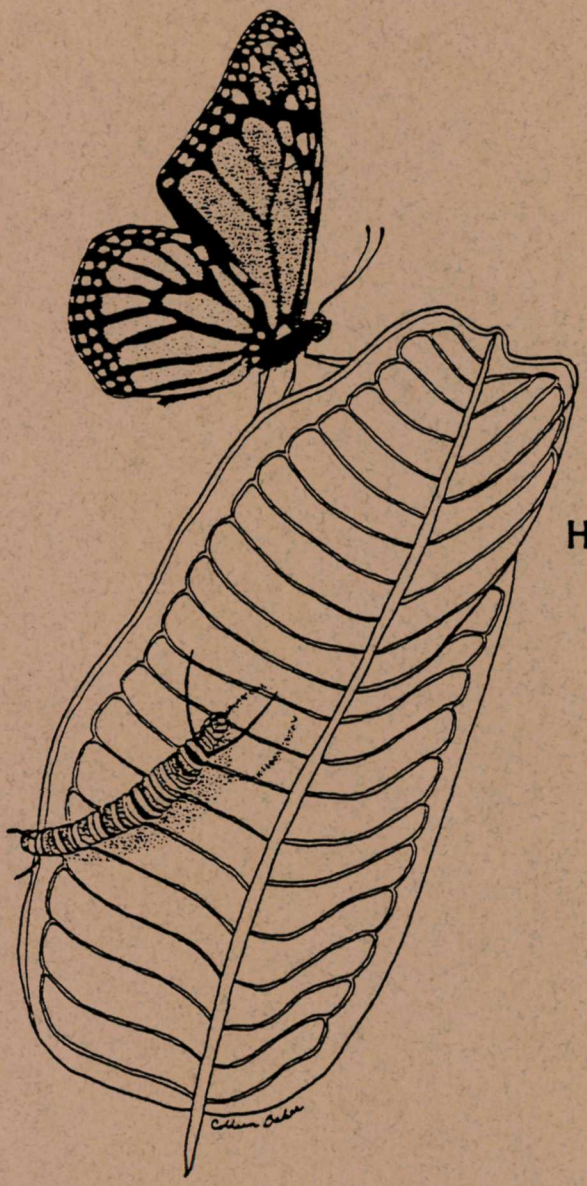
The Effects of Consuming Ozone-injured Milkweed Leaves on Feeding, Growth, and Development of Monarch Butterflies



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THE EFFECTS OF CONSUMING OZONE-INJURED MILKWEED LEAVES ON FEEDING, GROWTH, AND DEVELOPMENT OF MONARCH BUTTERFLIES

by

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The influence of ozone air pollution on the plant-insect relationship of the monarch (*Danaus plexippus*) and two of its host plants, common milkweed (*Asclepias syriaca*) and bloodflower (*Asclepias curassavica*), was investigated under controlled environment conditions. Preliminary fumigations of bloodflower with ozone (up to 0.15 ppm) revealed considerable changes in physiological parameters such as primary and secondary metabolites that are relevant to insect nutrition. Soluble sugars were found to be significantly decreased whereas amino acid and total phenol concentrations were significantly increased.

Based on these experiments, a series of insect assays were performed to test changes in the feeding preference, growth, and nutritional indices of monarch larvae on ozone-treated host plants. In dual-choice tests, a strong feeding preference of 3rd instar larvae was noted for ozone-treated *Asclepias curassavica* leaves, whereas 4th instar larvae showed no preference between treatments on either host plant. Visible damage (i.e., purple stippling) did not deter the feeding larvae. Larvae fed fumigated tissue throughout their development grew and developed significantly faster than the corresponding controls fed on untreated host plants. Relative growth rate (RGR) and relative consumption rate (RCR) were greatest on fumigated foliage of either species, but none of the other nutritional indices (ECI, ECD, OR AD) was altered by fumigation. Regression of RGR against RCR showed the lines for insects fed ozone-treated leaves to be the same as those for insects fed control leaves. These results indicate that the greater rate of growth on fumigated leaves is due primarily to a greater rate of consumption (i.e., ozone increased "acceptability" of the host more than it did "suitability"). While overall larval performance seemed to be improved on ozone-treated plant tissue in these tests, we have no information on the long-term effect of these changes on population dynamics of this insect. Also, no information is available concerning effects on adult fecundity or longevity.

The results of this study warrant further investigation with this plant-insect system in the field, with open-top chambers. In ambient air under natural conditions additional environmental factors could considerably amplify changes in plant metabolites and result in a greater effect on monarch larvae. Also, effects of ozone-induced changes in plant reproduction and/or senescence could be investigated.

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16. Abstract (Limit: 200 words)

The influence of ozone air pollution on the plant-insect relationship of the monarch butterfly (Danaus plexippus) and two of its host plants, common milkweed (Asclepias syriaca) and bloodflower (Asclepias curassavica), was investigated under controlled environment conditions. Preliminary fumigations of bloodflower with ozone revealed considerable changes in physiological parameters relevant to insect nutrition. Based on these experiments, a series of assays tested changes in the feeding preference, growth, and nutritional indices of monarch larvae on ozone-treated host plants. A strong preference of 3rd instar larvae was noted for ozone-treated Asclepias curassavica leaves, whereas 4th instar larvae showed no preference between treatments on either host plant. Visible damage (purple stippling) did not deter feeding larvae. Larvae fed fumigated tissue throughout their development grew and developed significantly faster than the corresponding controls fed on untreated host plants. Relative growth rate and relative consumption rate were greatest on fumigated foliage of both species, but no other nutritional indices were altered by fumigation. No effect was observed on duration of pupal stage or final dry weight of adults.

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ABBREVIATIONS USED IN TEXT

PAR .... photosynthetically active radiation in $\mu E\ m^{-2}s^{-1}$

T ..... temperature in degrees Celsius

RH ..... relative humidity in percent

CSTR ... continuous flow stirred tank reactors

RuBPCO ribulosebisphosphate carboxylase/oxygenase

O_3 ..... ozone

ppm .... parts per million; multiply by 1.997 to obtain $mg \cdot m^3$ O_3 at 20°C and standard air pressure (= $ml \cdot l^{-1}$)

ppb .... parts per billion; multiply by 1.997 to obtain $\mu g \cdot m^3$ O_3 at 20°C and standard air pressure (= $\mu l \cdot l^{-1}$)

CHO ... soluble carbohydrates

aa ..... amino acids

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THE EFFECTS OF CONSUMING OZONE-INJURED MILKWEED LEAVES ON FEEDING, GROWTH, AND DEVELOPMENT OF MONARCH BUTTERFLIES

1. RESEARCH OBJECTIVES AND APPROACH

Plant-herbivore relationships represent a dynamic equilibrium between plant defense against herbivory and insect feeding adaptation on host plants. Recent studies have shown that environmental stresses such as air pollution can shift the balance of these relationships (Hughes 1988). Ozone, a major pollutant in civilized regions and elevated altitudes, shows insidious effects on plants. Visible foliar damage is merely the end of a long process in which the plant has attempted to adjust to the environmental stress. However, before the appearance of symptoms, drastic metabolic changes have occurred within the plant, largely as a result of scavenging and detoxification processes. Therefore, the question arises as to whether these metabolic changes might affect the plant-insect interaction significantly, especially because many of these changes involve metabolites upon which insects depend.

The primary objectives of the research conducted were (1) to study the biochemical effects of ozone injury on two milkweed species, and (2) to determine the indirect effect of ozone on monarch performance under controlled laboratory conditions.

Plants of two milkweed species, the bloodflower (*Asclepias curassavica*) and the common milkweed (*Asclepias syriaca*), were subjected to various levels of ozone to produce the visible symptoms encountered in natural habitats (i.e., purple stippling; Duchelle and Skelly 1981). At the same time, primary and secondary metabolites (soluble sugars, proteins, amino acids, cardenolides, starch and phenols) were analyzed to reveal chronic and "invisible" changes in the fumigated plants that could be relevant to herbivore nutrition or behavior or both. This phytocentric approach allows a more profound understanding of the interaction of the air pollutant with the plant than is provided by other approaches; eventually, both the observed and the potential effects on such herbivores as monarch larvae can be much better discussed and understood. Based on these investigations of changes in plant chemistry, a variety of insect assays were performed to assess the effects of ozone on feeding and growth of monarch butterflies.

2. MATERIALS AND METHODS

2.1 HOST PLANTS AND BUTTERFLIES

2.1.1 Asclepias curassavica (Bloodflower)

For the preliminary exposure experiments, *Asclepias curassavica* plants were propagated from stem cuttings in sand. Because the analysis of foliage showed no important difference in data homogeneity between seed-grown plants and cloned plant material, and because the propagation from stem cuttings produced plants of uneven growth and shape, all subsequent plants were grown from seeds (Thompson and Morgan Ltd., Ipswich). Cuttings and seedlings were transferred after six weeks to 5-in pots. Cornell Mix A (Boodley and Sheldrake 1977) containing 40 g Osmocote 9-6-12 (N-P-K) per kg mixture was used initially as a soil substrate.

Following transfer from the greenhouse to the CSTR fumigation chambers, the plants developed symptoms of nutrient deficiency; this problem was traced to insufficient development of the roots under the conditions of growth in the greenhouse. A change of soil substrate to Redi-Earth™ and a careful fertilization regime with Osmocote Sierrablend after sufficient root development in the greenhouse yielded healthy plants that remained vigorous even after being moved to the artificial light and high temperature/humidity conditions of the fumigation chambers. All plants were grown in charcoal- and Purafil-filtered air to remove common air contaminants including ozone. Temperature and humidity in the greenhouse was held at 28 ± 4°C and 50–70%, respectively. The plants were watered twice per day and fertilized weekly with Peters 20-20-20 (N-P-K) solution (2.4 $g \cdot l^{-1}$). Supplemental lighting was provided by metal halide high intensity discharge lights.

2.1.2 Asclepias syriaca (Common Milkweed)

Plants of *Asclepias syriaca* could not be propagated successfully from stem or root cuttings on a routine basis. Therefore, seeds (Seed Service, Woodstock, Illinois) were stratified for at least one month at 4°C in humid and sterilized vermiculite. This type of stratification was the only procedure of those tried that enabled successful germination of the seeds after purchase; any type of scarification (acid, mechanical scratching or breaking of testa) or biochemical treatment (ethylene, giberellic acid) was not satisfactory. The best germination was obtained from freshly collected, naturally (winter) stratified wild seeds (Wildflower Garden, Cornell, Ithaca). The seeds were germinated in vermiculite and transplanted twice to Redi Earth™ in 5-in pots. After the roots were nicely developed, the plants were given 4 g Osmocote Sierrablend every two weeks.

2.1.3 Danaus plexippus (Monarch Butterfly)

For the insect assays, a colony was established from two pairs of monarch butterflies obtained from Dr. L.P. Brower. Once established, the colony was divided into two groups whose generations overlapped to provide insects at all times. The adults were kept in an insect cage (0.6 x 0.6 x 0.6 m) in the greenhouse at 28 ± 3°C and were fed with a 20% (v/v) honey solution (soaked foam pads). Egg deposition was allowed on either *Asclepias curassavica* or *Asclepias syriaca* seedlings or on a larger *Asclepias curassavica* shoot that had been rehydrated under pressure to overcome wilting. Seedlings with eggs were transferred to a growth chamber (24°C, 60% RH) for hatching while shoots were placed under a glass bell jar with high humidity until hatching occurred. The eggs hatched within three to five days and the larvae were transferred to fresh shoots in food crispers (35 cm x 24 cm x 12 cm). Fresh leaf tissue was provided daily. Moisture buildup in the boxes was kept to a minimum to prevent diseases. When prepupae were first seen (9–10 d), all larvae were transferred into styrofoam cups where they began to migrate up to the lids for pupation. After the adult butterflies had emerged (9–10 d) in the cups, they were put in the insect cage where they reached fertility after another four days. For the insect assays, egg deposition was allowed for 24 h to obtain enough larvae of similar age and reared separately from the colony.

2.2 OZONE FUMIGATION

2.2.1 Fumigation Chambers and Ozone Generation

Plants were exposed in continuous-flow stirred tank reactors (CSTRs, 1.2m^3 each) (Heck et al. 1978) to filtered air and ozone-polluted air (Figure 1). The environmental conditions in the CSTRs were maintained at 28 ± 2°C, 70 ± 15% RH, and 550–600 $\mu E \cdot m^{-2} \cdot s^{-1}$ photosynthetic photon flux density (PPFD) for 16 h per day. The flow rate of the chamber air was about 2.8 $m^3 \cdot min^{-1}$ corresponding to a 2.3-fold air exchange every minute. Ozone (O_3) was generated by an oxygen-supplied discharge ozone generator (OREC 03V5-0 or Griffin GTC-0.5B). The O_3 output from the generator was captured first in a stainless steel cylinder and distributed over a manifold with flow meters to the chambers. Ozone concentration in each chamber was monitored sequentially for a period of 5 min every 20 min by means of a Monitor Labs ozone monitor (Model 8410) connected to a sequential sampling valve (Scanivalve Corp., San Diego, CA). The monitor was calibrated regularly before and after the fumigation experiment to check for baseline shifts. In addition to this, span checks (zero and 0.15 ppm) were performed at least weekly.

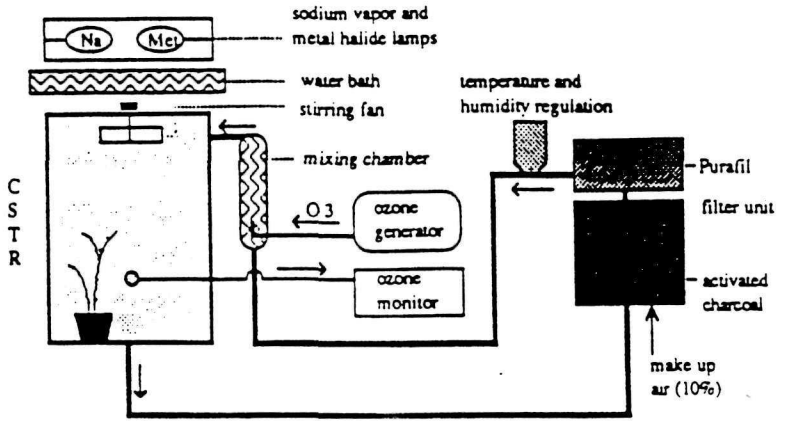


Figure 1. CSTR diagram (one chamber shown).

2.2.2 Summary of Fumigations Conducted

Randomized plants of the same age were exposed daily for 7 h between 0900 and 1700 seven days a week. To assess the effects of ozone fumigation, a series of different fumigation regimes was tried (Table 1). One major problem encountered was to provide real replication for statistical inference. Because the number of replicates (on a chamber basis) was governed by the availability of CSTRs, the degrees of freedom obtained for statistical inference varied from experiment to experiment (see Table 3). In a specifically designed experiment series (fumigations 5, 6 and 10), replicates through time were obtained by repetition of the entire fumigation twice (three fumigations total).

2.3 PLANT ANALYSIS

As part of the study, plant tissue was analyzed in all fumigation experiments to reveal ozone-induced effects on plant biochemistry. Leaf tissue was sampled uniformly using leaf age as one classification in addition to time and ozone concentration. At the same time, visible ozone damage was estimated using either a percentage-based or a twelve-point grading system that takes into account the distinguishing ability of the human eye as a logarithm of the light intensity (Horsfall and Barratt 1945). To assure consistence in the sampling of leaves of the same physiological age, the nodes were numbered (Figure 2). The starting node (node 0) was determined differently for the two plant species. For *Asclepias syriaca*, the leaf pair/node following the distal-most internode measuring more than 1 cm was considered as node 1, whereas for *Asclepias curassavica* the leaf pair/node following the distal-most node in which the angle of the unfolding leaves exceeded 90° was considered as node 1. According to this system, different leaf age classes could be sampled as an additional classification (see fumigation 4). Age class A referred to leaf/node 1, age class B to leaf/node 4, and age class C to leaf 6, and are in the following described also as the youngest/assimilate-importing, mature/exporting, and mature but senescing/exporting leaves.

Table 1. Summary of fumigations conducted during the investigation period.

Fumigation No.	Purpose of fumigation[1]	Plant species[2]	Duration (days)	O_3 applied (ppb)	Number of plants/CSTR	#CSTRs used control/O_3	Biochemical analysis[3]	Age class	Remarks
1	system test	A.c.; A.s.	4	0; 40; 80; 120	10	1/3	Pr	-	stomatal conductance
2	recovery of CHO	A.c.; A.s.; Sb	7	0; 42; 83; 125	10	1/3	C; Pr; Aa	B; V3	-
3	system test	A.c.	4	0; 50; 100; 150	3 / 3	1/3	-	-	cancelled
4	biochem. dynamics	A.c.	1; 2; 4; 8; 16	0; 43; 84; 134	9	1/3	C; Pr; Aa	A; B; C	stomatal conductance
11	biochem. dynamics	A.c.	0; 1; 2; 4; 8; 16	0; 43; 75; 121	9	1/3	C; Pr; Aa; Ca; St; Phe	B	-
5, 6, 10	replication	A.c.	10	0; 23; 43; 65	10	1/3	C; Ca (Aa; Pr)	B	stomatal conductance
7	system test	A.c.	8	0; 30; 50; 80	5	1/3	-	-	GSH/GSSG
8	visible symptoms	A.c.	1	0; 100; 200; 300	10	1/3	-	A; B	-
9	visible symptoms	A.c.; A.s.; Sb	0, 1, 2	0; 100; 200	9	1/2	C	B; V3	-
12	FP; RGR/RCR	A.c.	12	0; 130	10	3/3	C; Pr; Aa	B	preliminary test
13	FP	A.c.	4	0; 130	10	3/3	-	-	cancelled
14	growth & development	A.c.	13	0; 120	15	3/3	C; Pr; Aa	B	-
15	FP	A.c.	10	0; 115	10	3/4	C; Pr; Aa; Ca; Phe	B	-
16	FP; RGR/RCR	A.c.	17	0; 150	10	3/4	C; Pr; Aa; Ca; Phe	B	-
17	growth & development	A.c.	16	0; 180	10	3/4	C; Pr; Aa	B	-
18	FP; RGR/RCR	A.s.	14	0; 165	10	3/4	C; Pr; Aa; Phe	B	-
19	FP; RGR/RCR	A.s.	19	0; 180	10	3/4	C; Pr; Aa; Ca	A; B	-
20	purple stippling	A.s.	4	0; 150-300	4	3/4	Phe	B	histology

[1] FP... feeding preference; RGR/RCR...relative growth and consumption rate.
[2] A.c.: *Asclepias curassavica*; A.s.: *Asclepias syriaca*; Sb: soybean.
[3] C...CHO; Pr...proteins; Aa...amino acids; Ca...cardenolides; St...starch; Phe...phenols.

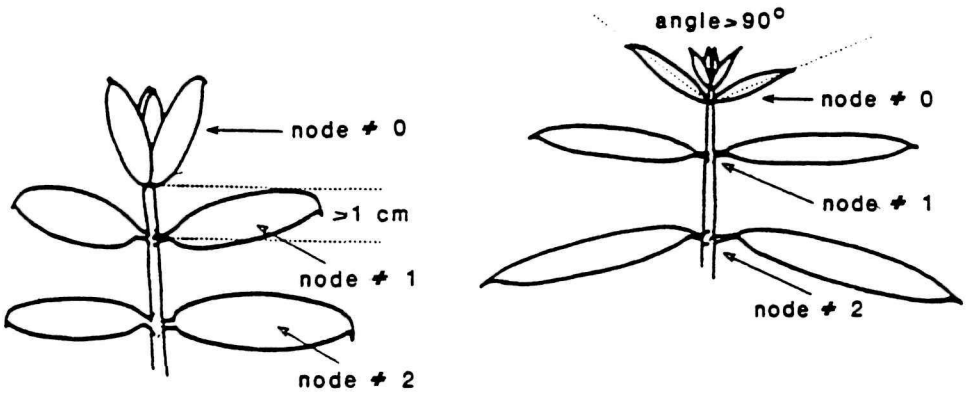


Figure 2. Determination of leaf/node number for sampling of *A. syriaca* (left) and *A. curassavica* (right). See text for more detail.

The sampled leaf tissue was immediately frozen in liquid nitrogen, lyophilized, and stored air-tight in a freezer. Samples with plenty of leaf tissue were ground in a Wiley mill using a 60-μm mesh. Single leaf samples were ground in a mortar using liquid nitrogen. About 1 kg (dw) of *A. curassavica* tissue was ground and served as a standard reference throughout all analyses performed. Samples were analyzed in duplicates and were redone when the difference of the duplicates exceeded 10%. The methods for analyzing the plant tissue are given in Table 2. Some modifications to the methods described in these papers were made, mainly to adjust for the range of the specific compound in the plant species used. Because a variety of extraction solvents (ethanol, methanol) with varying water content are described in literature for soluble sugars and amino acids, an 80% EtOH was used for both compounds after tests proved no difference in extraction yield.

2.4 INSECT ASSAYS

To assess different aspects of the overall performance of monarch butterfly larvae on fumigated plant tissue, a number of different insect assays were applied. The feeding preference test permitted the larvae to choose between eight leaf discs of 1.54-cm^2 area each in a petri dish. Four each were cut from control and fumigated leaves and placed alternately on moist filter paper. For this assay, 3rd and 4th instar larvae were used, because the insects seem to lose selectivity in the later instars (Lewis and van Emden 1986). The tests were run until the larvae had consumed about 50% of the total leaf area offered, which occurred in

Table 2. Analysis of total soluble compounds in plant tissue.

Analysis of	Method	Extraction	Reference
sugars (CHO)	colorimetric, anthrone	EtOH 80%	Yemm and Willis 1954
starch	enzymatic/colorimetric	residue	Haissig and Dickson 1979
proteins	colorimetric	NaOH	Bradford 1976
amino acids	colorimetric, ninhydrin	EtOH 80%	Yemm and Cocking 1955
cardenolides	colorimetric, TNDP[1]	EtOH 95%	Malcolm et al. 1988
glutathione (GSH)	colorimetric, DTNB[2]	EDTA/TCA[3]	Sedlak and Lindsay 1968
phenols	colorimetric, Folin-Denis	acetone	Hagerman 1988

[1]...2,2',4,4'-tetranitrodiphenyl.

[2]...5,5'-dithiobis(2-nitrobenzoic acid).

[3]...ethylene diamine tetraacetic acid (Na_2)/trichloro acetic acid buffer.

15–24 hr. This allowed the maximum resolution in detecting a preference (e.g., the extreme case would be if all of one treatment were consumed but none of the other, leaving 50% of the total leaf area). The remaining leaf area was measured with a leaf area meter and the leaf area consumed was then calculated.

For growth and development studies, first instar larvae were transferred from a synchronized colony to plants in the CSTRs that had already received 4–8 days of fumigation. Thus, the larvae were feeding during the fumigation experiment on intact plants. While this does not permit assessment of direct effects of ozone on the insects, it can be defended (1) as representing what occurs in the field; (2) as being practical (a very extensive facility would be required to include both direct and indirect effects in a single test); and (3) published literature does not show insects generally to be affected by such low concentrations of ozone. After pupation on the plants, the larval development time, pupal weight, pupal development time, and adult weight could be determined.

The nutritional indices served as a means to detect more subtle effects in terms of larval feeding and food conversion to biomass. For this, early 5^{th} instar larvae, which undergo the most pronounced growth rate between all larval instars, were fed with fumigated leaf tissue. Their growth rates were determined by using two different formulas: (1) the relative growth rate (RGR; Waldbauer 1968, modified by Farrar et al. 1989), and (2) the mean relative growth rate (MRGR; Miller and Miller 1986). The formulas differ slightly in their mathematical approach:

$$(1) \quad RGR = \frac{G}{T\,A}$$

G = dry weight gain of larva during feeding period; T = duration of feeding period (hours); A = mean dry weight of larva during feeding period.

$$(2) \quad MRGR = \frac{\ln w_e - \ln w_i}{T}$$

w_i = initial dry weight of monarch larva; w_e = end dry weight of larva after feeding; T = duration of feeding period (hours).

Furthermore, two variations of RGR (and RCR) were calculated. The first method used the geometrical mean weight of the larva as "A" (designated as RGRG or RCRG), while the second method used the initial weight (designated as RGRI or RCRI). Use of the initial weight rather than geometrical mean has recently been recommended for short-term bioassays such as ours in order to better detect differences related to behavioral responses (as opposed to postingestive effects) (Farrar et al. 1989).

To determine consumption and food utilization, (3) the relative consumption rate (RCR) and (4) the efficiency of conversion of ingested food to body substance (ECI), (5) the efficiency of conversion of digested food to body substance (ECD) and (6) the approximate digestibility of food (AD) were determined:

$$(3) \quad RCR = \frac{F}{T\,A}$$

F = dry weight of food eaten.

$$(4) \quad ECI = \frac{w_e - w_i}{F}\ 100$$

w_i = initial dry weight of monarch larva; w_e = end dry weight of larva after feeding.

$$(5) \quad ECD = \frac{w_e - w_i}{F - w_{fec}}\ 100$$

w_{fec} = dry weight of feces.

$$(6) \quad AD = \frac{F - W_{fec}}{F} 100$$

The RCR represents the rate of food consumed per time unit, whereas the ECI is an overall measure of an insect's ability to utilize the food that it ingests for growth. ECI varies with both the digestibility of the food and the proportion of digestible food that is converted to body substance and energy. The ECD provides more information about the use of food for energy, because, for example, the term will decrease as the proportion of digested food metabolized for energy (denominator) increases. The AD finally provides information about the digestibility of the food offered (Waldbauer 1968; Miller and Miller 1986). Initial dry weights of larvae were estimated from calibration lines (one for each host plant). L5 larvae reared on the appropriate host (36 and 41 larvae for *A. curassavica* and *A. syriaca*, respectively) were individually weighed, dried, and reweighed. Dry weight was regressed against fresh weight to provide the calibration line (correlation coefficients were 0.982 and 0.988 for *A. curassavica* and *A. syriaca*, respectively).

2.5 STATISTICAL INFERENCE

2.5.1 Biochemical Changes in Plants Due to Ozone Fumigation

The experiments were laid out in a split-unit design, with chambers as the whole units, where different ozone treatments were applied, and plants as the subunits, with different exposure periods. For one experiment (fumigation 4), there was a further level of subdivision: the age classes as a subunit of the plant.

For each experiment, four plants were used in each exposure time subunit. The number of tissue samples produced in the experiment with one plant sample in each exposure time subunit was too large to analyze all at once. Therefore, the analysis was done in four blocks with one plant from each exposure time subunit in each block to prevent analytic errors. For the analysis of total phenols, which was only done in fumigation 11, it was necessary to pool the four plants in each exposure time subunit. Hence, we do not have an experimental error term for this analysis, but by pooling the first two and the second two exposure periods, we were able to obtain an estimate of the experimental error.

With four levels of ozone and five (fumigation 4) or six (fumigation 11) exposure periods (i.e., fumigation days), we were able to fit response surface models for each of the variables measured (CHO, amino acids, etc.). None of the plant constituents showed responses that were more complicated than could be fit with a quadratic response surface; most of them could even be well described by simpler models. For all response variables, the effect of ozone could be well described by a straight line (i.e., regression line), if the exposure period was held constant.

Because there was no error term for ozone concentration (i.e., there was no replication on a chamber basis), even the "within subunit" error terms were not usable to derive an error term for the simple effect of ozone when exposure time is held constant (Table 3).

Table 3. Error terms and degrees of freedom (DF) in a multiway ANOVA in fumigations 4 and 11.

	Source	df
Block A	Ozone concentration	3
	Error A	0
Block B	Fumigation period	4
	Ozone concentration x fumigation periods	12
	Error B	60
Block C	Age class	2
	Ozone concentration x age class	6
	Fumigation period x age class	8
	Ozone concentration x fumigation period x age class	24
	Error C	118
Corrected total		237

To interpret the results of the experiments, a linear regression analysis of the means of age class and fumigation period was run against ozone concentration. For these regressions, there are several possible error terms. One possible approach is to run each regression against ozone concentration individually, where each age class/fumigation period combination would be a separate regression. Since there would be only two degrees of freedom for the error of each regression, the ability to detect effects of ozone with such an analysis would be very low. Also, the 15 regressions could not be pooled because the three age classes are not independent; therefore, the age classes were not pooled. However, the plants can be

considered as independent because they were taken out of the chambers on different days. The form of the regression line is:

$$\hat{Y} = \bar{y} + b(x - \bar{x})$$

$\hat{Y}$ is predicted value; $\bar{y}$ is mean value of analyzed compounds (CHO, amino acids, etc.);

$\bar{x}$ is mean ozone concentration amoung four treatments; x is ozone concentration to predict $\hat{Y}$.

Where a significant interaction between the linear effects of exposure period and the linear effect of ozone concentration could be detected, a regression line was fit separately for each exposure period. Although the five or six regressions run for each response variable are not independent, they do answer the question of primary interest, i.e., the effect of ozone. Each regression alone is valid and has a valid error term. Because these regressions are not independent, the different regression slopes cannot be compared.

Two different approaches are used to present the results in this report. First, a simple regression analysis of each block is presented as a scatterplot in conjunction with information about the regression line (slope, intercept, and probability value of the slope to be significantly different from zero). When statistically significant regressions were found, the computed regression line was drawn in the scatterplot. Secondly, the results are summarized in 3-D surface plots (quadratic) for a better overview of the treatment effects. Because the latter presentation serves only as a visualization of the data, no mathematical description of the surface is given.

2.5.2 Insect Assays

The feeding preference assays required more than a paired sample t-test because there were only three control chambers as opposed to four treatment chambers, and, to make six pairings to increase the replication size, both leaves at the node were used from control plants. Strictly speaking, these opposite leaves are not independent. Therefore, the randomized pairings for the preference trials were analyzed for a serial correlation structure of the pairings. For *Asclepias curassavica*, it was assumed that the mean correlation between the opposite leaves (mean of all plants within one chamber) was the same in all chambers. Therefore, the mean difference of the feeding preferences between control and ozone treatment could be corrected by an estimate of the correlation between the pairings. For *Asclepias syriaca*, the pairing scheme required a weighted analysis to account for different variances caused by unequal sample sizes.

For both host plants, a weighted ANOVA, using the sample size as weight, was performed for statistical analysis of the metabolites in the leaf tissue samples. For *A. curassavica*, two independent fumigation experiments (15 and 16) were averaged, whereas for *A. syriaca*, only one fumigation experiment was available (18).

Finally, the growth and development tests and the assays for the determination of the nutritional indices food consumption tests were analyzed with t-tests (estimated s^2).

3. RESULTS AND DISCUSSION

3.1 PHYSIOLOGICAL ASPECTS OF OZONE FUMIGATION

Treatment of the host plants with ozone produced considerable physiological and biochemical changes. It became clear in the course of the investigations that the extent of ozone impact depended strongly on a variety of factors other than ozone concentration alone (see Darrall 1989 for further discussion). Light quality and quantity and plant nutrition proved to impose most of the problems encountered, such as poor plant growth, changes in root:shoot ratio and nutrient deficiency/toxicity symptoms. At the same time, extensive purple stippling of ozone treated leaves could not always be obtained before ozone-induced premature leaf senescence, therefore making correlation studies between the development of visible symptoms and changes in plant metabolism or insect performance almost impossible.

3.1.1 Humidity and Gas Exchange of Plant

In the initial fumigations, the relative humidity (RH), a major factor controlling the stomatal aperture (Fitter and Hay 1987), was kept too low (<60%) during fumigation. Consequently, the stomatal conductance was relatively low and the plants showed no symptoms or only very slight symptoms. Because ozone also causes the stomates to close (Table 4), even less ozone was allowed to enter the leaf mesophyll. As Reich and Amundson (1985) have shown, tree species with higher stomatal conductances had a higher potential for pollutant uptake and thus exhibited greater negative responses to ozone treatment. Therefore, RH was kept between 75 and 90% in our subsequent fumigations so as to increase gas exchange and to represent the more susceptible end of the spectrum such as would be common in southern and eastern United States.

Table 4. Effect of ozone on stomatal conductance (s/cm) of different-aged leaf pairs of *Asclepias curassavica* after 1 h and 7 h of fumigation on the 14th day of fumigation.

$[O_3]$	Control	0.134 ppm	2P < (t-test)
After 1 h fumigation			
Leaf pair/node #1 (age class A)	0.717	0.635	0.1692
Leaf pair/node #4 (age class B)	0.748	0.507	0.0077
After 7 h fumigation			
Leaf pair/node #1 (age class A)	0.584	0.273	0.0001
Leaf pair/node #4 (age class B)	0.386	0.180	0.0003

3.1.2 Plant Nutrition

Nutrition seems to be another important factor influencing the response of the plant to ozone but is still subject to scientific controversy (Darrall 1989). In our first experiments we used a low-nutrient substrate (Cornell Mix A) consisting mainly of peat moss. To improve plant growth and vigor, we later switched to Redi Earth + Osmocote slow-release fertilizer. Plants grown under the higher nutrient conditions were found to be far less sensitive to ozone. Therefore, the subunit "low and high nutrition" (LN-plants and HN-plants) is included in the following discussion of the results. These are grossly defined categories because the nutrient mixes as well as the soil medium were different and not well controlled.

3.1.3 Lighting

The plants also responded drastically to changes in light intensity and light quality, especially when underfertilized. The change of irradiance from the greenhouse to the CSTR environment required the plant to adjust its photosynthesis, resulting in a new net photosynthesis rate (Grange 1987). Further, the assimilate translocation rate seems also to be affected by changes in radiant energy input. Because adjustment of the assimilate translocation rate is much slower than the adjustment of the net photosynthesis rate, starch and sucrose may accumulate in the leaves (Troughton et al. 1977). As light quality mediated effects, short internodes, small leaves and even chlorosis of older leaves were identified and are considered to be caused by the overabundance of red light (red/far red = 2.4–2.6; slightly overcast sky = 1.4–1.5)

(Keiller and Smith 1989) by affecting the phytochrome photoequilibrium. Red light seems also to inhibit the uptake of nutrients, especially iron, in the rhizosphere, which might explain the nutrient deficiency symptoms found in the first experiments (Amundson, pers. comm.). Consequently, the plants were pretreated with additional artificial light in the greenhouses for phytochrome equilibration, and, together with a careful fertilization regime, the plants seemed to do much better in the environment conditions of the CSTRs.

3.1.4 Purple Stippling and Premature Senescence

The development of visible symptoms was strongly dependent on the factors discussed in sections 3.1.1 to 3.1.3. Hence, only the plants that had undergone low fertilization showed bronzing and strong purple stippling of the upper leaf surface (Table 5), whereas well-fertilized plants showed almost no visible symptoms. At the same time, premature senescence advanced rapidly when leaves became stippled.

Table 5. Estimation of purple stippling and senescing of low-fertilized *Asclepias curassavica* plants after 15 d of fumigation (7 h/d).

$[O^3]$	0.0 ppm	0.043 ppm	0.084 ppm	0.134 ppm
Purple stippling range (%)	0	0–20	21–40	61–80
Senescence-aborted leaves (%)	20.3	26.8	52.6	47.5

3.2 BIOCHEMICAL CHANGES IN *Asclepias curassavica* DUE TO OZONE FUMIGATION

Our first full-scale fumigation experiment (fumigation 4) was performed with plants in Cornell Mix A (low fertilization; LN-plants). All three age classes of leaves were investigated. The experiment was repeated later (fumigation 11) with the modification of using Redi Earth in combination with a fertilization regime (high fertilization; HN-plants). This time, only age class B, the most relevant for insect feeding, was investigated. Both experiments were designed to yield information about the dose–response relationship of ozone on

plant metabolites (as compared with the appropriate low- or high-fertilization control plants). In addition to these experiments, a series of three identical 10-day fumigations with very low ozone concentrations was conducted to obtain replication through time (fumigations 5, 6, and 10).

3.2.1 Soluble Carbohydrates (CHO) and Starch

Ozone fumigation of plants resulted in a consistent depletion of soluble carbohydrates in the leaf tissue. Statistically, there was a significant interaction between the linear effects of exposure period and ozone concentration for CHO (Figures 3 to 5). The significant linear regression of CHO against ozone level was not observed in the low level fumigations of LN-plants (Figure 6). Except for an apparent increase in starch with increasing ozone after the first period of fumigation, starch concentration was not significantly affected (Figure 7).

All age classes investigated showed significant decreases in soluble carbohydrates after four days of fumigation (Figure 3). The effect occurred earlier in the youngest leaves (two days) and was strongest after eight days (i.e., a total of 48 h of ozone fumigation) in middle-aged leaves. The surface plots illustrate the general trend of CHO-depletion with increasing ozone concentration (Figure 4).

The effect of ozone on carbohydrate metabolism has been well studied in the past for several species of plants and has been reviewed recently by Koziol et al. (1988). The impact of ozone on net carbon gain ranges from inhibition of gas exchange through stomatal closure, reduction of the photosynthetically active leaf surface through cell damage (purple stippling), and increase of maintenance respiration to inhibition of the CO_2-fixation process as a result of a limitation in the amount of ribulosebisphosphate carboxylase/oxygenase (RuBPCO) present in the leaves (Amthor and Cumming 1988; Lehnherr et al. 1987, 1988; Rowland-Bamford et al. 1989). A decrease in CHO accompanying increased respiration following ozone fumigation has been reported for other plant species (e.g., Leone et al. 1966; Leone and Brennan 1970). It is therefore not surprising to find a decrease in soluble, nonstructural carbohydrates in ozone-treated milkweed.

Apart from the ozone-mediated effects that are nicely expressed in their positive linear regression with increasing ozone concentration, the leaves seemed generally to contain most soluble CHO after eight days of fumigation (Figure 4). Beyond this point, the CHO generally became depleted over all treatments, as seen in the surface plot. Because this coincided with the development of nutrient deficiency symptoms of the leaves, the trend might be a consequence of nutrient deficiency in combination with adjustment of the photosynthetic rate and increased CHO-allocation to the roots. That the plants in well-fertilized substrate (Figure 5) seemed to respond similarly but with a delay of about one week supports this possibility. Also, these plants did not respond with visible damage of the upper leaf surface (see 3.1.4). Nevertheless, the effect of ozone upon CHO concentration in the leaves seemed not to be altered much except for the delay by this period of time.

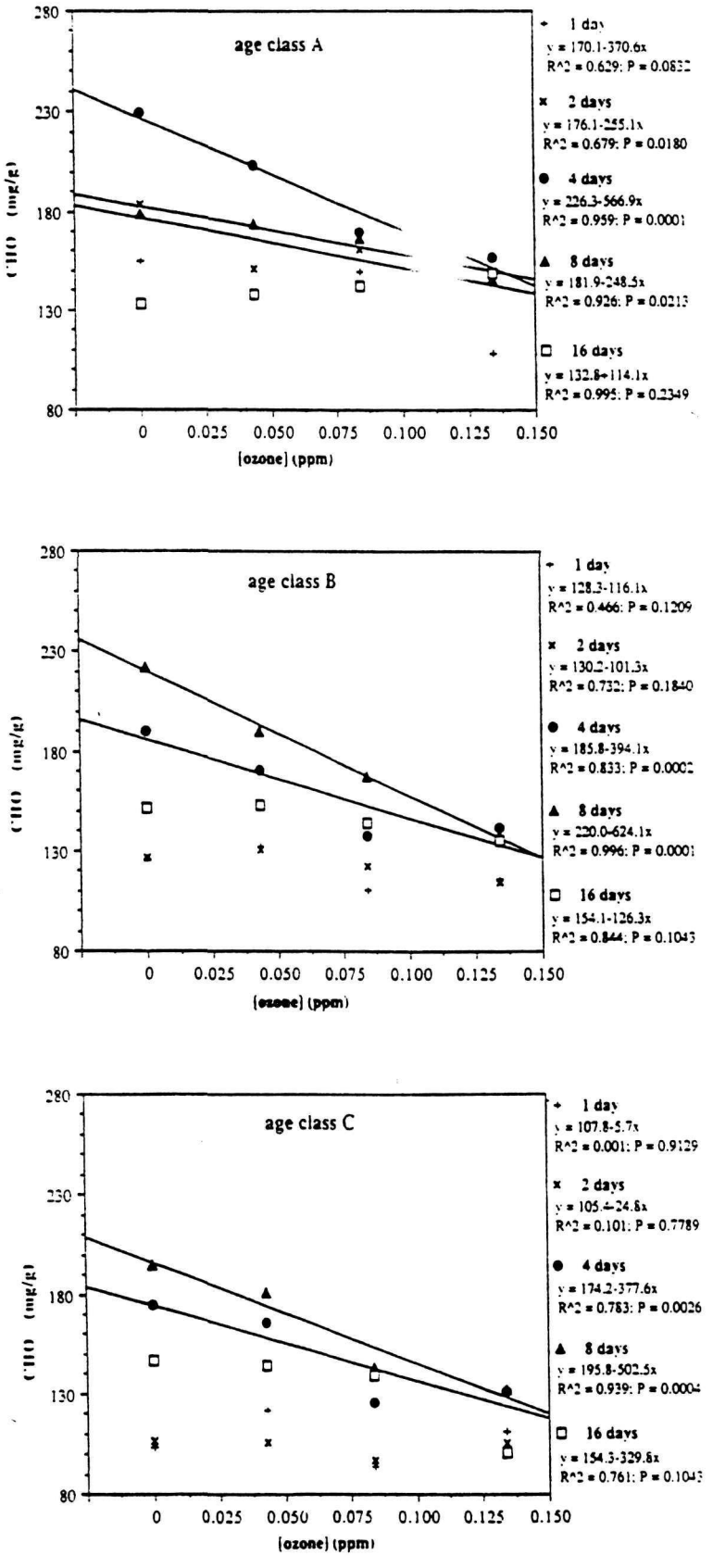


Figure 3. Soluble carbohydrates (CHO) in leaves of different age classes (A: young/importing, B: mature/exporting, C: mature/senescing) after 1, 2, 4, 8, and 16 d of fumigation with ozone in low-nutrient soil substrate (fumigation 4; only significant regression lines are shown).

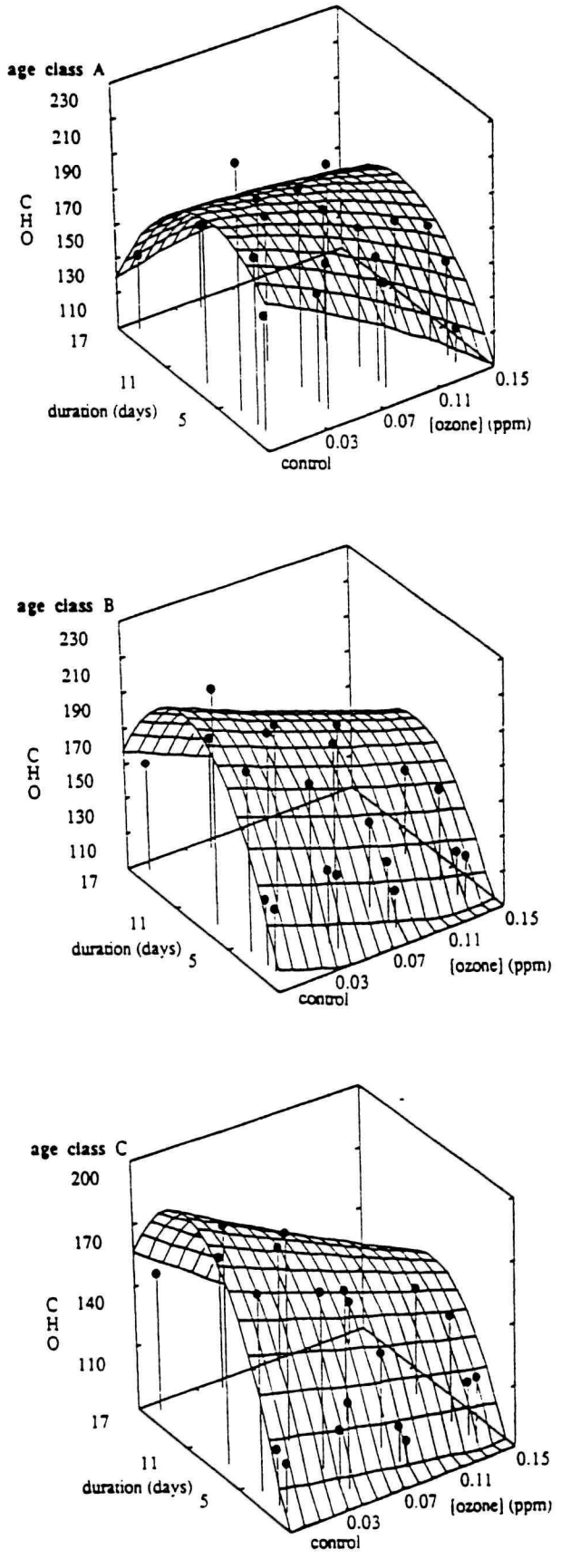


Figure 4. Interaction between the effect of exposure duration (days of fumigation) and ozone concentration on soluble carbohydrate concentration in leaves of *Asclepias curassavica* grown in low-nutrient soil substrate (fumigation 4; quadratic surface plot).

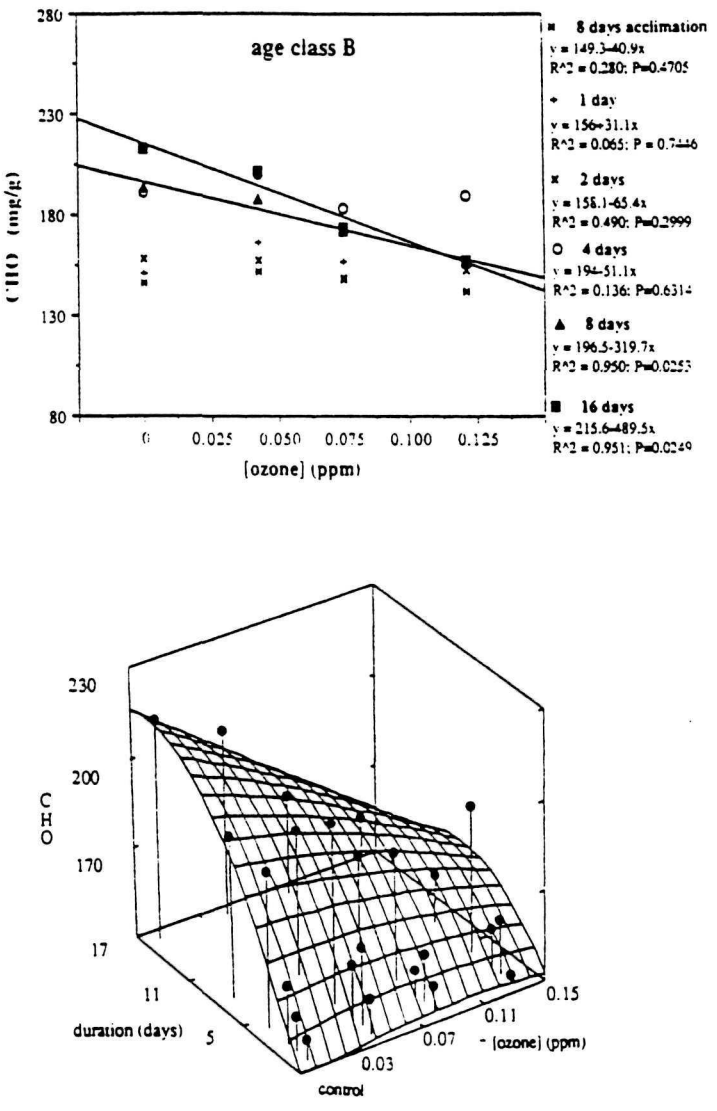


Figure 5. Top: Soluble carbohydrates (CHO) in leaves of age class B (mature/exporting) after an acclimation period of 8 d (=0), and 1, 2, 4, 8, and 16 d of fumigation with ozone in high-nutrient soil substrate (only significant regression lines shown). Bottom: Interaction between the effect of exposure duration (fumigation days) and ozone concentration on soluble carbohydrate concentration in leaves of *Asclepias curassavica* grown in high-nutrient soil substrate (quadratic surface plot).

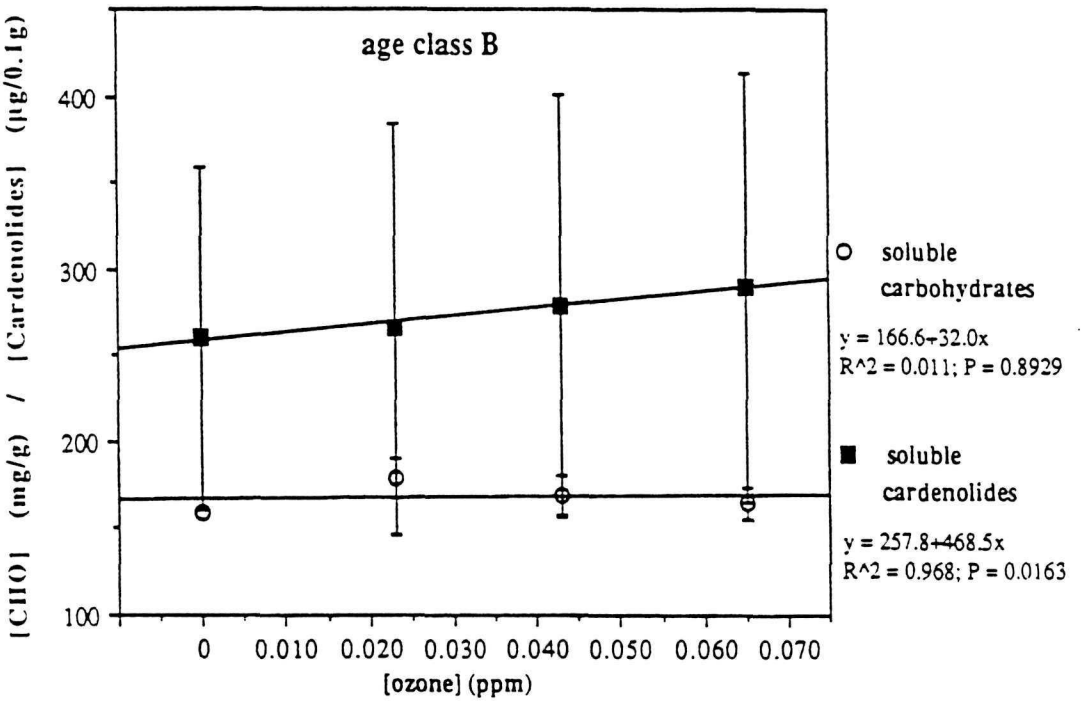


Figure 6. Effect of low level ozone on soluble carbohydrates (CHO) and cardenolides in mature leaves of LN-plants (fumigations 5, 6, and 10).

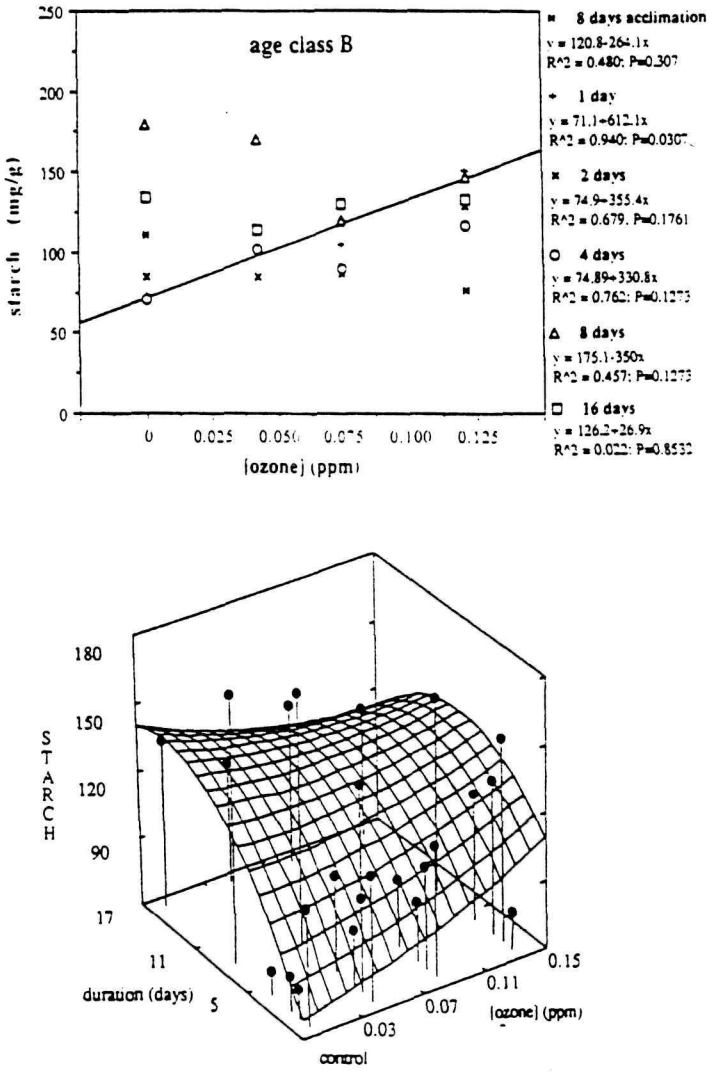


Figure 7. Top: Soluble starch in leaves of age class B (mature/exporting) after an acclimation period of 8 d (=0), and 1, 2, 4, 8, and 16 d of fumigation with ozone in high-nutrient soil substrate (only significant regression lines shown). Bottom: Interaction between the effect of exposure duration (fumigation days) and ozone concentration on starch concentration in leaves of *Asclepias curassavica* grown in high-nutrient soil substrate (quadratic surface plot).

3.2.2 Proteins and Amino Acids.

For the soluble proteins, no significant interaction between ozone level and exposure periods was noted except in the youngest leaves (age class A) of LN-plants (Figures 8 and 9), where a significant increase in protein concentration after 16 d of fumigation was observed. Again, the surface plots reveal a linear effect of time, with soluble protein content gradually decreasing as the experiment progressed. This effect has also been observed in other studies in which controlled environment chambers were used (Beckerson and Hofstra 1979). In high-nutrient plants (Figure 10), the slope values of 8 and 16 d of fumigation, although above $p = 0.05$, indicate a decreasing trend of protein concentration in middle aged leaf tissue as ozone level was increased. Also, high-nutrient plants contained overall about 20% more protein than low-nutrient plants. The overall depletion trend through time was not so clearly visible in high-nutrient plants.

In contrast to the proteins, some linear response of the amino acid concentration to ozone could be found in low-nutrient plants (Figure 11). For the youngest leaf age class, a significant increase could be detected after 7 h of fumigation. Surprisingly, there was no significant effect during the next six days, but on the 8^{th} and 16^{th} days of ozone treatment there was again a significant linear relation with amino acid concentration greater at the higher ozone concentrations. For the other age classes, the effect was only significant on the 8^{th} day of ozone treatment. As observed for proteins, there was a general decrease in amino acids with time (Figures 12 and 13) that was unrelated to fumigation. No effect of ozone on amino acid concentration could be observed for high-nutrient plants (Figure 13) and the general depletion of amino acids with time was not significant, indicating that the trend can be countered with better nutrition.

Proteins are structural components of cells, act as major H^+ ion buffers, and many have enzymatic activity. Oxidation of the highly reactive amino acid residues, which provide the molecular bonding to form the secondary and tertiary structure of the protein, can result in protein breakdown (Rowland et al. 1988). Either the concentration of free amino acids or free proteins has been reported to increase after ozone fumigation (Tingey et al. 1973; Beckerson and Hofstra 1979) and increase is considered to be a direct effect of the breakdown of structural proteins (Rowland et al. 1988).

In contrast, a decrease in protein concentration due to ozone air pollution has been observed by Agrawal et al. (1985) on soybeans and was considered to be the result of a decrease in the rate of protein synthesis or an increase in the rate of protein degradation, or both. Watanabe and Kondo (1984) found a decrease in proteinase inhibitors when treating leaves with hydrogen peroxide. This might lead to an increased activity of protease which catalyzes proteolysis, which has been associated with leaf senescence. Considering the amount of proteins in leaf tissue, it is quite possible that more subtle changes in total protein concentration cannot be detected and, therefore, serve as a immediate explanation of the increase in amino acids found.

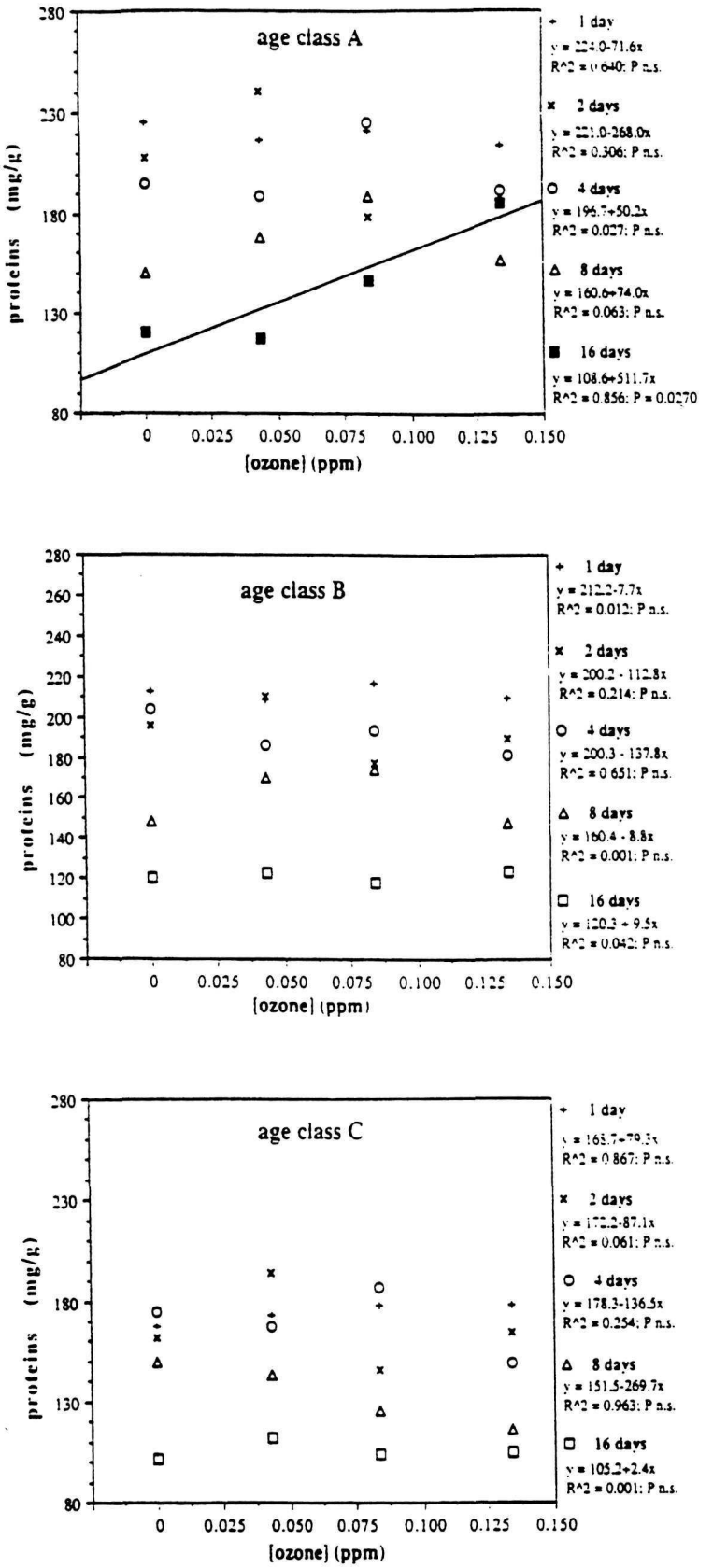


Figure 8. Soluble proteins in leaves of different age classes (A: young/importing, B: mature/exporting, C: mature/senescing) after 1, 2, 4, 8, and 16 d of fumigation with ozone in low-nutrient soil substrate (only significant regression lines shown).

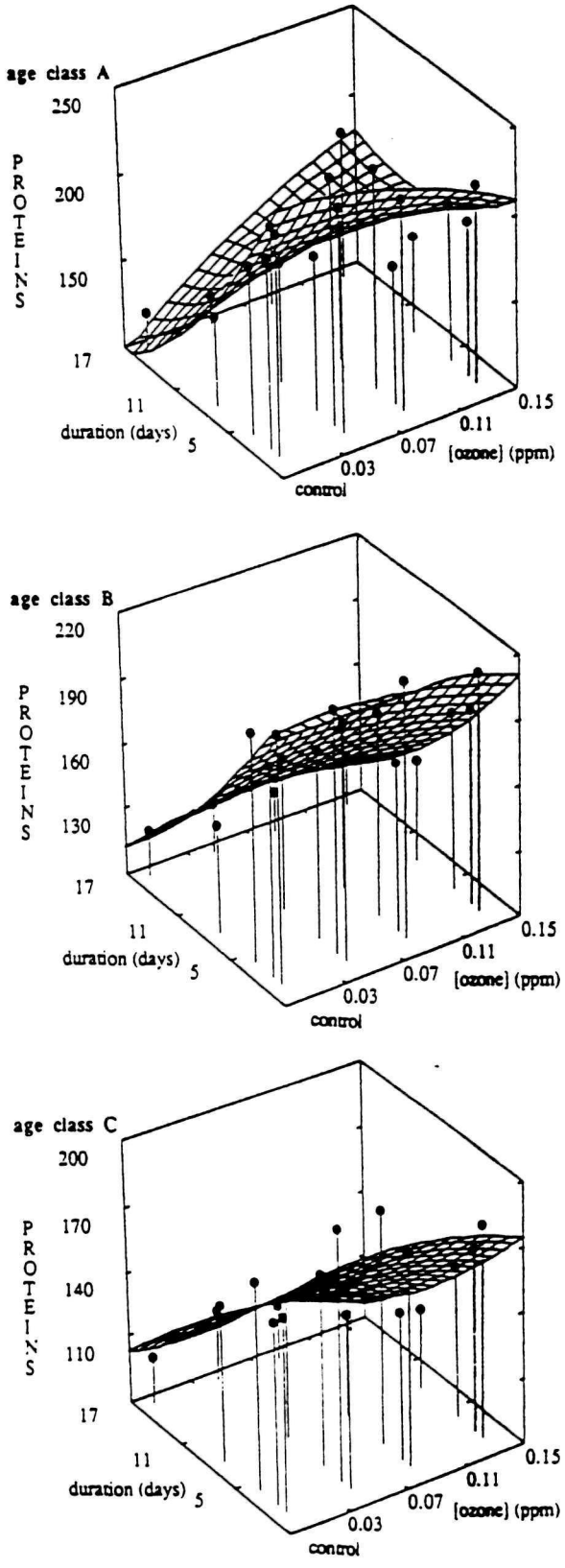


Figure 9. Interaction between the effect of exposure duration (fumigation days) and ozone concentration on soluble protein concentration in leaves of *Asclepias curassavica* grown in low-nutrient soil substrate (quadratic surface plot).

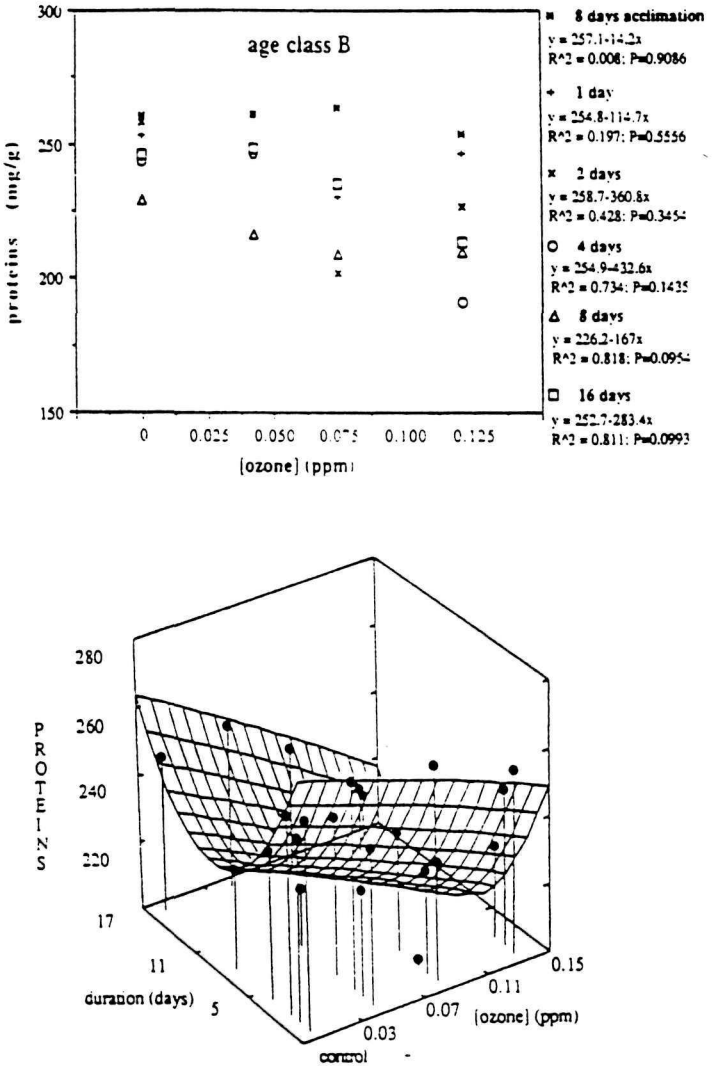


Figure 10. Top: Soluble proteins in leaves of age class B (mature/exporting) after an acclimation period of 8 d (=0), and 1, 2, 4, 8, and 16 d of fumigation with ozone in high-nutrient soil substrate (only significant regression lines shown). Bottom: Interaction between the effect of exposure duration (fumigation days) and ozone concentration on soluble protein concentration in leaves of *Asclepias curassavica* grown in high-nutrient soil substrate (quadratic surface plot).

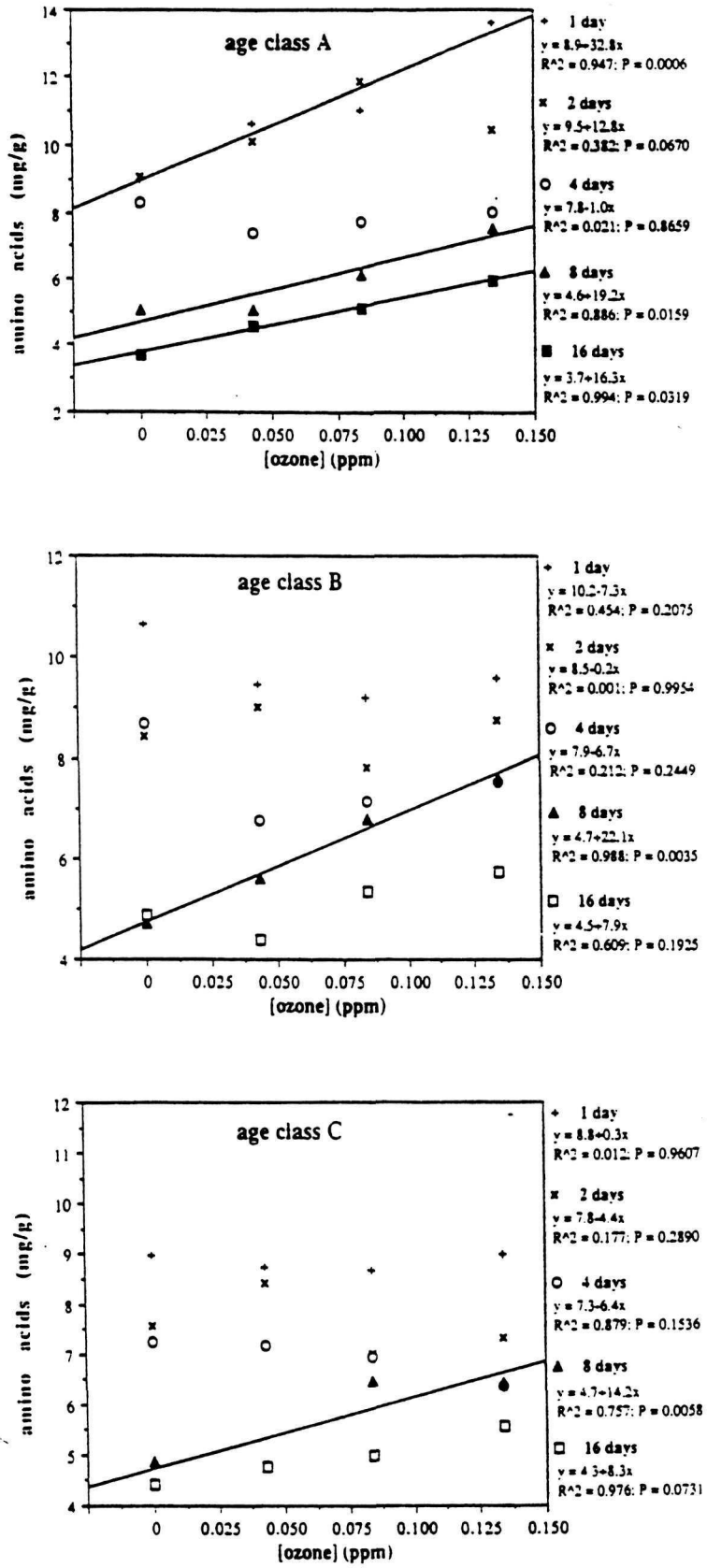


Figure 11. Soluble amino acids in leaves of different age classes (A: young/importing, B: mature/exporting, C: mature/senescing) after 1, 2, 4, 8, and 16 d of fumigation with ozone in low-nutrient soil substrate (only significant regression lines shown).

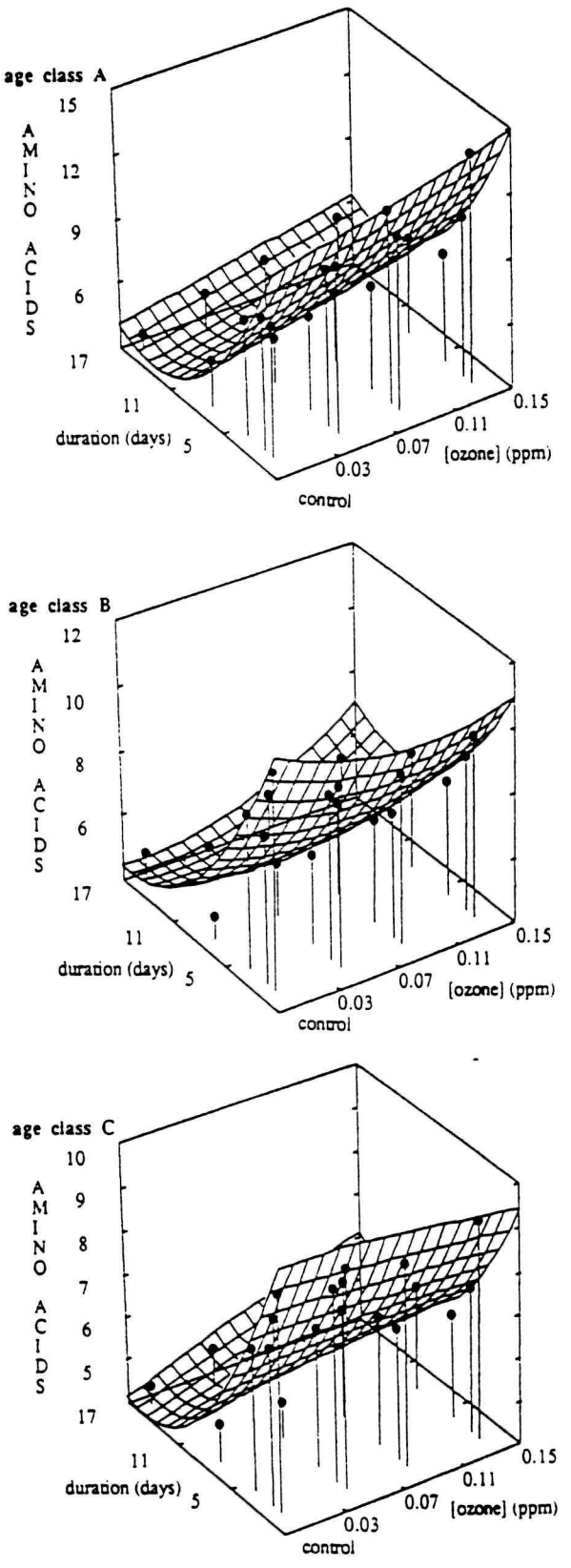


Figure 12. Interaction between the effect of exposure duration (days of fumigation) and ozone concentration on soluble amino acid concentration in leaves of *Asclepias curassavica* grown in low-nutrient soil substrate (quadratic surface plot).

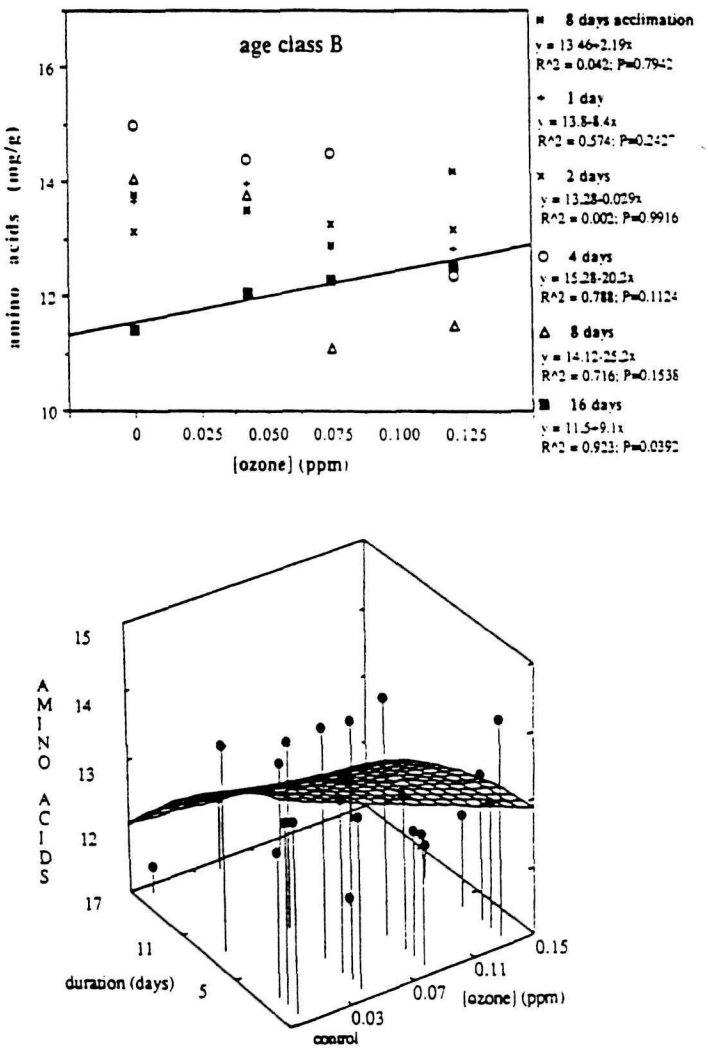


Figure 13. Top: Soluble amino acids in leaves of age class B (mature/exporting) after an acclimation period of 8 d (=0), and 1, 2, 4, 8, and 16 d of fumigation with ozone in high-nutrient soil substrate (only significant regression lines shown). Bottom: Interaction between the effect of exposure duration (fumigation days) and ozone concentration on soluble amino acid concentration in leaves of *Asclepias curassavica* grown in high-nutrient soil substrate (quadratic surface plot).

3.2.3 Cardenolides and Phenols

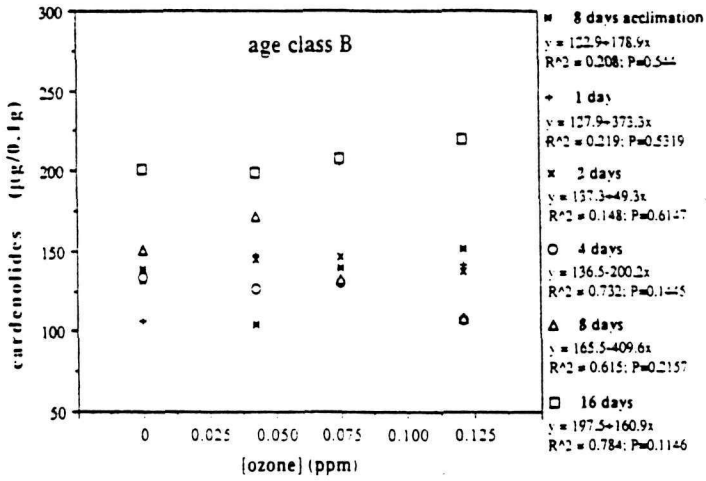
As for the secondary plant metabolites, no effect of ozone treatment was observed on the gross cardenolide concentration in HF-plants (Figure 14). Overall, exposure time had a strong effect, indicating an ozone-independent accumulation of cardenolides with maturing of the leaves. However, in low-nutrient plants of the triple-replicated experiment (Figure 6), a significant increase with very low levels of ozone was observed. Unlike the cardenolides, a very strong and consistent effect of ozone and exposure time on the concentration of total phenols was measured in HN-plants (Figure 15) fumigated for 8 and 16 d.

Because the concentration of many of the secondary plant compounds (e.g., flavonoids) can determine the nutritional quality of food for humans, concerns about the interaction of air pollutants with the secondary plant metabolism have led to a variety of investigations. Ozone seemed to induce flavonoid synthesis (Keen and Taylor 1975; Hurwitz et al. 1979), cause accumulations of anthocyanins (Nouchi and Odaira 1973) and caffeic acid (Howell et al. 1971) and generally increased polyphenols (Menser and Chaplin 1969; Howell 1974). The occurrence of purple stippling, a well-known visible symptom of ozone injury also observed in this study, is related to changes in phenol metabolism. Phenols and derivatives are located in the vacuoles and chloroplasts of plant cells. Because ozone impairs the integrity of membranes, the phenols may be oxidized by oxidative enzymes to their respective quinones, which themselves can polymerize with amino acids, amines, and sulfhydryl groups of proteins to form the pigment seen as the "purple stippling" (Howell 1974). Secondary metabolism can be influenced by a number of extrinsic factors in addition to air pollutants. It is thought that the major soil nutrients and incident light intensity are predominantly involved in resource allocation for the various precursors of secondary compounds (Waterman and Mole 1989). Also, the induction of premature senescence observed in this study is related to changes in the secondary plant metabolism, namely the ethylene–polyamine biosynthetic pathway (Pell 1988).

3.3 EFFECTS OF CONSUMING OZONE-TREATED LEAVES ON MONARCH BUTTERFLIES

3.3.1 Feeding Preference

The dual-choice experiments with monarch larvae revealed a strong preference of 3rd instar larvae for ozone-treated *Asclepias curassavica* leaf discs ($p \leq 0.0096$) (see Figure 16). On *Asclepias syriaca*, and for 4th instar larvae on *Asclepias curassavica*, no preference could be detected. Analysis of the tissue offered to the larvae showed decreases in soluble carbohydrates ($p \leq 0.00397$) and proteins ($p \leq 0.0845$) and an increase in phenols ($p \leq 0.0739$) in ozone-treated *A. curassavica* plants. However, no effect could be seen on the concentration of amino acids or gross cardenolides. For *A. syriaca*, significant decreases were noted for proteins ($p \leq 0.042$) and amino acids ($p \leq 0.0702$), and phenol level increased ($p \leq 0.0205$). No effects on CHO or cardenolide levels were detected.



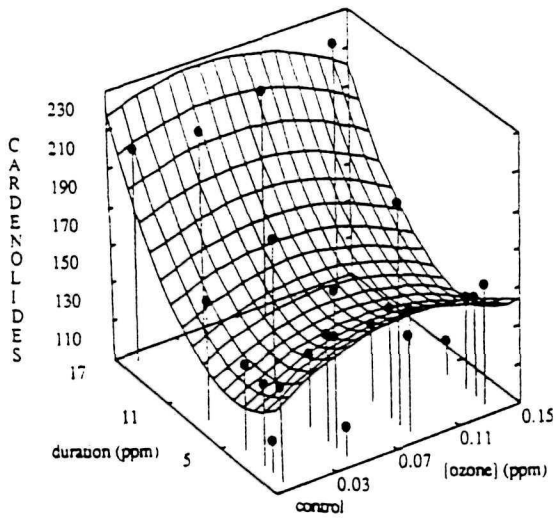


Figure 14. Top: Soluble cardenolides (digitoxin eq.) in leaves of age class B (mature/exporting) after an acclimation period of 8 d (=0), and 1, 2, 4, 8, and 16 d of fumigation with ozone in high-nutrient soil substrate (only significant regression lines shown). Bottom: Interaction between the effect of exposure duration (fumigation days) and ozone concentration on soluble cardenolide concentration in leaves of *Asclepias curassavica* grown in high-nutrient soil substrate (quadratic surface plot).

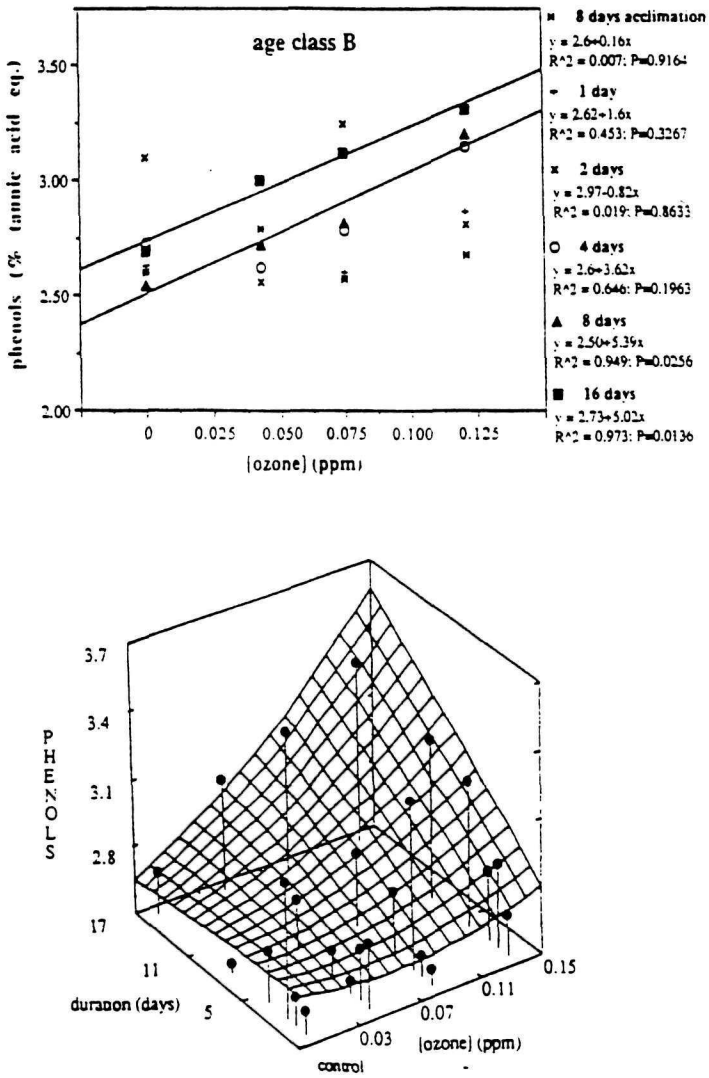


Figure 15. Top: Soluble phenols (expressed as % tannic acid eq.) in leaves of age class B (mature/exporting) after an acclimation period of 8 d (=0), and 1, 2, 4, 8, and 16 d of fumigation with ozone in high-nutrient soil substrate (only significant regression lines shown). Bottom: Interaction between the effect of exposure duration (fumigation days) and ozone concentration on soluble phenol concentration in leaves of *Asclepias curassavica* grown in high-nutrient soil substrate (quadratic surface plot).

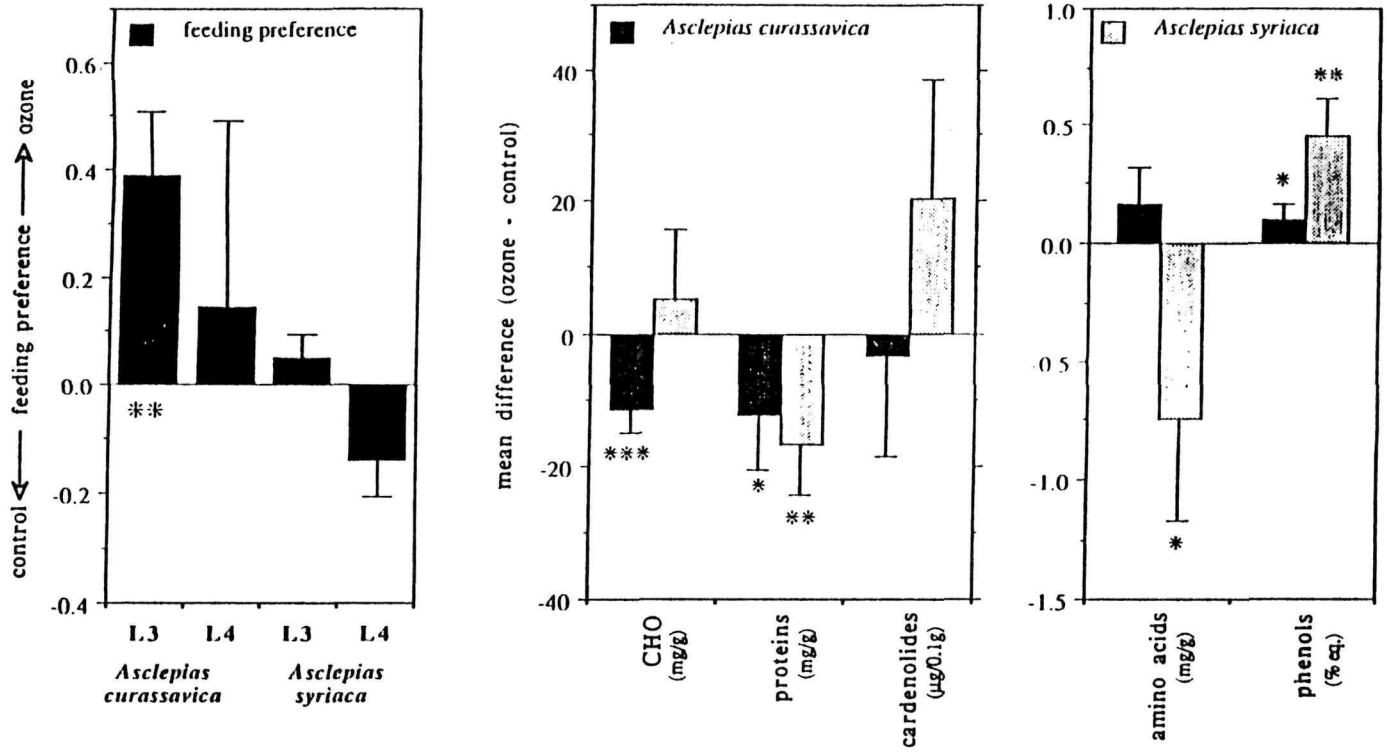


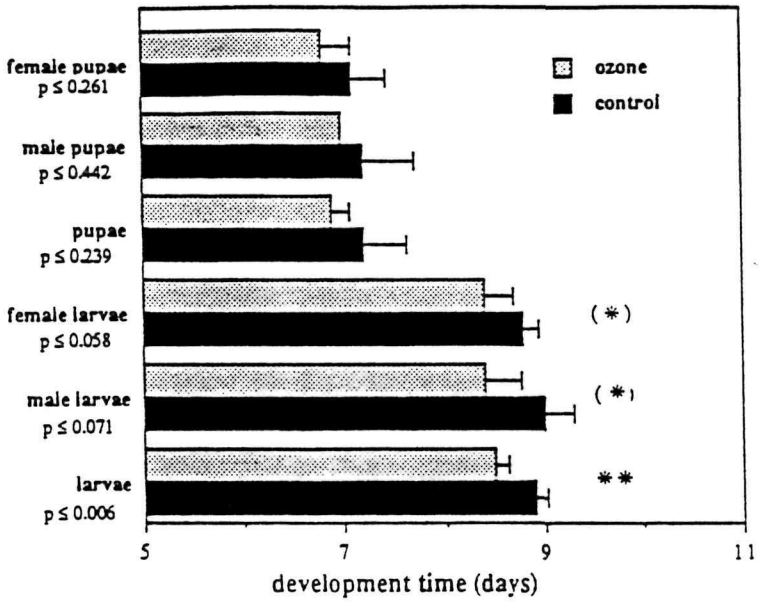
Figure 16. Feeding preference of 3rd (L3) and 4th (L4) instar larvae on ozone-treated leaf tissue shown as mean % difference (left); mean difference in soluble carbohydrates (CHO), proteins, cardenolides, amino acids, and phenols (right) in ozonated leaf tissue offered in the feeding preference test (units are mg/g dry wt of leaf tissue for CHO, proteins, and amino acids; micrograms/0.1g for cardenolides; % tannic acid equivalents for phenolics) (* $p \leq 0.1$; ** $p \leq 0.05$; *** $p \leq 0.01$; weighted ANOVA).

Jones and Coleman (1988) observed that adults and larvae of the willow leaf beetle *Plagiodera versicolora* preferred to feed on previously fumigated cottonwood leaf tissue (0.20 ppm ozone for 5 h). Also, the gypsy moth showed a preference for leaves from ozone-exposed oak seedlings (Jeffords and Endress 1984), and the Mexican bean beetle preferred ozone-treated soybean leaves (Endress and Post 1985). However, the mechanisms of the increased feeding preferences were not identified. Hughes and Voland (1988) presented strong evidence that the preference of Mexican bean beetles for SO_2-fumigated soybean leaves is due primarily to increased sugar content of these leaves. The feeding preference found in this study, however, cannot be explained simply on the basis of changes in any of the constituents measured, including sugars and amino acids, when the differences in behavior and metabolites between the two host plants are considered. Since *Asclepias curassavica* emits a strong smell, other chemicals such as volatiles might be involved which may be modified under the highly oxidative conditions of ozone-enriched air and, therefore, could change the feeding preference of earlier instars.

3.3.2 Growth and Development

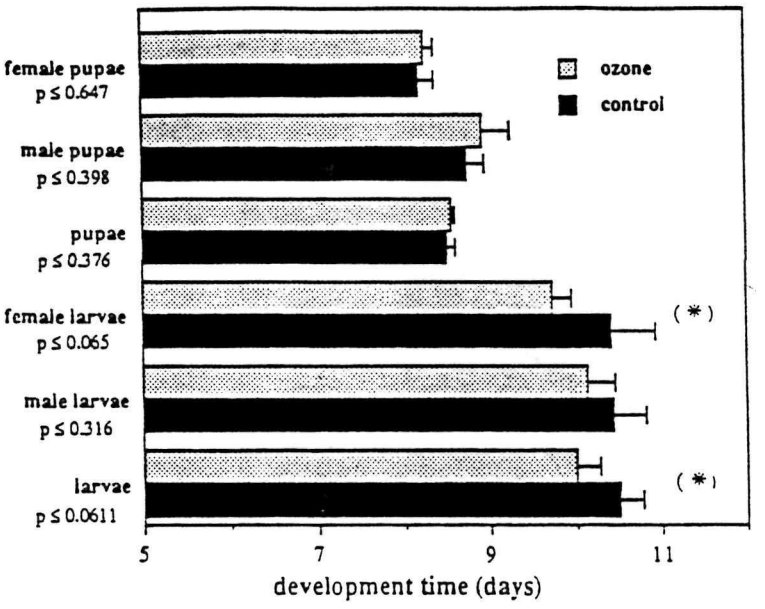
Larvae placed on plants during fumigation showed subtle effects on development time (Figure 17, top, and Figure 18, top), but no effects on growth were observed for either host plant investigated. Larval development was faster on fumigated plants of both species than on the respective controls. However, duration of the pupal stage was not affected and the difference was not significant for time to adulthood. Because the larvae were feeding during the fumigation experiment, the sampling of leaf tissue for biochemical analysis was limited. The fumigation of host plants with 0.18 ppm ozone resulted in a heavy purple stippling in *Asclepias currasavica* plants after 8 d (Figure 17, bottom), whereas *Asclepias syriaca* was less seriously affected even after 11 d of fumigation (Figure 18, bottom). In *A. currasavica* plants, the CHO were significantly decreased in the ozone treatment, whereas the amino acids and the proteins were unaffected. In contrast, the *A. syriaca* plants showed an increase in CHO and a substantial decrease in soluble proteins, both of which were also observed in the tissue of the feeding preference assay (Figure 16).

Trumble et al. (1987) found also a substantially faster development of the tomato pinworm (*Keiferia lycopersicella*) on ozone-treated plants, which were higher in proteins and total assimilable forms of nitrogen shortly after fumigation. Also, the development rate of *Aphis fabae* was shown to increase in ambient air near a motorway containing oxides of nitrogen and sulfur; this increased rate of development was correlated with an increase in soluble amino acids (Bolsinger and Flückiger 1989).



Treatment	Ozone (ppm)	Purple stippling (%)	CHO (mg/g)	Amino acids (mg/g)	Proteins (mg/g)
Control	0	0	150.0	7.81	216.3
Ozone	0.18	47.5	140.5	8.20	212.3
			$p \leq 0.0125$	$p \leq 0.257$	$p \leq 0.716$

Figure 17. Development times of larvae and pupae in the growth and development experiment on *Asclepias curassavica* (top) and ozone concentration (one hour mean, visible damage, and leaf biochemistry after eight days of fumigation (=2 d after transfer of 1st instar larvae on plants) (bottom) (t-test).



Treatment	Ozone (ppm	Purple stippling (%)	CHO (mg/g)	Amino acids (mg/g)	Proteins (mg/g)
Control	0	0	219.9	12.96	276.3
Ozone	0.18	14	259.5	13.4	227.4
			p≤0.088	p≤0.813	p≤0.0045

Figure 18. Development times of larvae and pupae in the growth and development experiment on *Asclepias syriaca* (top) and ozone concentration (one hour mean, visible damage, and leaf biochemistry after 11 d of fumigation (= day of transfer of 1st instar larvae on plants) (bottom) (t-test).

The development rate effects observed in this study cannot be explained by changes in the plant chemicals analyzed. Although all possible precautions were taken (random assignment of CSTRs to treatment) and temperature differences between the single CSTR chambers were minimized (less than 1°C), it is quite possible that treatment artifacts have been created because this type of experiment is very sensitive to effects of temperature. In addition, leaf temperature of ozone-treated plants might have been increased due to lowered leaf transpiration (less evaporative cooling) (see Table 4), which also could have contributed to the increased development rate observed. For example, Perring et al. (1986) found that water stress induced an increase in plant-canopy temperature which was associated with an increasing abundance of Banks grass mites (*Oligonychus pratensis*).

3.3.3 Nutritional Indices

Because the rates of development tended to be greater on ozone-treated leaf tissue, changes in the nutritional indices might be expected. Larvae fed ozone-treated leaf tissue tended to have increased growth rates (RGR or MRGR) on both *Asclepias curassavica* and *Asclepias syriaca* leaves (Table 6). The effects tend to be slightly more pronounced using initial weights of the larvae compared with the geometrical mean weights. On both species of milkweed, RCR and RGR were significantly greater on fumigated plants than on control plants. Regression analyses (Blau et al. 1978) revealed that with each species the control and fumigated lines shared a common intercept and slope (Figure 20). In contrast, none of the nutritional indices investigated (ECI, ECD, AD) were different when ozone-treated leaves were fed to 5^{th} instar larvae. Considered together with the effects on growth and development, these analyses suggest that increased feeding stimulation may be the primary cause of the enhanced rates of growth and consumption on fumigated plants (as opposed to increased nutritional value of the hosts). Comparisons of soluble carbohydrates, amino acids, proteins, and phenols in the control and ozonated foliage of each species showed a greater variation in soluble carbohydrates with a slightly higher mean concentration and significantly more phenolics in ozonated leaves (Figure 19). A significant increase in amino acids and decrease in proteins was found in treated leaves of *A. curassavica*.

When the different plant metabolites are plotted for the two treatments versus MRGR and RCR (Figures 21 to 28), the effects of the metabolites on MRGR and RCR can be explored visually. Because most of the data clusters yield regressions of poor characteristics, neither regression analysis nor any other descriptive statistics are given except for the RGR versus total phenols of *Asclepias curassavica* (Figure 24). However, some of the data clusters suggest changes in the rates of consumption or growth, or both, with increasing amount of a soluble compound. In *Asclepias curassavica*, MRGR seemed to increase with higher CHO (Figure 21) or amino acids (Figure 22) and decrease with increasing protein concentration (Figure 23). As mentioned above, RGR increased with increasing phenol levels. Although phenols are generally regarded as feeding deterrents, it seems that they can be beneficially utilized as nutrients by certain insects (Neville and Luckey 1971; Bernays and Woodhead 1982). Even an increase in the growth rate has been reported, when phenolics were added to locust diets. It has been suggested that phenolic compounds may replace or spare, e.g., phenylalanine, for

the production of cuticular tanning or proteins for growth (Bernays et al. 1983). For the consumption rates in *A. curassavica*, the data cluster seemed to indicate a slight increase in consumption with increasing sugar content (Figure 21). The amino acids, the proteins, and the phenols seemed not to influence feeding behavior (Figures 22, 23, and 24).

Table 6. Nutritional indices for 5th instar larvae of *Danaus plexippus*.

Species	Treatment	RCRI	RCRG	RGRI	RGRG	MRGR	ECI	AD	ECD
A. syriaca	Control	5.492	3.656	1.231	0.806	0.782	0.220	0.481	0.466
	Ozone	7.039	4.187	1.800	1.059	1.010	0.253	0.483	0.532
	P	0.025	0.033	0.057	0.062	0.064	0.241	0.954	0.202
A. curassavica	Control	7.870	4.242	2.404	1.292	1.212	0.307	0.530	0.587
	Ozone	9.117	4.578	2.940	1.473	1.362	0.325	0.532	0.620
	P	0.011	0.040	0.026	0.027	0.028	0.250	0.849	0.138

The data clusters of *Asclepias syriaca*, however, allow even less speculation about possible interactions between MRGR or RCR and plant constituents. The concentration of soluble carbohydrates showed no effects on the consumption rate, but the growth rates of larvae feeding on ozonated leaves seemed to be somewhat higher at a given sugar concentration (Figure 25). Increasing concentrations of soluble amino acids seemed to affect the feeding rate negatively but also yielded higher growth rates (Figure 26). Whereas the proteins seemed not to affect feeding rates and growth of larvae (Figure 27), the highest growth rates were found on leaf tissue containing the most phenolic compounds, which are found in ozone-treated leaves (Figure 28).

It must be emphasized again that the interpretations above are not supported by sufficient replication. Therefore, these data should not be used to explain the indirect effects of ozone on the nutritional indices of monarch larvae.

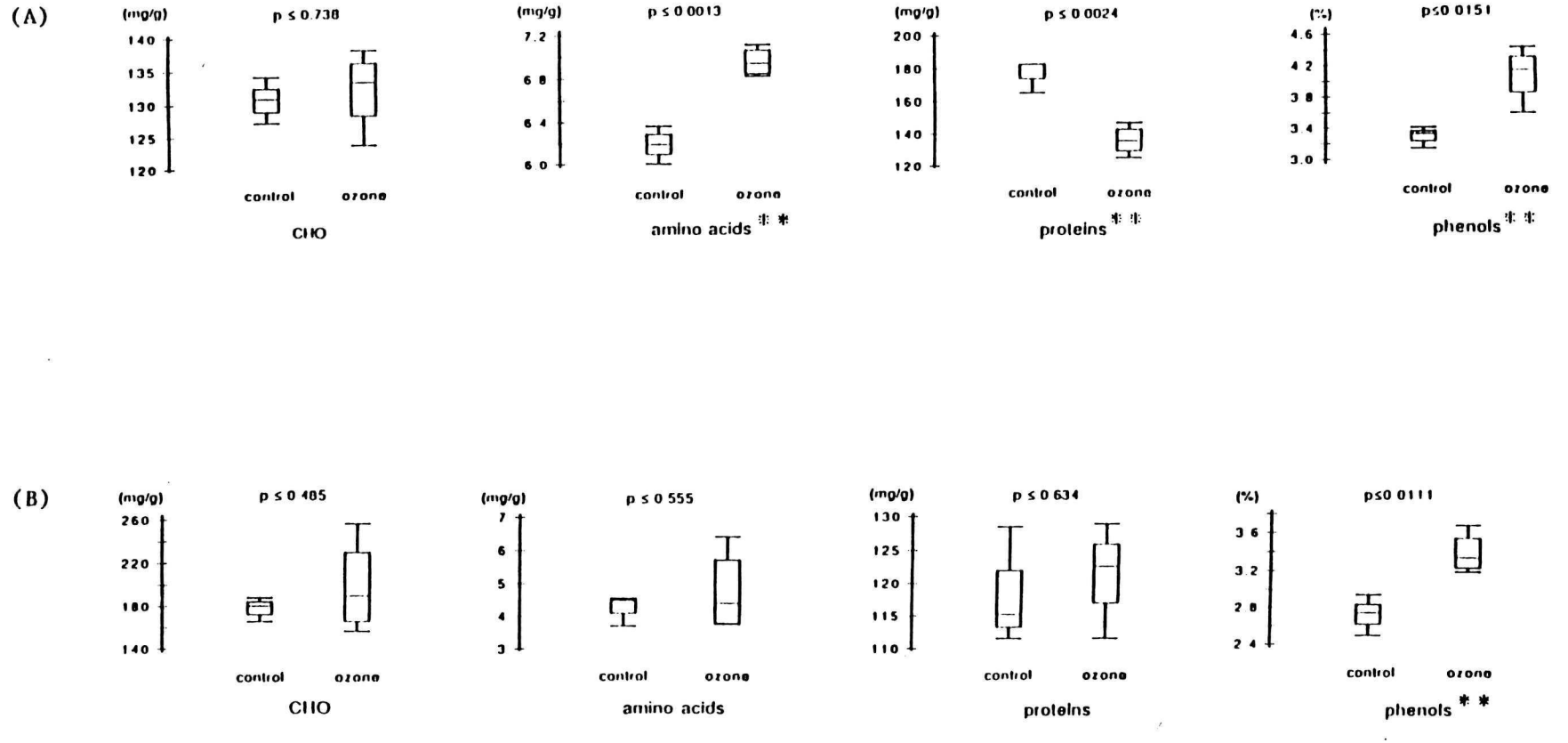


Figure 19. Analysis of soluble carbohydrates (CHO), amino acids, proteins, and phenols of control and ozonated leaves from plants used to determine nutritional indices for 5th instar larvae on (A) *Asclepias curassavica* and (B) *Asclepias syriaca*. Data were analyzed by t-test. Ozone doses were 0.15 ppm for total of 118 h (*A. curassavica*) and 0.165 ppm for total of 98 h (*A. syriaca*).

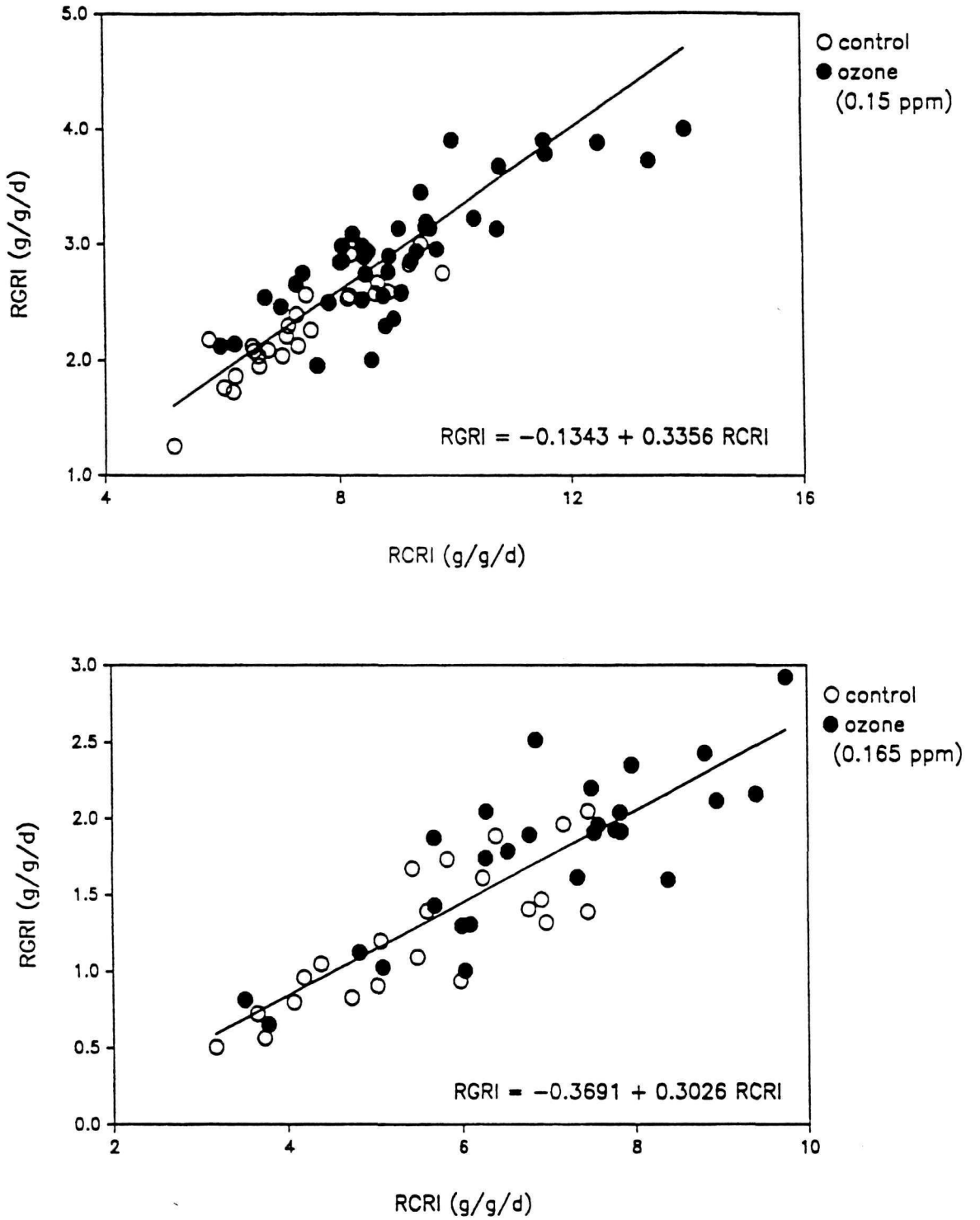


Figure 20. Growth (RGRI) of 5th instar larvae as a function of consumption rate (RCRI) in *Asclepias curassavica* (top) and *Asclepias syriaca* (bottom).

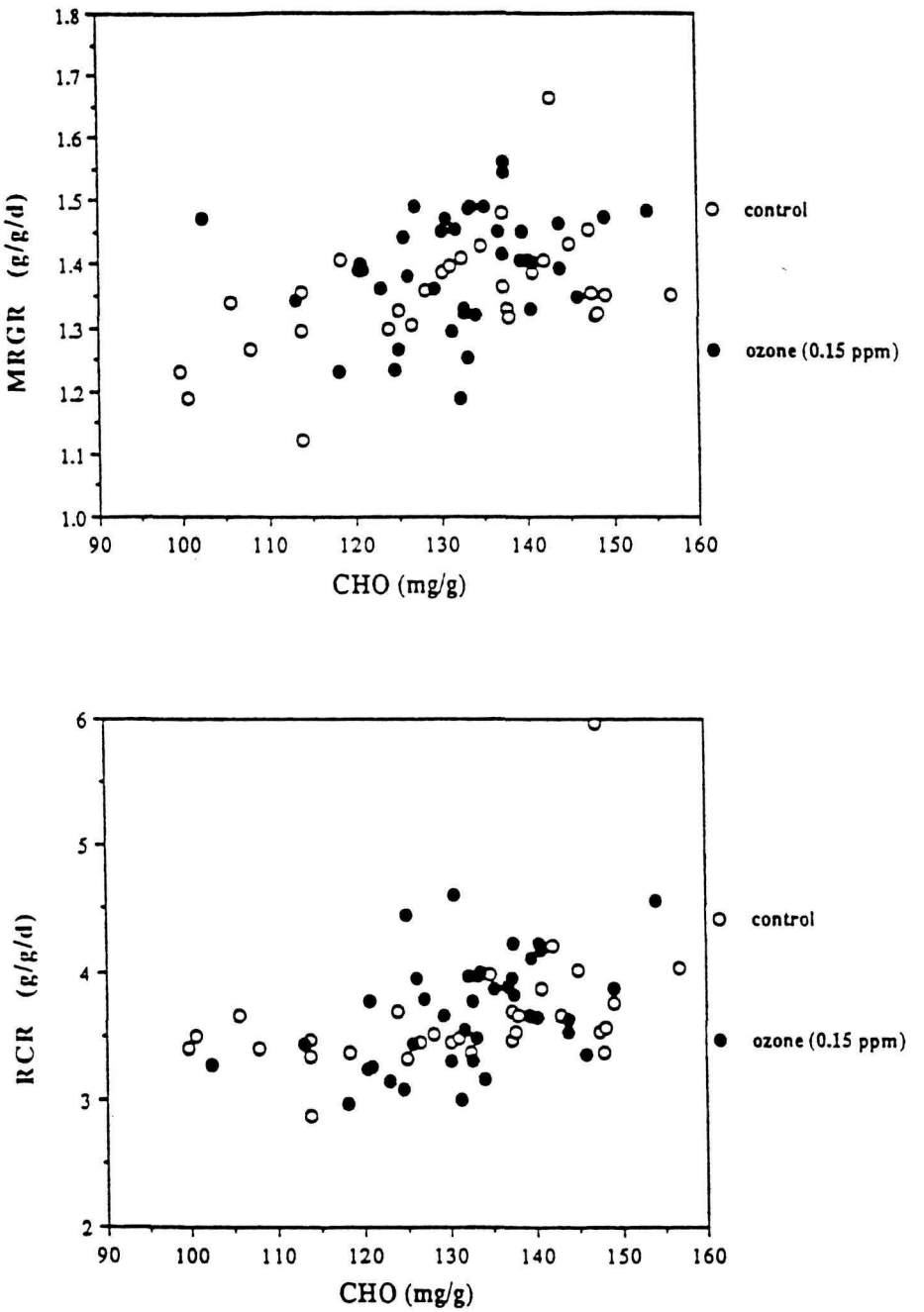


Figure 21. Growth rate (MRGR) and consumption rate (RCR) as functions of the soluble carbohydrate concentration in control and ozone-fumigated leaf tissue of *Asclepias curassavica*.

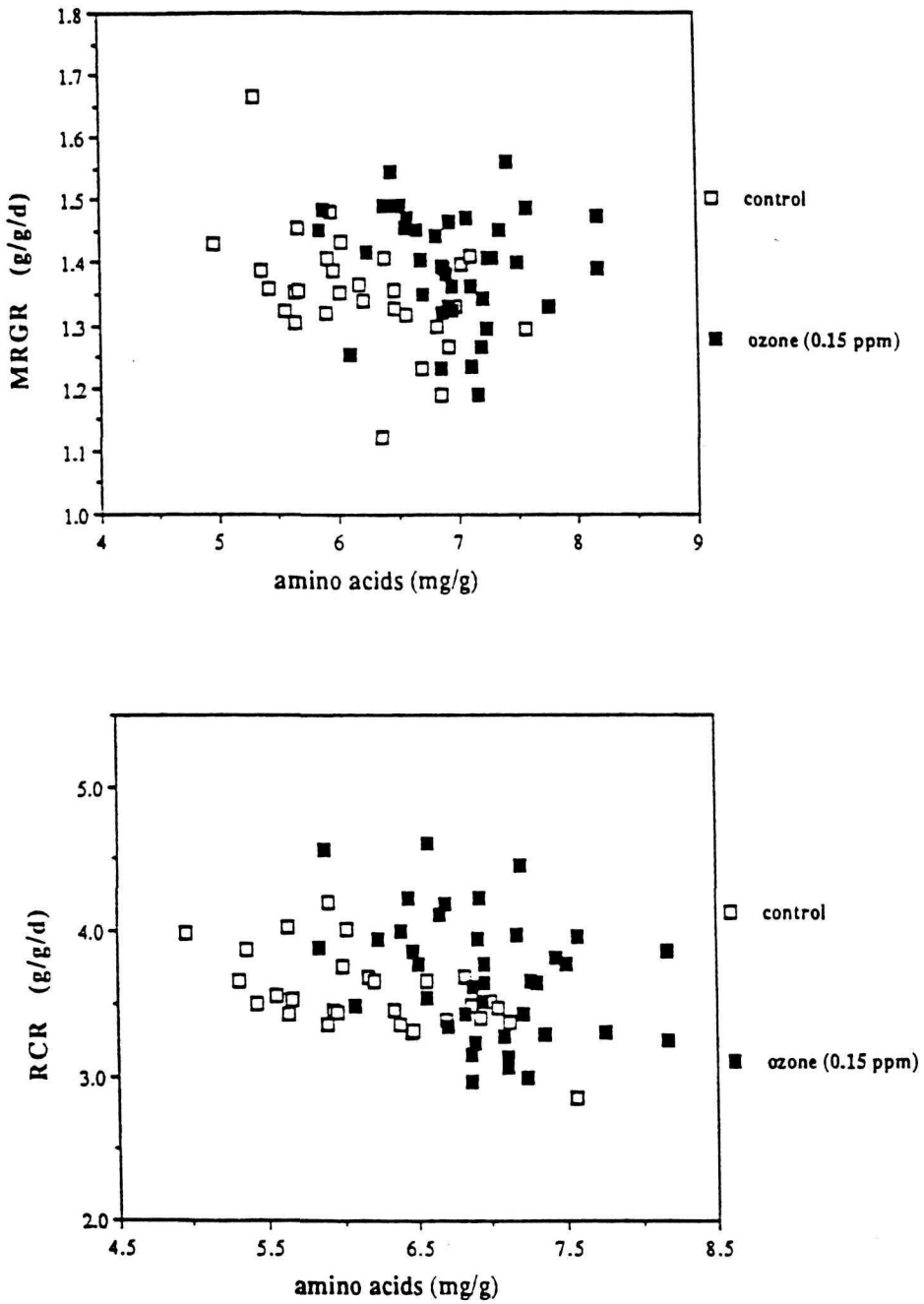


Figure 22. Growth rate (MRGR) and consumption rate (RCR) as functions of the soluble amino acid concentration in control and ozone-fumigated leaf tissue of *Asclepias curassavica*.

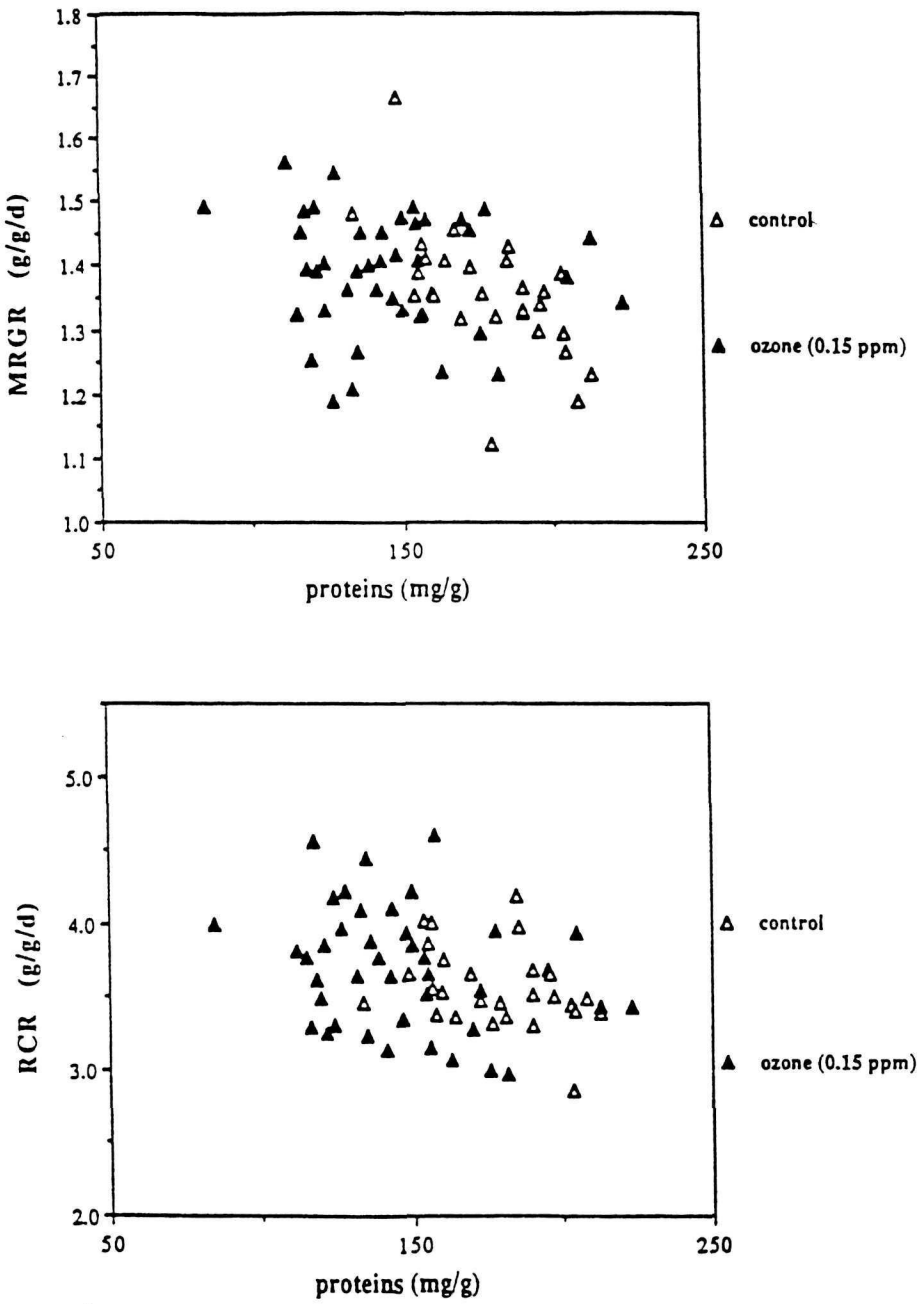


Figure 23. Growth rate (MRGR) and consumption rate (RCR) as functions of the soluble protein concentration in control and ozone-fumigated leaf tissue of *Asclepias curassavica*.

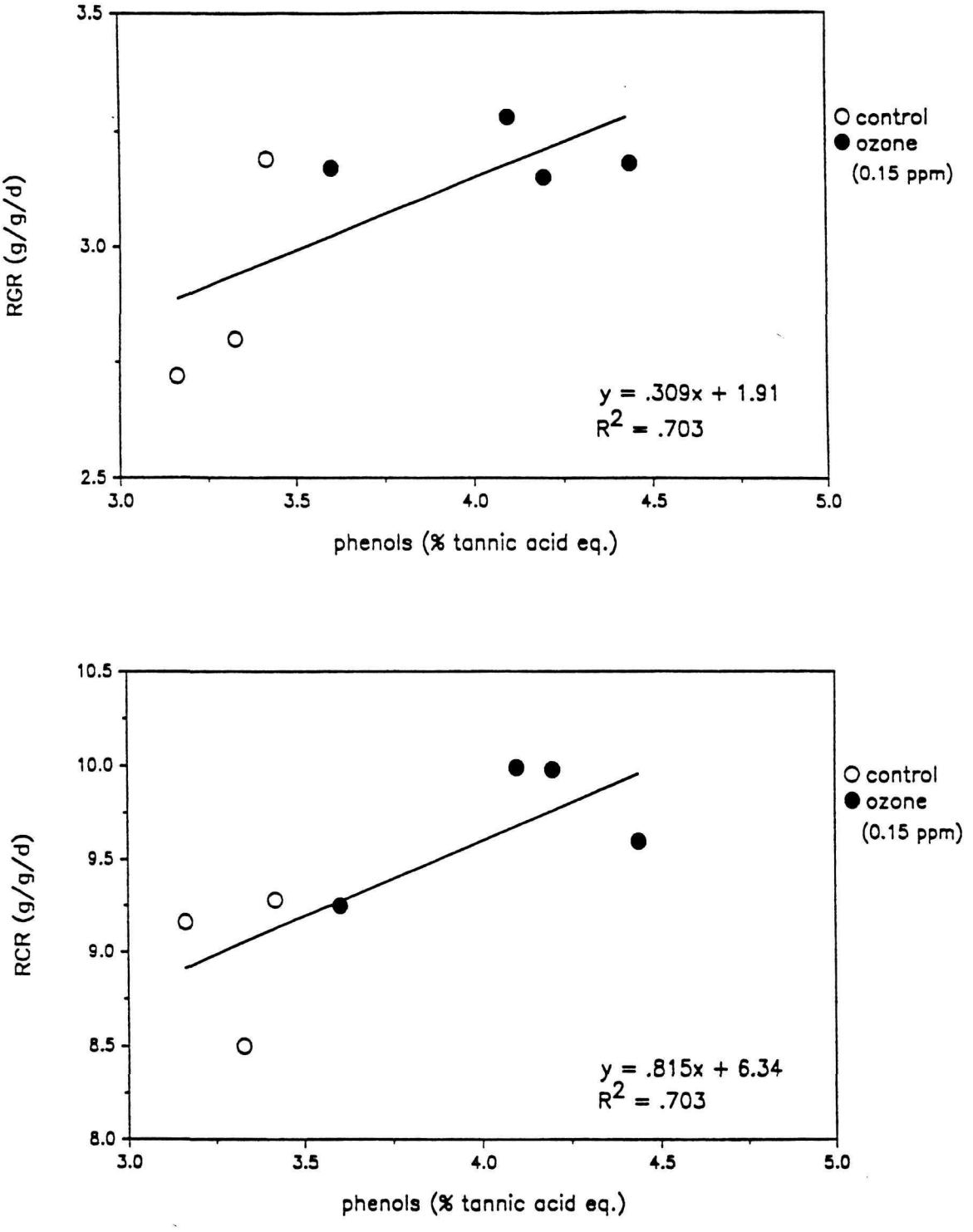


Figure 24. Growth rate (RGR) and consumption rate (RCR) as functions of total phenol concentration in control and ozone-fumigated leaf tissue of *Asclepias curassavica* (probability that slope = 0 is 0.077 and 0.043 for RGR and RCR, respectively).

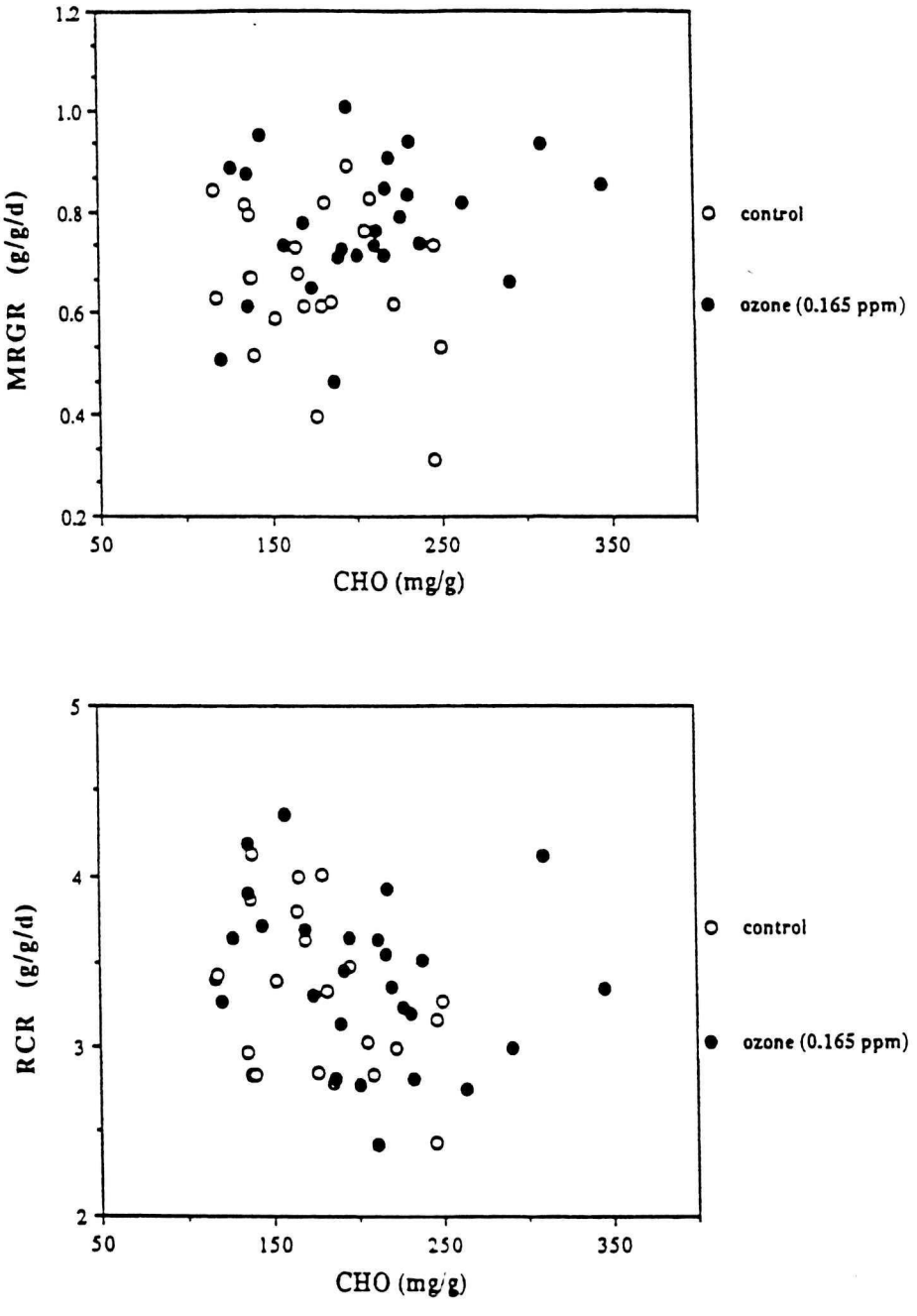


Figure 25. Growth rate (MRGR) and consumption rate (RCR) as functions of the soluble carbohydrate concentration in control and ozone-fumigated leaf tissue of *Asclepias syriaca*.

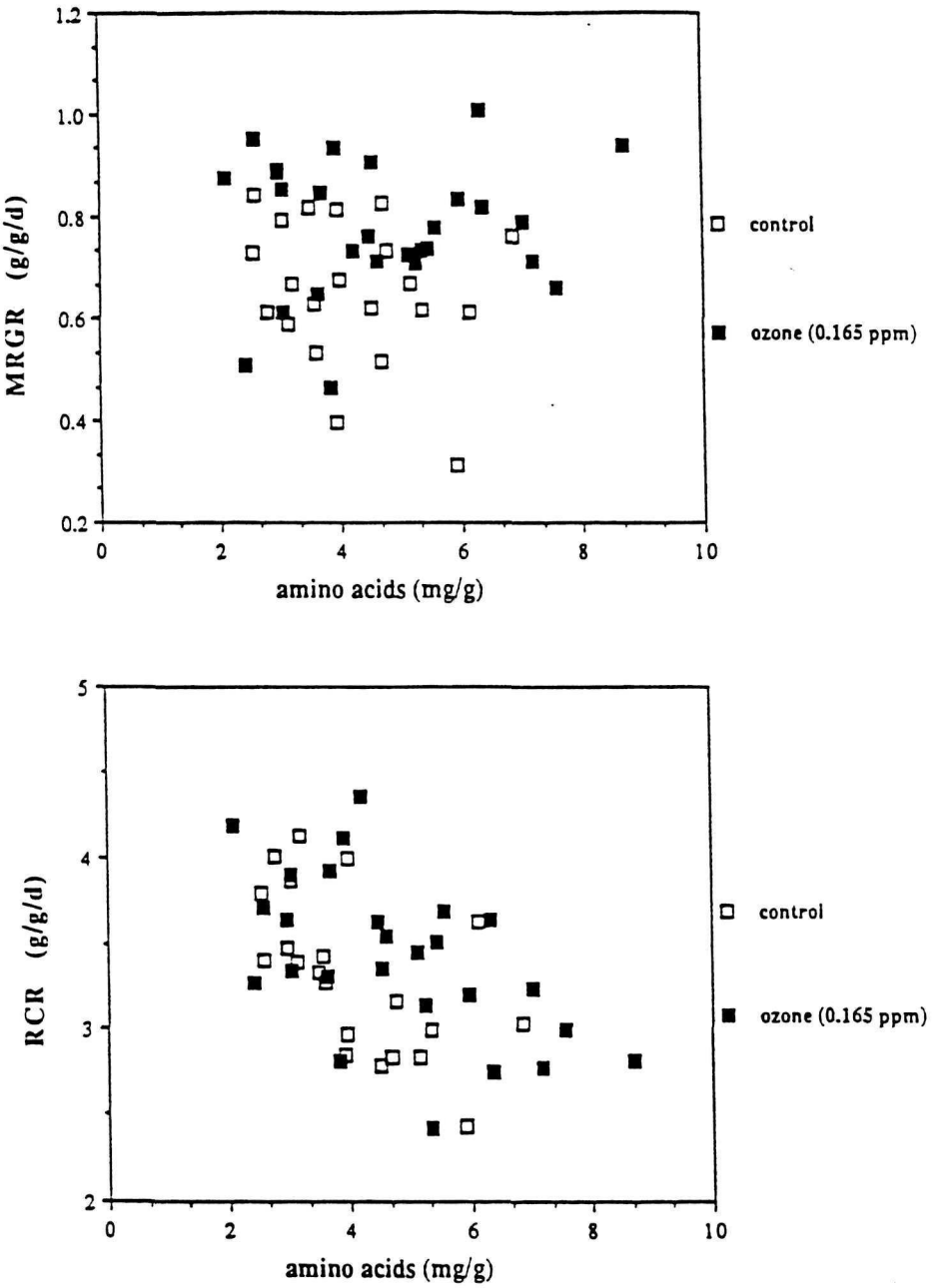


Figure 26. Growth rate (MRGR) and consumption rate (RCR) as functions of the soluble amino acid concentration in control and ozone-fumigated leaf tissue of *Asclepias syriaca*.

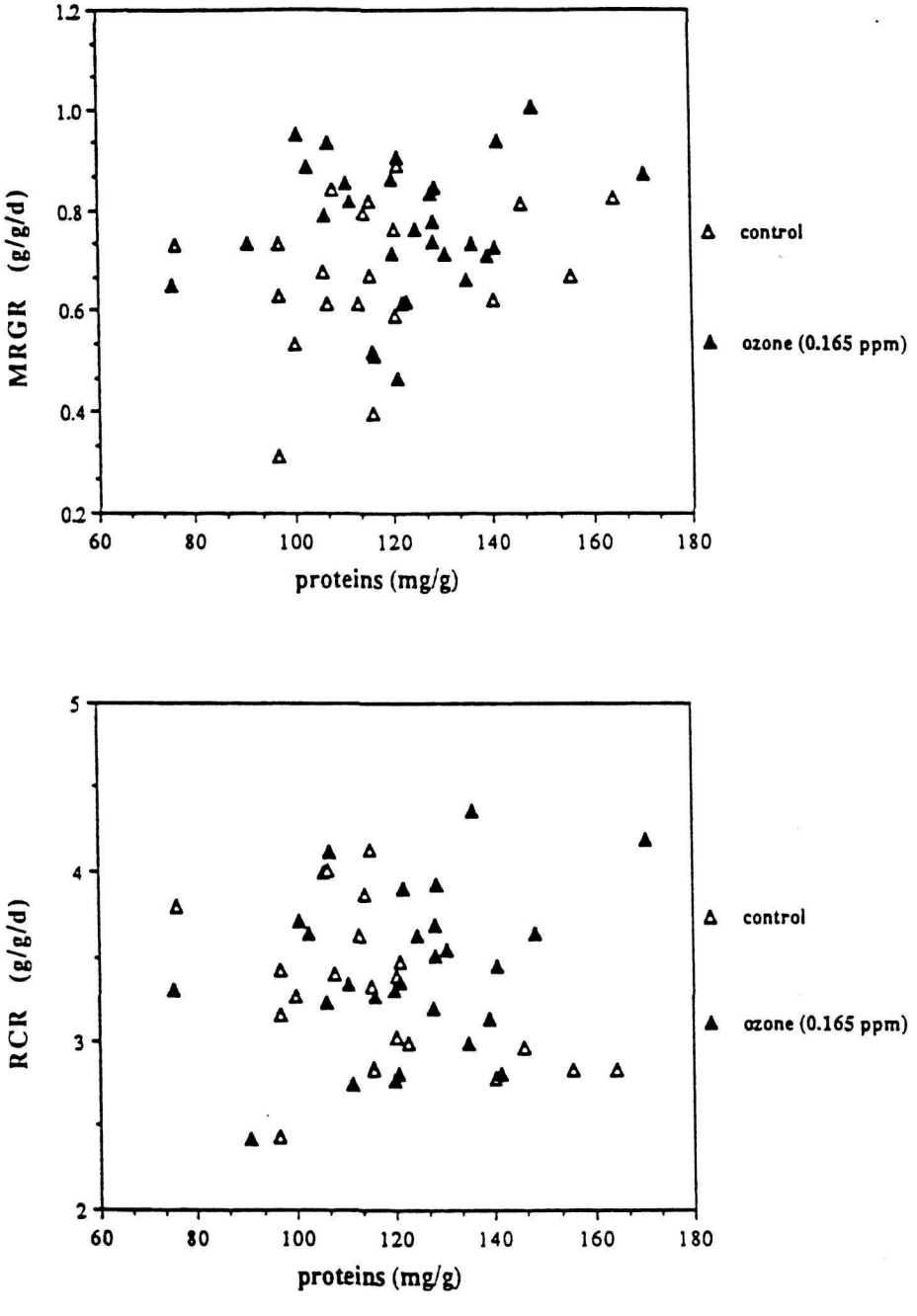


Figure 27. Growth rate (MRGR) and consumption rate (RCR) as functions of the soluble protein concentration in control and ozone-fumigated leaf tissue of *Asclepias syriaca*.

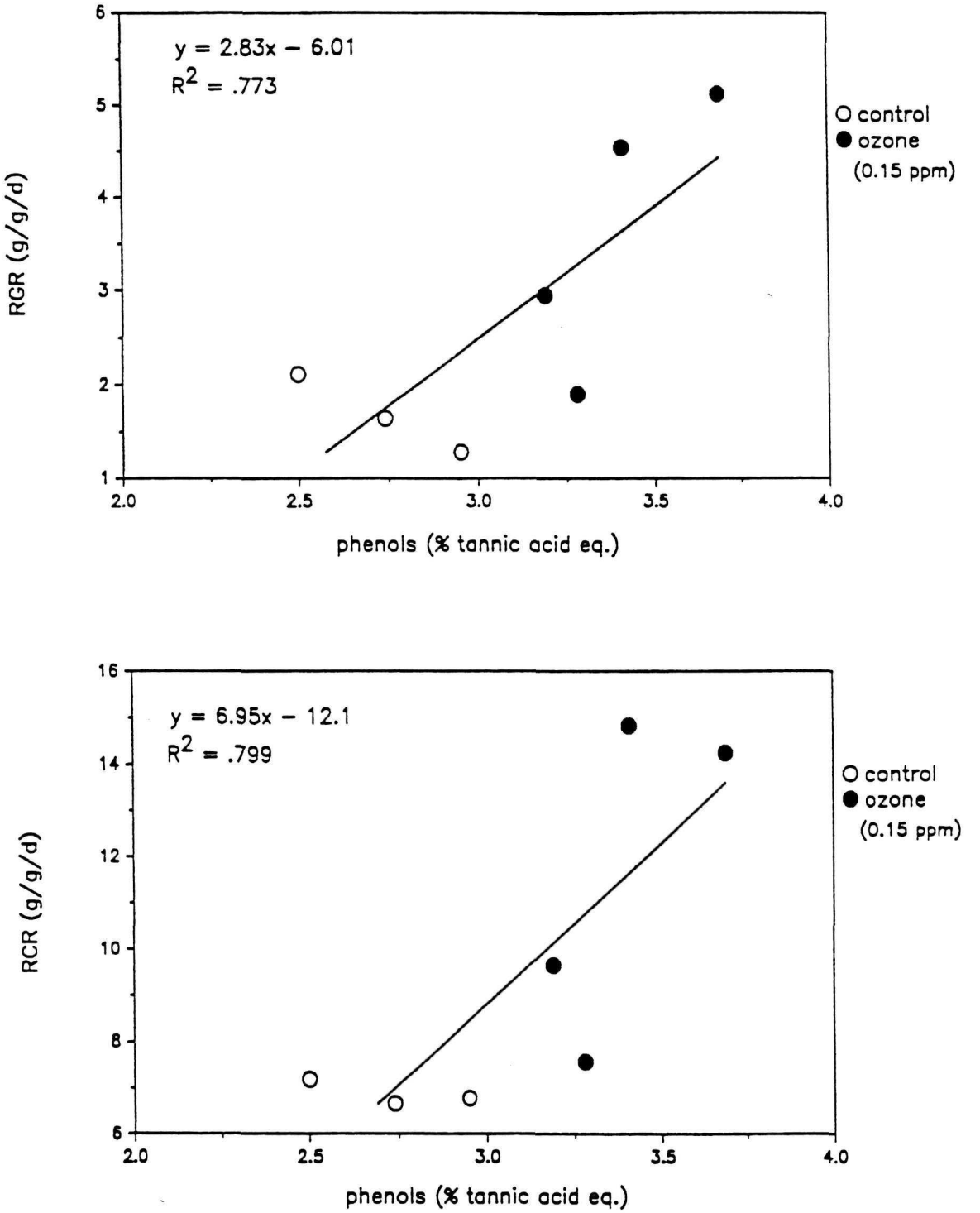


Figure 28. Growth rate (RGR) and consumption rate (RCR) as functions of total phenol concentration in control and ozone-fumigated leaf tissue of *Asclepias syriaca* (probability that slope = 0 is 0.042 and 0.031 for RGR and RCR, respectively).

4. SUMMARY

The objectives of this study were to investigate the possible effects of ozone-induced changes in milkweed on the behavior and reproduction of the monarch butterfly. In a phytocentric approach, changes in primary and secondary metabolites as an effect of ozone fumigation were studied. Then, effects of ozone fumigation on performance of monarch larvae were studied and related to the known changes in metabolites.

1. Under laboratory conditions, the fumigation of *Asclepias curassavica* and *Asclepias syriaca* with ozone revealed significant changes in primary and secondary metabolites. Soluble sugars and proteins generally decreased and amino acids and total phenols increased at ozone levels between 0 and 0.15 ppm. The cardenolides, which are stored in monarch larvae and determine the emetic potency of adults for avian predators, showed variable responses, being lower in ozone-treated leaves at 4–8 d but higher at 16 d.

2. Environmental conditions, such as relative humidity, light quantity and quality, and plant nutrition, appeared to strongly modify plant response to ozone in terms of biochemical changes, development of visible damage, and premature senescence. Well-fertilized plants responded more slowly to ozone treatment in terms of metabolic changes and premature senescence than the control plants and showed very little visible damage (i.e., purple stippling) compared to the controls.

3. Third instar larvae of the monarch butterfly preferred to feed on ozone-treated *Asclepias curassavica;* purple stippling did not appear to deter feeding. No change in feeding preference on ozone-treated *Asclepias syriaca* leaves was detected in these tests.

4. Larvae reared on host plants with concomitant fumigation developed more rapidly under the ozone treatment. No effect was observed on duration of pupal stage or final dry weight of the adults.

5. RGR and RCR of 5^{th} instar larvae were significantly greater on fumigated plants than on controls. For both species of milkweed, significant regressions were found for RGR against RCR with common intercepts and slopes for the two treatments on a given species.

5. CONCLUSIONS

The data presented in this study demonstrated that, under the laboratory conditions of the fumigation chambers, monarch larvae performed slightly better on ozone-treated host plant tissue than on untreated host plant tissue. These findings are similar to those of other studies with tomato pinworms (Trumble et al. 1987) and Mexican bean beetles (Endress and Post 1985; Jones and Coleman 1988), where increased feeding preferences and growth rates were also observed on ozone-treated host plant tissue. Clearly, ozone can cause shifts in metabolism of milkweed that affect the monarch butterfly. It is also clear that the effects on the plant can depend greatly on environmental conditions. Especially, the impact of plant nutrition needs to be better

defined. The studies also showed some differences between short- and long-term responses of the plants. While chamber studies are essential to elucidating mechanisms, there is now a need to move to field studies with open-top chambers that will permit assessment of effects under more natural conditions of plant growth and pollutant exposure. Such field studies will also permit better exploration of the importance of accelerated leaf senescence to monarch survival as well as the effect of ozone on nectar composition, which is critical to pollen germination, and hence to seed production by the milkweed.

6. SCIENTIFIC OUTPUT

Poster presentations:

Hughes, P.R.; Bolsinger, M.; Stolte, K.W. Effect of ozone on the monarch butterfly/milkweed relationship. 20th Annual Air Pollution Workshop. Pennsylvania, USA; 1988.

Bolsinger, M.; Lier, M.; Stolte, K.; Hughes, P.R., Ozone induced changes in primary and secondary metabolites; relevance to insect herbivores. 21st Annual Air Pollution Workshop. California, USA; 1989.

Papers in preparation:

Bolsinger, M.; Lier, M.E.; Lansky, D.M.; Hughes, P.R. Influence of ozone air pollution on plant-herbivore interaction. Part 1: Biochemical changes in ornamental milkweed (*Asclepias curassavica*, Asclepiadaceae) induced by ozone. To be submitted for publication in *Environmental Pollution*.

Bolsinger, M.; Lier, M.E.; Hughes, P.R. Influence of ozone air pollution on plant-herbivore interaction. Part 2: Effects of ozone on feeding preference and growth and consumption rates of monarch butterflies (*Danaus plexippus*). To be submitted for publication in *Environmental Pollution*.

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United States Department of the Interior

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April 23, 1991

N3615 (475)

Memorandum

To: Milkweed Biomonitoring Participants (Distribution List Attached)

From: Botanist, Research Branch, Air Quality Division

Subject: Final Report on Ozone-Milkweed-Monarch Butterfly Interaction

Attached is a peer-reviewed final copy of "The Effects of Consuming Ozone-Injured Milkweed Leaves on Feeding, Growth, and Development of Monarch Butterflies" by Patrick Hughes, Martin Bolsinger, and Monica Lier of Boyce Thompson Institute. The report is a preliminary assessment of how ozone injury alters primary and secondary metabolites in milkweed leaves (Asclepias syriaca L.) and the subsequent effects on monarch larval growth.

There are many unanswered questions regarding the impact of ozone on common milkweed and the subsequent ramifications for the monarch butterfly. Of primary interest is the impacts of accelerated leaf senescence on survival of larva hatched on ozone-sensitive plants and the effects on fecundity and survival after many generations of consuming ozone-injured leaves.

We welcome any comments you may have regarding this report and also any suggestions for monitoring ozone-milkweed-monarch butterfly interactions in the field. Please contact me if you have any questions regarding this report.

for Kenneth W. Stolte

Enclosures