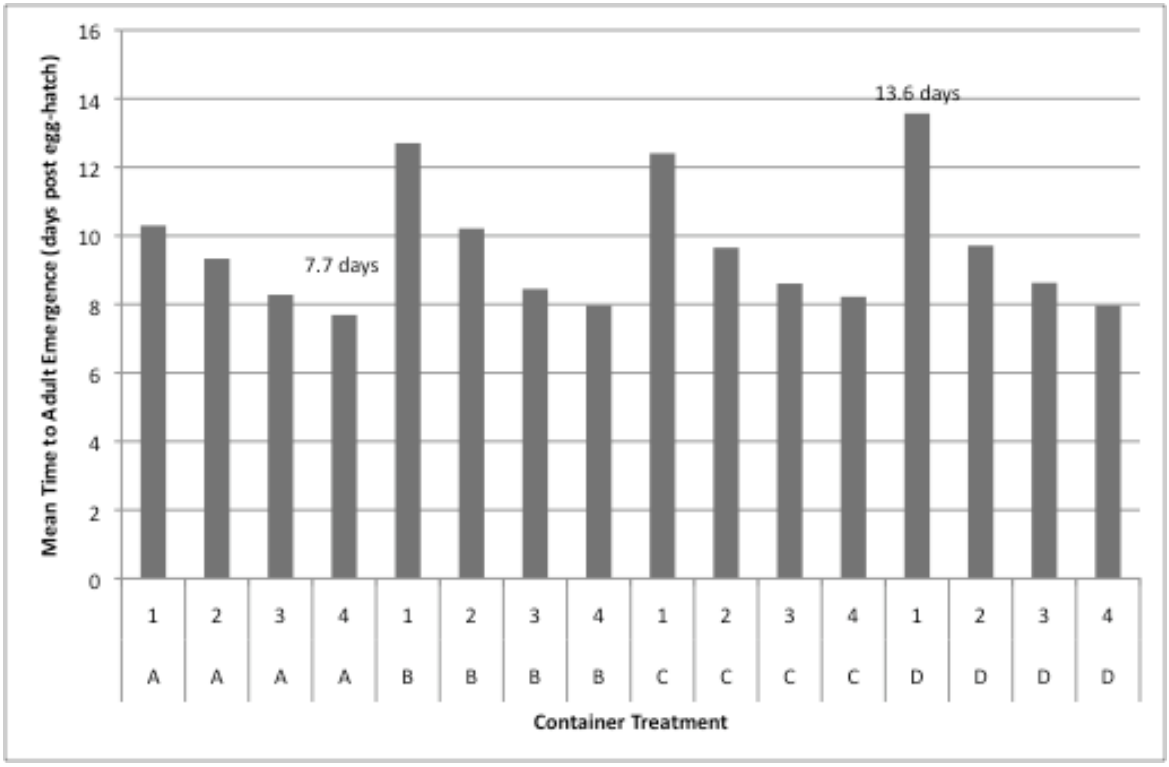
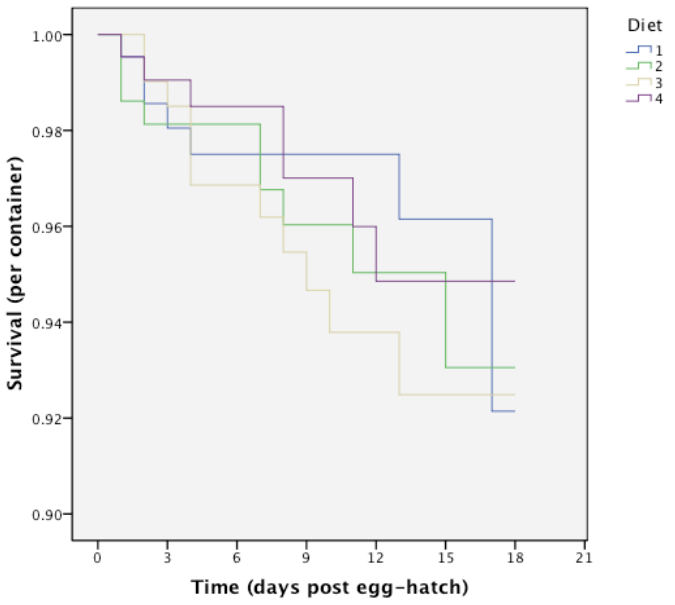
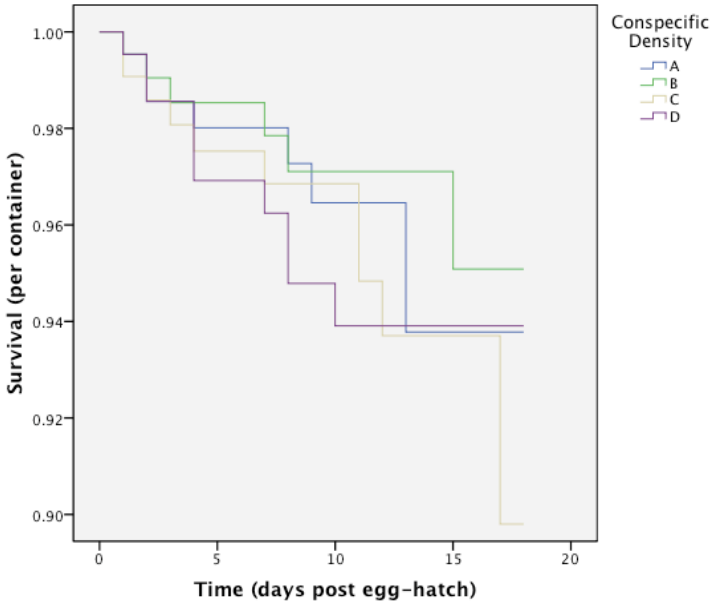


Figure 2 Mean development time to adult emergence across all container treatments. Values for the shortest and longest mean development time are labeled for treatment 4A and 1D respectively. Refer to Table 1 for nomenclature of treatments (numbers 1-4 indicate diet level and letters A-D indicate conspecific density level). Each treatment was replicated three times (N=3).



Diet: 1: 2.0mg, 2: 3.6mg, 3: 7.2mg, 4: 20.0mg

Figure 3 Survival Analysis Curve using diet level as a potential predictor of mean lifespan (per container type) across all replicates (N=3). Survival is representative of the experimental larvae (10 experimental larvae per container) reared under the various diet conditions. Each Diet was added (in constant amounts) to respective containers daily. The graph was obtained by performing a Kaplan Meier Survival Analysis (p=0.921; no difference in survival across diet).



Conspecific Density: A: zero larvae, B: 20 larvae, C: 40 larvae, D: 80 larvae

Figure 4 Survival Analysis Curve using conspecific density (CD) as a potential predictor of mean lifespan (per container type) across all replicates (N=3). Survival is representative of the experimental larvae (10 experimental larvae per container) reared under the various CD conditions. Half of each conspecific density treatment (e.g. 10 of 20 larvae for CD level B) were added as 1st-Instar larvae whereas the other half was added as 3rd or 4th-instar larvae. The graph was obtained by performing a Kaplan Meier Survival Analysis (p=0.414; no difference in survival across CD).

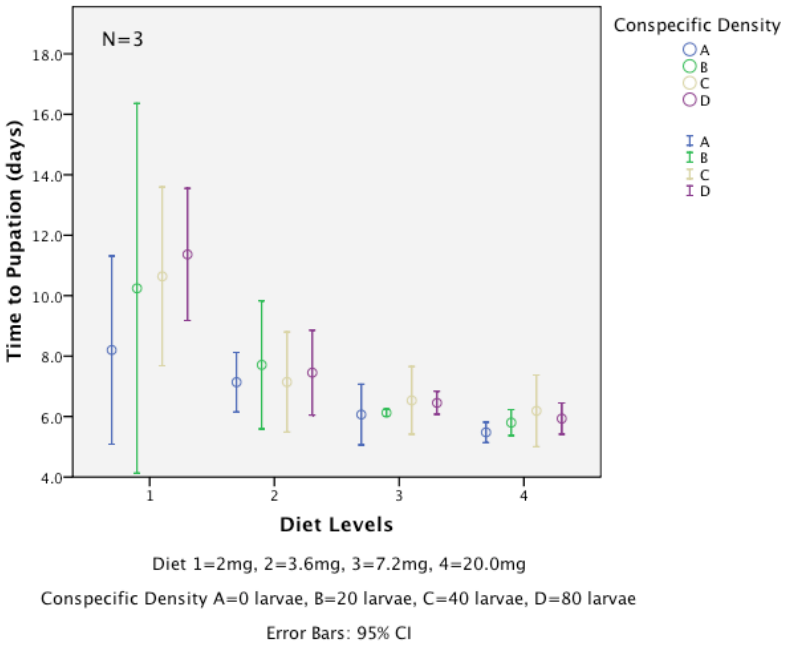


Figure 5 Mean within-container time to pupation (days) across the various diet level and conspecific density (CD) conditions. Data are comprehensive of the whole 18-day time duration of the larval development experiment. Mean points are representative of three biological replicates of each container type with each biological replicate consisting of 10 technical replicates (10 experimental larvae). Each container type has a distinct diet and CD identification, and error bars represent standard deviation from the means.

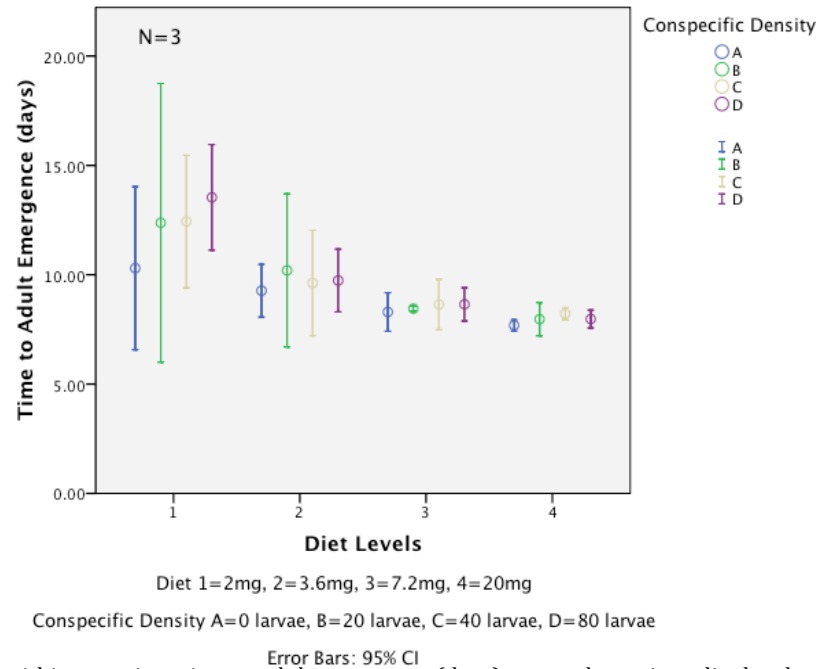


Figure 6 Mean within-container time to adult emergence (days) across the various diet level and conspecific density (CD) conditions. Data are comprehensive of the whole 18-day time duration of the larval development experiment. Mean points are representative of three biological replicates of each container type with each biological replicate consisting of 10 technical replicates (10 experimental larvae). Each container type has a distinct diet and CD identification, and error bars represent standard deviation from the means.

Table 3 Nomenclature for container treatments used in the larval development experiment (experiment 1). Each treatment is identified by diet and conspecific density level.

Conspecific Density / Diet (per daily dose)	ID A: zero larvae	B: 20 larvae	C: 40 larvae	D: 80 larvae
ID 1: 2.0 mg	Container identification code: 1A	1B	1C	1D
2: 3.6 mg	2A	2B	2C	2D
3: 7.2 mg	3A	3B	3C	3D
4: 20.0 mg	4A	4B	4C	4D

Table 4 Nomenclature for container treatments used in oviposition assays (experiment 2). Each treatment is identified by diet and conspecific density level.

Conspecific Density / Diet (per dose)	ID a: zero larvae	b: 10 larvae	c: 80 larvae
ID 1: 2.0 mg	1a	1b	1c
2: 3.6 mg	2a	2b	2c
3: 7.2 mg	3a	3b	3c
0: no food	0a	0b	0c

Table 3 Estimated marginal means of time to pupation (per container type in days) based on diet and conspecific density independently obtained from a Two-Way completely randomized analysis of variance (ANOVA). Means take the average of the three biological replicates of each container type, each replicate consisting of 10 technical replicates (10 monitored larvae).

			95% Confidence Interval	
Diet	Mean Time to Pupation (days)	Standard Deviation	Lower Bound	Upper Bound
1 (2mg)	10.1 [a]	2.85	9.60	10.62
2 (3.6mg)	7.4 [b]	1.67	6.85	7.87
3 (7.2mg)	6.3 [c]	1.15	5.78	6.81
4 (20mg)	5.9 [c]	0.94	5.34	6.36
Conspecific Density				
A (zero larvae)	6.7 [a]	1.74	6.21	7.23
B (20 larvae)	7.5 [a]	2.67	6.96	7.98
C (40 larvae)	7.6 [a]	2.53	7.11	8.14
D (80 larvae)	7.8 [b]	2.72	7.29	8.31

Different letters indicate significant difference ($p < 0.05$).

Table 4 Estimated marginal means of time to adult emergence (per container in days) based on diet and conspecific density independently obtained from a Two-Way completely randomized analysis of variance (ANOVA). Means take the average of the three biological replicates of each container type, each replicate consisting of 10 technical replicates (10 monitored larvae).

			95% Confidence Interval	
Diet	Mean Time to Adult Emergence (days)	Standard Deviation	Lower Bound	Upper Bound
1 (2mg)	12.2 [a]	2.84	11.59	12.74
2 (3.6mg)	9.7 [b]	1.80	9.13	10.28
3 (7.2mg)	8.5 [c]	1.14	7.93	9.09
4 (20mg)	8.0 [c]	0.80	7.39	8.54
Conspecific Density				
A (zero larvae)	8.9 [a]	1.84	8.31	9.47
B (20 larvae)	9.7 [a]	2.67	9.17	10.32
C (40 larvae)	9.7 [a]	2.35	9.15	10.31
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		4	1.5	0.001*			D	-0.3	0.790
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Post-hoc Tukey comparisons between treatment levels of diet and conspecific density (CD) and associated significance values. Multiple comparisons were analyzed for diet and CD independently. p-values < 0.05 were considered statistically significant.

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		4	0.2	< 0.001*			D	-0.4	0.044*
	2	3	0.1	0.007*		B	C	-0.0	0.998
		4	0.1	< 0.001*			D	-0.0	0.935
	3	4	0.0	0.290		C	D	-0.0	0.962

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	2	90.8	9.96		B	95.0	10.000
	3	91.7	11.15		C	93.3	9.847
	4	96.7	6.51		D	92.5	7.538

	Level	Mean (emergence success rate [%])	Standard Deviation		Level	Mean (emergence success rate [%])	Standard Deviation
Diet	1	98.2	4.12	CD	A	99.2	2.89
	2	99.2	2.89		B	100.0	0.00
	3	99.2	2.89		C	95.7	5.27
	4	97.5	4.52		D	99.2	2.89