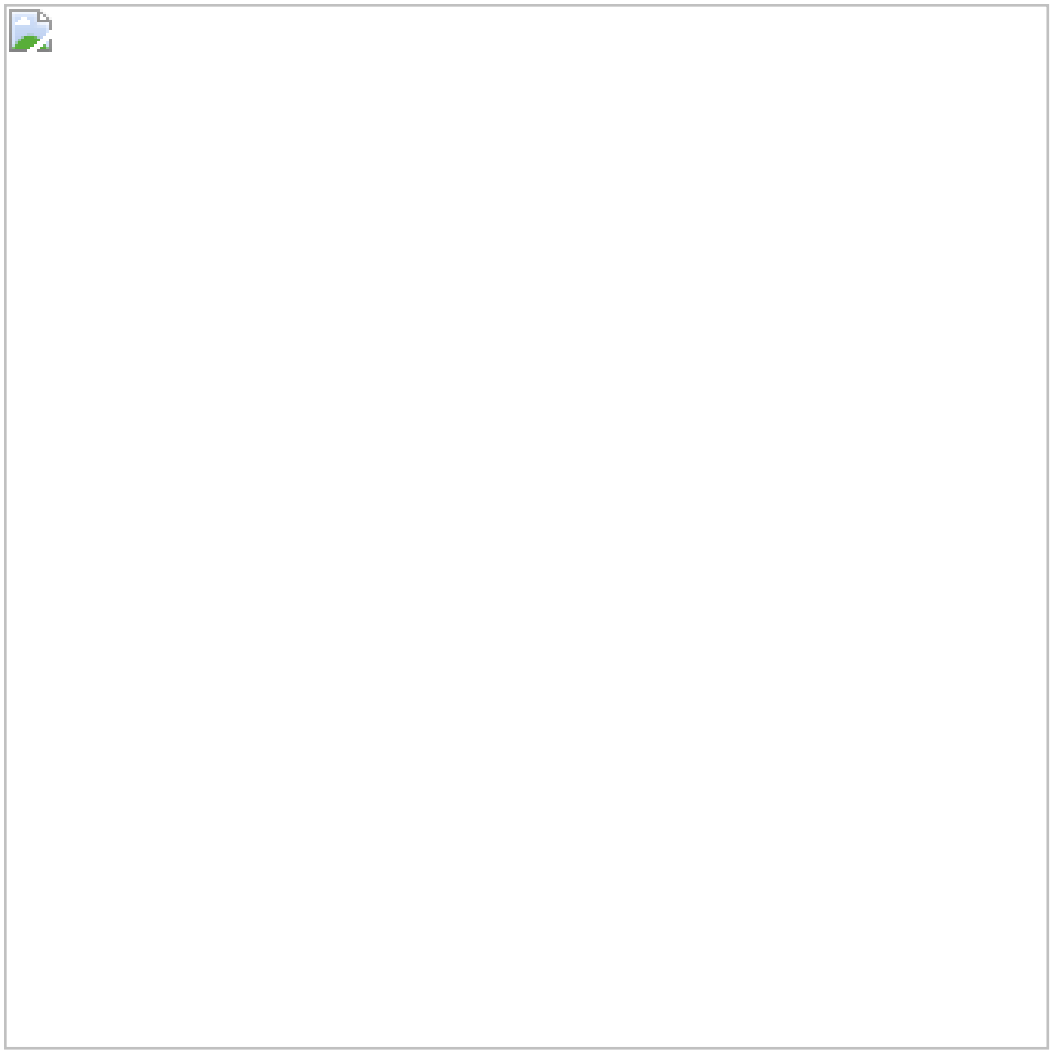

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Diminution of Intracellular Symbiont of Aphid Maintained on Artificial Diet: A Morphological Study

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ABSTRACT—When pea aphids, *Acyrthosiphon pisum*, were raised on an artificial diet which mimics the amino acid composition of the total tissue of aphids, their intracellular symbionts in the bacteriocyte gradually decreased in number as the host generation proceeded, accompanied with a decrease of the host's fecundity. It is possible that host sacrificed its symbionts to supplement the artificial diet that is nutritionally deficient. This inevitably incurs aposymbiosis, which will, in turn, lead to the host's sterility, as observed repeatedly.

INTRODUCTION

Most aphid species harbor prokaryotic intracellular symbionts in the cytoplasm of their bacteriocytes, or mycetocytes [1]. The bacteriocyte symbiont is proposed to play important metabolic roles in the host insect. This has often been discussed in conjunction with the fact that the phloem sap on which aphids feed is nutritionally poor with a high carbon/nitrogen ratio and unbalanced amino acid composition [2, 7, 13]. In fact, it has been demonstrated that the pea aphid, *Acyrthosiphon pisum*, exploits its bacteriocyte symbionts to convert and recycle amino acids [12, 14, 15, 17]. Also, aposymbiotic pea aphids which are produced by the treatment with antibiotics or heat show retarded growth with no fecundity [8, 11], suggesting that the bacteriocyte symbiont is essential to these insects feeding on the nutritionally poor diets.

In the present study, in an effort to further demonstrate the significance of bacteriocyte symbiont to the host, insects were maintained on an artificial diet, and the changes of their symbionts and embryos were examined under a light microscope. As a result, it was demonstrated that feeding aphids on the artificial diet induces gradual diminution of the symbiont, which is followed by reduction of host's fecundity.

MATERIALS AND METHODS

Insects: A long-established parthenogenetic clone of pea aphids, *A. pisum* (Harris) was used in the present study. The stock culture of aphids was maintained on young broad bean plants, *Vicia faba* (L.) at 15°C with photoperiod of 16 h.

Artificial Diet: The artificial diet employed here was prepared according to Febvay *et al.* [3] except that β-alanine and ornithine were omitted [16]. Its amino acid composition had been directly derived from that of hydrolyzate of the whole tissue of pea aphid. The chemical composition of the diet is summarized in Table 1. In the diet the deficiency of tyrosine caused by its low solubility was compensated by addition of β-alanyltyrosine [3]. The solution was filtered through an 0.2 μm filter and aseptically enclosed between the two layers of stretched Parafilm membranes as described by Srivastava and Auclair [18]. The prepared diet sachets were stored at −20°C up to one month. The cages used for experiments were plastic dishes (5.5 cm dia, 1.5 cm high). All experiments were performed at 15°C with photoperiod of 16 h. Diet sachets were renewed every third day [16].

Histochemical Procedures: For paraffin tissue sections, aphids were decapitated and fixed in ethanol formalin solution. The fixed insects were washed in 70% ethanol, dehydrated and cleared through ethanol-xylene series and embedded in Paraplast plus (Monoject). Tissue sections of 3 μm thick were prepared on a rotary microtome and mounted on gelatin-coated microscope slides. The sections on the slides were dewaxed and hydrated through xylene-ethanol-water series, and stained with Ehrlich's haematoxylin. The tissue sections were immunohistochemically stained with anti-symbionin antiserum by which prokaryotic intracellular symbionts were specifically stained in deep brown [4].

RESULTS

Fourth instar nymphs of pea aphid that had been maintained on broad bean shoots were transferred to artificial diet, and their progeny, maintained on the artificial diet, were examined for the density of the bacteriocyte symbiont by immunohistochemistry using antiserum raised against symbionin, a stress protein specifically produced by aphid symbiont [9, 10]. In the following experiments, the first generation born on the artificial diet was referred to as G1. The next generation born by G1 on the artificial diet was called G2. G3, which was born by G2 on the artificial diet, was less in number, and consistently sterile.

As shown in Figure 1A, sagittal thin sections of control insect that had been kept on the plant demonstrated the presence of many bacteriocytes, each of which was filled with

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TABLE 1. Composition of Artificial Diet

L-Amino Acids(mM)	
Non-essential amino acids	
Alanine	20.06
Asparagine, H_2O	19.88
Aspartic acid	6.63
Cysteine,HCl, H_2O	2.44
Glutamic acid	10.15
Glutamine	30.49
Glycine	22.19
Proline	11.23
Serine	11.83
Tyrosine	2.13
Essential amino acids	
Arginine, HCl	14.06
Histidine	6.49
Isoleucine	12.56
Leucine	17.56
Lysine, HCl	19.22
Methionine	4.85
Phenylalanine	10.29
Threonine	10.67
Tryptophan	2.09
Valine	16.29
β-Alanyltyrosine	7.50

Other Chemical Constituents			
Vitamins (mg/100 ml of diet)		Others (mg/100 ml of diet)	
p-Aminobenzoic acid	10	$CuSO_4$, $5H_2O$	0.47
L-Ascorbic acid	100	$FeCl_3$, $6H_2O$	4.45
Biotin	0.1	$MnCl_2$, $4H_2O$	0.65
D-Calcium pantothenate	5	NaCl	2.54
Choline chloride	50	$ZnCl_2$	0.83
Folic Acid	1	Calcium citrate	10
i-Inositol	42	Cholesteryl benzoate	2.5
Nicotinamide	10	$MgSO_4$, $7H_2O$	242
Pyridoxin HCl	2.5	KH_2PO_4	250
Riboflavin	0.5	Sucrose (mM)	845
Thiamine di-HCl	2.5	pH adjusted to 7.5 with KOH	

symbionts. Figure 1A also shows that in the control insect its embryos also contained bacteriocytes filled with symbionts. Figure 1B represents a similar thin section of an adult aphid of G1 that was born and raised on the artificial diet. It is evident that in this insect not only the number of bacteriocyte but also the density of symbiont in each bacteriocyte was markedly decreased. This inclination was further pronounced in the insect of G2, in which both the number of bacteriocyte and the symbiont density in the remaining bacteriocytes decreased to the extremity (Fig. 1C).

Figure 2 is to further demonstrate the difference of bacteriocyte in between those kept on the plant (A) and G2 (B). Bacteriocytes of the control insect were entirely filled with the symbionts (A). In a striking contrast, in the aphid maintained on the artificial diet for two generations, the density of symbiont in the bacteriocyte was much lower, and some cells apparently lost symbionts completely, as indicated by arrow heads in Figure 2B. It was also noted that those bacteriocytes with less density of symbiont were also decreased in cell volume.

In telescoping generations due to parthenogenesis, aphid is infected with symbionts at an early embryonic stage in the ovariole of its mother which is predominantly at nymphal stages [6]. To see the effect of the artificial diet on the transmission of symbiont, we examined the nymphs born and maintained on the artificial diet by immunohistochemistry. As shown in Figure 3A, the second instar nymph of control insect was rich in bacteriocyte, each of which was filled with

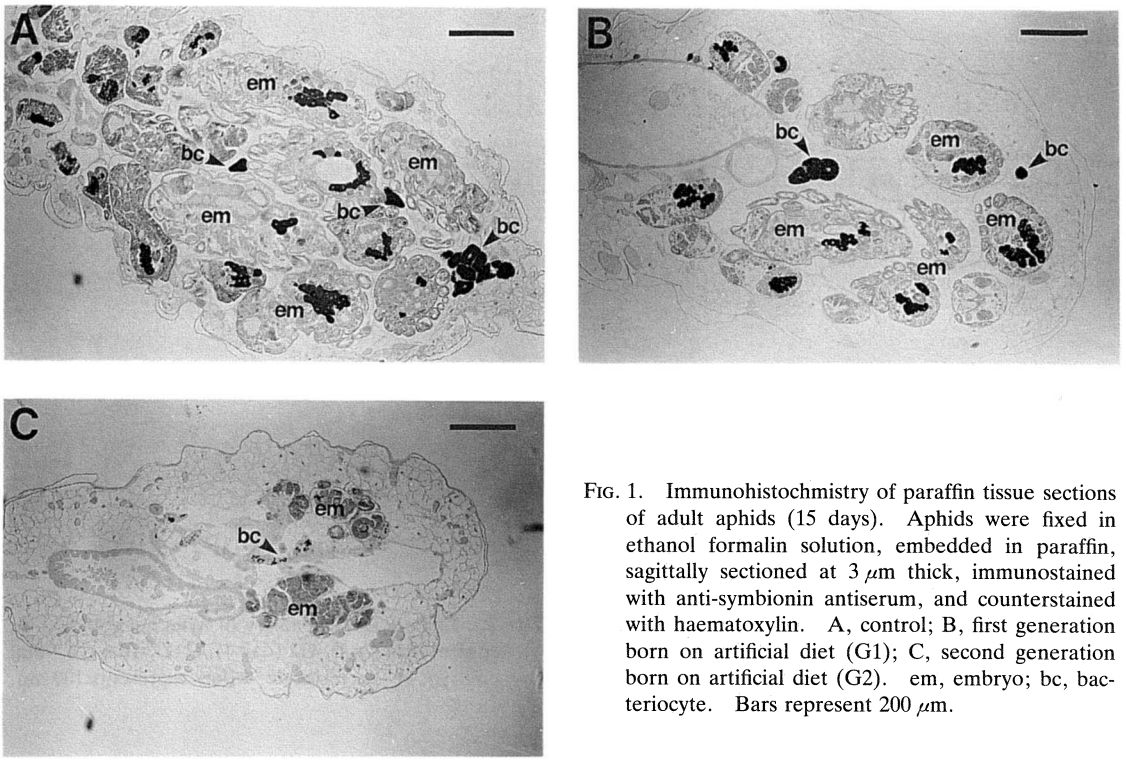

FIG. 1. Immunohistochmistry of paraffin tissue sections of adult aphids (15 days). Aphids were fixed in ethanol formalin solution, embedded in paraffin, sagittally sectioned at 3 μm thick, immunostained with anti-symbionin antiserum, and counterstained with haematoxylin. A, control; B, first generation born on artificial diet (G1); C, second generation born on artificial diet (G2). em, embryo; bc, bacteriocyte. Bars represent 200 μm.

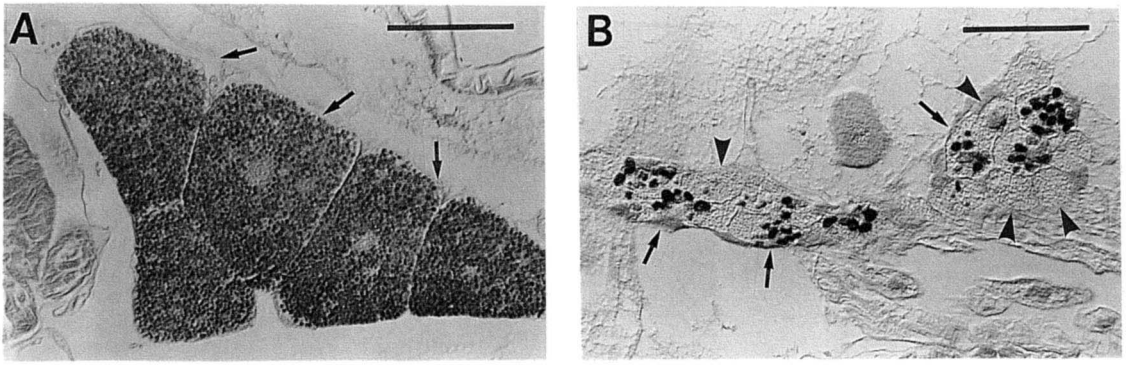

FIG. 2. Bacteriocytes of adult aphids. Adult aphids (15 day) were fixed, sectioned, and stained as under Fig. 1. A, control; B, G2. Arrows and arrow-heads indicate bacteriocytes with and without symbionts, respectively. Bars represent 50 μm. Note that in B bacteriocytes were decreased in both cell volume and symbiont density.

symbionts. In the figure also depicted were the early embryos just in process of infection with symbionts (see later). Figure 3B represents the G2 nymph at the same stage, in which both the number of bacteriocyte and the symbiont density were evidently decreased. Such the differences were more striking when the G3 nymph was examined (Fig. 3C). It was apparent that in neither nymph itself nor embryos was symbionin-stained endosymbiont visible. Such the G3 nymphs did not proceed beyond the third instar, and thus were sterile.

In Figure 4, parts of photographs in Figure 3 were magnified in order to focus on the infection with symbionts. Figure 4A shot three embryos at early developmental stages that happened to be in the process of symbiont infection, where symbiont lumps were just invading the embryos at their posterior poles. Figure 4B represented a similar shot of the G2 nymph in which an embryo was being infected with symbionts. However, it was noted that the density of symbiont invading was much lower, probably reflecting its lower density in the nymphal bacteriocyte. There were a few developing embryos in the G3 nymph, in which presumptive bacteriocyte anlage was observed (Fig. 4C). However, because of lack in infecting symbiont, bacteriocyte was not developed. Probably, this, in turn, prevented embryos from further development, leading to the sterility of the G3 insect.

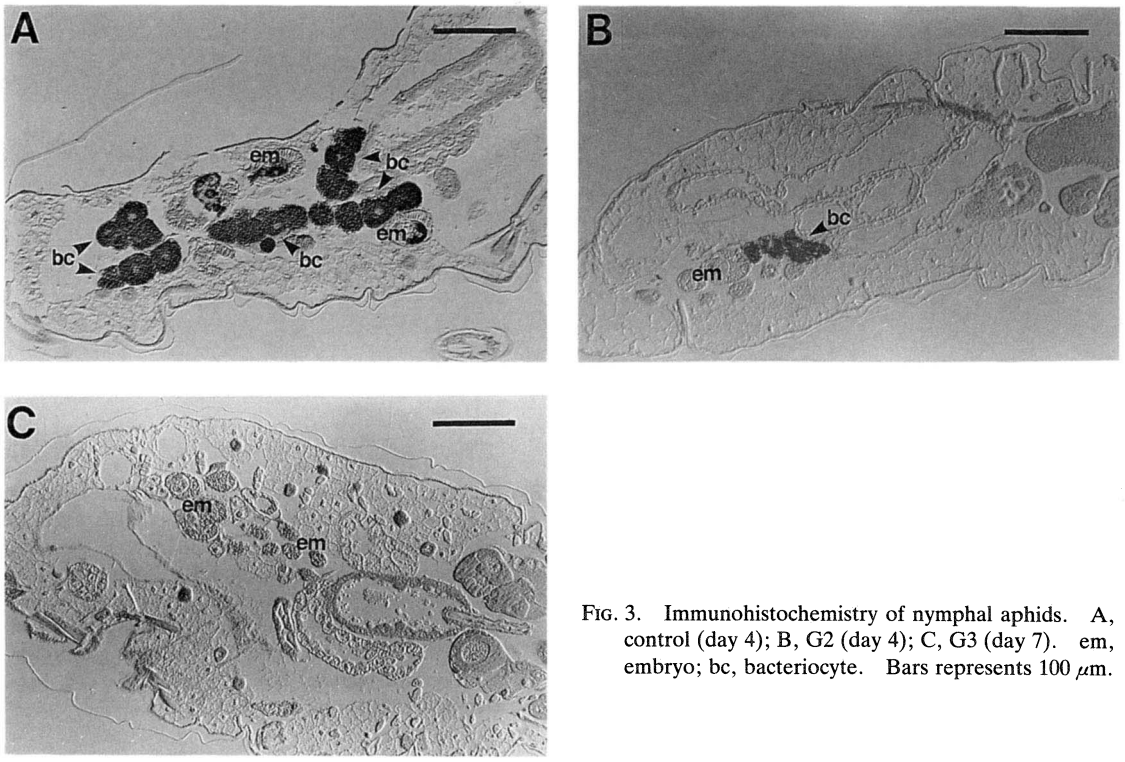

FIG. 3. Immunohistochemistry of nymphal aphids. A, control (day 4); B, G2 (day 4); C, G3 (day 7). em, embryo; bc, bacteriocyte. Bars represents 100 μm.

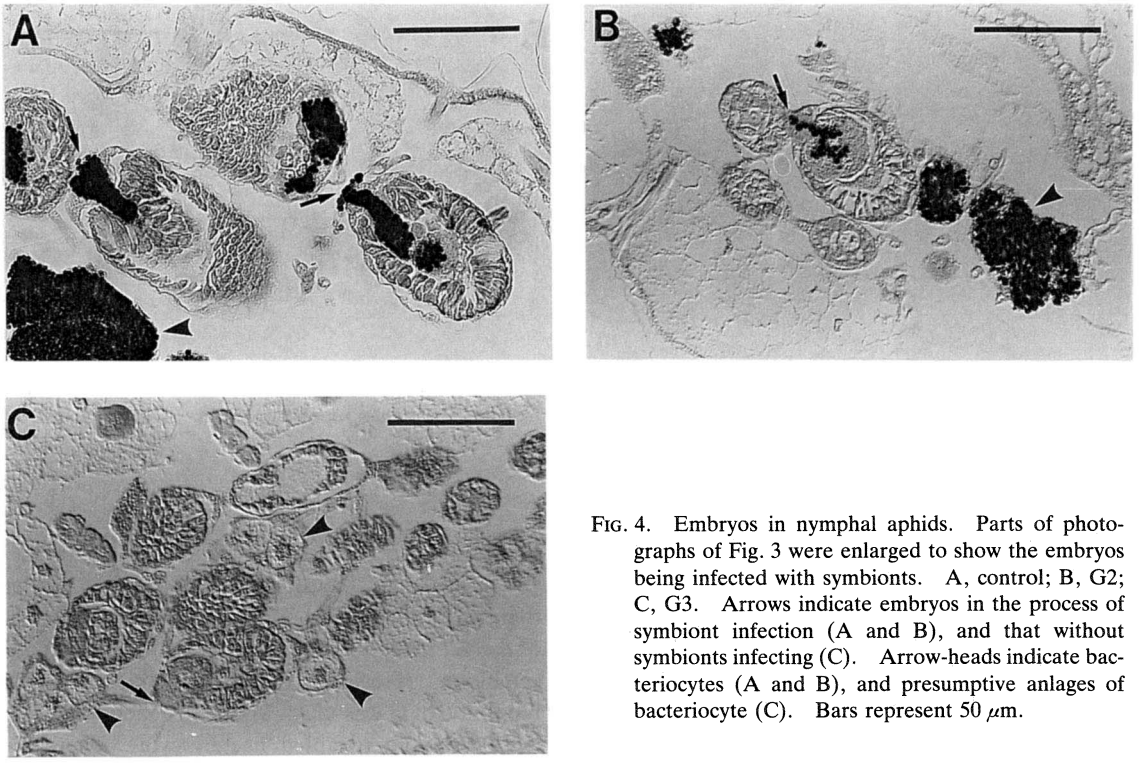

FIG. 4. Embryos in nymphal aphids. Parts of photographs of Fig. 3 were enlarged to show the embryos being infected with symbionts. A, control; B, G2; C, G3. Arrows indicate embryos in the process of symbiont infection (A and B), and that without symbionts infecting (C). Arrow-heads indicate bacteriocytes (A and B), and presumptive anlages of bacteriocyte (C). Bars represent 50 μm.

DISCUSSION

It has been repeatedly reported that deprivation of bacteriocyte endosymbiont by antibiotics or heat treatment leads to the sterility of the host aphid [8, 9, 11]. The present study further evidenced that aposymbiosis caused by nutritional abnormality is also accompanied with the sterility. It is comprehensible that aposymbiosis is always paralleled by

the host's sterility whatever causes aposymbiosis. Probably, it is just because symbiont supplies host with some nutrients without which host cannot support the embryonic development.

Therefore, more important point to be considered in the present study is why the artificial diet causes aposymbiosis. In this context, it should be emphasized that the artificial diet employed in this experiment had been prepared in accordance with the amino acid composition of the total tissue of pea aphid, and thus, compared with the phloem sap of broad bean, was well balanced in terms of amino acid composition [3]. Actually, aposymbiotic aphids that had been produced by antibiotic treatment, which never produced progeny on any other diets including the natural plant, bore nymphs, though very few in number, on the same artificial diet used here [16]. This may be taken to suggest that so far as aphid ingests nutritionally rich and well-balanced diets, its bacteriocyte symbiont is not necessarily essential to growth and reproduction, or is even a nuisance because it appropriates nutrients. The present result that on the artificial diet aphid tended to loose the symbiont may be consistent with this assumption. However, the result that such the aposymbiosis caused by the artificial diet was also followed by the sterility suggested that the diet tested here was still not good enough to support the full development of embryos.

An alternative explanation for the diminution of bacteriocyte symbiont on the artificial diet may simply be that host aphid consumes its symbiotic system on its own physiological demands. Our previous study demonstrated that when aphid is kept away from food, it reduces not only the size and number of the bacteriocyte but also the symbiont density in the cell to survive the adverse conditions. Taken this into consideration, it is not surprising that aphid consumes its symbionts when the artificial diet on which it feeds does not suffice its physiological demands. Catabolites obtained at the cost of the symbiont will, on the one hand, sustain the host's life by supplementing the artificial diet that is nutritionally deficient, but on the other, such incurred aposymbiosis will lead to the host's sterility.

All these considered, if any artificial diet can diminish the bacteriocyte symbiont without loosing the host's fecundity, then it must be a perfect diet, which will permit full understanding of the nutritional role played by the symbiont in the aphid physiology.

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