Influence of Dietary Lipids on Survival of *Phyciodes phaon* Butterflies (Lepidoptera: Nymphalidae)[1]

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Abstract The Phaon crescent butterfly, *Phyciodes phaon* (Edwards), can be reared by placing newly-eclosed larvae on a pinto bean-based artificial diet; however, the adults fail to reproduce. Addition of 10% (w/w) of freeze-dried leaves of the host plant, *Phyla nodiflora* (L.) Greene, to the artificial diet approximately doubled the number of adults produced, and females oviposited viable eggs. Addition of wheat germ oil, linseed oil, or olive oil to the artificial diet without host plant tissue also increased survival to the adult stage, but adult females failed to oviposit. Larvae and adults reared from the artificial diet with addition of any one of the oils contained a higher quantity of linolenic acid in their body lipids than those reared on the artificial diet without additional oil. Leaves of the larval host plant are especially rich in the polyunsaturated fatty acids, linoleic acid and linolenic acid; whereas, the pinto bean diet is relatively low in these fatty acids, especially linolenic acid. Addition of synthetic β-sitosterol, stigmasterol, and campesterol, the sterols identified in the host plant leaves, to the artificial diet improved neither larval nor adult survival, and adult females did not oviposit. The factor (or factors) present in host plant leaves and acquired during larval feeding that enables reproduction of adults remains to be identified.

Key Words *Phyciodes phaon* Lepidoptera, Phaon crescent butterfly, dietary lipids, dietary sterols, artificial diet, pinto bean diet

Artificial diets have been developed for rearing only a few butterflies. Webb and Shelton (1988) published a diet for rearing the cabbageworm butterfly, *Pieris rapae* (L.), and commercial, semiartificial diets (Bio-Serv, One 8th St., Frenchtown, NJ 08825) are available for rearing the painted lady butterfly, *Vanessa cardui* (L.) and monarch butterfly. Artificial diets for one or more butterflies might be useful to schools for teaching developmental biology, conservation, and ecology. Diets for butterflies also might benefit butterfly houses that raise and/or display butterflies for the public. Ecological and behavioral traits of a butterfly that may facilitate development of an artificial diet include year-round activity, lack of diapause, available supply of the natural host plant for initial colony development and study, and ease of rearing and maintenance of a colony. The Phaon crescent, *Phyciodes phaon* (Edwards), is a small colorful butterfly that meets these criteria; it is active all year in Gainesville, FL, ovipositing on the underside of leaves of *Phyla nodiflora* (L.) Greene, a small creeping ground-cover plant, and it mates and oviposits readily in the laboratory or in outdoor screen cages. After preliminary trials with several diet formulas, we decided to con-

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centrate our efforts on a pinto bean diet used by Guy et al. (1985). A pinto bean-based diet was developed by Shorey and Hale (1965) for rearing the cabbage looper, *Tricoplusia ni* (Hübner) and other noctuid moths, and various modifications in components and preparation have been published (Vail et al. 1973, Guy et al. 1985). A colony of Phaon crescents was maintained continuously on the host plant, *P. nodiflora*.

Genc (2002) found that a few Phaon crescents can be reared on a completely artificial diet (Guy et al. 1985), designated herein as the PB diet, composed of pinto bean meal, wheat germ, torula yeast, casein, vitamins, antifungal and antimold agents, and a gelling agent. Adult Phaon crescent females reared from larvae feeding on the PB diet, however, did not oviposit (Genc 2002), and survival to the adult stage was poor.

Addition of 10% (w/w, 5.1 g) of freeze-dried host plant leaves to the PB diet composition increased about 2-fold the number of adults successfully reared compared to PB diet (Genc 2002); adults mated and females oviposited on the host plant. Thus, factors from host plant leaves needed by the adult butterflies are acquired during larval feeding.

Some adults reared from larval feeding on the PB diet had crumpled wings that were not completely expanded, possibly symptomatic of a deficiency in polyunsaturated linoleic and linolenic acids known to be essential fatty acids for some insects, including Lepidoptera (Fraenkel and Blewett 1946, Grisdale 1973). Sterols also are essential dietary nutrients for insects, and cholesterol typically is added to diets to satisfy the sterol requirement (Gilbert 1967). The PB formula does not require addition of lipids as such, but sterols and other lipids may be present in the pinto bean meal, wheat germ, and torula yeast.

No information is available on the fatty acids and sterols in the larval host plant *P. nodiflora*. Phytophagous insects usually convert plant sterols into cholesterol (Svoboda et al. 1975, Feldlaufer et al. 1995). A few insect species, however, are known to have an absolute requirement for a particular sterol in their food (Kircher et al. 1967, Norris and Baker 1967, Mondy and Corio-Costet 2000).

Herein, we report that adding various oils to the PB diet increased larval survival and adult emergence; however, addition of sterols was without effect. We also made quantitative measurements of the major fatty acids and sterols in the host plant, and the fatty acid composition of Phaon crescent larvae and adults reared on the PB diet and on the host plant.

Materials and Methods

Insects and host plant. A colony of *P. phaon* was maintained in the laboratory at 27°C on a 16:8 (L:D) cycle on the host plant, *P. nodiflora* collected from the University of Florida campus and vicinity. Newly-eclosed larvae were picked up with a camel-hair brush and deposited on artificial or semi-artificial diets in experiments.

Artificial diets. We used the pinto bean (PB) diet formula employed by Guy et al. (1985) comprising pinto bean meal (19 g), wheat germ (14 g), torula yeast (8 g), casein (7 g), gelcarin (3 g), methyl paraben (0.5 g) and sorbic acid (0.3 g). These components were mixed and added to 182 ml cold water with stirring in a mechanical mixer. The mixture was stirred continuously and heated slowly to 70°C on a hot plate. One ml formaldehyde was added and stirring was continued without heating for about 3 min; 0.9 g ascorbic acid was added, and after 3 additional min without heat, we

added tetracycline (0.01 g), BioServ Vitamin Mix #F8095 for Lepidoptera (0.8 g), and propionic acid (0.3 ml). This procedure made about 250 ml of diet. The mixture was poured into paper cups and allowed to cool and gel at room temperature. The gelled diet was stored in a refrigerator until needed.

A major diet modification to the aforementioned PB formula was the addition of 10% freeze-dried host plant leaves. A second modification was the addition of 0.75 ml of wheat germ oil, olive oil, or linseed oil. Wheat germ oil was purchased from Nutritional Biochemicals Corp., linseed oil from BioServ, and olive oil from a local supermarket. A third modification tested was addition of sterols to the PB diet. Synthetic sterols (β-sitosterol, stigmasterol, and campesterol) purchased from Nutritional Biochemicals Corp. were dissolved in diethyl ether and added to the dry casein component of the PB diet. After ether had evaporated thoroughly in a hood, the dry casein-sterol mixture was added to the other dry PB components and the diet processed as above. Newly-eclosed larvae (50 per replicate, 3 replicates per diet test) were added to diets and the number of adults reared and ability of females to lay eggs were recorded as measures of diet quality. Most diet trials were not replicated over time; if a diet modification (typically in 3 replicates run simultaneously) did not improve adult production or promote reproduction, we abandoned that modification. With each diet trial, however, we always included 3 replicates with 10% host plant tissue in the diet as a positive control, and PB diet with no modifications as a negative control.

Extraction of lipids. Mature, last instars were removed from each diet and starved 24 h before lipid extraction to eliminate gut contents. They were subjected to extraction of lipids or frozen until extraction. Adults were frozen within 24 h after eclosion for later extraction. Frozen samples were held only a few days at −20°C before extraction. Lipids were extracted from fresh host plant leaves, the dry components of the artificial diet, and from Phaon crescent larvae and adults by the method of Folch et al. (1957). Approximately 1 to 2 g of fresh or frozen plant or animal tissue were extracted with 20 ml of chloroform:methanol (2:1, v/v) by grinding the insects or leaves in a mortar. The chloroform:methanol extract was filtered through Whatman #1 filter paper into a separatory funnel, and an additional 2 ml of chloroform:methanol solvent was poured over the residue and added to the filtrate. The chloroform:methanol extract was shaken in a separatory funnel with an equal volume of 3% aqueous sodium chloride solution to remove methanol and small water soluble molecules. The bottom chloroform layer containing lipids was saved, and a small quantity of anhydrous sodium sulfate was added to dry the extract. The lipid extract was filtered to remove the sodium sulfate and evaporated to dryness with a gentle stream of nitrogen. Fatty acids were liberated from complex lipids and transesterified by the procedure of Luddy et al. (1960) as follows. Samples were saponified overnight with methanolic KOH (1 ml of 0.5 N KOH:2 ml methanol). After standing overnight at room temperature, samples were acidified with approximately 1 ml 0.5 N HCl, about 1 ml deionized water was added, and the fatty acid methyl esters were formed with the methanol already in the samples. The esters were extracted into pentane with gentle shaking. The pentane layer was removed and dried with a small amount of anhydrous sodium sulfate, and 1 to 2 μl were injected into a gas chromatograph for analysis.

Isolation of sterols. Sterols in the lipid extract of the host plant were partially purified by thin layer chromatography (TLC) on glass plates coated with silica gel G. Approximately 5 to 10 μl of lipid extract in chloroform were applied to the TLC plate in each of a series of spots. In order to concentrate the sample on the TLC plate, multiple applications of sample were made, waiting each time until the chloroform of

the previous application had evaporated in order to keep the spots small. Cholesterol was run as a standard to locate the sterol migration. The plate was placed upright in a vessel that contained a shallow layer of chloroform for ascending TLC. Development of the plate in the solvent required about 45 min. The plate was allowed to dry in a fume hood until free of chloroform. A second plate containing known cholesterol and a few spots of the extract was developed in the same chamber, and the sterol position on the plate was located with iodine vapor in an iodine chamber. When spots were evident (usually after 10 to 15 min), the plate was removed from the iodine chamber, and the spots were outlined with a pencil. A band of silica gel at the Rf value for the cholesterol standard was scraped from the plate into a small beaker, and sterols were extracted with a few ml of pentane.

Gas chromatography. Fatty acid methyl esters (FAMEs) were analyzed on a Varian model 3700 gas chromatograph equipped with a flame ionization detector. The methyl esters were separated by a 4-mm × 1.3-m column packed with 10% Silar 10C coated on 100/120 mesh Gas Chrom Q. The column was held at 140°C for 30 min, and then programmed to 150°C at 5°C per min. Injector and detector temperatures were 170 and 180°C, respectively. Nitrogen was used as a carrier gas with a flow rate of 30 ml/min. Identification of the esters was made by comparing retention times with FAME standards (Applied Science Laboratory Inc., State College, PA). FAME quantitative mixture K 108 (Applied Science, C14:0, C16:0, C18:0, C18:1, C18:2, C18:3) showed the chromatographic technique to be ±4% of the expected values.

Sterols were analyzed on a Shimadzu GC 14A gas chromatograph equipped with a flame ionization detector. Injector and detector temperatures were 250 and 320°C, respectively. Helium was used as a carrier gas at a flow rate of 27 cm/s. The capillary column was 0.25 mm × 25 m coated with 0.2 µm thickness of RSL-150 non polar coating. The column was initially at 200°C, and upon sample injection, the temperature increased at 4°C/min to 300°C, with a 20 min hold at 300°C. Identification of the plant sterols was made by comparing retention times with authentic standards (Applied Science Laboratory Inc., State College, PA), and by formation and GC analysis of acetate and trimethylchlorosilyl (TMCS) ether derivatives. Acetate derivatives of the sterols were formed by adding a few drops of acetyl chloride (Fischer Scientific) to a solution of the sterol(s) and holding at room temperature for 1 to 2 h before gas chromatography. The volume was then reduced by a gentle stream of nitrogen gas, water was added, and the sterol acetates extracted into pentane. Trimethylchlorosilyl (TMCS) ether derivatives were made by adding about 50 µl of the TMCS reagent (Applied Science Laboratory, Inc.) to sterol samples. The TMCS derivatives formed immediately, and the samples were chromatographed without further treatment. The sterols in the host plant tissue and PB diet were quantified with cholesterol as an internal standard.

Statistical analyses. Binary logistic regression analyses and a Chi-square test (Harrell 2001, Hosmer and Lemeshow 2000) were used to test the hypothesis of no treatment differences in adults produced. When a significant Chi-square value was obtained, the means for adults produced on each diet were separated by transforming them from a non-linear to linear function and a least squares estimate of diet-specific probabilities, **P,** was calculated by inverting the log odds model.

Results

Survival on PB diet with added oils. Survival of larvae to the adult stage was enhanced by addition of linseed oil (78% adults), olive oil (76% adults), or wheat germ

oil (79% adults) to the basic PB diet formula. Percent adult production was not statistically different among the oil-fortified diets nor from percent of adults produced on PB diet plus 10% host plant leaves (88% adults) (Table 1). The PB diet with no host plant leaf tissue produced 56% adults, which was statistically different from all other diets in Table I (χ^2 = 33.59, df = 4, P = <0.001). Even though each of the oils improved survival to the adult stage, adult females did not lay eggs. Only adults reared on the living host plant or on PB diet with 10% host plant leaves were able to reproduce.

Fatty acid composition of larvae and adults and their diet. The fatty acids in the larvae and adults were C14:0 (myristic, a trace amount) C16:0 (palmitic), C16:1 (palmitoleic), C18:0 (strearic), C18:1 (oleic), C18:2 (linoleic) and C18:3 (linolenic acid) (Table 2). The predominant fatty acids were C16 and the three 18 carbon fatty acids. Individuals reared on PB diet with olive oil, wheat germ oil, or linseed oil had about the same fatty acid patterns with increased amount of C18:3 linolenate in their body lipids.

The fatty acid that is most influenced by diet composition is C18:3, linolenic acid. This polyunsaturated fatty acid varied from 1.6 to 4% of the total fatty acids in adults and larvae, respectively, produced on PB diet, to as much as 30% or more in larvae and adults produced on PB diet with added oils, PB diet with 10% host plant leaves, or living host plant leaves (Table 2). Fresh tissue of *P. nodiflora* (n = 5 samples) contained the following fatty acids, with each expressed as a percent of the total fatty acids: C14:0, 0.95%; C16:0, 21.4 % ± 3.1%; 18:0, 1.2% ± 0.3%; C18:1, 4.0% ± 3.8%; C18:2, 15.0% ± 1.7%; and C18:3, 58.9% ± 5.9%. Freeze-dried leaves (data not shown) had similar percentages of fatty acids as did fresh leaves. Our data show that the host plant is especially rich in C18:3 fatty acid. The dry components of the pinto bean diet contain C14:0, 1.9%; C16:0, 14.8%; C18:0, 1.9%; C18:1, 14.9%; C18:2, 52.7%, and C18:3, 13.7%. Thus, the PB diet mix is rich in C18:2 fatty acid, but has only a modest amount of C18:3 fatty acid. Phaon larvae feeding upon the PB diet are unable to selectively accumulate the higher level of C18:3 (typically 30% or more of all fatty acids) they acquire from the host plant or from PB diet with added oils.

Plant sterols. The principal sterols in the host plant are β-sitosterol, stigmasterol, and campesterol (Table 3). The same three sterols were detected in the artificial diet

Table 1. Survival and adult emergence of adult Phaon crescents reared on PB diet with the added components indicated

Addition to PB diet	Mean adults per replicate*	Percent adult emergence
Linseed oil	39.0 ± 1.4a	78
Olive oil	38.0 ± 1.4a	76
Wheat germ oil	39.5 ± 0.7a	79
10% dried host plant leaves	44.0 ± 1.4a	88
No addition to PB diet	28.0 ± 0.0b	56

Each of three replicates of each diet received 50 neonates.

* Analyses of means by Minitab revealed that Chi-square = 33.59, df = 4, P < 0.001, indicating that production of adults varied significantly among means. Means were separated by pair-wise comparison of binary logistic regression coefficient, and means in the same column followed by the same letter are not different at α = 0.01 level.

Table 2. Fatty acid methyl esters (FAMEs) in body lipids of Phaon crescent larvae and adults as influenced by diet. Newly-emerged larvae were placed on each diet. Larvae taken for FAME analysis were in the last instar; adults were newly emerged. N = number of replicate samples

	FAMEs in larvae reared on the following diets (percent of total fatty acids)				FAMEs in adults when larvae were reared on the following diets (percent of total of total fatty acids)			
	Living host plant	PB diet	PB + linseed oil	PB + 10% host plant	Living host plant	PB diet	PB + linseed oil	PB + 10% host plant
FAME	N = 5	N = 6	N = 6	N = 6	N = 9	N = 11	N = 11	N = 11
C14:0	t*	t	t	t	t	t	t	t
C16:0	25.5	25.8	14.5	24.5	18.8	23.5	16.6	21.7
C16:1	t	13.9	7.7	4.6	t	10.0	3.8	6.5
C18:0	1.7	4.9	4.9	8.9	1.3	6.3	2.1	9.6
C18:1	11.3	26.9	24.9	16.7	12.6	37.2	36.7	25.6
C18:2	16.8	20.6	17.5	10.0	18.1	19.9	10.9	17.3
C18:3	44.0	4.0	23.3	33.8	48.0	1.6	29.7	18.9

* t = trace, too small for integrator to measure.

Table 3. The retention time from gas chromatography (GC), percent of total sterols found in tissue, and µg/g in tissue of β-sitosterol, stigmasterol, and campesterol found in fresh *Phyla nodiflora* leaves and in the dry components of the PB diet

	Phyla nodiflora fresh leaves			PB diet		
Sterol	RT (min)	% of total	µg/g	RT (min)	% of total	µg/g
β-sitosterol	27.4	55.6	105.7	27.4	60.0	3.1
Stigmasterol	26.2	27.7	52.6	26.3	20.1	1.0
Campesterol	25.7	16.7	31.8	25.7	19.9	1.0

mix, but in much lower quantity (Table 3). The artificial diet was enriched by adding enough β-sitosterol, stigmasterol and campesterol to approximate the amount in the host plant, but the percent of larvae reaching the adult stage on the augmented diet was not significantly different from that of adults produced on the PB diet, and females failed to reproduce. Thus, addition of sterols did not improve the artificial diet.

Discussion

The fatty acid composition of Phaon crescents reared on their host plant or PB diet containing 10% host plant tissues is not unusual. Many lepidopterans and other

insects have a fatty acid pattern like that of the Phaon crescents in this study, with C16:0, C18:1, C18:2, and C18:3 as the main fatty acids (Schaefer 1968, Ito and Nakasone 1967, Levinson and Navon 1969, Chippendale and Reddy 1972, Cookman et al. 1984). The diet consumed by Phaon larvae clearly influences the level of fatty acids, particularly the polyunsaturated ones, in the body lipids of both larvae and adults. A relatively inexpensive addition of a nutritive oil to the PB diet substantially increased the percentage of polyunsaturated fatty acids in the lipids of larvae and adults, and increased the percentage of adults reared, although these adults did not reproduce. The high level of C18:3 in the host plant, and in the PB diet with added oils is reflected in relatively high levels of C18:3 in larvae and adults reared on those diets. The high percentage of C18:3 linolenic acid in the lipids from host plant leaves suggests that the beneficial effect of added oils on adult production is largely due to the increase in available polyunsaturated fatty acids in the diet when one of the three oils is added.

Based on GC retention times of the free sterols, and their acetate and trimethylchlorosilyl ether derivatives, the sterols in the host plant *P. nodiflora* are β-sitosterol, stigmasterol and campesterol. These are common sterols in many plants, and phytophagous insects such as lepidopteran larvae ingest these plant sterols and usually convert them to cholesterol (Svoboda et al. 1975, Feldlaufer et al. 1995). These same sterols are present in low levels (about 5 μg/g) in the dry mix of the PB diet, whereas, the host plant is relatively rich in these three phytosterols, with a total of 189 μg/g fresh tissue. Addition of 10% (w/w) host plant tissue (i.e., 5.1 g of freeze-dried plant tissue) to the PB diet could add about 964 μg plant sterols to the PB diet, substantially enriching the dietary sterols. Addition of these three sterols together to the diet gave very poor adult production, and the few females produced did not oviposit. Failure to find benefit either in percent adult reared or in their reproduction from addition of the sterols might be due to poor bioavailability of the sterols. The plant tissue may contain compounds that help emulsify highly insoluble lipids such as free sterols and, thus, aid in their absorption from the midgut, and/or conversion to cholesterol. Additional nutritional and biochemical studies will be required to determine why the host plant is necessary for reproduction of Phaon crescents.

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