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Artificial diet rearing system for the silkworm *Bombyx mori* (Lepidoptera: Bombycidae): effect of vitamin C deprivation on larval growth and cocoon production

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Abstract
An artificial diet containing 2% L-ascorbic acid was given to silkworm (*Bombyx mori*) larvae throughout larval life, or only in some larval instars in order to make a comparison of larvae fed on a diet without L-ascorbic acid throughout larval life. Obtained results show that, when complete L-ascorbic acid deprivation is done during the larval cycle, it affects larval growth and cocoon production. Furthermore, L-ascorbic acid absence from larval food, particularly during the first and last instars, generates beneficial effects to cocoon production without affecting the survival rate or delaying the larval cycle.

Key words: L-ascorbic acid; vitamin C; artificial diet; *Bombyx mori*; silkworm

INTRODUCTION

L-ascorbic acid (vitamin C) has always been regarded as indispensable for the growth and development of *Bombyx mori*. In fact, ascorbic acid is present in large amounts in mulberry leaves (Bresci, 1951; Lombardi, 1964), the exclusive food for the silkworm, and insects are incapable of synthesizing it.

The availability of ascorbic acid for the silkworm (*B. mori*) larvae fed on fresh mulberry leaves has probably always been overestimated. The content of vitamin C in mulberry leaves is largely dependent on climatic and seasonal conditions (with a maximum content in late springtime, or at the beginning of summer under temperate climatic conditions) and on the position of the leaf on the shoot (with the maximum content in the apex and median leaves and minimum content near the branch insertion) (unpublished data). Furthermore, the choice of the mulberry variety affects the content of ascorbic acid in the mulberry leaves. Leaf preservation, even if it is protected from direct sunlight, is responsible for diminishing ascorbic acid content at a rate of at least 20% in 24 h. While it is difficult to precisely estimate the amount of ascorbic acid ingested by silkworm larvae fed fresh mulberry leaves, the availability of artificial diet from the 1960s, permitted researchers to confirm the importance of L-ascorbic acid for the silkworm physiology on the basis of classical experiments carried out by Ito (1961). Drying of mulberry leaves causes a complete degradation of ascorbic acid, so mulberry leaves cannot be considered as a source of ascorbic acid for larvae fed on artificial diet containing even a high percentage of dried, pulverized mulberry leaves (unpublished data). Ascorbic acid is usually added to silkworm food (enrichment) in a quantity generally varying from 1–2% of the dry weight of the artificial diet, which is considered as optimum content of this vitamin (Ito, 1978).

Artificial diet is routinely cooked and sterilized by an autoclaving process, and it is generally accepted that heat and light under oxygen presence are responsible for a strong degradation of the vitamin to the oxidized form (dehydroascorbic acid),

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which is even less stable than the reduced form. Although it is likely that the silkworm larvae are able to utilize dehydroascorbic acid as a vitamin C source (Ito, 1978), the actual needs of the silkworm larvae remain unknown. Furthermore, Ito demonstrated that L-ascorbic acid deprivation in the first 20 d of rearing affects larval growth and body weight (Ito, 1961), but it remains to be clarified if vitamin C is required throughout the complete larval stage or if some instars are less sensitive to its absence from the food.

This point is of particular interest for at least two reasons. Firstly, it has recently been hypothesised that vitamin C has an adverse effect on silkworm feeding (Cui et al., 2003). In fact, it was demonstrated by an electrophysiological method that the sensilla styloconica SS-I and SS-II on the maxillary tubercle of the silkworm are sensitive to L-ascorbic acid (Cui et al., 2001). Developing an artificial diet without ascorbic acid, could be essential in rearing first instar larvae of pure strains not adaptable to artificial diet rearing. The second reason concerns the use of artificial diet for practical purposes such as silkworm rearing in bulk. In this case, ascorbic acid introduction in the diet represents a cost and its elimination or reduction, particularly in the last two larval instars, may be helpful in producing low-cost diets.

The object of this study was to verify which are the periods, if there are any, of larval life when the silkworm is particularly sensitive to vitamin C deprivation.

MATERIALS AND METHODS

Silkworm strains. To carry out the experiments, the double-cross larvae [(119×118)×(120×121)] and single-cross larvae (118×120) were employed, both produced from parental lines preserved in the germplasm collection of the Sericulture Specialized Unit of Padua of the Experiment Institute for Agrarian Zoology. Parental lines used to constitute the above-mentioned double-cross larvae and a single-cross hybrid were reared on the artificial diet produced by the same institution. However, these parental lines cannot be considered as being polyphagous.

Artificial diet. The powder, containing 25% dried and pulverized mulberry leaf out of the diet dry weight, was hydrated (1 dry powder : 2.6 water), mixed and cooked in an autoclave for about 40 min at 105°C. The diet was cooled to room temperature and then maintained in a refrigerator (5°C) until its utilization. Diet containing vitamin C (named "2% diet"), was added with 2% pure grade L-ascorbic acid (Sigma, Italy, 99% purity), while diet not-containing vitamin C (named "0% diet") was added with 2% maize starch to substitute the 100% composition. The artificial diet composition is shown in Table 1.

Table 1. Composition of the artificial diet

Ingredients	Quantities/100 g dry weight[a]
Dried mulberry leaf powder	25.0 g
Defatted soybean meal	36.0 g
Wheat meal	15.0 g
Corn starch	4.0 g
Soybean fiber	5.0 g
Citric acid	4.0 g
Ascorbic acid	2.0 g
Salt mixture	3.0 g
Agar	4.2 g
Vitamin mixture	399.0 mg
Sorbic acid	200.0 mg
Propionic acid	691.0 mg
Chloramphenicol	10.0 mg
β-sitosterol	500.0 mg

[a] The powder was hydrated in the ratio of 1 g dry powder : 2.6 g of water.

Silkworm rearing. Rearing was carried out in a climatized room where temperature, relative humidity and photoperiod were controlled. Temperature was set at 25±1°C, relative humidity (R.H.) at 85±5% during the first instar, 80±5% during the second instar, 75±5% during the third instar, 70±5% during the fourth instar, and 65±5% during the fifth instar. R.H. was lowered to 60±5% during the moulting period to permit diet desiccation. The photoperiod was 16 h dark : 8 h light. Bed cleaning was performed once during the the first, second and third instar stages at the end of the moulting period, twice in the fourth instar stage and daily in the fifth instar stage. The same schedule was followed with regard to feeding: diet was given "ad libitum" during the first four instar stages while a weighed amount was given daily to larvae in the fifth instar stage on the basis of previous experiments carried out to establish their food needs. Larvae were reared in plastic boxes of dif-

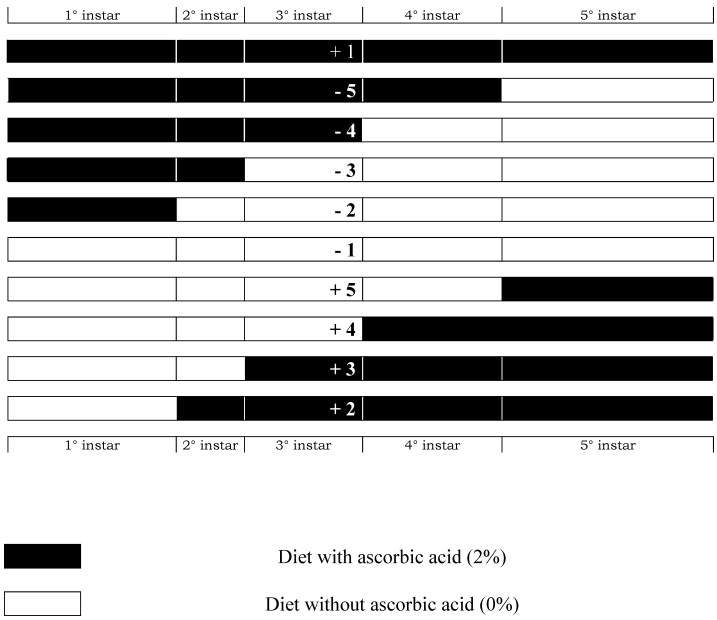

Fig. 1. Experimental groups. Ten experimental groups are shown: +1 (positive control), +2, +3, +4, and +5 are larvae reared on diet containing ascorbic acid (2%), respectively, from 1st, 2nd, 3rd, 4th, and 5th instar. −1 (negative control), −2, −3, −4, and −5 are larvae reared on diet without ascorbic acid (0%), respectively, from 1st, 2nd, 3rd, 4th, and 5th instar. Various instar durations are represented by the means of a different bar length.

ferent sizes according to their age and number. Mounting was performed on plastic frameworks.

Experimental plan. Two experiments were carried out; the former with the single hybrid (118×120), the latter with the double-cross larvae [(119×118)×(120×121)]. Both of the trials were identically planned (Fig. 1). Ten groups of larvae (three replications for each group) were fed under different diet regimens with regard to ascorbic acid content. One group was fed with diet containing ascorbic acid throughout larval life (positive control); while another group was fed with diet deprived of ascorbic acid throughout larval life (negative control). The other groups were fed diets containing or not-containing ascorbic acid starting from definite periods of larval life (the beginning of the different instars). Before these definite periods, larvae were reared in bulk on diet containing or not-containing ascorbic acid. Replications were formed by a decreasing number of larvae in the subsequent instars (100 for each replication in the first instar, 70 in the second, 45 in the third, 35 in the fourth and 20 in the fifth). The following parameters were recorded: duration of each larval instar, percentage of early and late larvae for each moult, mortality rate during larval life and the first week after spinning, cocoon weight, shell weight, silk ratio. Economic parameters regarding silk production and pupal mortality were estimated 7 d after larval spinning. Production data were separately collected for male and female cocoons after pupal sexing according to morphological features of the abdomen (Sakaguchi, 1978).

Analysis of pH. A pH analysis was performed on the diet containing ascorbic acid (2%) and that deprived of the same vitamin (0%). For each diet, 20 samples of about 5 g of wet diet were collected and suspended in 250 ml of deionized water. Evaluation of pH was carried out with a portable pH-meter (SA230, Orion Research) at room temperature (25°C).

Statistical analysis. Data were analysed by *t*-test (comparison between two groups) or by ANOVA, followed by the Tukey-Kramer's test. Preliminary test for assessing homogeneity of variance was carried out by Bartlett's test. The computer software Statistica 6.0 was used. Appropriate transformation (arcsine) was performed on data collected as percentages.

RESULTS

One of the most interesting results of this study was the larval mortality in the groups reared on

Table 2. Larval mortality (%) in the groups reared on a diet with or without ascorbic acid in the different larval instars of the double-cross larvae [(119×118)×(120×121)]

Experimental groups	Mortality[a]					
	I instar	II instar	III instar	IV instar	V instar	Cocoon stage
+1[b]	1.7±1.5 a	1.7±1.3 ab	2.0±0.6 b	0.0±0.0 b	0.0±0.0 b	3.3±3.0 b
−1[c]	1.3±1.2 a	2.3±1.2 ab	14.0±5.6 a	51.3±12.5 a	12.7±4.0 b	25.7±4.6 a
+2		5.0±1.7 a	0.0±0.0 b	0.0±0.0 b	0.0±0.0 a	3.3±3.0 b
−2		1.0±1.7 b	0.0±0.0 b	0.0±0.0 b	40.0±13.2 a	23.3±11.6 b
+3			0.0±0.0 b	0.0±0.0 b	1.3±2.3 b	4.0±4.8 b
−3			1.0±1.7 b	1.0±1.7 b	12.0±8.0 b	9.3±2.3 b
+4				0.0±0.0 b	0.0±0.0 b	2.7±4.6 b
−4				0.0±0.0 b	2.3±4.0 b	4.7±4.0 b
+5					0.0±0.0 b	0.0±0.0 b
−5					2.7±4.6 b	0.0±0.0 b

[a] Average±standard deviation; [b] Positive control; [c] Negative control.
Values marked with different letters are significantly different at $p<0.05$. Angular transformation (arcsine) was performed on measurements before carrying out ANOVA test and Tukey's test. Non-transformed values are shown in the table.
+1, +2, +3, +4, +5=larvae reared on diet with ascorbic acid (2%) respectively from 1st, 2nd, 3rd, 4th, and 5th instar.
−1, −2, −3, −4, −5=larvae reared on diet without ascorbic acid (0%) respectively from 1st, 2nd, 3rd, 4th, and 5th instar.

diets containing or not-containing ascorbic acid in the different instars. Table 2 shows data concerning the double-cross larvae [(119×118)×(120×121)]. Similar data were obtained (not shown) in the analogous experiment carried out on the single-cross larvae (118×120). The absolute values of mortality in the different instars were slightly different in the two strains, but the same general trend was observed.

In the first and second instars, in the negative control, ascorbic acid deficiency in the diet did not affect larval mortality, while from the third instar, mortality increased substantially in comparison to the positive control. These findings are in agreement with Cui et al. (2003). Furthermore, deprivation of ascorbic acid in the diet did not affect larval mortality directly in the same instar when deprivation occurred, but after two or three instars that followed, significant effects were observed. For example, larvae reared on the diet without ascorbic acid from the second ("−2") and third ("−3") instars began to show higher mortality, in comparison to the positive control, from the fifth instar, while those reared on the diet without ascorbic acid from the fourth ("−4") or fifth ("−5") instars did not show significant variations in their mortality at all.

On the other hand, when larvae reared in the previous instars on the diet not-containing ascorbic acid were transferred to the diet containing ascorbic acid in later instars, they seemed to recover completely with regard to mortality, with the exception of group "+2" (diet without ascorbic acid during the first instar, diet with ascorbic acid from the second instar onward), where larval mortality during the second instar (immediately after the change of the type of diet) was slightly higher than that in the positive control (although the difference was not significant). It is noteworthy that, in this group, mortality during the second instar was even a little higher than in the negative control, probably due to the fact that ascorbic acid has a negative impact on feeding habit (Cui et al., 2003) and the younger the larvae are the more negative the impact is. To explain the apparently complete larval recovery following the change of diet from not containing to containing L-ascorbic acid, it has to be underlined that mortality in previous instars caused a type of natural selection between individuals tolerant to ascorbic acid deficiency and non-tolerant ones. For this reason, groups of ascorbic acid-deprived larvae established at the beginning of the fourth ("+4") or fifth ("+5") instars, made up by so called "tolerant individuals", were probably able to immediately restore their normal physiological behaviour once they were fed again on a diet containing ascorbic acid.

On the other hand, both larval growth and development were significantly affected only after a pro-

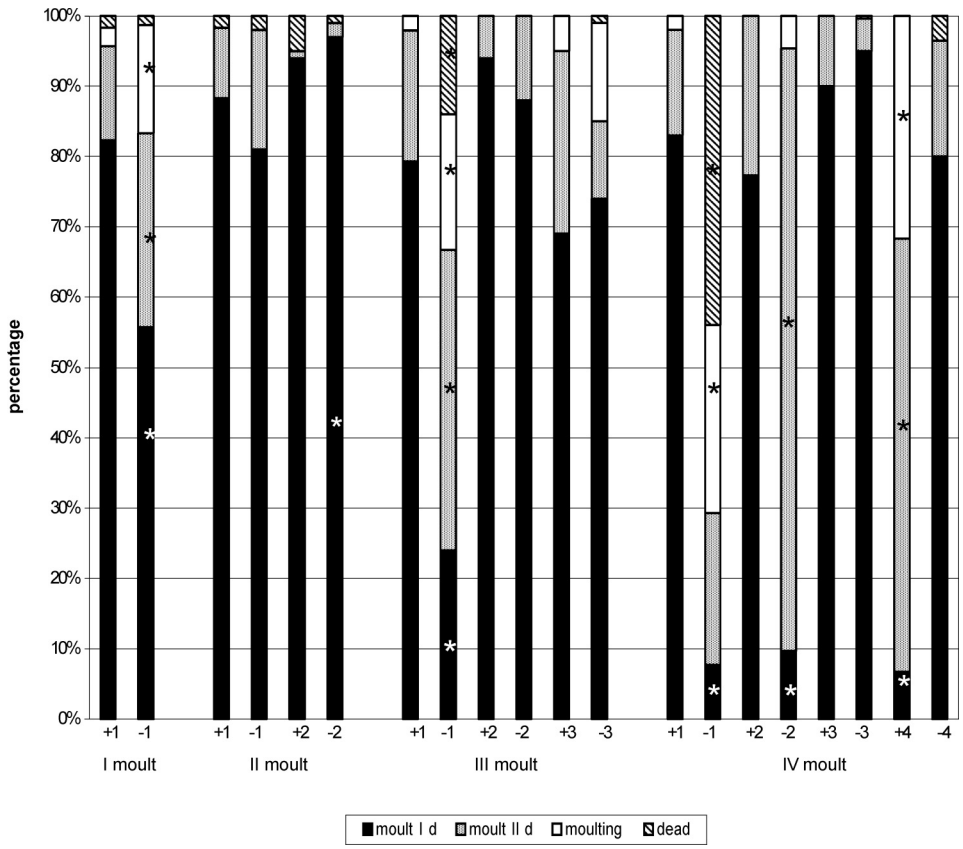

Fig. 2. Behaviour of different groups of the double-larvae [(119×118)×(120×121)] at moulting. Moult I d=percentage of larvae that moulted on the first day of moult. Moult II d=percentage of larvae that moulted on the second day of moult. Moulting=larvae that were still moulting on the second day of moult. Dead=larvae that did not successfully moult and died during the moulting process. Bars with asterisk indicate values significantly different from the positive control (+1) ($p<0.05$) (Tukey-Kramer's test). Statistical analysis was carried out after angular transformation (arcsine) of the percentage values. Not-transformed values are shown in the graph. In the figure, moulting is shown together also in the cases where groups did not begin moulting on the same day. See the text for further details regarding delays of different groups in comparison to the positive control.

longed period of ascorbic acid deprivation. However, with regard to the moulting behaviour and the weight of newly moulted individuals, some differences between the two observed strains were recorded. In Fig. 2, the delay in larval development is estimated at every moult and for each experimental group in the double-cross larvae [(119×118)×(120×121)]. It is possible to observe that, already at the end of the first instar (second moulting day), the percentage of moulted larvae is significantly lower in the ascorbic acid-deprived larvae (negative control) than in the positive control group. This result does not correspond to the findings of Cui et al. (2003). It is also quite different from larval performances of the single-cross larvae (118×120). During the second instar, larval growth was comparable both in the positive and negative controls, while from the third instar, retardation at larval moults was evident again in the negative control in comparison to the positive control, accompanied by remarkable larval mortality. Another interesting datum is that the group deprived of ascorbic acid from the second instar ("−2") had significantly better growth performances in comparison to the positive control (moulted individuals on the first day of moult). Nevertheless, from the fourth moult, the gained advantage on positive control groups fell significantly. On the other hand, group "+2", after the second instar stage, recuperated any growth gap with the positive control. Group "+3" had a delay of 1 d with regard to positive control (not visible in Fig. 2) at the third moult, while group "−3" showed an immediate delay in growth (one day at the third moult, not shown in Fig. 2), highlighting the fact that the third instar stage could be a critical period

Table 3. Comparison among larval weights of just moulted larvae fed a diet with ascorbic acid (group "+1" or positive control) or without ascorbic acid (group "−1" or negative control) from the first instar (single-cross larvae 118×120)

	Larval (10) weight (g) at moulting[a]			
	I moult	II moult	III moult	IV moult
+1 (positive control)	0.056±0.009 a	0.341±0.010 a	1.660±0.059 a	8.680±0.090 a
−1 (negative control)	0.063±0.001 a	0.296±0.003 a	1.203±0.023 b	6.110±0.073 b

[a] Average±standard error.
Data shown within the same row and followed by different letters are significantly different ($p<0.05$ level by *t*-test).

for the silkworm in need of ascorbic acid together with the first instar. Group "+4" showed obvious growth retardation at the last moult (which started 2 d later in comparison to the positive control, not shown in Fig. 2), while group "−4" showed no delay in moulting behaviour at the last moult.

With regard to the last instar duration, only the negative control ("−1") showed a last instar stage longer than the other groups (8 d instead of 7 d); this extra day has to be added to the other 2 d which represent the delay of the negative control in comparison to the positive control from the third moult.

As stated above, in the single-cross larvae (118×120) there were no differences among positive and negative controls at the first moult, with differences beginning to be evident only from the third moult.

Table 3 shows the larval weight at the various moults in the single-cross larvae (118×120). It is worth noting that no significant differences in weight were recorded at the first and second moults. A decreasing trend was already visible at the second moult, but differences began to be significant from the third moult, when deprived larvae were lighter than larvae fed on the diet containing ascorbic acid. With regard to the double-larvae, results concerning the weight at the first moult were rather unclear. In fact, the experiment was repeated many times, obtaining contradictory data. Specimens belonging to the group of negative controls showed mostly lighter weight or, in some cases, the same weight as specimens belonging to the positive controls. According to Cui et al. (2003), a suitable concentration of ascorbic acid in the diet would increase the weight of the silkworm body.

Table 4 illustrates economic parameters, measured in terms of silk production, obtained for the double-larvae. Similar parameters were obtained for the single-cross larvae.

Prolonged deprivation of ascorbic acid from the silkworm diet negatively affected the economic parameters of silk production both in the male and female (see group "−1").

The most interesting result of this study is that feeding silkworms with a diet containing ascorbic acid from the first instar did not significantly increase silk production either in the male or female. Particularly, the groups deprived of ascorbic acid from the beginning of the fourth and fifth instars (group "−4" and "−5") were completely comparable to the positive control, with regard to their silk production both in the male and female. Furthermore, both groups "+2" in the single-cross larvae (data not shown) and "+ 3" in the double-cross larvae gave good productive results (slightly better in comparison to the positive control), supporting Cui's findings about a negative effect of ascorbic acid on silkworm feeding behaviour during the first instars.

The last observation regards the pH values recorded in the two different diets that were almost the same, as shown in Table 5.

DISCUSSION

From the data, it is evident that ascorbic acid plays a key role in silkworm physiology, and that this role changes according to the developmental stage.

During the first instar stage, a stimulatory effect of ascorbic acid on silkworm voluntary feeding was postulated by Ito (1961). According to Cui et al. (2003) and their studies "*in vivo*" and "*in vitro*", the negative effect on voluntary ingestion by silkworm larvae would overcome its physiological pos-

Table 4. Comparison of cocoon weight, shell weight and silk ratio among different larval groups of the double-cross larvae [(119×118)×(120×121)] reared on a diet with or without ascorbic acid

Groups	Male			Female		
	Cocoon weight[a]	Shell weight[a]	Silk ratio	Cocoon weight[a]	Shell weight[a]	Silk ratio
+1	1.402±0.133 bc	0.307±0.042 abc	0.219±0.019 a	1.778±0.200 abc	0.337±0.041 abc	0.188±0.008 ab
−1	1.218±0.343 c	0.201±0.078 d	0.165±0.050 b	1.555±0.298 c	0.230±0.047 d	0.152±0.037 c
+2	1.554±0.125 ab	0.333±0.053 a	0.220±0.026 a	1.873±0.137 ab	0.358±0.033 ab	0.191±0.040 a
−2	1.530±0.245 ab	0.270±0.089 cde	0.172±0.060 b	1.565±0.321 c	0.266±0.052 cd	0.176±0.043 abc
+3	1.450±0.162 ab	0.331±0.049 ab	0.228±0.017 a	1.914±0.201 a	0.369±0.046 a	0.192±0.011 a
−3	1.560±0.201 a	0.326±0.053 ab	0.207±0.018 a	1.825±0.240 abc	0.344±0.047 ab	0.189±0.011 a
+4	1.395±0.223 abc	0.293±0.045 bcd	0.210±0.015 a	1.690±0.259 bc	0.315±0.054 bc	0.187±0.018 ab
−4	1.569±0.214 a	0.341±0.049 ab	0.218±0.031 a	1.988±0.201 a	0.374±0.042 ab	0.188±0.009 ab
+5	1.248±0.198 c	0.242±0.060 cd	0.200±0.033 a	1.602±0.410 c	0.271±0.085 cd	0.168±0.024 bc
−5	1.506±0.102 ab	0.335±0.029 ab	0.223±0.023 a	1.896±0.183 ab	0.355±0.047 ab	0.187±0.012 b

[a] Average weight (g)±standard deviation.
Data shown within the same row and followed by different letters are significantly different ($p<0.05$ level by Tukey-Kramer's test).
+1, +2, +3, +4, +5=larvae reared on diet with ascorbic acid (2%) respectively from 1st, 2nd, 3rd, 4th, and 5th instar.
−1, −2, −3, −4, −5=larvae reared on diet without ascorbic acid (0%) respectively from 1st, 2nd, 3rd, 4th, and 5th instar.

Table 5. pH value of the diet with ascorbic acid (2%) or without ascorbic acid (0%)

	Without ascorbic acid (0%)	With ascorbic acid (2%)
pH value[a]	4.99±0.035 a	4.96±0.021 a

[a] Average±standard deviation.
Data followed by different letters are significantly different ($p<0.05$ level by *t*-test).

itive effect. In our experiments, Cui's findings were not fully confirmed. In the case of double-cross larvae, the body weights of the first moulters were never heavier in the negative control when compared to the positive control, and in the case of the simple-cross larvae, the body weights of the first moulters were not significantly different for both the negative and positive controls. In both strains, negative controls did not anticipate the first moult, but a slight delay was recorded in the case of double-cross larvae, while negative and positive controls moulted in the same period in the case of single-cross larvae.

This different behaviour recorded in the two different strains puts forward the hypothesis that silkworm strains may show different tolerances to the deprivation of ascorbic acid during the first instar stage. In some strains, the deprivation of ascorbic acid may result in a strong beneficial effect on voluntary ingestion so that it can compensate the negative effect on the larval physiological processes during the short period of the first instar and first moult. Cui's findings (2003) of an adverse effect of ascorbic acid on feeding may be supported by the data on the silkworm mortality in group "+2" in the second instar, which is slightly higher than those in positive and negative controls (although not significant). It seems that larvae have to become accustomed to the food that positive control larvae have already accepted. Also consistent with Cui's findings are the production results. In fact, apart from the long-term deprivation of ascorbic acid, which occurred in groups "−1", "−2" , "+4", "+5", in all the other groups productive results show a general improvement in comparison to the positive control, which was fed a diet containing ascorbic acid throughout the larval life. This may support the hypothesis that an artificial diet is better accepted by larvae when it does not contain ascorbic acid.

With regard to groups fed on ascorbic acid diet only in the last instars ("+4" and "+5"), they could not recuperate their productivity and delay in growth. However, their mortality in the last phases of larval life and their transformation into pupae were surprisingly similar to those of the positive control. This phenomenon may involve two as-

pects: the former is the selection of individuals tolerant to ascorbic acid absence from the diet; this selection could have occurred during rearing in bulk until formation of the "+4" and "+5" groups; the latter is an immediate recovery of all the physiological functions regulated by ascorbic acid presence. These two aspects (possibility of selection for tolerance to ascorbic acid absence and efficiency in using the chemical when present) may be taken into account in view of a reduction of ascorbic acid quantity in the artificial diet formulation.

From our data, it is clear that there is a no-return line represented by the third instar. We cannot feed larvae from the first instar on a diet not containing ascorbic acid longer than to the third instar without facing growth retardation and remarkable mortality later on. Furthermore, it is not possible to begin deprivation in the third instar stage and continue it until the end of larval life without encountering the same kind of problems. It may depend on the fact that silkworm larvae can tolerate only a restricted period of time without ascorbic acid corresponding to the duration of the first and second instars (about 10 d) or fourth and fifth instars (about 12 d). It could depend on the particular needs of the third instar stage larvae, which represents the transition period from young instar to grown instar. This hypothesis is supported by the experimental results that show an immediate delay in the growth of group "−3", which moulted a day after the positive control.

The diet pH should be between 4.5 and 5.0 for adaptability to silkworm nutrition. This condition is not altered by ascorbic acid elimination from the diet thanks to the buffering action of other ingredients in the artificial diet.

Under normal conditions of storage, ascorbic acid appeared not to be indispensable in preventing alteration of the diet.

In summary, on the basis of our studies, it seems possible to eliminate ascorbic acid from the formulation of artificial diet, at least for fifth instar stage nutrition, without affecting the production results or altering larval mortality and the length of the larval cycle. Further studies may be required to evaluate the ascorbic acid needs in different silkworm strains.

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