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Use of *Tenebrio molitor* (Coleoptera: Tenebrionidae) powder to enhance artificial diet
formulations for *Coleomegilla maculata* (Coleoptera: Coccinellidae)

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Abstract

The predatory lady beetle *Coleomegilla maculata* has potential to control several arthropod pests on crop plants in greenhouses and high tunnels. However, an effective artificial diet is needed in order to mass produce *C. maculata* in sufficient quantities for augmentative releases. The objectives of this study were to develop a semi-solid insect-free artificial diet, evaluate the diet effects on *C. maculata* fitness, and determine if adding extracts (consisting of dry powder of whole pupae) of the yellow mealworm, *Tenebrio molitor*, could improve the suitability of the diet formulations and the life parameters of *C. maculata*. Although *C. maculata* completed development and reproduced on two meridic artificial diet formulations (M1 and M2), neither one was as effective as a control diet mix consisting of *Ephestia kuehniella*, *Artemia* sp. Eggs, and bee pollen. Incorporation of *T. molitor* extracts into the diet formulation (5 and 7% in diets T1 and T2, respectively), significantly improved *C. maculata* larval survival, shortened post-embryonic development time, increased fecundity and egg viability as compared to the meridic diet formulations. Adults feeding on diets T1 and T2 (containing *T. molitor* extracts) produced more eggs and had a better survival than those feeding on the control diet mix. Also, egg viability was significantly higher in diets T1 and T2 than in the control. Demographic parameters showed that the overall fitness of *C. maculata* fed the meridic diet containing *T. molitor* extracts was superior to beetles fed the control mix. The role of feeding stimulation by *T. molitor* added components as responsible for the diet improvement is discussed.

Keywords: ladybird beetles; mass rearing; in vitro rearing; development; fecundity; life cycle; life table

1. Introduction

Coleomegilla maculata De Geer (Coleoptera: Coccinellidae) inhabits landscapes in North, South, and Central America (Gordon, 1985; Munyaneza and Obrycki, 1998; Krawiec and Obrycki, 2000). It is a generalist predator of aphids, lepidopteran eggs and larvae, chrysomelid eggs and larvae, spider mites, and other soft-bodied prey (Michaud and Jyoti, 2008; Hodek and Evans, 2012), and can complete development on plant pollen (Smith, 1960, 1965; Michaud and Grant, 2005; Lundgren et al., 2011). There is interest in using this ladybird beetle for augmentative biological control of plant pests in protected culture, i.e., high tunnels, greenhouses, plantscapes and interiorscapes (Riddick et al., 2014a, 2014b, and 2014c). Its polyphagous tendencies are an advantage when there are multiple pest species on the same crop plant. Another advantage is that in the absence of prey, *C. maculata* can survive on plant material such as pollen and nectar.

Mass production of *C. maculata* is necessary for augmentative biological control to provide enough quantities of immature and/or adult stages to control pests on plants on a commercial scale. One approach to facilitating mass production of *C. maculata* is through the provision of inexpensive, nutritious artificial diets that promote development, growth and reproduction of *C. maculata* over consecutive generations. Artificial diets devoid of insect components, or meridic diets (Grenier and De Clercq, 2003), would greatly reduce costs associated with rearing *C. maculata*, in lieu of natural prey, because natural prey (such as most aphid species) often require live host plants as food

(Hagen, 1987; Riddick and Chen, 2014). No such standalone, arthropod-free artificial diet is currently available for lady beetles, but research has been ongoing (Smirnoff, 1958; Atallah and Newsom, 1966; Silva et al., 2010; Sighinolfi et al., 2013).

Exploring the effectiveness of artificial diets containing plant protein, rather than insect protein, is another line of research with potential. In efforts to identify a highly effective artificial diet, Riddick et al. (2014c) tested the usefulness of a powdered formulation of *Spirulina platensis* (Nordstedt) Geitler (Cyanophyceae: Phormidiaceae), a protein- and vitamin-rich cyanobacteria microalga, as a supplement to a suboptimal meridic artificial diet for *C. maculata*. Unfortunately, *S. platensis* was not a satisfactory supplement; since it did not improve the quality of the suboptimal artificial diet for *C. maculata* (Riddick et al., 2014c).

Difficulties in developing effective meridic diets arise from nutritional requirements that are particular to insects. For example, insects require higher quantities of the amino acids proline and tyrosine than vertebrates (Wigglesworth, 1972; Carter et al., 2006; Lundgren, 2009). Levels of these two amino acids present in vertebrate and vegetable protein are not adequate for insect nutrition and must be added to meridic diet formulations (Morales-Ramos et al., 2014). Adequate levels of these amino acids are found in the protein of natural prey and non-prey food such as pollen (Lundgren et al., 2005). Additionally, artificial diets that contain insect components are useful when predators depend on chemical cues to stimulate feeding (De Clercq et al., 2004). Morales-Ramos et al. (2014) suggested that insect protein may be added to artificial diet formulations from insect species that are produced commercially. The yellow mealworm *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) is

commercially available in the United States, and some North and South American, European and Asian countries (van Huis et al., 2013). In some countries *T. molitor* powder extracts are commercially available, which could be easily incorporated into artificial diet formulations (Morales-Ramos et al., 2014).

The objectives of this study were: 1) to compare the biological and demographic parameters on *C. maculata* fed semi-solid meridic artificial diets with a control mix consisting of natural ingredients and 2) to determine if adding powdered pupae of the yellow mealworm, *T. molitor*, to diet formulations could improve their suitability and the life parameters of *C. maculata*. As such, we tested the hypothesis that supplementing a suboptimal meridic artificial diet with dry extracts from *T. molitor* improves the biological and demographic parameters of *C. maculata*.

2. Materials and Methods

2.1 Colonies Origin and Maintenance

The stock colony of *C. maculata* was initiated from field collections conducted in the spring and summer of 2010 in Stoneville, Mississippi. Adults were maintained in a 2-section modular cage, which provides an upper space for feeding and a lower space for oviposition (Fig. 1A). The upper section was constructed from plastic containers (312 x 230 x 102 mm) (Product 295C, Pioneer Plastics Inc., North Dixon, KY), which were modified by adding 10 screened windows (nylon screen with 500 µm openings) on the sides (30 mm diam.) and 8 openings on the cover (52 mm diam.) that could be used as windows or water sources (Fig. 1A). The bottom section was constructed from

cylindrical plastic containers (191 x 202 mm diam.) (Product 289C, Pioneer Plastics Inc., North Dixon, KY) and modified by adding a side opening with screwed cap to allow the exchange of oviposition substrate (Fig. 1B). The stock colony was fed with a combination of food sources including Entofood® (Koppert Biological Systems Inc., Howell, MI), ground bee pollen granules (product 2530, NOW Foods, Bloomingdale, IL), *Lygus hesperus* (Knight) (Heteroptera: Miridae) eggs, ground lyophilized *T. molitor* pupae, honey, and a meridic artificial diet as supplement. Water was provided by saturated water crystals of cross-linked potassium polyacrylate and polyacrylamide copolymer (T-400, Terawet Inc., San Diego, CA) placed in screened devices on the cover of the cage. The oviposition substrate consisted of stacked and folded paper sheets (Kimwipes®, Kimberly-Clark LLC, Roswell, GA) placed standing vertically inside the bottom section of the cage. Egg masses were cut from the paper sheets and deposited in Petri dishes every day for development. First instars were transferred to cages constructed from square hinged plastic boxes (334 x 233 x 50 mm) (product 700C, Pioneer Plastics Inc., North Dixon, KY) and modified by adding screened widows (nylon screen with 100 µm openings) to the sides and top and two openings with screwed cap for access to the inside. Cages were filled with 14 crumbled sheets of Kimwipes® paper to provide shelter space and increase surface area. Boxes were closed and sealed on the sides using laboratory labeling tape to prevent first instars from escaping. The stock colony of *C. maculata* was maintained in an environmental chamber at 26°C, 75% RH, and 14 h photophase. The colony of *T. molitor* was established with stock provided by Southeastern Insectaries, Perry, GA. *Tenebrio molitor* rearing procedures were as described by Morales-Ramos et al. (2012).

2.2 Diet Preparation

Diet formulations tested in this study were modifications of a colloidal diet for predatory mites described by Rojas and Morales-Ramos (2014). Four diet formulations were compared; two meridic (M1 and M2) and two which included whole lyophilized and ground *T. molitor* pupae (T1 and T2). Diet formulations, including ingredient list and quantities, are presented in Table 1. To prepare a 100g portion of the diets, solid ingredients in Table 1 were mixed with no water at room temperature. The B-complex vitamin solution (Table 2) and salt solutions A, B, and C (Table 3) were added to 10 ml of reverse osmosis (RO) water and agitated at room temperature. The mixture of solids was added to the water and mixed thoroughly. Diet consistency was similar to peanut butter. Adults and larvae of *C. maculata* can feed directly on the diet mix, but first instars require the diet to be finely spread to prevent the larvae from becoming trapped and drowned within the diet mix.

Diets T1 and T2 contained dried *T. molitor* pupae preparation. To obtain the pupae preparation, *T. molitor* pupae from the stock colony were frozen at -28°C and lyophilized using a freeze-drier (FreeZone 12, Labconco Co., Kansas City, MO). Lyophilized pupae were grounded using a high speed spice and nut grinder (model SG-10, Cuisinart, South Shelton, CT). Particle size was not uniform and ranged from approximately 280 to 900 µm.

The control diet (C) consisted of a 2:1 mix of Entofood® and ground bee pollen granules, respectively. The product Entofood® is a mix of intact (frozen) eggs of *Ephestia kuehniella* (Zeller) and *Artemia* sp. At ca. 16% and 83%, respectively. The two

control diet components were mixed dry and presented directly to adults and larvae of *C. maculata*.

The approximate ratios of lipid, protein and carbohydrate were estimated for the diet formulations (Table 1) by using the matrix operation described by Morales-Ramos et al. (2014). Nutrient information of natural diet ingredients was obtained from the USDA nutritional database (USDA, 2010). Nutrient content of *T. molitor* was based on Finke (2002) and preliminary analyses of dry pupae done in our lab (M. G. R., unpublished). Nutrient data of *E. kuehniella* eggs was obtained from Specty et al. (2003), bee pollen nutrient content was obtained from Herbert and Shimanuki (1978), and nutrient content of *Artemia* eggs was inferred from Riddick et al. (2014b). Only graphic comparisons of nutrient ratios were accomplished and statistical analysis was not attempted due to the uncertain variability of the data used.

2.3 Immature Development and Survival in Isolation

Newly eclosed first instars were randomly selected from the stock colony and placed individually in tightly fitted dishes (9 x 50 mm) (Falcon 35-1006, Becton Dickinson Co., Franklin Lakes, NJ) modified with a screened window (9 mm diam.). The bottom of the dishes was partially lined with Kimwipes® paper with saturated water crystals provided as a source of water. A total of 320 dishes, each containing a single first instar, were randomly divided in 5 groups. Each group of 64 isolated larvae was provided with a different diet treatment consisting either of diets M1, M2, T1, T2, or control. Diets were presented in detached caps of 1.5 ml centrifuge tubes (Fisherbrand 02-682-550, Fisher Scientific Co. LLC, Horizon Ridge, CT) to minimize microbial contamination from frass particles. Food was replenished as it was consumed or

replaced by new diet every 4 days. Dishes with larvae were maintained in an environmental chamber at 26°C, 75% RH, and 14 h photophase until all the beetles completed development.

Larvae were monitored daily for development and mortality. Every molting event was recorded and exuviae were removed from dishes until the pupal stage was attained. Monitoring continued during the pupal stage until adults emerged. The duration of each instar and pupae of each individual was recorded. Newly emerged (teneral) adults were sexed and weighed in a precision balance (Mettler-Toledo AB104-S, Mettler-Toledo AG, Greifensee, Switzerland), placed individually in small Petri dishes. The number of individuals reaching the adult stage, and their sex, and weight were recorded.

Analysis of variance (ANOVA) was used to analyze development data because the number of observations per treatment were the same and no deviations from normality were detected. Time in each stadium and pupal development time were compared among treatments using the Tukey-Kramer HSD test. Immature survival was compared among treatment groups using the Z test, which is designed for analysis of categorical data and eliminates the bias due to the restriction of values between 0 and 1. ANOVA and the Tukey-Kramer HSD test were also used to analyze and compare teneral adult weight among treatments. No deviations from normality were detected on the date of teneral adult weight.

2.4 Adult Fecundity and Egg Viability

Adults were maintained in isolation and fed the same diet as in the larval stage for 3 days. At 4 days of age adult females were paired with males from the same diet treatments described above and transferred to square plastic boxes (74 x 74 x 27 mm) (product 006c, Pioneer Plastics Inc., North Dixon, KY) modified with a screened window (22 mm diam.) on the cover. The total number of pairs per treatment corresponded to the number of surviving females from the previous experiment. This was done to utilize the data for life table analysis.

Pairs were also provided 3 crumpled paper (Kimwipes®) squares (approximately 40 x 40 mm) as oviposition substrate. Food was presented on small petri dish covers (40 mm diam.). The control diet mix was placed directly on the dish covers and artificial diets were first spread on paper squares, which were placed on the dish covers. Diet was replaced as it was consumed, or every 4 days. Dishes were replaced with clean ones weekly. Boxes with adult pairs were kept in an environmental chamber at the same conditions described above for a period of 68 days. Pairs were monitored daily for mortality and oviposition. Data collected consisted of number of total eggs oviposited per day, number of eggs cannibalized and date of female death. Dead males were replaced by adding males reared with the same treatment diet. Eggs from each of the treatments were collected daily and were placed in plastic Petri dishes, dated and maintained at the same environmental conditions until hatching. The number of first instars and eclosion date were recorded for each oviposition date and treatment. Newly eclosed first instars were removed to prevent them from feeding on the remaining unhatched eggs.

Data consisting of total oviposition per female during a 68-d period (starting at emergence) were analyzed using ANOVA and means were compared using the Tukey-Kramer HSD test. Egg cannibalism was recorded as the total number of cannibalized eggs divided by the total oviposition in each treatment. Egg viability for each treatment was calculated by dividing total eclosion by total numbers of eggs surviving cannibalism for each treatment. Egg cannibalism and egg viability were compared among treatments using the Z test. Egg development time was determined for each egg that completed development and compared among treatments using ANOVA and Tukey-Kramer HSD test. Linear regression analysis was used to determine the impact of female weight on fecundity within treatment groups.

The age-dependent fecundity and female age-dependent survival data were used to calculate fertility tables. Life tables were also calculated using the immature development and survival data. Combined life and fertility tables were calculated by modifying the generalized spreadsheet provided by Portilla et al. (2014). This was accomplished by multiplying initial survival (l_x) adult values by egg viability and immature survival and by adding total development time to the starting adult age for each of the treatments. Combined life and fertility tables provide an analysis of the complete life cycle. Demographic parameters such as net reproductive rate (R_0), intrinsic rate of increase (r_m), generation time (G), and doubling time (DT) were automatically calculated according to Carey (1993) using the modified Portilla et al. (2014) spreadsheet. The jackknife method (Manly, 1997; Efron and Tibshirani, 1993) was used on demographic parameters to obtain 20 estimates of the parameters as described by Maia et al. (2000) for statistical comparisons. ANOVA was used to analyze

the 20 estimates per treatment of the demographic parameters and means were compared among treatments using the Tukey-Kramer HSD test.

2.5 Immature Development and Survival in Groups with Combined Treatments

A second experiment was performed to determine if providing a combination of control and artificial diets would improve immature survival and shorten development time. In this experiment artificial diet formulations were combined with control mix in two new treatments (T1a and T2a). Only treatments T1, T2 and the control (C) were retained from the previous experiments for comparisons of immature development and survival in groups. Treatments T1a and T2a consisted of control mix provided to first instars for the first 4 days and diets T1 or T2 for the rest of the development, respectively.

Newly eclosed first instars from the stock colony were collected to make groups of 20 individuals. Because of limitations on production capabilities of the stock colony, repetitions consisting of 1 group per treatment were established at different dates. Groups were randomly assigned to treatments until a total of 9 repetitions were established.

Groups were reared in tri-compartment plastic boxes (170 x 84 x 42 mm) (product 182C, Pioneer Plastics Inc., North Dixon, KY), which were modified by cutting a hole between each compartment, gluing the cover permanently, adding screened windows in both short sides, and 3 screened openings at the bottom of the same design as those on the covers of the cages used for the stock colony. One of the screened openings (central) was used to house saturated water crystals and the other two as

ventilation. Each compartment was filled with 10 crumbled paper squares as described above to increase surface area and provide shelters for larvae. Food consisting of the corresponding diet treatment was presented on Kimwipes® paper strips. For treatments T1a and T2a, control mix was provided for the first 4 days, then removed and replaced by T1 and T2 diet, respectively. Food was replenished as consumed or replaced every 4 days. Groups were maintained in an environmental chamber at the conditions described above.

Larvae groups were monitored daily until the adult stage was reached. The date of emergence of each adult was recorded for each group. Data consisting of total development time and total number of adults completing development was used to determine total development time and immature survival, respectively. Cannibalism was removed from survival data. Total development time and immature survival were analyzed using ANOVA and the means were compared using the Tukey-Kramer HSD test. Data on immature survival was transformed using square root and arcsine before being analyzed to reduce bias due to binomial distribution (Zar, 1999).

3. Results

3.1 Immature Development and Survival in Isolation

Development time did not differ significantly between males and females in all treatments, but the weight of teneral adults was significantly higher in females ($F = 35.87$, $df = 165$, $P < 0.0001$). Beetles feeding on the control diet reached the adult stage significantly faster than those feeding on the artificial diets ($F = 57.94$, $df_1 = 4$, $df_2 = 162$,

$P < 0.0001$) (Table 4). Development time did not differ significantly among most artificial diets, except between diets M1 and T2, where the later was significantly shorter. Second, third, and fourth stadia were significantly shorter in beetles fed with the C diet mix than in those fed with all other diet treatments ($F = 20.08, 18.25, \text{ and } 26.3$, respectively; $df_1 = 4, df_2 = 162; P < 0.0001$). The length of the first stadium differed significantly only between treatments C and T1 and the pupal stage length did not differ among treatments.

The weight of teneral females was on average 2 mg higher than that of males across diet treatments (Fig. 2) and this difference was significant ($F = 35.87, df = 165, P < 0.0001$). Adult females resulting from immature feeding on control diet weighed significantly more (14.69 ± 0.98 mg) than females resulting from immature feeding on all the other treatments ($F = 16.38, df_1 = 4, df_2 = 73, P < 0.0001$). There was little difference in teneral adult female weight among artificial diet treatments except between diet M1 (9.1 ± 1.22 mg) and both T diets (12.37 ± 1.97 and 12.32 ± 1.93 for T1 and T2, respectively), where adults resulting from immature feeding on diet M1 weighed significantly less.

No significant differences were observed in sex ratio among diet treatments. The observed female proportions ($p \pm \text{standard error}$) were $0.43 \pm 0.2, 0.51 \pm 0.09, 0.48 \pm 0.09, 0.46 \pm 0.08$, and 0.42 ± 0.06 for treatments M1, M2, T1, T2, and C, respectively. The Z test did not detect significant deviations from the expected 1:1 (0.5) sex ratio for a sexually reproducing insect.

The highest immature survival was observed in the C treatment (0.922) followed by T2 and T1 (0.578 and 0.453, respectively) ($Z = 9.2, 8.47, 5.72$, and 4.49 for

comparisons against M1, M2, T1, and T2, respectively; $N = 128$; $P < 0.0001$) (Fig. 3). The lowest survival was observed in the M1 treatment followed by the M2 treatment (0.109 and 0.273, respectively) (Fig. 2). Survival in the C treatment was significantly higher than in all the other treatments. There was no significant difference in immature survival between treatments T1 and T2. Both T diets (with *T. molitor*) showed higher immature survival than the M diets (without insect components) ($Z \geq 2.49$, $N = 128$, $P \leq 0.0064$) (Fig.3). The M diets differed on immature survival, diet M2 showing significantly better survival than M1 ($Z = 2.59$, $N = 128$, $P = 0.0048$).

Although the highest survival was observed in the control diet, many of the pupae from beetles fed the C diet molted defectively resulting in adults with malformations including deformed elytra and legs. Some of these deformities resulted in difficulties walking, moving, or mating. Defective molting was 40.68, 0, 5.71, 0, and 10.81% in diet treatments C, M1, M2, T1, and T2, respectively. The Z test indicated that defective pupae molting was significantly more frequent in the C treatment than in all the artificial diet treatments ($Z = 2.12, 3.66, 4.02$, and 3.13 ; $N = 66, 94, 88$, and 96 ; $P = 0.0174, 0.0001, < 0.0001$, and 0.0009 , for comparisons between treatments M1, M2, T1, and T2, respectively).

3.2 Fecundity and Egg Viability

A significantly higher proportion of eggs oviposited by females feeding on diets T1 (0.31 ± 0.008) and T2 (0.32 ± 0.01) hatched successfully and produced healthy larvae as compared with eggs produced by females feeding on M1 (0.006 ± 0.004), M2 (0.008 ± 0.002), and C (0.1 ± 0.008) diets ($Z \geq 14.6$, $P < 0.0001$) (Fig. 3). Egg viability was significantly higher in the C treatment than M1 and M2 treatments ($Z \geq 5.5$, $P <$

0.0001). Developmental time for eggs did not differ among treatments with a mean length of 3.02 ± 0.01 days.

Total number of eggs oviposited per female during the 68-d experimental period (mean $\pm$ standard deviation) was significantly higher in treatment T1 (300.6 ± 184.6 eggs) than in treatments C (127.2 ± 219.4 eggs), M1 (124.3 ± 112.2 eggs), and M2 (116.0 ± 133.4 eggs) ($F = 2.76$, $df_1 = 4$, $df_2 = 73$, $P = 0.0342$) (Fig. 4). Total oviposition did not differ significantly between treatments T1 and T2 (188.4 ± 148.0 eggs) and among treatments T2, M1, M2, and C. Female weight had no significant correlation with total fecundity in all treatments except in treatment T2 where it had a marginally significant impact ($R^2 = 0.34$, $F = 7.89$, $df = 15$, $P = 0.0132$). Females that showed malformations due to defective molting were more likely to die within the first two weeks without producing progeny. The proportion of females surviving to the third week alive was significantly lower in the C treatment (0.54) as compared to diet treatments M2 and T1 (0.94 and 1, respectively) ($Z = 2.9$ and 3.04 ; $N = 44$ and 40 ; $P = 0.0019$ and 0.0012 , for comparisons between treatments M2 and T1, respectively). Adult malformations are a real phenomenon associated with the treatment and deformed females should be part of the estimate. Most likely, the high rate of early female mortality without progeny production impacted the estimate of fecundity of treatment C. No significant difference was detected in the proportion of females reaching the third week alive among treatments M1 and T2 and the rest of the treatments. Although the duration of the experiments did not allow the comparison of adult longevity among treatments, adult female survival at 68 d of age was significantly impacted by diet. The survival of 68 d-old adult females was significantly lower in the C treatment (0.15 ± 0.07) than in all the

artificial diet treatments ($Z = 3.24, 3.15, 4.33$, and 2.26 ; $P = 0.0006, 0.0008, < 0.0001$, and 0.012 for diets M1, M2, T1, and T2, respectively). Adult female survival at 68 d of age was no different among artificial diet treatments except between T1 (0.86 ± 0.10) and T2 (0.47 ± 0.12) ($Z = 2.24, P = 0.0125$). Female survival at 68 d of age was 0.61 ± 0.12 in the M2 treatment and all females survived to 68 d in the M1 treatment. However, only 3 females completed development in the M1 diet thus comparisons with this treatment's adult data should be taken cautiously.

The proportion of cannibalized eggs differed significantly among all treatments ($Z \geq 2.29, P \leq 0.011$). Egg cannibalism in descendent order was $0.38 \pm 0.008, 0.27 \pm 0.008, 0.22 \pm 0.006, 0.18 \pm 0.008$, and 0.13 ± 0.017 in treatments C, T2, T1, M2, and M1, respectively. The highest egg cannibalism was observed in the control diet and the lowest in the M1 diet.

3.3 Life and Fertility Tables

Although immature survival to the adult stage in the control diet was the highest among all treatments, the intrinsic rate of increase (r_m) of complete data sets was higher in both T diets. The r_m values of both M diets were negative indicating a declining population in beetles fed with the meridic diet formulations. Demographic parameters from the treatment complete data sets are presented in Table 5. Reproductive output from the fertility table alone ($L_x M_x$ from data consisting of development time, fecundity, and adult mortality), which represents production of female eggs per female, was the highest in diet T1, followed by diet T2 (Figure 5A). Diet treatments M1, M2, and C had similar reproductive output in the fertility table. Adding the life table information (egg viability and immature survival) to the fertility table provides the reproductive output

information (L_xM_x) as adult female progeny per female. Plots of L_xM_x expressed as adult progeny per female showed more dramatic differences among the diet treatments (Figure 5B).

Comparison with treatments M1 and M2 was considered irrelevant because none of the M treatments were able to maintain a viable *C. maculata* population which resulted in negative r_m values. Consequently, statistical analysis of demographic parameters using jackknife was accomplished among treatments C, T1, and T2 only. Demographic parameters were all significantly different among the C, T1, and T2 diet treatments (Table 6). Generation time (G) was the shortest in treatment C and the longest in treatment T1; doubling time (DT) was the shortest in treatment T1 and the longest in treatment C; R_0 and r_m values were the highest in treatment T1 and the lowest in treatment C (Table 6). Overall, beetles in treatment T1 had the fastest population growth indicating that diet T1 was superior to all the other diets tested.

3.4 Diet Nutrient Composition

The matrix graphic analysis of major nutrient components showed that all artificial diet formulations had similar lipid, protein and carbohydrate ratios (Fig. 6). The position of the points close to the center of the graph indicated that ratios of lipid, protein and carbohydrate are nearly equal in all artificial diet formulations. The control mix, on the other hand, showed a clear protein bias indicating that its nutrient content is substantially different than that of the artificial diets (Fig. 6). A preliminary attempt to match the control mix major nutrient ratios with a meridic artificial diet formulation (using the same ingredients in Tables 1, 2, and 3 at different ratios) yielded negative results

and beetles feeding in this formulation were unable to complete development (M.G.R. and J.A.M.-R., unpublished) (diet P, Fig. 6).

There were some differences in a small number of nutrients among the artificial diet formulations. For instance, diet M2 had higher content of tyrosine (6.63 mg/100g) than diets M1, T1, and T2 (3.74, 4.14, and 4.44 mg/100g, respectively) and diet T2 had less proline (5.6 mg/100g) than diets M1, M2, and T1 (9.2, 8.93, and 8.97 mg/100g, respectively). But in general, all artificial diets were mostly equivalent in the content of nutrients that are reported to be important for insect development and reproduction (Cohen, 2004; Morales-Ramos et al., 2014). There was not sufficient information available to make a detailed comparison of the control mix with the artificial diets. Detailed nutrient analyses of *E. kuehniella* and *Artemia* spp. eggs and bee pollen are lacking at the moment.

3.5 Immature Development and Survival in Groups with Combined Treatments

In general, immature survival in groups was lower than when larvae were in isolation. However the *Z* test did not detect any statistically significant changes on group versus isolated immature survival in treatments C and T1. Treatment T2 did show a significant reduction on immature survival ($Z = 2.62$, $P = 0.0044$) when in groups as compared with isolated larvae feeding on the same diet.

Providing control mix for the first 4 days and T diets (T1 and T2) for the rest of the post embryonic development (treatments T1a and T2a) significantly improved immature survival (78.33 ± 7.07 and 80.56 ± 9.5 %, respectively) as compared with treatments using T1 and T2 diets exclusively (36.67 ± 24.87 and 38.89 ± 25.22 %, respectively).

respectively) ($F = 16.52$, $df_1 = 4$, $df_2 = 40$, $P < 0.0001$). Survival in treatments T1a and T2a did not differ significantly from that of the control (86.11 ± 10.54 %) (Fig. 7A). Treatments T1a and T2a also showed a significant reduction in post-embryonic development time (19.36 ± 0.68 and 19.37 ± 0.66 d, respectively) as compared with treatments T1 and T2 (21.66 ± 1.02 and 21.63 ± 0.32 d, respectively), but beetles feeding on the C diet still developed significantly faster than all the other treatments (17.83 ± 0.56 d) ($F = 38.93$, $df_1 = 4$, $df_2 = 40$, $P < 0.0001$) (Fig. 7B).

4. Discussion

Results clearly show that both artificial diets with *T. molitor* components (T1 and T2) were superior to the control mix diet in producing *C. maculata*. Both meridic diets (M1 and M2) were ineffective at maintaining a viable *C. maculata* population despite providing sufficient nutrition for immature development. Intrinsic rate of increase (r_m) values were negative in both M diets indicating a decline in population with every generation. This also yielded negative values of doubling time (DT). To convert DT values from negative to positive, $\ln(0.5)$ is divided by the r_m value instead of $\ln(2)$. This operation results in the same values with positive sign, which can be interpreted as halving times (time in d require to reduce the population in half). Egg viability was an important factor in the effectiveness of the T diets and the failure of the M diets.

The control mix used in this study has been considered an adequate diet for *C. maculata* based on the high rates of immature survival (J.A.M.-R. unpublished). Other studies have found *E. kuehniella* eggs and bee pollen separately to be suitable foods for

C. maculata based on immature survival (Michaud and Grant, 2005). Our studies show that the control mix is a sub-optimal diet for *C. maculata* producing a high percentage of malformed adults and reducing egg viability and adult longevity. Also females fed on the control diet mix cannibalized a higher percentage of eggs. Other artificial diet studies have associated the increase of cannibalism with sub-optimal diets in *Geocoris pinctipes* (Say) (Cohen, 1985) and *Podisus maculiventris* (Say) (De Clercq and Degheele, 1992, 1993). Egg cannibalism was not taken into account for the calculation of demographic parameters of life and fertility tables because levels of egg cannibalism may change in a colony with the frequency of egg collection or rearing temperature (J.A.M. unpublished). However, the lower egg cannibalism observed in the T diets as compared to the control, further justify our conclusions.

Adults feeding on the control mix also had the lowest survival at 68 d of age. Lundgren and Wiedenmann (2004) reported high adult mortality in *C. maculata* feeding exclusively on corn pollen of different varieties and the mortality was negatively correlated with the percentage of organic matter in the pollen. The authors discussed the possibility that mortality of adult *C. maculata* was due to the lack of a micronutrient present in insufficient quantities in the corn pollen. It is unlikely that micronutrient deficiency would have played a role in the high adult mortality observed in our study because the C diet only contained 33% bee pollen and most of its content was of animal origin (arthropod eggs). However, there are similarities in the observed *C. maculata* adult mortality and defective molting during emergence in this study and Lundgren and Wiedenmann (2004) when pollen was provided as food. An alternative explanation for the increase of adult mortality in pollen-based diets is the presence of phytochemicals

such as flavonoids, alkaloids, tannins, and polyphenolics (Roulston and Cane, 2000; Lundgren and Wiedenmann, 2004). Those chemicals may be present in too small amounts to be lethal, allowing insects to complete development. This may be especially true for bee-pollen because it is mixed with a substantial amount of nectar (Roulston and Cane, 2000). Continuous consumption of pollen by *C. maculata* may favor the accumulation of phytochemicals and additive sub lethal effects resulting in defective molting or premature adult death. Future research should focus on testing the hypothesis that sub lethal levels phytochemicals present in pollen may impact *C. maculata* survival.

The matrix graphic analysis did not find differences in nutrient content among the artificial diet formulations that could explain the differences observed in performance among the meridic (M1 and M2) and *T. molitor*-enriched (T1 and T2) artificial diets. Feeding stimulation rather than nutrition seems to be the factor determining diet quality. The presence of feeding stimulation is one of the main factors listed by Cohen (2004) that makes artificial diets successful. Feeding stimulants may be compounds other than nutrients (token stimuli) and may be present in small concentrations (Cohen, 2004). Feeding stimulation is important because the quantity of food ingested is as important as the quality of the food consumed on providing adequate nutrition for insect development and reproduction (Morales-Ramos et al., 2014). Diets T1 and T2 were likely enhanced by the presence of feeding stimulant compounds from the *T. molitor* extracts.

Food treatment combinations of control mix for the initial 4 d and then T1 or T2 diets greatly improved immature survival and reduced development time as compared

with the uncombined T treatments. Providing control mix for such a short period of time could result in a large increase in population growth with a minimal expense on conventional food for *C. maculata*. Providing conventional food to the first instars seem to impact the survival and development during the other 3 stadia. It is possible that high immature mortality in artificial diet treatments is due to difficulties experienced by first instars feeding on the formulation. In this case, mortality of immature stages could be due to insufficient food consumed early in the development. Diet presentation is often a major factor impacting diet effectiveness (Morales-Ramos et al., 2014). First instars of *C. maculata* may not be able to feed on a gel, but could easily consume small food particles such as moth and shrimp eggs and pollen. In a laboratory study, *C. maculata* first instars had higher survival when feeding on small particles of powdered dry *Artemia* sp. eggs as compared with whole eggs (Riddick and Wu, unpublished). Future research will be focused on developing dry powder versions of the artificial diets presented in this publication and determining the optimal particle size for first instar survival.

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Figure Titles

Fig. 1. Adult cage design for *C. maculata* rearing. A) Full assembled cage showing the upper section (a), lower section (b), detachable cover windows (c), and water sources with saturated water-absorbing polymer (d). B) Lower cage section with open access showing a stack of paper sheets as of oviposition substrate (arrow).

Fig. 2. Adult (live) weight of male and female *C. maculata* fed on five different diet treatments. Bars represent means and brackets represent standard error of the mean. Bars with the same letter (capital for females and lower case for males) are not significantly different after Tukey-Kramer HSD test at $\alpha = 0.05$.

Fig. 3. Immature survival and egg viability of *C. maculata* fed on five different diet treatments. Bars represent means and brackets represent standard error. Bars with the same letter (capital for immature survival and lower case for egg viability) are not significantly different after Z test at $\alpha/2 = 0.025$.

Fig. 4. Fecundity of *C. maculata* expressed as total oviposition per female during a 68-d period (starting at emergence) feeding on 5 different diet treatments. Circles represent means and brackets standard error of the mean. Means with the same letter are not significantly different after Tukey-Kramer HSD test at $\alpha = 0.05$.

Fig. 5. Reproductive output of *C. maculata* fed on five different diets. A) Adult LxMx from fertility table representing female eggs per female / d. B) Full life cycle LxMx from combined life and fertility tables representing adult female progeny per female / wk.

Fig. 6. Ternary plot of lipid (Lip), protein (Prot), and carbohydrate (Carb) content of five artificial diets and the control mix (C). Artificial diets included two meridic formulations (M1 and M2), two *T. molitor* enriched (T1 and T2), and one protein enriched meridic diet (P), which failed to produce adult beetles.

Fig. 7. Survival rate (A) and development time (B) of groups of twenty *C. maculata* larvae reared on three different diets (T1, T2, and C) and two combinations (T1a and T2a) consisting of 2 d on C diet and the remaining time on T1 or T2 diets. Circles represent means and brackets standard deviation. Means with the same letter are not significantly different after Tukey-Kramer HSD test at $\alpha = 0.05$. Proportions in "A" were square root and arcsine converted for analysis and converted back for plotting.

Figure 1

Figure 2

Figure3

Figure 4

Figure 5

Figure 6

Figure 7

Table 1. Main components (g / 100g) of four artificial diets.

Diet component	Diets			
	M1	M2	T1	T2
Lipid:				
Fish oil	0.45	0.45	0.43	0.43
Soy lecithin (powder)	8	7.97	7.61	7.69
Egg lecithin (powder)	1.53	1.52	1.46	1.47
Cholesterol	0.08	0.08	0.076	0.077
Ergosterol	0.05	0.05	0.048	0.048
Carbohydrate:				
Glucose	5.7	5.68	5.42	5.48
Fructose	11.4	11.36	10.85	10.96
Sucrose	4.27	4.25	4.06	4.11
Honey	1.42	1.41	1.35	1.37
Protein:				
Soy protein	1.38	1.37	1.31	1.33
Casein hydrolysate	1.38	1.37	1.31	1.33
Yeast hydrolysate	7.57	7.54	7.2	7.28
Albumin (dry egg white)	3.45	3.44	3.28	3.32
Collagen	1.48	1.47	1.41	1.42
Beef globulin (powder)	4	3.99	3.8	3.85
L-Tyrosine	0	0.75	0	0
L-Proline	1	1	0.95	0
Vitamin:				
Vitamin A	0.07	0.07	0.066	0.067
Vitamin E (α -tocopherol)	1.15	1.15	1.09	1.11
Vitamin C	0.23	0.23	0.22	0.22
B-complex vitamin solution	0.31	0.31	0.29	0.3

Mineral:				
Solution A	1.97	1.96	1.87	1.89
Solution B	1.97	1.96	1.87	1.89
Solution C	1.97	1.96	1.87	1.89
Undefined ingredients:				
Egg yolk	20.18	20.11	19.21	17.48
Milk	2.3	2.29	2.19	2.21
Beef liver	3.04	3.03	2.89	2.92
<i>T. molitor</i> pupae	0	0	4.76	6.73
Preservatives:				
Sorbic acid	0.14	0.14	0.13	0.13
Citric acid	2	1.99	1.9	1.92
Other:				
L-carrageenan	1.09	1.09	1.04	1.05
RO Water	10.42	10	10	10
RO = reverse osmosis.				

Table 2. Formulation of B vitamin complex solution.

Vitamin	Amount in mg / 100g
Biotin (B7)	1.2
Calcium panthothenate (B5)	42
Choline chloride (B4)	10,000
Cobalamin (B12)	2.5
Folic acid (B9)	140
Myo-inositol (B8)	625
Nicotinamide (B3)	100
Pyridoxine (B6)	7
Riboflavin (B2)	85
Thiamine (B1)	10
RO Water	88,987.3

RO = reverse osmosis.

Table 3. Formulation of minerals solution

Chemical	Amount in g / 100g
Solution A:	
CaCl ₂	1.2
CoCl ₂	0.2
FeCl ₂ ·6H ₂ O	0.8
ZnCl ₂	0.2
RO water	97.6
Solution B:	
K ₂ HPO ₄	2.96
Na ₂ HPO ₄	0.4
RO water	96.64
Solution C:	
MgSO ₄	4.8
CuSO ₄	0.2
MnSO ₄	0.04
RO water	94.96

RO = reverse osmosis.

Table 4. Impact of diets on *C. maculata* post-embryonic development time in days

Diet	Stadium				Pupa	Total
	First	Second	Third	Fourth		
M1	3.5 ± 0.8ab	3.0 ± 0.8a	4.0 ± 1.0a	7.6 ± 1.8ab	3.0 ± 0.5a	21.1 ± 3.1a
M2	3.3 ± 0.5ab	2.8 ± 0.7a	3.5 ± 1.5a	7.1 ± 1.8a	3.1 ± 0.5a	19.7 ± 1.6ab
T1	3.7 ± 0.9a	2.8 ± 0.7a	3.5 ± 0.5a	6.2 ± 1.1b	3.1 ± 0.3a	19.3 ± 2.0ab
T2	3.4 ± 0.6ab	2.9 ± 0.5a	3.2 ± 0.5a	6.4 ± 0.8ab	3.2 ± 0.4a	19.1 ± 1.3b
C	3.2 ± 0.5b	2.0 ± 0.5b	2.3 ± 0.5b	4.8 ± 0.6c	3.4 ± 0.5a	15.7 ± 1.2c

Mean ± standard deviation. Means with the same letter within columns are not significantly different after Tukey-Kramer HSD test at $\alpha = 0.05$.

Table 5. Demographic parameters of *C. maculata* fed on five different diets obtained from the complete treatment data sets of life and fertility tables

Diet	Ro	G	r_m	DT
M1	0.036	65.69	-0.046	-14.94
M2	0.136	60.23	-0.0308	-22.53
T1	20.251	61.19	0.0559	12.41
T2	15.768	59.02	0.0527	13.16
C	5.184	53.26	0.0341	20.33

Ro = net reproductive rate = adult female progeny per female; G = generation time in days; r_m = intrinsic rate of increase; DT = doubling time in days.

Table 6. Results of the statistical analysis of demographic parameters of *C. maculata* among three diet treatments using 20 data points generated by the Jackknife method.

Demographic	Diet treatment			
parameter	C	T1	T2	<i>F</i>
<i>Ro</i>	5.19 ± 0.51c	20.32 ± 1.17a	15.82 ± 1.05b	1329.9
<i>G</i>	53.24 ± 0.59c	61.18 ± 0.38a	59.02 ± 0.64b	1124.9
<i>r_m</i>	0.034 ± 0.002c	0.056 ± 0.001a	0.053 ± 0.002b	945.3
<i>DT</i>	20.48 ± 1.54a	12.40 ± 0.26c	13.16 ± 0.39b	459.8

Mean ± standard deviation, $df_1 = 2$, $df_2 = 57$, $P < 0.0001$.

Parameter *Ro* = net reproductive rate = adult female progeny per female; *G* = generation time in days; *r_m* = intrinsic rate of increase; *DT* = doubling time in days.