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Source: Florida Entomologist, 93(1) : 56-62

Published By: Florida Entomological Society

URL: https://doi.org/10.1653/024.093.0108

AN ARTIFICIAL DIET FOR *DIAPREPES ABBREVIATUS* (COLEOPTERA: CURCULIONIDAE) OPTIMIZED FOR LARVAL SURVIVAL

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ABSTRACT

The root weevil *Diaprepes abbreviatus* (L.) has been reared since 1992 on an artificial diet first reported in 1982. Recently, we have shown that several ingredients included in the original diet have little or no effect on insect performance. Here we examined the effects of 2 principal drivers (cottonseed meal and wheat germ) on weight gain and survival of larval *D. abbreviatus* in varying proportions along with a non-nutritive filler (cellulose). We employed a geometric design to evaluate amount and proportion combined with response surface models to identify optimal proportions for larval weight gain, larval survival, and development rate. All larval responses measured lead to the conclusion that cottonseed meal is the only major nutritive component, in addition to standard vitamin and salt mixes, required for a successful artificial diet for rearing larvae of *D. abbreviatus* to pupation.

Key Words: Diaprepes root weevil, artificial diet, response surface model, mixture optimization

Supplemental material online at http://www.fcla.edu/FlaEnt/fe931.htm#InfoLink1.

RESUMEN

El curculionido *Diaprepes abbreviatus* (L.) ha sido criado desde el año 1982 sobre una dieta artificial. Recientamente, hemos probado que varios ingredientes de esa dieta original tienen poco o ningun efecto sobre el desempeño del insecto. En el presente estudio, estudiamos los efectos que resultaron cuando se variaron las proporciones de dos ingredientes principales (harina de semillas de algodón y gérmen de trigo) y un relleno sin valor nutritivo (celulosa) sobre el peso y supervivencia de larvas de *D. abbreviatus*. Se empleó un diseño geométrico junto con modelaje de superficie de respuesta para evaluar cantidad y proporción y entonces identificar proporciones optimas para ganancia de peso, supervivencia y taza de desarrollo de las larvas. Todas las respuestas larvales medidas llevan a la conclusión de que la harina de semilla de algodon sea el unico ingrediente nutritivo, además a las mezclas estandares de vitaminas y sal, requerido para una dieta artificial exitosa para criar larvas de *D. abbreviatus* hasta la pupación.

Translation provided by the authors.

The ability to rear insects on artificial diets is often an essential component of research and development of pest control strategies. The root weevil *Diaprepes abbreviatus* (L.) (Coleoptera: Curculionidae) has been continuously reared since 1992 on an artificial diet developed by Beavers (1982) with procedures described by Lapointe & Shapiro (1999). That diet was shown to contain ingredients with little or no effect on the growth or survival of *D. abbreviatus*; 3 ingredients (corn starch, cottonseed meal and wheat germ) were identified as principal drivers of larval weight gain and survival (Lapointe et al. 2008). The application of response surface methods (RSM) based on geometric experiment designs is particularly appropriate for determining optimal responses to insect diet composition and visualizing complex interactions of diet mixture components (Clancy & King 1993). Our ongoing objective is to define a diet that will produce large numbers of weevils of normative weight, i.e., comparable to feral *D. abbreviatus*. To accomplish this, we constructed a 3-component geometric design that included 2 principal drivers (cottonseed meal, wheat germ) identified previously (Lapointe et al. 2008) and cellulose as a non-nutritive filler that could be scaled to produce the desired response. We report here the effect of varying proportion and amount of diet components (cottonseed meal, wheat germ, and cellulose) on larval survival and weight gain.

Mixtures, such as insect diets, require a special statistical approach based on mixture polynomials developed by Scheffé (Cornell 2002). Mixtures are inherently constrained because the proportion of one component of a mixture cannot be varied without simultaneously varying the proportion of all other components. Scheffé polynomials also do not contain an intercept because it is impossible to generate a mixture that contains all ingredients set to zero. The result is that a degree of freedom is lost and the intercept is contained in the linear coefficients (Anderson & Whitcomb 2005). These peculiar qualities of mixtures require appropriate experiment designs, particularly when attempting to improve insect diets where the objective is to identify component combinations that result in optimal signal (response) and not to simply compare a particular set of treatment combinations. For the latter, ANOVA and post-hoc means tests may be adequate. But if the experimenter's need is a more complete description of the experiment design space and identification of trends and optima, then mixture designs are required.

Clancy & King (1993) used RSM to look at the effect of calcium, magnesium, and phosphorous on western spruce budworm performance. However, they did not use a mixture design based on the Scheffé polynomial and experienced the difficulty of independently varying cations and ions (see Niedz & Evens (2006) regarding experimental designs for exploring cation and anion effects). As a result, their study ignored the effect of proportionality and their design was constrained and inherently confounded, as the authors recognized.

RSM seeks to identify trends and optimal combinations, often the objective of experimentation in general and of mixtures in particular. A paradox of RSM is it's ability to produce compelling visual imagery of responses that are easily interpreted by readers without requiring an understanding of the underlying principals of design and modeling (Anderson & Whitcomb 2005). The pairing of mixture designs with RSM now available in modern statistical software programs is particularly powerful and appropriate for use in diet development. Here we use a mixture design combined with RSM to determine the effect of proportionality and amount of diet ingredients on survival and weight gain of *D. abbreviatus.*

Materials and Methods

All stages of *D. abbreviatus* were reared at the U.S. Horticultural Research Laboratory, Fort Pierce, FL (Lapointe & Shapiro 1999; Lapointe et al. 2008). Eggs were collected from caged adults on wax-paper strips (Wolcott 1933) and allowed to hatch in plastic containers. Standard procedure for preparing purchased diet for larval development was as follows: 40 L of water were combined with 725 g agar and heated to near boiling. While stirring, 9.5 kg of commercially prepared insect diet (product no. F1675, Bio-Serv, Inc., Frenchtown, NJ, similar to that developed by Beavers (1982)) were added to the water/agar mixture, mixed, and heated to boiling. Methyl paraben (9 g dissolved in 10 mL 95% EtOH, Sigma-Aldrich) and 9 g of benzoic acid (Sigma-Aldrich) in solution with boiling deionized water were added as preservatives. After 10 min of boiling, ~15 mL of diet was dispensed into 30-mL plastic cups and allowed to cool and dry in a laminar flow hood. Neonate larvae were surface sterilized for ~2 min in 0.25% hypochlorite solution (Clorox™), rinsed with deionized water, and placed in cups with diet. Each diet cup was infested with approximately 12 neonate larvae (≤24 h old) and 10 cups were infested for each experimental diet (treatment) for a total of approximately 120 neonate larvae per diet. Diet cups were capped and placed in trays enclosed in zip-lock plastic bags and held in a dark environmental chamber at 25°C and 60-70% RH. Humidity within the diet cups held in sealed plastic bags probably exceeded 95% (Lapointe 2000). Cups were opened 28 d after infestation and larvae were counted and weighed. A total of 30 larvae per treatment were randomly selected and transferred to fresh diet cups to complete development (1 larva per cup). Larvae pupated in the diet cups. Cups were inspected on weekdays to determine date of pupation. The date and time of an observed event (e.g., death, pupation) were calculated as the midpoint between the times of the observed change and the previous inspection. Time to pupation was recorded for 6 months from date of infestation with neonates. Insects that failed to pupate within 6 months were discarded. Survival was determined by the number of larvae alive at transfer and by the number of larvae that successfully pupated (survival of pupae to adult in this trials and in previous experiments was uniformly high, approaching 100%). Survival from neonate to adult was calculated by multiplying the percent survival of neonates to 28 d by the percent survival of larvae from transfer to adult.

A previous study showed that the F1675 diet could be diluted with cellulose with no significant reduction in insect weight gain or survival (Lapointe et al. 2003). Subsequently, we showed that at least 3 ingredients in the F1675 diet have little to no effect on survival or development (casein, soy protein isolate, and sucrose) (Lapointe et al. 2008). Therefore, we constructed a modified D-optimal mixture design sufficient to satisfy a Scheffé cubic polynomial response surface model (Cornell 2002) for 3 diet ingredients (cellulose, cottonseed meal, and wheat germ) in addition to constant amounts of salt and vitamin mixtures (Table 1). All diets contained 2.5% Vanderzant vitamin mix (bio-mix #9796, Bio-Serv, Frenchtown, NJ), 2.5% Wesson salt mix (bio-mix #9798, Bio-Serv), 1.5% methyl paraben and 0.5% sorbic acid (Sigma-Aldrich). In addition to those needed to satisfy model terms, points were added to estimate lack of fit (LOF). Several points were duplicated to attain sufficient degrees of freedom (*df*) to estimate pure error across the design space and to minimize leverage for all points (Weisberg 1985). The resulting design (Fig. 1) had 5 model, 9 lack of fit, and 6 pure error degrees of freedom (Myers & Montgomery 2002). The LOF diet blends were chosen so that they could be used to satisfy higher order model coefficients if necessary.

TABLE 1. PROPORTION OF 3 DIET INGREDIENTS VARIED IN A 3-COMPONENT BLEND EXPERIMENT TO MEASURE LARVAL GROWTH OF *D. ABBREVIATUS*. CL = CELLULOSE, CM = COTTONSEED MEAL, WG = WHEAT GERM.

Blend	CL	CM	WG
1	0.70	0.00	0.30
2	0.97	0.00	0.03
3	0.90	0.05	0.05
4	0.70	0.20	0.10
5	0.97	0.00	0.03
6	0.70	0.00	0.30
7	0.88	0.12	0.00
8	0.79	0.10	0.11
9	0.97	0.03	0.00
10	0.70	0.15	0.15
11	0.83	0.00	0.17
12	0.70	0.20	0.10
13	0.79	0.21	0.00
14	0.79	0.10	0.11
15	0.70	0.10	0.20
16	0.70	0.30	0.00
17	0.88	0.00	0.12
18	0.97	0.03	0.00
19	0.83	0.17	0.00
20	0.70	0.30	0.00
21	0.79	0.00	0.21

RESULTS AND DISCUSSION

A summary of the ANOVA, lack-of-fit tests, the best fitting models and the R^2 statistics for several responses are presented in Table 2. Some model fits were improved by backward regression and are designated as "reduced". Some responses required transformation as per Box-Cox analyses (Box & Cox 1964). The remaining diagnostics were all within acceptable limits, i.e., the data appeared normal and displayed a constant variance, there were no outlier-*t* points, no points that exceeded a Cook's distance of one (Cook & Weisberg 1982), and the predicted versus actual value plots showed close agreement (data not shown). The 3 statistics (R^2, R^2_{adj} and R^2_{pred}) were clustered with a difference less than 0.2. The lack-of-fit tests were not significant, indicating that additional variation in the residuals could not be removed with better models. The models (Table 2) were significant ($\alpha = 0.05$), indicating significant factor effects on the response variables, and were considered of sufficient quality to navigate the experimental design space and to predict new observations. The ANOVAs revealed multiple significant terms that are indicative of important component effects and interactions. The regression coefficients are reported in coded terms. Thus, they are directly comparable and provide information on how each term contributes to the shape of the response surfaces.

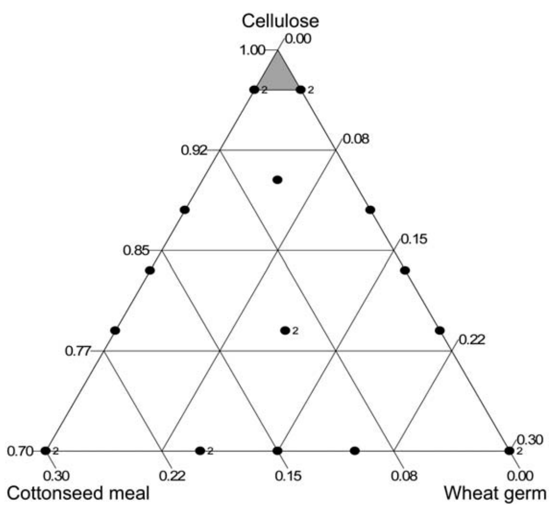

Fig. 1. Design space for a 3-component blend experiment showing coordinates of experimental and validation diet blend proportions (black points). Points accompanied by "2" were replicated. Shaded area not sampled by the experiment design.

Survival of early instars from neonate (24 h) to 28 d of age ranged from 19 to 102 of the approximately 120 neonates used to infest each diet. The response surface model was significant (P = 0.0017) but the R^2_{adj} value was 0.45, indicating that the model explained approximately one-half of the observed variance. The linear response model (Fig. 2A) indicated that larval survival to 28 d was maximal (predicted value of 77%) at the highest content of cottonseed meal (the cottonseed meal apex in Fig. 1) and minimal (predicted value of 26%) at the wheat germ apex.

Standard rearing procedure for *D. abbreviatus* includes a transfer of larvae from cups containing multiple larvae to individual cups at 28 d of age. Survival of larvae from transfer to adult emergence ranged from 0 to 97%. The response surface was highly significant (P < 0.0001) and the R^2_{adj} value was 0.99 (Table 2). In contrast to neonate survival to 28 d, survival of larvae from transfer to adult was predicted by the model to be optimal at a blend that included both cottonseed meal and wheat germ (range of predicted diet proportions were 0.84 - 0.86, 0.07 - 0.13, and 0.04 - 0.09 for cellulose, cottonseed meal, and wheat germ, respectively) (Fig. 2B).

A response surface was also constructed for the calculated survival of neonates to adult (percent survival of neonates to transfer multiplied by percent survival from transfer to adult). Survival of neonates to adult varied from 0 to 74%. The response surface (Fig. 2C) was highly significant (P < 0.0001) and the R^2_{adj} value was 0.75 (Table 2). The model suggests that binary blends of cottonseed meal and cellulose provide optimal larval survival. In addition, the curvature along the cel-

TABLE 2. P-VALUES, REGRESSION COEFFICIENTS, AND RESPONSE SURFACE MODEL FITTING DIAGNOSTIC STATISTICS FOR 5 MEASURES OF D. ABBREVIATUS PERFORMANCE ON 3-COMPONENT ARTIFICIAL DIET BLENDS. CL = CELLULOSE, CM = COTTONSEED MEAL, WG = WHEAT GERM, RC = REGRESSION COEFFICIENT, SQRT = SQUARE ROOT, P = PROBABILITY OF A GREATER F VALUE (P-VALUES IN BOLD ARE SIGNIFICANT, A = 0.05).

	Survival									
	To transfer @4 wk		Transfer to adult		Neonate to adult		Weight at 4 wk		Days to pupation	
	P	RC[a]	*P*	RC[a]	*P*	RC[a]	*P*	RC[a]	*P*	RC[a]
Model	**0.0017**		**<0.0001**		**<0.0001**		**<0.0001**		**0.0215**	
Linear Mixture	**0.0017**		**<0.0001**		**<0.0001**		**<0.0001**		**0.0215**	
Cellulose		47.47		-3.39		-0.40		0.39		145.33
Cottonseed Meal		92.47		4.40		7.58		5.48		124.98
Wheat Germ		31.22		4.36		4.49		5.48		136.44
CL · CM			**<0.0001**	16.02	**0.0018**	14.70				
CL · WG			**<0.0001**	9.76	**0.0412**	8.79				
CM · WG			**<0.0001**							
CL · CM · (CL - CM)			**<0.0001**	15.64						
CL · WG ˇ (CL - WG)			**<0.0001**	23.87						
Lack of Fit	0.659		0.673		0.183		0.873		0.581	
Model Type	Linear		Reduced Cubic[b]		Reduced Quadratic[b]		Linear		Linear	
Transformation[c]	None		Sqrt (#Adults+0.97)		Sqrt (#Adults+0.74)		Sqrt (larval weight)		None	
R^2	0.506		0.999		0.802		0.840		0.422	
R^2_{adj}	0.451		0.998		0.752		0.822		0.340	
R^2_{pred}	0.320		0.997		0.665		1.788		0.187	

[a]Presented in coded form by placing values between -1 and +1 to allow direct comparison.
[b]Model reduction by backward elimination
[c]Transformation determined by Box-Cox plot analysis

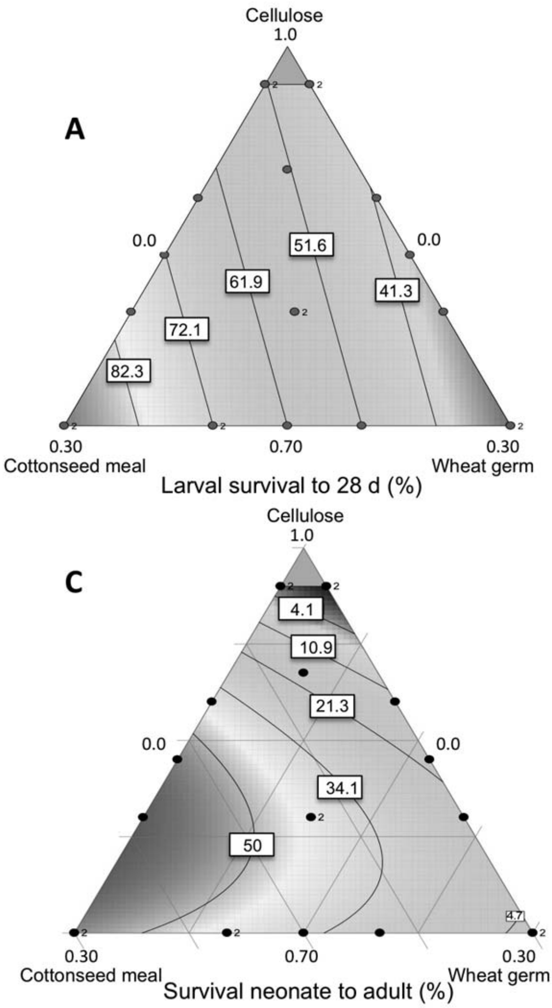

Fig. 2. Predicted 3-dimensional surface response plots for 4 measures of larval *D. abbreviatus* performance reared on diets with varying proportions of 2 major diet components (cottonseed meal and wheat germ) and a non-nutritive filler (cellulose): survival of neonate larvae to transfer at 28 d (A), mean larval survival from 28 d to pupation (B), survival of larval *D. abbreviatus* from neonate to adult (C), and mean weight of 28-d-old larvae (D). Values on plots are original scale.

lulose-cottonseed meal axis suggests that cottonseed meal can be reduced as a percentage of the blend from 30% without reducing larval survival.

Larval weights at transfer (28 d) ranged from 0.6 to 48.5 mg. The response surface (Fig. 2D) was highly significant ($P < 0.0001$) and the R^2_{adj} value was 0.82 (Table 2). The linear model suggests that larval weight at 28 d is positively correlated with nutrient content, either cottonseed meal or wheat germ or a mixture of the 2 with no blending effect (interaction) (Fig. 2D).

The dataset for larval development was truncated at 180 d. The development period on diets that produced >0% survival ranged from 119 to 146 d. The response surface (Fig. 3) was significant ($P = 0.02$) and the R^2_{adj} value was a relatively low 0.34 (Table 2). Nonetheless, the predicted R^2 was in reasonable agreement with the adjusted R^2_{adj} and the response signal was judged adequate to navigate the design space and draw general inference. Cottonseed meal had the largest effect on development time (see regression coefficients in Table 2). In this context, faster development is preferred.

Beavers (1982) reported the recipe for what became the F1675 diet produced by BioServ, Inc. for "citrus root weevil", i.e., *D. abbreviatus*. In that article, Beavers alluded to diets developed for the boll weevil, the plum curculio, and the cerambycid *Dectes texanus* as providing a basis for his recipe. No explanation was provided as to how the particular combination and proportion of ingredients

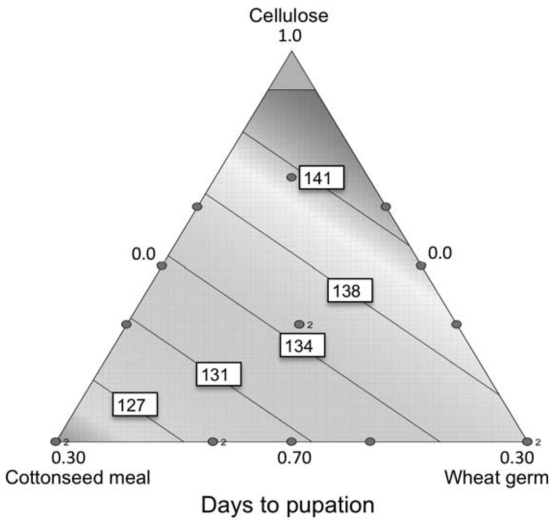

Fig. 3. Predicted 3-dimensional surface response plot of development for larval *D. abbreviatus* reared on diets with varying proportions of 2 major diet components (cottonseed meal and wheat germ) and a non-nutritive filler (cellulose). Values on plot are original scale.

acceptable to *D. abbreviatus* were determined. Since then, *D. abbreviatus* has been produced for approximately 25 years by the USDA and more recently by the state of Florida without modification of the original diet. Many thousands of colony-reared *D. abbreviatus* have been used in experiments spanning fields such as biological control, genomics, plant resistance, ecology, chemical ecology, and others. We have previously demonstrated that certain ingredients in the F1675 diet (casein, cornstarch, soy protein isolate, sucrose) have no significant effect on *D. abbreviatus* development as measured by multiple responses (Lapointe et al. 2008). Our objective in the current study was to determine the proportions of 2 principal nutritional components (cottonseed meal and wheat germ) of a simplified diet plus a non-nutritive filler (cellulose) that would allow for scaling of larval weight gain to produce adults of normative weights. The results of these studies showing adult weights and survival compared with field-collected specimens will be published separately. Here we examined the effect of varying proportions of cellulose, cottonseed meal, and wheat germ on larval weight gain and survival.

Larval weight gain from egg eclosion to 28 d appeared to be equally responsive to the proportions of cottonseed meal and wheat germ in the diet (Fig. 2D). However, both predicted values for larval survival during the first 28 d (Fig. 2A) and development rate (the inverse of days to pupation, Fig. 3) were greatest at the cottonseed vertex. Wheat germ appears to be detrimental to survival compared with cottonseed meal despite the fact that larvae that survived on diets with high wheat germ content gained weight at a rate similar to high cottonseed meal diets. The best combination of early instar survival and weight gain occurred at the cottonseed vertex (30% cottonseed meal, 70% cellulose). The best response surface model for survival of larvae from 28 d to pupation (Fig. 2B) included a cubic term that describes curvature resulting from blending effects (interactions) (Table 2). It's possible that this effect is an artifact of the curvature resulting from leverage of design points near the cellulose vertex. Nonetheless, the estimate of lack of fit was not significant (Table 2). The response surface shows a bias towards cottonseed meal compared with wheat germ for survival of late instars that was not apparent in the response of early instars (Fig. 2A). A similar surface was generated by modeling larval survival from neonate to adult (Fig. 2C), calculated as the product of the measurements of survival from neonate to transfer (Fig. 2A) and from transfer to pupation (Fig. 2B).

The response surface models demonstrated that larval survival to pupation and larval weight gain for *D. abbreviatus* are responses that can be independently manipulated. Diets that produce large insects do not necessarily produce the greatest number of insects (Lapointe et al. 2008). All larval responses measured lead to the conclusion that cottonseed meal is the only major nutritive component required for a successful artificial diet for rearing larvae of *D. abbreviatus* to pupation. Our experience here and in previous experimentation (Lapointe et al. 2008) is that survival of pupae to adult emergence is invariably close to 100%. Therefore, we conclude that a cottonseed meal diet containing cellulose as a scalable non-nutritive filler with standard vitamin and salt mixes is optimal within the design space described by the ingredients of the original diet described by Beavers (1982). It remains to be determined how diets within the 3-component design space described here affect adult responses such as adult weight. That analysis will be the subject of a separate manuscript.

Acknowledgments

We thank Peter D'Aiuto, Anna Sara Hill, PeiLing Li, Alexander Pheneger, Kelsey Stevens, and Eldridge Wynn, USDA-ARS, Ft. Pierce, FL for technical assistance. This article reports the results of research only. Mention of a trademark or proprietary product is solely for the purpose of providing specific information and does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

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