Use of Nutrient Self-Selection as a Diet Refining Tool in *Tenebrio molitor* (Coleoptera: Tenebrionidae)[1]

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Abstract A new method to refine existing dietary supplements for improving production of the yellow mealworm, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), was tested. Self-selected ratios of 6 dietary ingredients by *T. molitor* larvae were used to produce a dietary supplement. This supplement was compared with existing supplement formulations mixed with wheat bran at 1:4 ratio and a control consisting of wheat bran alone for food utilization efficiency, larval growth, development time, immature survival, and fecundity. Ingredients of dietary supplements included dry potato as a source of carbohydrate; dry egg white and soy protein as a source of protein and; peanut, canola, and salmon oil as a source of lipid. A supplement consisting of dry potato alone significantly improved food utilization, growth, development time, survival, and fecundity compared with the wheat bran-only control group. The addition of protein to the supplement significantly shortened development time and improved food conversion efficiency and fecundity compared with the supplement with potato alone. The addition of lipid did not provide any significant improvements. The supplement derived from self-selected ratios of the basic ingredients provided a significant increase in fecundity compared with previously developed supplements and the control. Self-selected ratios of the basic ingredients by *T. molitor* larvae had an effect on the adult stage that resulted in significantly higher progeny production.

Key words yellow mealworm, nutrition, rearing, development, fecundity, production, food conversion.

The yellow mealworm, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), is mass produced and sold in the United States for a variety of purposes. The larvae of *T. molitor* are one of the most common foods for captive mammals, birds, reptiles, and amphibians because they are easy to propagate, harvest, and feed (Martin et al. 1976, Barker et al. 1998, Finke 2002). One of the biggest markets for *T. molitor* is as feed for wild birds in backyards feeders (W. L. T., unpublished).

Commercial availability of insect protein provides an opportunity for developing new technologies for mass production of biological control agents. The potential of *T. molitor* as factitious prey for insect predators has been explored (Saint-Cyr and Cloutier 1996, De Clercq et al. 1998, Grundy et al. 2000, Costello et al. 2002, Lemos et al. 2003, Pappas et al. 2007, De Bortoli et al. 2011). Another application is as a host for

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in vivo mass production of entomopathogenic nematodes (Shapiro-Ilan et al. 2002, 2008). The low level of technology required for *T. molitor* production makes it ideal for small industry. In some cases, or for certain species, the quality (e.g., virulence or efficacy) of in vivo-produced nematodes may be superior to those produced in vitro (Gaugler and Georgis 1991, Shapiro and Lewis 1999, Cottrell et al. 2011). Entomopathogenic nematodes are currently produced commercially using *T. molitor* as a host; these nematodes are being sold for control of the small hive beetle, *Aethina tumida* (Murray) (Coleoptera: Nitidulidae), in the southeastern United States (W. L. T., unpublished).

The commercial success of mass-produced biological control agents depends greatly on lowering the costs of production. Research efforts to reduce the cost of *T. molitor* production have been focused on optimizing reproduction and reducing labor (Morales-Ramos et al. 2011a, 2012). Production costs also can be improved by optimizing the *T. molitor* diet so that better food conversion, immature survival, and adult reproduction are achieved. Significant improvement can be obtained on development time, immature survival and food utilization by adding simple food supplements containing dry potato and dry egg white to the basic *T. molitor* diet that consists of wheat bran (Morales-Ramos et al. 2010, 2011b). In other studies, the virulence of the entomopathogenic nematodes *Heterorhabditis indica* Poinar, Karunakar, and David and *Steinernema riobrave* Cabanillas, Poinar, and Raulston increased when the *T. molitor* host was fed with a supplement containing 20% lipid as compared with low lipid (5%) supplements (Shapiro-Ilan et al. 2008). In a subsequent study, virulence of the nematode *S. carpocapsae* (Weiser) increased when the *T. molitor* host was fed a supplement containing 5% peanut oil, 0.15% cholesterol, and 0.16% manganese sulfate in addition to potato and egg white (Shapiro-Ilan et al. 2012). A supplement containing optimal amounts of dry potato, protein and lipids could potentially improve insect production efficiency and nematode virulence simultaneously.

Existing research provides valuable information on the impact of nutrition on development and immature survival of *T. molitor*. However, this information is not sufficient to develop an optimal dietary supplement for mass production. Life table analysis is a requisite to obtain a meaningful evaluation of dietary supplements for optimal mass production because it evaluates the impact of development, survival, and reproduction as a whole. Proportions of individual diet components must be evaluated individually and then in combination using life table analysis. Developing a supplement containing optimal proportions of even a few components requires years of research by using this conventional approach. One alternative option could be to allow the insect itself, *T. molitor,* to identify the optimal proportions of these diet components. The ability of *T. molitor* to self-select optimal proportions of 2 food components has been demonstrated (Morales-Ramos et al. 2011b). The term dietary self-selection specifically refers to the consumption of different types of food items by selection to balance the optimal (or near optimal) levels of nutrients required by an organism (Waldbauer and Friedman 1991, Behmer 2009).

Several supplement versions have been produced and tested for impact in *T. molitor* development, growth, and reproduction and for impact on entomopathogenic nematode virulence (Shapiro-Ilan et al. 2012). These supplements were developed after an extensive series of trial-and-error experiments based primarily on pupal weight and food utilization efficiency (J. A. M.-R., unpublished). However, a recent study showed that pupal weight is not correlated with fecundity in *T. molitor* (Morales-Ramos et al. 2012) and, therefore, is not a good parameter for diet evaluation. Dietary self-selection

could be used as a tool to refine existing dietary supplements and improve development time, survival, growth, and reproduction of *T. molitor*. The objectives of this study were to refine a dietary supplement for *T. molitor* based on self-selected consumption ratio of 6 food ingredients and compare it to previously tested supplements for impact on development, growth, and reproduction.

Materials and Methods

Colony maintenance. The *T. molitor* colony used in this study was originally established in 2005 from stock donated by Southeastern Insectaries Inc. (Perry, GA), and it has been continuously grown in the National Biological Control Laboratory (Stoneville, MS) for the last 7 years. The rearing methods were as described by Morales-Ramos et al. (2012) using stacked trays with screened bottoms (500 µm pore size) to grow the larvae, boxes with screened bottoms (850 µm pore size) to hold the adults, and collection of first instars in a second tray at the bottom. The colony was fed exclusively with wheat bran to the completion of this study. Water was provided to the adults twice a week, and no water was provided to the larvae. Larvae of *T. molitor* have the ability to take up water dissolved in subsaturated air (Dunbar and Winston 1975). The environment in the rearing room was maintained in darkness at 27°C and 65% RH.

Self-selection. A self-selection experiment was designed to determine relative consumption of 6 basic food ingredients mixed at different ratios within 6 different formulations. The basic ingredients included dry potato flour, dry egg white, soy protein, peanut oil, canola oil, and salmon oil. These ingredients were used to prepare 6 nutritional supplements (Table 1). Supplement "DP" consisted entirely of dry potato and provided a high carbohydrate choice. Supplements "EW-20" and "Soy-20" consisted of 20% of high protein ingredients (egg white and soy protein, respectively) thereby providing 2 high protein choices consisting of animal and vegetable protein, respectively. Supplements "Pe-10", "Ca-10", and "Sa-10" contained 10% of lipids consisting of peanut oil, canola oil, and salmon oil, respectively (Table 1), thus, providing high lipid choices from vegetable and animal origins. Each one of the supplement

Table 1. Composition of the supplements included in the multiple-choice test for dietary self-selection by *T. molitor*. Percentage of basic ingredients by weight.

	Basic Ingredients					
Supplement	Dry potato	Dry egg white	Soy protein	Peanut oil	Canola oil	Salmon oil
DP	100	0	0	0	0	0
EW-20	80	20	0	0	0	0
Soy-20	80	0	20	0	0	0
Pe-10	80	5	5	10	0	0
Ca-10	80	5	5	0	10	0
Sa	80	5	5	0	0	10

formulations was nutritionally beneficial to *T. molitor* and superior to a diet of wheat bran alone as determined in preliminary tests (J. A. M.-R., unpublished).

The dry Ingredients (and oil in some preparations) of each supplement were mixed in the proportions shown in Table 1. The mixtures were then combined with reverse osmosis (RO) water at a 1:2 ratio (33.33%) in a KitchenAid® mixer (Model K45SSWH, KitchenAid Portable Appliances, St. Joseph, MI) for 20 min. Subsequently, the supplement mixtures were extruded with a bench-top extruder (Carousel Line, Savage Bros. Co., Chicago, IL) using a star pattern nozzle. The star pattern creates ridges that are easy to chew by *T. molitor* larvae. Extruded supplement pieces were placed on top of aluminum foil and dried in a vacuum oven (Yamato DP43, Yamato Scientific America Inc., Santa Clara, CA) at 50°C and a negative pressure of 80 KPa for a period of 48 h. The dry extruded supplement was manually broken into small pieces (between 2 and 3 cm) and stored at room temperature in plastic containers labeled with the corresponding supplement formulation.

The 6 supplement formulations were presented simultaneously to groups of 30 8-wk-old *T. molitor* larvae. Multiple-choice arenas were constructed from plastic dishes (120 × 25 mm, Pioneer Plastics 53C, Pioneer Plastics, North Dixon, KY). A shallow depression (5 mm deep X 50 mm diam) was created at the center of the dishes by gluing the covers of small tight-fittting dishes (50 × 9 mm, Falcon Brand, No. 351,006, Fisher, Pittsburgh, PA) to the outer bottom and cutting the plastic in between with a rotary tool (Fig. 1A). The bottom of the shallow depression was replaced by a nylon screen (350 µm openings) to allow frass particles to fall trough. Frass particles were collected in another plastic dish of the same dimensions glued to the bottom of the modified dish (Fig. 1A).

The food supplements were placed inside the inverted bottoms of small Petri dishes (39 × 12 mm, Falcon Brand, Fisher, Pittsburgh, PA) (Fig. 1A) that were distributed around the arena to prevent the different supplements from mixing by the movements of the larvae (Fig. 1B). Inverted dishes placed this way provided a gap in the edge closer to the central depression of the arena. This gap allowed *T. molitor* larvae to access the supplements (Fig. 1D), but the gap was small enough to prevent the supplement particles from falling to the central depression. Between 1.6 - 2.1 g of supplement was placed in each of the inverted dish bottoms, and the central depression was filled with 5 g of wheat bran (Fig. 1C).

Six inverted dishes each containing 1 of the 6 supplements described in Table 1 were placed in each of the multiple-choice arenas. A total of 21 arenas was prepared each with a different random distribution of the 6 supplement treatments (Fig. 1C). The distribution of the supplements was randomly assigned using the random number generator of MS Excel. The weight of each of the supplement portions in each of the 21 arenas was recorded. Thirty 8-wk-old *T. molitor* larvae were introduced into each of the 21 arenas. The initial weight of the larvae groups was recorded for each of the arenas. The 21 arenas were then placed in an environmental chamber at 27 ± 1°C, 65 ± 5% RH with no lights for a period of 60 d.

The arenas were monitored weekly to ensure that none of the supplements or bran was consumed completely. If one of the supplements was heavily consumed, the weight of the remaining supplement was recorded, removed and a new portion of supplement was added recording its weight. Similarly, if the wheat bran was depleted, more bran was added, and the additional weight was recorded. At the end of the 60-d period the weight of each of the remaining supplements and wheat bran was

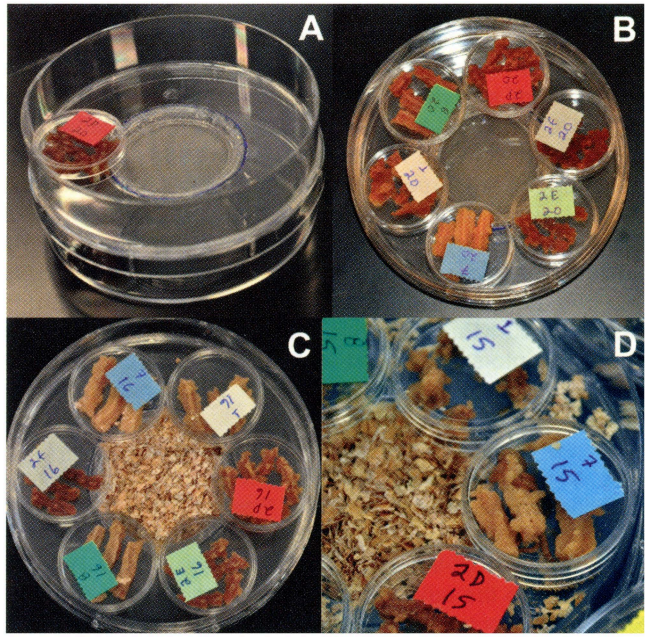

Fig. 1. Multiple-choice arena design. (A) Stacked dishes showing central depression with screen and overlapping up-side-down mini dish with supplement. (B) Arena with full complement of supplement choices each in their own mini dish. (C) The central depression is filled with 3 g of wheat bran. (D) *Tenebrio molitor* larvae had access to the supplement choices through the overlapping edge of the surrounding mini dishes.

recorded. The weight of the frass collected in the bottom dish was recorded as well as the weight of the *T. molitor* larvae after the 60-d period.

The consumed weight of supplements and bran was determined by subtracting the remaining weight from the initial weight. Similarly, the weight gained by each group of *T. molitor* larvae was determined by subtracting initial weight from the final weight. Data consisting of weight consumed from each supplement were analyzed using ANOVA. Means of consumption in mg were compared among supplements using the Tukey-Kramer HSD test of JMP software (SAS Institute 2008).

The weight consumed of individual food ingredients (within each supplement) was determined by multiplying consumption of the entire supplement by the proportion of each ingredient as presented in Table 1 and then adding the total from all the supplements for each of the ingredients. The proportion consumed of each individual ingredient was calculated as individual ingredient consumed / total supplement consumed. Because supplements contained at least 80% dry potato, the relative consumption of this ingredient could not have been lower than 80%. The level of dry potato used was based on preliminary results showing that lower concentrations of dry potato resulted in lower pupal weights and longer developmental times (J. A. M.-R., unpublished). In this study we emphasized the relative consumption of

protein (supplements EW-20 and Soy-20) and lipid (supplements Pe-10, Ca-10, and Sa-10).

A new supplement formulation was created using the means proportions consumed of individual ingredients obtained in the experiment described above. This new supplement was named "S", and its nutritional potential was evaluated and compared with supplements that were previously determined to be satisfactory for *T. molitor* growth, development, and reproduction.

Supplement evaluation. Supplement "S" was compared with 5 supplements as described by Shapiro-Ilan et al. (2012) where their suitability for in vivo nematode production was assessed. Formulations of all the supplements are presented in Table 2. Supplements also were compared with a control "C" with no supplements. Supplement "DP" consisted exclusively of dry potato and was used as a secondary control to assess the impact of protein and lipid on *T. molitor* biology. Protein was added to the formulation in supplement "Pr" and lipid plus protein was added in supplement "PrO". Supplements "PrN" and "PrON" included a nematode virulence-enhancing combination of ingredients, which consisted of cholesterol and manganese sulfate at 0.07 and 0.15%, respectively (Table 2). In supplement "PrN", simple lipids were excluded from the formulation. All supplements were fed to *T. molitor* larvae in combination with wheat bran at a 1:4 ratio (20% supplement) except for the control where only bran was provided.

The supplement mixtures were flattened between aluminum foil and wax paper using a roller. The wax paper was carefully removed leaving the flat supplement mixture on the aluminum foil. The flattened supplement mixtures on their foil bases were then dried in the vacuum oven at 50°C and a negative pressure of 80 KPa for a period of 48 h. Dry supplement mixtures were then broken into smaller pieces (1 - 5 mm) suitable for precise ratio adjustment as they were mixed with bran in experiments with small numbers of larvae.

Four-week-old larvae from the stock colony were separated according to sizes by using a series of sieves from standard No. 20 to No. 35. Larvae passing through sieve No. 30 (600 μm) and remaining with sieve No. 35 (500 μm) were selected for the experiment. This group included fourth, fifth, and sixth instars (J. A. M.-R., unpublished).

Table 2. Composition of the supplements compared for impact on *T. molitor* population growth. Percentage of basic ingredients by weight.

	Basic ingredients				
Supplement	Dry potato	Dry egg white	Soy protein	Peanut oil	NV-Mix*
DP	100	0	0	0	0
Pr	85	10	5	0	0
PrO	80	10	5	5	0
PrN	84.78	10	5	0	0.22
PrON	79.78	10	5	5	0.22
S	83	13	2	2	0

*Nematode virulence enhancing mix = MnSO4 and Cholesterol 8.5: 4 parts.

Larvae were randomly divided in 210 groups of 5 larvae each. Larvae groups were randomly assigned to 6 treatments and the control consisting each of 30 repetitions. Treatments consisted of the supplements described in Table 2 mixed with bran at 1: 4 ratios and the control consisted of bran only.

Each group of 5 larvae was weighed at the beginning of the experiment, and their initial weight was recorded. Groups were transferred to stacked Petri dishes separated by a nylon screen standard No. 45 (350 µm openings). The screen allowed frass particles to fall to the second dish at the bottom whereas keeping the larvae and food in place. Each dish was provided with 3.2 g of wheat bran except for the 30 dishes in the control group, which were provided with 4 g of wheat bran. Each of the dishes belonging to the supplement treatments (30 dishes per treatment) also was provided with 800 mg of the corresponding supplement. No water was provided to the larvae.

All dishes were maintained at 27 ± 1°C, 65 ± 5% RH, and darkness until all larvae had pupated. The weight of the pupae was recorded and, when all larvae had pupated, the weights of the remaining food and the frass collected in the second dish also were recorded. Live weight gain (LWG) was determined by subtracting the initial larval weight from the accumulated weight of live pupae from each dish. Food consumed (FC) was calculated by subtracting the weight of the remaining food from the weight of the food provided. Food assimilated (FA) was calculated by subtracting the frass weight from the weight of the food consumed. Weight of food converted was equal to the live weight gain (LWG). Food conversion (Conv) was calculated as Conv = LWG * 100 / FA and efficiency of food conversion (EFC) was calculated as EFC = LWG * 100 / FC (Waldbauer 1968). These 2 parameters are expressed in percentages, and therefore, their statistical analysis tends to be biased. For this reason, we used 3 transformations for the statistical analysis. (1) Live weight (in mg) per gram of food consumed = LWG / (FC * 0.001); (2) food consumed (in g) per gram of live weight produced = FC / LWG, and; (3) food required to complete development (in mg) = FC / Live pupae. These 3 transformations of EFC were analyzed using ANOVA, and the means were compared among treatments and control by Tukey-Kramer's HSD test (SAS Institute 2008). Because we were interested in biomass produced as living insects, measures of LWG represent wet weight estimates.

Newly-eclosed first instars were obtained by using the method described by Morales-Ramos et al. (2011b). Groups of 25 adult *T. molitor* were placed in stacked plastic boxes (140 X 103 X 36 mm) separated by nylon screen standard No. 20 (850 µm openings). Adults were provided with 4 g of wheat bran and 125 µL of RO water twice a week. Eggs were glued to the bran flakes by the beetles. Eclosing first instars instinctively travel to the bottom where they fall through the screen to the second box at the bottom of the stack. First instars were collected daily and randomly placed in 7 groups of equal numbers. Each group corresponded to 1 of the 6 supplement treatments or control. First instars were placed in small Petri dishes (55 × 15 mm) in numbers not exceeding 11 larvae per dish to reduce the impact of larval density, which is known to affect developmental rate in *T. molitor* (Tschinkel and Willson 1971, Weaver and McFarlane 1990). Each dish with larvae was provided with 800 mg of wheat bran and 200 mg of the corresponding supplement, except in the control group where dishes were provided with 1 g of bran and no supplement. First instars were collected daily for a period of 22 days until the final count of first instars reached 400 for each of the treatments and control.

Dishes with larvae were maintained at 27 ± 1°C, 65 ± 5% RH, and no lights until all completed development. Dishes were placed in trays with all treatments and the

control together to avoid potential position effects within the environmental chamber. Dishes were monitored for larval mortality every 3 wks for a period of 100 d. Food was added as required, and dead larvae were removed and recorded. After 100 d, dishes were monitored daily to record pupation date. Pupae were sexed, weighed, and placed in smaller dishes (39 × 11 mm), labeled with treatment, eclosion, and pupation dates and returned to the same chamber to complete development. Pupae also were monitored daily until the emergence of adults. Eclosion, pupation and emergence dates for each individual of each treatment were recorded. These data were used to calculate development times of larvae, pupae, and total (from first instar to adult) for each individual. The total number of living adults in each treatment was subtracted from the initial number of first instars to determine survival.

Development time data were analyzed using ANOVA, and means were compared among treatments and control using Tukey-Kramer's HSD test at $\alpha = 0.05$. Survival rates were compared using contingency analysis, and ChiSquare was used to compare survival among treatments and the control. Data consisting of survival rates from first instar to adult, development time, and sex ratio of each treatment and control were used to calculate life tables.

Diet effects on age-dependent fecundity were studied in groups of 6 adults (3 females, 3 males). Pupae resulting from the previous study were used to obtain newly-emerged adults for this experiment. Pupae collected from each of the supplement treatment groups were sexed and allowed to complete development at the conditions described above. Newly-emerged adults were grouped according to treatment, sex, and emergence date. Adult supplement treatment groups were created from individuals of the same emergence date and larval supplement treatment origin. Seven groups per treatment and control (a total of 49) were created. Each adult group was assigned the same treatment that they had received during development to assure treatment continuity during the whole life cycle.

Groups were placed in stacked boxes separated by No. 20 nylon screen as described above. Each box was provided initially with 4 g of wheat bran and 1 g of one of the supplement formulations in Table 2, except for the control group, which was provided with 5 g of wheat bran and no supplement. Boxes also were provided with 125 μL of RO water twice a week. Additional food was provided every 45 d in the corresponding ratios of bran to supplement until the end of the experiment. Boxes with adult groups were maintained at the same conditions as described above for a period of 154 d (= 22 wk).

Boxes were monitored daily for the presence of first instars in the bottom box. First instars were counted and recorded. Because egg development takes 7 d at 27°C (J. A. M.-R., unpublished), the dates of each larval count was 7 d after the oviposition date. Oviposition dates were determined by subtracting 7 d from the larval count dates to assign the oviposition data to the correct female age. Dead adults were sexed, recorded, and removed to keep track of the number of live females in each box at any given age. Daily oviposition per female was calculated as the number of larvae at day "i" divided by the number of live females at day "i - 7".

Data consisting of progeny produced per female per day were analyzed by 2 methods. The first method was ANOVA of daily progeny per female during the first 90 days. Means of progeny produced per female per day were compared among treatments and control by the Tukey-Kramer HSD test. The second method consisted of GLM analysis of progeny produced per female per day during 16 weekly intervals. The model included the variable a dummy variable "diet" and the numerical variable "week".

Daily progeny produced per day during a period of 112 d was divided in 16 7-d intervals (weeks), which were given a value from 1 - 16. In this way, the effect of female age was controlled by the numerical variable "week". Least square means of treatments and control were compared by the Tukey-Kramer HSD test for LS-means.

Results

Self-selection. Mean (± SD) consumption of wheat bran per experimental unit was 6.71 ± 0.61 g and mean (± SD) total supplement consumption was 2.69 ± 0.21 g. The self-selected diet of *T. molitor* larvae consisted mostly of wheat bran and a mean (± SD) of 28.7 ± 2.9% of dietary supplements. However, there were significant preferences among the 6 supplements provided. The proportion consumed of each of the supplements was consistent through the 21 replicates. Larvae consumed significantly more of the "EW-20" supplement than all of the others, and supplements "DP" and "Pe-10" were consumed in significantly higher rate than "Soy-20", "Ca-10", and "Sa-10" ($F = 792.5$; $df_1 = 5$, $df_2 = 119$; $P < 0.0001$) (Fig. 2). Results indicate that *T. molitor* larvae had a significant preference for supplement EW-20 as compared with supplement "Soy-20" as a source of protein. Similarly, larvae showed a significant preference of supplement "Pe-10" as compared with supplements "Ca-10" and "Sa-10" as a source of lipid.

Consumption of individual supplement ingredients, calculated as a function of supplement consumption and supplement composition, is presented in Table 3. The mean relative consumption of each ingredient was used as a base to create supplement "S" (see Materials & Methods). The mean relative consumption of the dry potato ingredient

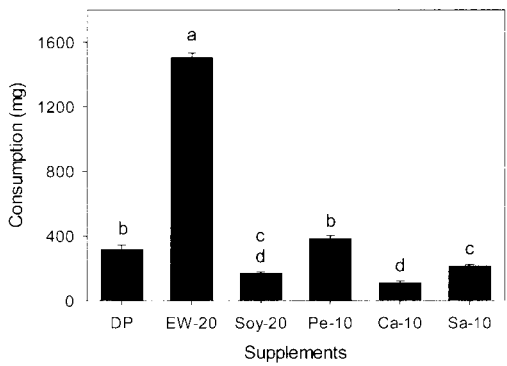

Fig. 2. Mean consumption (mg) of selected diet supplements by *T. molitor* larvae in a multiple-choice experiment. Brackets represent standard error of the mean; bars with the same letter are not significantly different after Tukey-Kramer HSD test at $\alpha = 0.05$. Supplements formulations: DP = 100% dry potato; EW-20 = 80% dry potato, 20% dry egg white; Soy-20 = 80% dry potato, 20% soy protein; Pe-10 = 80% dry potato, 5% dry egg white, 5% soy protein, 10% peanut oil; Ca-10 = 80% dry potato, 5% dry egg white, 5% soy protein, 10% canola oil; Sa = 80% dry potato, 5% dry egg white, 5% soy protein, 10% salmon oil.

was 82.3%. Relative consumption of the protein-based ingredients egg white and soy was 12.49 and 2.57%, respectively. The total relative consumption of protein-based ingredients was 15%. Relative consumption of lipid-based ingredients was 2.61% and from this percentage 1.43% consisted of peanut oil. Canola and salmon oils had relative consumptions of 0.38 and 0.8%, respectively. For this reason peanut oil was the only lipid-based ingredient used in the formulation of supplement "S".

Supplement evaluation. Supplement "S" did not improve food utilization significantly in *T. molitor* when compared with all of the existing supplement formulations, but food utilization was significantly better in larvae feeding on supplement "S" than in those feeding on supplement "DP" and those feeding on bran alone (Table 4). Larvae feeding on bran only showed significantly less efficient food utilization than larvae feeding on bran plus a supplement. However, larvae feeding on the supplement consisting of dry potato alone (DP) were significantly less efficient utilizing the food than larvae feeding on more complex supplements in addition to bran (Table 4).

Food conversion efficiency was significantly lower in the control group than in all treatment groups. Larvae feeding only on wheat bran were required to consume significantly more food to complete development than larvae feeding on bran plus a supplement (F= 29.15; df_1 = 6, df_2 = 203; $P < 0.0001$). Similarly, more food was required to produce 1 g of live pupae in the control group than in all treatment groups (F= 44.59; df_1 = 6, df_2 = 203; $P < 0.0001$) (Table 4). Larvae feeding on supplement "DP" required more food to complete development, produced less live pupal weight per 1 g of food, and had a lower food conversion efficiency than larvae feeding on supplements "Pr", "PrO", "PrN", and S (Table 4).

Addition of dry potato to the diet of *T. molitor* significantly increased larval growth. Pupal weight was significantly higher in the "DP" treatment than in all other treatment and control groups (F= 33.56; df_1 = 6, df_2 = 2432; $P < 0.0001$) (Fig. 3). Adding a protein and a lipid source decreased growth significantly, but larvae still grew larger than in the control group. Larvae feeding exclusively on wheat bran (control) developed into pupae that were significantly smaller than those in the other treatment groups. Pupal

Table 3. Relative consumption of individual supplement components by 8-wk-old *T. molitor* larvae during a 60-d period in a multiple-choice test of 6 formulations.

Supplement component	Consumed (mg)	Percentage consumed*
Dry potato	2218.1 ± 179.2	82.34 ± 0.78
Dry egg white	336.0 ± 27.6	12.49 ± 0.63
Soy protein	69.0 ± 9.7	2.57 ± 0.34
Peanut oil	38.4 ± 8.0	1.43 ± 0.28
Canola oil	10.9 ± 6.5	0.38 ± 0.24
Salmon oil	21.5 ± 3.9	0.80 ± 0.17
All oils combined	70.3 ± 9.9	2.61 ± 0.31

Mean ± standard deviation, n = 21.

*Percentage from total supplement consumption excluding wheat bran.

Table 4. Food utilization by *T. molitor* larvae on selected diet supplements.

Supplement*	Conversion efficiency**	Weight gained per g of food†	Food per g of weight gained‡	Food required for development§
C	12.3 ± 0.6c	123.4 ± 6.3c	9.0 ± 0.64b	1041.9 ± 68.4a
DP	20.6 ± 0.4b	206.4 ± 3.5b	4.9 ± 0.09a	752.8 ± 16.3b
Pr	23.7 ± 0.4a	237.3 ± 4.3a	4.3 ± 0.08a	613.2 ± 18.3c
PrO	23.8 ± 0.3a	238.0 ± 3.3a	4.2 ± 0.07a	597.6 ± 11.6c
PrN	23.3 ± 0.5a	233.5 ± 4.6a	4.3 ± 0.10a	615.4 ± 15.1c
PrON	22.6 ± 0.5ab	225.7 ± 5.2ab	4.5 ± 0.14a	635.2 ± 17.7bc
S	23.3 ± 0.5a	233.3 ± 4.7a	4.4 ± 0.11a	613.5 ± 17.5c

Mean ± SEM, $n = 30$. Means with the same letter are not significantly different after Tukey-Kramer HSD test at $\alpha = 0.05$.

*C = control (no supplement); DP = 100% dry potato; Pr = 85% dry potato, 10% dry egg white, 5% soy protein; PrO = 80% dry potato, 10% dry egg white, 5% soy protein, 5% peanut oil; PrN = 84.78% dry potato, 10% egg white, 5% soy protein, 0.22% MnSO4 and cholesterol (8.5:4); PrON = 79.78% dry potato, 10% dry egg white, 5% soy protein, 5% peanut oil, 0.22% MnSO4 and cholesterol (8.5:4); S = 83% dry potato, 13% dry egg white, 2% soy protein, 2% peanut oil.

**In percentage = (Live weigh gained * 100) / food consumed.

†In mg of live pupae.

‡In g.

§In mg = total food consumed from first instar to adult.

weight was not significantly different among treatments feeding on supplements that contained protein (Pr, PrO, PrN, PrON, and S) (Fig. 3).

Supplement "S" did not shorten the development time of *T. molitor* as compared with other supplements containing protein. However, development time was significantly shorter in groups feeding on supplements containing protein as compared with those feeding on dry potato as supplement (DP) and those feeding on wheat bran alone (control) ($F = 290.63$; $df_1 = 6$, $df_2 = 2432$; $P < 0.0001$) (Fig. 3). Larval mortality was significantly higher in the control group than in all the treatment groups fed with supplements ($X^2 = 137.37$, $df = 6$, $P < 0.0001$). Immature survival was 0.657, 0.879, 0.909, 0.889, 0.906, 0.872, and 0.911 in the control, "DP", "Pr", "PrO", "PrN", "PrON", and "S" groups, respectively. There was no significant difference in immature survival among the groups feeding on supplements.

The supplement formulation derived from self-selected ratios of *T. molitor* larvae (S) significantly impacted adult fecundity. Indirect estimates of fecundity expressed as first instars produced per female per day were significantly higher in females feeding on supplement "S" than on females from all other treatment groups except "PrON" ($F = 140.58$; $df_{1, 2} = 6$, 4403; $P < 0.0001$) (Fig. 3). Mean progeny per female per day was 7.1 ± 3.3 and 6.9 ± 3.2 in the "S" and "PrON" treatments, respectively. The other 3 treatments that included supplements with protein produced 5.9 ± 2.8, 6.1 ± 3.0, and 6.3 ± 3.3 first instars per female per d of treatments "Pr", "PrO", and "PrN", respectively. These 3 treatments produced more than the control (3.3 ± 2.5) and treatment "DP" (4.2 ± 2.4), which included only dry potato as supplement (Fig. 3). All treatments with supplements produced significantly more progeny than the control with no supplements.

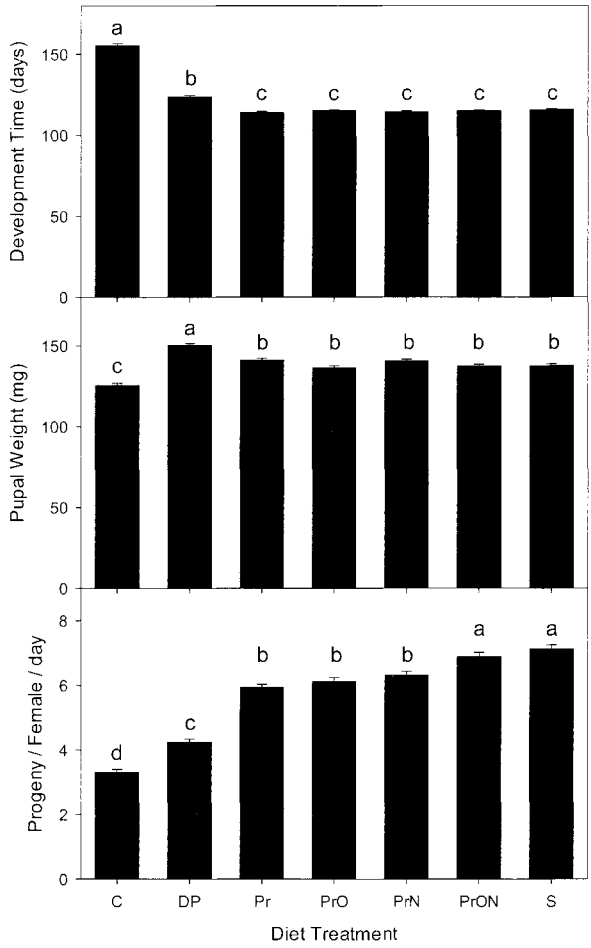

Fig. 3. Supplement effect on development time in days (Top), growth as measured by pupal weight (Middle), and fecundity as measured by progeny produced per female / day for a period of 90 d (Bottom) of *T. molitor*. Bars with the same letter are not significantly different after Tukey-Kramer HSD test at α = 0.05. Supplements formulations: C = control (no supplement); DP = 100% dry potato; Pr = 85% dry potato, 10% dry egg white, 5% soy protein; PrO = 80% dry potato, 10% dry egg white, 5% soy protein, 5% peanut oil; PrN = 84.78% dry potato, 10% egg white, 5% soy protein, 0.22% $MnSO_4$ and cholesterol (8.5:4); PrON = 79.78% dry potato, 10% dry egg white, 5% soy protein, 5% peanut oil, 0.22% $MnSO_4$ and cholesterol (8.5:4); S = 83% dry potato, 13% dry egg white, 2% soy protein, 2% peanut oil.

Adult *T. molitor* feeding on supplements produced progeny for a longer period of time than adults feeding on wheat bran alone. Females feeding on wheat bran alone reached 80% of their potential reproduction 65 d after emergence and the mean total progeny produced per female was 104.05 ± 39.89. Females feeding on bran plus supplements "DP", "Pr", "PrO", "PrN", "PrON, and "S" Reached 80% of their reproductive potential 84, 92, 86, 92, 93, and 95 d after emergence and total progeny per female was 115.62 ± 43.26, 139.48 ± 37.52, 171.05 ± 51.11, 150.86 ± 37.28, 170.95 ± 44.59, and 175.81 ± 50.92, respectively (Fig. 4).

Discussion

The results indicate that a nutritional supplement formulated from self-selected rates of *T. molitor* larvae (S) significantly improved fecundity as compared with other previously developed diet supplements. Although the data indicate that no improvement occurs in food utilization efficiency, growth, development and survival during the larval stage, the supplement seems to have induced significant changes in larval development that resulted in more fertile adults. Adding protein and lipid to the supplement provided significant benefits in all biological parameters as compared with the dry potato alone supplement. However, the addition of lipid to a supplement containing protein (PrO) did not provide any significant improvements. Supplement "PrO" comprises the same components as supplement "S", but in different proportions. It seems that adding a small proportion of lipid to the diet is beneficial, but higher concentrations become less advantageous and possibly slightly detrimental. The addition of 20% lipid to a supplement containing the basic ingredients of "Pr" significantly increased larval susceptibility to infection by the nematode *H. indica* as compared with larvae feeding on a supplement identical to "PrO" (Shapiro-Ilan et al. 2008), which contains only 5% lipid. In another study, *T. molitor* larvae fed on a supplement identical to "PrO" were significantly more susceptible to *S. carpocapsae* than larvae fed with a supplement identical to "Pr" (Shapiro-Ilan et al. 2012). Adamo et al. (2010) determined that a physiological trade-off occurs between immune system and digestion when some insects feed on a high lipid diet. A high lipid diet reduced immune response to the bacterium *Serratia marcescens* Bizio in the cricket *Gryllus texansis* Cade and Otte (Adamo et al. 2010).

It seems that supplement "S" has a better lipid balance than "PrO" and this balance was obtained as the result of self-selection by *T. molitor* larvae. It would have been extremely difficult to obtain the correct lipid proportions by trial and error or by a series of experiments with various lipid concentrations. The use of self-selection methods to obtain optimal dietary balance has been advantageous in the case of *T. molitor* and could be applicable to other insects with self-selection potential.

The addition of the nematode virulence-enhancing components cholesterol and $MnSO_4$ in combination with lipid (supplement PrON) provided a similar improvement in fecundity as the self-selection-based supplement. The addition of either lipid (PrO) or virulence-enhancing mix (PrN) alone did not improve fecundity as compared with the supplement containing only protein (Pr) in addition to dry potato. Shapiro-Ilan et al. (2012) did not report any significant improvement in fecundity in *T. molitor* feeding on the supplement with cholesterol and MnSO4. This apparent discrepancy may be due to the length of the oviposition period evaluated in each experiment. Shapiro-Ilan et al. (2012) evaluated 60 d of oviposition as compared with 90 d in this study. Studies of adult biology of *T. molitor* showed that 80% of the total reproductive potential of

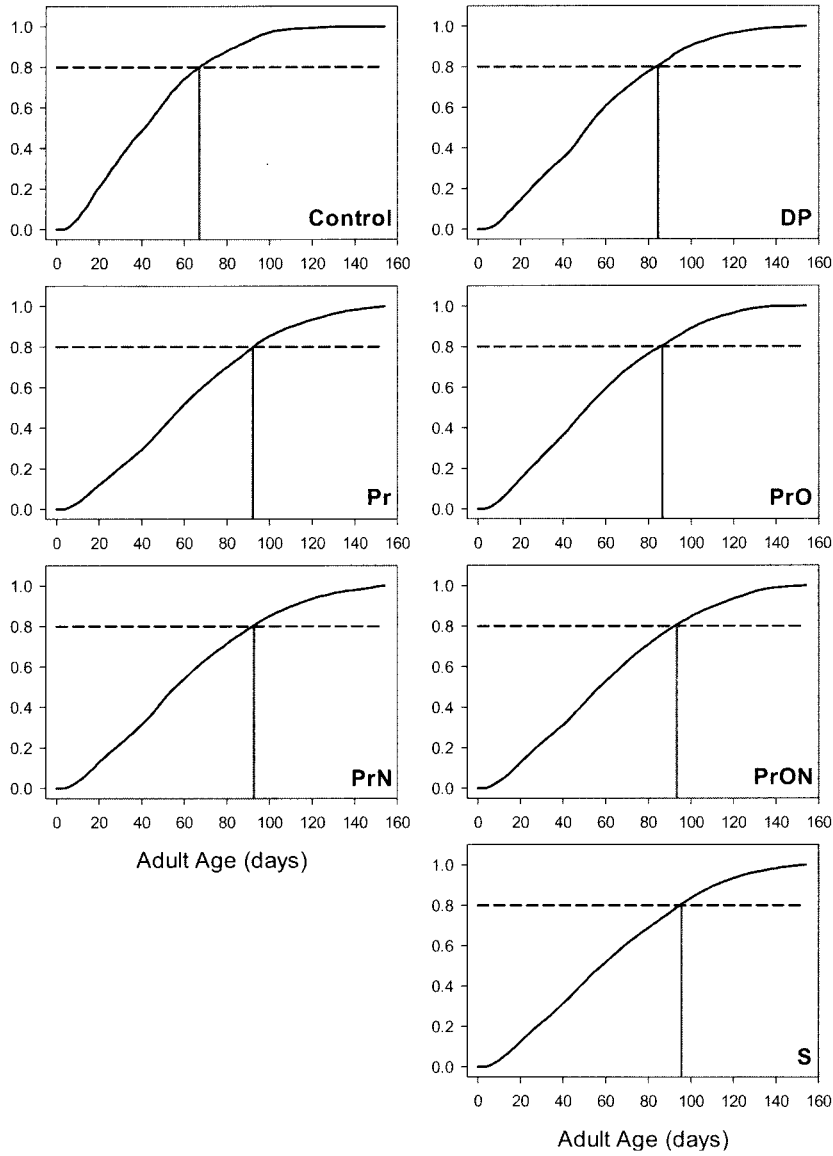

Fig. 4. Age-dependent reproductive potential proportions of *T. molitor* females reared with bran only and with 6 dietary supplements mixed with wheat bran in a 1:4 ratio (20% supplement). Curves represent the cumulative reproductive potential over time; dashed lines represent the 80% level of reproductive potential; vertical lines mark the intersection of curves with the 80% reproductive potential indicating the adult age at time of intersection. Supplement formulations can be found in Table 2.

adult females is realized by day 60 after emergence when fed exclusively on wheat bran (Morales-Ramos et al. 2012). However, in our study we determined that adults feeding on supplements were fertile for a much longer time than adults fed exclusively on wheat bran. In the case of supplements "PrON" and "S", females reached 80% of their reproductive potential at ages of 93 and 95 d, respectively, compared with an age of 65 d in females feeding exclusively in wheat bran. In a mass-production system the reproductive stock would be viable for a full month longer if the colony was provided with a supplement than if it was fed exclusively with bran. Supplement "PrON" provided significant benefits in reproduction compared with the other supplements tested with the exception of supplement "S". Supplement "PrON" could be used in a *T. molitor* mass-production system destined to be used for entomopathogenic nematode production with benefits in colony health and increased nematode virulence. Because the addition of cholesterol increase production costs, supplement "S" would be a better choice for a mass-production system destined to produce insect protein for diverse applications.

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