EVALUATION OF NUTRITIONAL QUALITY OF FOOD WITH THE RED FLOUR BEETLE *(Tribolium castaneum)*

I. Effects of Protein Concentration in the Diet on Larval Growth, and Developmental Time[1]

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ABSTRACT

To determine the effect of protein concentration in the diet on growth and development of *Tribolium castaneum*, larvae were reared from 8 to 24 days of age in diets containing graded amounts of protein provided by an optimum growth media for *Tribolium* (90%/o unbleached white wheat flour and 10%/o Torula yeast). Weight gain, assessed from 10 to 14 days of age, showed that growth response was linear and very sensitive to the amount of protein in the diet, from 0 to 6%/o. Maximum growth rate was obtained with 8%/o protein, and a decreasing growth response was observed above this level. Developmental differences, assessed by counting the different metamorphic forms in the cultures at 18, 20, 22, and 24 days of age, were significant when the diet contained from 0 to 4%/o protein, but a further increase above 6%/o had no noticeable effect on development. The results are discussed in terms of utilizing *Tribolium* in nutritional trials as a bioassay organism to assess protein quality of foods.

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INTRODUCTION

Growing concern of nutritionists and plant breeders with assessing nutritional quality of cereal and of legume food proteins, prompted us to consider the larva of the red flour beetle *Tribolium castaneum* as a small-scale, fast and inexpensive bioassay organism. Likewise, the food industry could also benefit if such a method could be developed. Its potential value and advantages in feeding trials have already been ascertained by earlier reports (1-4). In addition, increasing information in the literature[2] generated by extensive use of this organism as an experimental animal in many biological areas of research, has contributed in making it of promising value in nutritional studies.

In most nutritional trials with *Tribolium,* there has been some uncertainty as to what are the appropriate levels of dietary protein to use. Consequently, studies have been performed either without standardizing the level of protein in the diet (1, 2), or with fixed, arbitrarily chosen levels of protein (3, 4).

Some of these uncertainties on the protein requirements of *Tribolium* have arisen from suggested optimum levels for larval growth in the range of 24 to 38% (5) up to 48% (6) protein utilizing casein as the source. However, an improvement of more than 50% in larval growth (3, 7) or a reduction of more than 50% of the protein level with no change in larval performance has been observed (6) after supplementing the diets with 1 to 5% yeast. The synergistic effect conferred by unknown fractions of yeast (6), suggested in studies (8) with *Tenebrio molitor* to be inorganic zinc and possibly potassium, is in agreement with evidence (3) that casein for *Tribolium* nutrition, in comparison with other proteins, is deficient in substances provided by both a mineral mixture and yeast. In conjunction with the above, the importance of an adequate balance and amount of minerals in *Tribolium* diets has been demonstrated (3, 9).

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2 Tribolium Information Bulletin, edited by A. Sokoloff, California State College, San Bernardino, No. 1-17 (1958-1974).

In *Tenebrio molitor,* optimal larval growth has been reported (10) in the range of 2 to 32%/o dietary protein on a diet of protein isolated from autoclaved soybean. These results lead Davis and Sosulski (10) to suggest a 10%/o protein level as satisfactory in nutritional testing with this organism. In young rats, 10%/o protein in the diet is normally used in protein efficiency ratio (PER) determinations, and a range from 0 to 8%/o protein is accepted for relative protein value (RPV) determinations in assessing nutritional quality of foods. However, these values cannot be directly extrapolated to *Tribolium* in addition to the above - mentioned uncertainties in relation to the protein requirements of the organism. And, it is of primary importance in developing a nutritional quality assay with *Tribolium,* to have an appreciation of protein levels that would yield maximim sensitivity in test trials.

The present study was undertaken to determine the effects of different concentrations of dietary protein provided by an optimum growth media for *Tribolium castaneum* on growth and development of the insect.

MATERIALS AND METHODS

The *Tribolium castaneum* population used in this study was obtained from Dr. G.A.E. Gall, University of California at Davis, who designated (11) it "the foundation stock". The stock has been maintained by taking a monthly 48-hour egg collection from approximately 1000 adults to yield the next generation. All cultures are maintained in mason jars containing standard *Tribolium* media of 90%/o unbleached white wheat flour and 10%/o dried Torula yeast (diet 90-10) and housed in a refrigerator converted into an incubator at 33 $\pm$ 2°C and 70 $\pm$ 5%/o relative humidity.

Growth studies of the insect (11-13) and work preliminary to the present report indicated that the highest growth rate of the larvae is realized in the range of 10 to 14 days of age, counting day zero as the egg collection day. Therefore, this age range was selected as an appropriate and sensitive stage to perform growth response studies to different diets.

Larvae for nutritional studies were obtained by taking an 8 to 10-hour egg collection from approximately 500 adults under the same environmental conditions as the foundation stock.

When the larvae reached 8 days of age they were removed from the mass culture and randomly placed in groups of 70 to 100 larvae in 3 x 4 cm vials containing 4 to 5 g of the different test diets. After two days of this adaption period, the larvae were sieved from the media, five replicates of 10 larvae each were weighed to the nearest hundreth of a mg, and each replicate placed in a 2 x 5 cm shell vial containing 1 to 2 g of their respective test diet. When the organisms reached 14 days of age their weights were recorded again and then returned to their corresponding vial. Subsequently, at ages 18, 20, 22 and 24 days the developmental stage of surviving organisms in the diet was assessed by dumping the contents of each vial on a sheet of paper, recording the number of larvae, pupa and adults present, and then returning all the contents back to their respective vial.

The weight measurements were analyzed statistically by analysis of variance and differences between means were detected by least significant difference tests (L.S.D.); the discrete developmental observations were analyzed with χ^2 - tests of independence.

In the present study the foregoing general procedure was replicated two times, experiments one and two, with the following variations: In Experiment I the larvae were weighed and placed directly in their test diets at 10 days of age, without an adaptation period prior to the diet, and kept in this environment until 24 days of age, undergoing the different manipulations outlined in the general procedure. In Experiment II the larvae had a two-day period of adaptation to their test diets before weighing at 10 days of age, and were discarded after weighing at 14 days of age.

Nine different test diets were utilized in the study, containing 0, 2, 4, 6, 8, 10, 12, 14 and 16% protein. Their composition and preparation procedures are outlined in Table I. The diet was modified from Chirigos *et al.* (3) as follows: a) soybean oil was substituted for corn oil; b) a mineral mixture described by Medici and Taylor (9) was substituted for Drackett's mineral mixture No. 446; c) vitamins were added in a 10:1 ethanol:water solution 1.0 ml to 10 g of finished diet, instead of utilizing a 1:2 ethanol:water solution 2.0 ml to 10 g of diet, since it was found that the addition of

TABLE I

Preparation and composition of experimental test diets[1]

% of diet	Procedure
0 to 16	Protein 2
	Corn starch (filler)
2.0	Mineral premix[3]
	↓
	mortar
	↓
0.63	Vitamin premix[4] in 10:1 ethanol:water 1 ml per 10 g of diet
	↓
	mortar
	↓
	air-dried
	↓
1.0	Cholesterol
	+
3.0	Soybean oil dissolved in 2 ml of chloroform and 2 drops of ethanol per 10 g of diet
	↓
	mortar
	↓
	air-dried 24 hr
	↓
	sieved (80 mesh)
	↓
	stored in cold

1 Diet modified from Chirigos *et al.* (3), see text.
2 Provided by a mixture of 90% unbleached white wheat flour and 10% Torula yeast (diet 90-10, 18.54% protein).
3 Mineral control mixture of Medici and Taylor (9).
4 Vitamin premix (Chirigos *et al.* (3)).

more water than that utilized, created a sticky paste with the wheat flour, and d) the required amounts of cholesterol and soybean oil were dissolved in 2.0 ml of chloroform and two drops of 95°/o ethanol for every 10 g of diet.

Our experience with *Tribolium* husbandry indicates that for succesful utilization of the organism in feeding trials, conditions in the rearing environment should be maintained as constant as possible, and the organisms should be handled quickly and skillfully to avoid disturbances such as dehydration and injuries to their bodies.

RESULTS AND DISCUSSION

The effect of dietary protein on larval growth in Experiment II of the study is shown in Figure 1. Growth response was linear from the 0 to 6°/o protein level. The linear regression equations for weight gain on protein level describing this portion of the curve were: $Y_1 = 0.324 + 0.245\ X$ and $Y_2 = 0.113 + 0.258\ X$ for Experiments I and II, respectively. No significant difference between the slope of the regression lines was detected. Experiment I had a significantly ($P < 0.001$) higher intercept; since the larvae in this group had no adaptive period to the test diet prior to the ten-day weight, the prolonged stay in the culture media may have accounted for the observed additional residual growth, that shifted the growth response curve upwards.

Maximum growth rate was observed in the region from 0 to 2°/o protein level, pointing out the sensitivity of *Tribolium* larva to detect small changes in the amount of protein in the diet at very low levels of protein. The standard errors of the measurements for 14-day weight and weight gain (Fig. 1) from 0 to 6°/o protein were small, and significant differences at the $P < 0.05$ level were detected between all the values in this range.

The larvae achieved their maximum 14-day weight at the 8°/o protein level, although the weight gain obtained from the 6 to the 8°/o level was at a decreasing rate compared to the earlier linear growth period. The organisms did not utilize for growth the additional amount of protein provided above the 8°/o level.

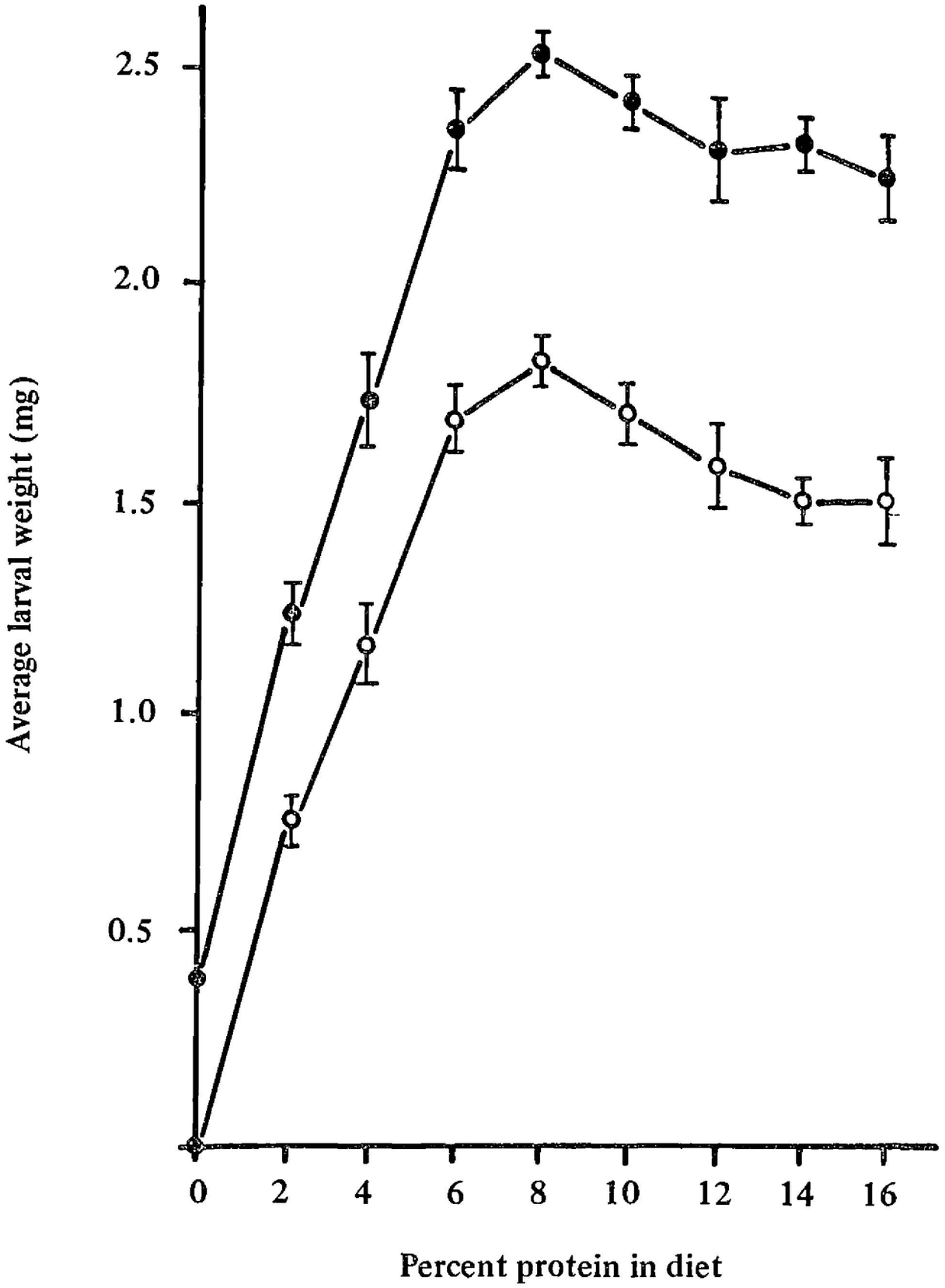

Fig. 1 Effect of dietary protein on larval weight at 14 days of age (⊢●⊣), and on growth response from 10 to 14 days of age (⊢○⊣) in *Tribolium castaneum.* Values are mean ± SE, n=8 (Data from Experiment II of the study).

The posibility that this last phenomenon was an artifact caused by mineral toxicity was ruled out, since even at the 16°/o protein level the amount of cations provided by the 2°/o mineral premix and yeast in the diet were below the toxic levels calculated from values suggested (9) for *Tribolium confusum.* In addition, the growth response observed in both experiments of this study at the 8°/o protein level and onwards, was as high or higher than the weight gain observed in our preliminary growth studies, where the larvae were kept in the plain 90-10 diet without any additional minerals.

As in other holometabolous insects, through the larval stage the main purpose in the life of *Tribolium* is to eat and grow in order to reach a threshold size where enough metabolic reserves have been accumulated to pupate and assure survival to the adult stage. As mentioned earlier, the *Tribolium* strain used in this study achieved its highest larval growth rate from 10 to 14 days of age. Consequently, food intake during this intense growth period approaching pupation is very high. The amount of food consumed per day is approximately the same as body weight (14). All these factors have contributed in making the organism very sensitive to the quality of the diet, as reflected in growth response and subsequent development.

A limiting effect of the protein level on the development of *Tribolium* (Table II) was detected at all ages studied at the 0 and 2°/o levels ($P < 0.01$), and after the 20th day the 4°/o protein level showed a significant effect. In general, an increase above the 6°/o dietary level of protein had no noticeable effect on development.

This observation showed that significant differences between feeding levels of 0 to 6°/o protein may be detected by checking the developmental stage of the organism through several days during the time they undergo their critical metamorphic changes. These results parallel the 14-day weight and weight gain observations in Figure 1. Above the 6°/o level the organisms achieved very similar 14-day weights, reflected in their similarity of development at a later stage, thus confirming the observations of Chirigos *et al.* (3) that larval weight at a fixed age close to pupation is inversely and highly correlated ($r= 0.92$) with the number of days to pupation.

The developmental criterion for assessing quality of the diet with *Tribolium,* although it extends the assay period some additional

TABLE II

Effect of dietary protein on *Tribolium* development[1]

Protein in diet	Age in days 18				20				22				24			
%	D[2]	L	P	A	D	L	P	A	D	L	P	A	D	L	P	A
0	24	76	0		40	60	0	0	40	60	0	0	40	60	0	0
2	2	96	2		14	78	8	0	14	68	16	0	20	42	32	6
4	6	66	28		6	28	66	0	8	20	48	24	14	14	14	58
6	4	52	44		6	28	64	2	8	26	18	48	8	26	0	66
8	2	72	26		4	24	46	26	6	22	4	68	8	20	0	72
10	6	66	28		10	20	44	26	10	20	6	64	14	16	0	70
12	8	66	26		8	26	30	36	14	20	0	66	24	10	0	66
14	6	52	42		18	26	38	18	22	22	0	28	30	20	0	50
16	2	66	32		6	30	28	36	8	26	2	64	24	16	0	60

1 Results represent percentage of adults (A), pupa (P), larva (L), and dead (D) organisms, found in five replicate samples of ten individuals each (data from Experiment I of the study).

2 No. of dead calculated as: 50 - No. of live forms.

Groups enclosed by a continuous line are not significantly different ($P < 0.10$).

days and is a little less precise than the weights criterion, is very simple to obtain and requires less equipment. Other authors (1-4) have confirmed these same observations in independent tests.

On the basis of the foregoing results the following conclusions were reached:

1. The optimum dietary protein level for maximal larval growth is achieved close to the 8%/o level.

2. To perform test of nutritional quality of a given protein at a constant level, such as PER determinations with rats, a level of 4 or 6%/o protein seems satisfactory.

3. To utilize a regression approach, such as RPV with rats, levels of protein in the range from 0 to 6%/o would be appropiate. The 8%/o protein level could also be included with little differences in the results.

4. The weight method, measuring either weight gain from 10 to 14 days of age, or the 14-day weight after placing the larva 4 days in the test diet are very sensitive ways to detect differences in nutritional quality between diets, and the trial can be completed in a short period of 4 to 6 days.

5. The developmental method, although not as sensitive as the weight method, and requiring six or eight additional days, is useful as a further confirmation of the latter, or, it can also be used under circumstances where the appropiate weighing equipment is not available. With this method the mean length of larval or pupal periods can be utilized as criteria to differentiate diets (1, 2), or the percent of larval, pupal or adult forms present at a fixed age-day.

6. It should be stressed that the *Tribolium* assay may prove to be of great significance in plant breeding programs where comparisons of nutritional quality of many lines could be carried out rapidly and utilizing only a few grams of sample.

REFERENCES

1. Loschiavo, S. R., A. J. McGinnis and D. R. Metcalfe. Nutritive value of barley varieties assessed with the confused flour beetle. *Nature 224,* 288 (1969).

2. Metcalfe, D. R., S. R. Loschiavo and A. J. McGinnis. Evaluation of cereal cultivars for feeding value with the confused flour beetle. *Tribolium confusum. Can. Ent. 104,* 1427 (1972).

3. Chirigos, M. A., A. N. Meiss, J. J. Pisano and M. W. Taylor. Growth response of the confused flour beetle, *Tribolium confusum* (Duval), to six selected protein sources. *J. Nutr. 72,* 121 (1960).

4. Applebaum, S. W. and A. M. Konijn. The utilization of starch by larvae of the flour beetle, *Tribolium castaneum. J. Nutr. 85,* 275 (1965).

5. Chirigos, M. A. Nutritional studies with the insect *Tribolium confusum* (Duval). Doctoral Thesis, as cited by R. Charbonneau and A. Lemonde (6) (1957).

6. Charbonneau R. and A. Lemonde. Unidentified growth factors in brewers yeast. I. Necessity of these factors for *Tribolium confusum* Duval. *Canad. J. Zool. 38,* 87 (1960).

7. Hogan, G. R. Development of *Tribolium castaneum* (Coleoptera: Tenebrionidae) in media supplemented with pyrimidines and purines. *Ann. Ent. Soc. Am. 65,* 631 (1972).

8. Fraenkel, G. Zinc and another inorganic growth factor required by *Tenebrio molitor. Fed. Proc. 16,* 386 (1957) (abstract).

9. Medici, J. C. and M. W. Taylor. Mineral requirements of the confused flour beetle, *Tribolium confusum* (Duval). *J. Nutr. 88,* 181 (1966).

10. Davis, G. R. F. and F. W. Sosulski. Protein nutrition of *Tenebrio molitor* L. XVI. Effects of dietary protein concentration on larvae of race. *F. Arch. Internat. Physiol. Biochim. 81,* 661 (1973).

11. Gall, G. A. E. Biochemical genetics of *Tribolium*. I. The foundation population. *J. Exp. Zool. 173,* 225 (1970).

12. Englert, D. C. and A. E. Bell. Genetic differences in the growth curve of *Tribolium castaneum. Growth 27,* 87 (1963).

13. Medrano, J. F. and G. A. E. Gall. Growth rate, body composition, cellular growth and enzyme activities in lines of *Tribolium castaneum* selected for 21-day pupa weight. *Genetics 83,* 379 (1976).

14. Medrano, J. F. and G. A. E. Gall. Food consumption, feed efficiency, metabolic rate and utilization of glucose in lines of *Tribolium castaneum* selected for 21-day pupa weight. *Genetics 83,* 393 (1976).

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