

Endocytobiote Control by the Host in the Weevil *Sitophilus oryzae*, Coleoptera, Curculionidae

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Abstract

In the rice weevil *Sitophilus oryzae*, the principal endocytobiote (SOPE) is located in a larval bacteriome and in ovaries (apical bacteriome, trophocytes and oocytes only). It was not found in other tissues and it never proliferates, except when eggs are irradiated (1,500 R), which results in oocyte and trophocyte destruction. This observation suggests a mechanism of control of the SOPE. We have demonstrated that this mechanism of control is of chromosomal origin. Firstly we have shown that it is possible to obtain, by selection, weevils with a high density of symbiotes per ovary ($111,000 \pm 4,000$), as well as weevils with a low density ($41,200 \pm 3,000$). Secondly, by crossing these two strains, we provide evidence of a chromosomal factor controlling the number of symbiotes per ovary. Thirdly, when males are irradiated, they induce in their female progeny a depressive effect on the number of endocytobiotes, despite the fact that they do not transmit the bacteria. These interactions between the insect and its symbiotes are necessary for the maintenance of the symbiocosme.

Keywords: Weevil (*Sitophilus oryzae*), symbiosis, symbiote control, selection, irradiation

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1. Introduction

Symbiosis is a widespread phenomenon, particularly among insects (Buchner, 1965; Nardon and Grenier, 1989). It presents various aspects, from ectosymbiosis to endocytobiosis, and affects the metabolism, morphology, cytology and behaviour of the host (Nardon and Grenier, 1993). Several reviews have been devoted to the role of symbiosis in insects (Nardon, 1987, 1988; Campbell, 1989; Douglas, 1989; Ishikawa, 1989; Hurst, 1993). In the integrated endocytobiosis, the symbiote, which is most frequently a Gram-negative bacterium, is located in specialized cells called bacteriocytes and forms a symbiotic tissue, or bacteriome, as in aphids and weevils.

This suggests that the host can control the location of its symbiotes as was already pointed out by Buchner (1965). In this work, we have experimentally demonstrated that the insect also controls the number of its symbiotes. Our model is the weevil *Sitophilus*, especially *S. oryzae*, which is a major pest of cereals through the world.

S. oryzae principal endocytobiontes (SOPE) were discovered by Pierantoni in 1927, and studied by several authors (Mansour, 1930; Scheinert, 1933; Schneider, 1956; Musgrave and Miller, 1953; Nardon, 1971). They are located in ovaries and in the larval bacteriome, and are transmitted to the progeny only by the female. They are always intracellular and lie free in the cytoplasm of host cells, without any surrounding host membrane. Thus, they are "perfectly" tolerated by the insect, which indicates a high degree of intimacy between the two partners. Hence, the partners constitute a new biological unit that is submitted to natural selection: the symbiome (Nardon and Grenier, 1993). Inside this microecosystem, several genomes are in interaction: nuclear, mitochondrial and bacterial genomes. SOPE do not grow outside the host; however, using PCR analysis and sequencing, we have recently identified this symbiote as γ Proteobacteria (Enterobacteriaceae).

Furthermore, some strains of *Sitophilus* were shown to also harbour a secondary symbiote of the *Wolbachia* group, which makes the symbiome *Sitophilus* much more complicated than previously described.

In this work, we shall only consider the principal endocytobiontes (SOPE), which are easily visible in light microscope, because of their relative big size (3 to 20 μm) (Fig. 1). In contrast, the secondary symbiote is not harboured in specialized cells or organ and cannot be observed by the same method.

In ovaries the SOPE are found in apical bacteriomes of ovarioles (Fig. 2), trophocytes and oocytes. In aposymbiotic strains, in the absence of bacteria, the apical bacteriomes do not differentiate, which suggests a genetical interaction between the bacteria and the insect. SOPE are never seen in testis.

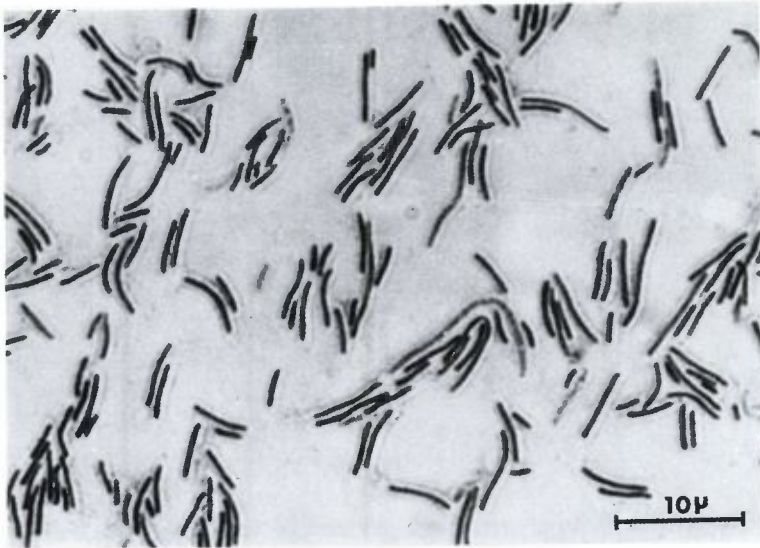


Figure 1. Aspect of the principal endocytobiont of *Sitophilus oryzae*, from a squash of ovaries. Stain: methylene blue.

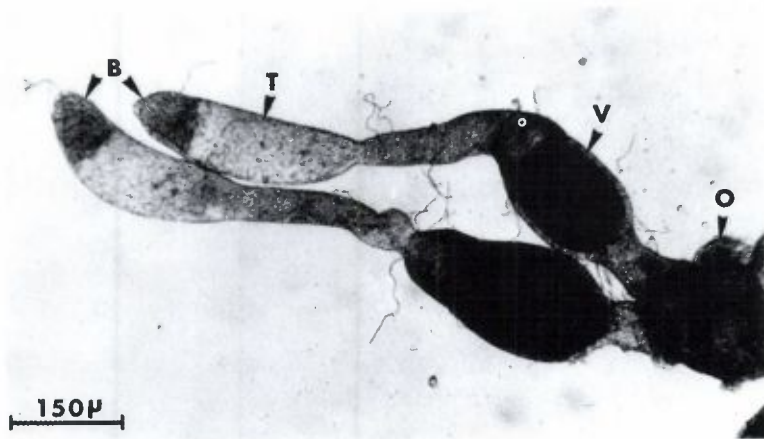


Figure 2. Vital observation in the Yeager's physiological medium, of an ovary of *S. oryzae*. B: apical bacteriomes; O: oviduct; T: tropharium; V: vitellarium.

2. Material and Methods

Insect rearing

The *S. oryzae* strain (SFr) used in this work is from French origin and it has

been reared on wheat since 1959 in the laboratory, according to the procedure described by Laviolette and Nardon (1963), at 27.5°C and 75% r.h. In experiments, the insects used are 2 to 4 weeks old.

Selection experiments

The French strain SFr has been used for selection experiments on development time, because its emergence curve was bimodal (Fig. 3). In the course of selection of a line with rapid development and a line with slow development, we discovered that the development time was related to the symbiote density. To determine the development time, the laying insects are placed in plastic boxes with 20 to 25 grains per insect, during 2 days. Under these conditions we have the highest fecundity and only one egg is laid per grain (Nardon et al., 1981). About one month later the young adults emerging from the grains are counted each day and placed in distinct boxes. The laying period being two days, to determine the development time until emergence of adults, we suppose that all the eggs are deposited in the middle of the oviposition period. When crosses are made, we need virgin females. To keep them virgin, the grains having received (or not) the eggs, are isolated in small special boxes (Nardon, 1964). The emerging adults are immediately sexed, males and females separated and placed on new grains, which are used as control of virginity, in order to verify that one male was not put by error with the females and vice-versa.

Typically, the adults of non-selected SFr weevils emerge from the grains between the 26th and the 44th days (Fig. 3). During the selection process, to obtain more rapid growth, only the adults born before the 29th or 32th day were chosen as parents for the following generation. To obtain a slower development only weevils born after the 34th day were chosen as parents.

Counting the symbiotes

Symbiotes have been counted in eggs, ovaries and larval bacteriome. Ovaries were chosen at nearly the same physiological state: previtellogenesis and onset of vitellogenesis. To count the symbiotes, four ovaries were gently homogenized in a Potter tube, in 0.2 ml of Yeager's solution [NaCl (10 g), KCl (1.5 g), CaCl₂, 1 H₂O (0.5 g), NaHCO₃ (0.18 g), MgCl₂ (0.17 g), glucose (1 g), fructose (0.4 g), maltose (15 g), NaH₂ PO₄ (0.01 g), in one liter of H₂O]. Six eggs ready to ovulate are taken inside oviducts and were also gently homogenized in 0.2 ml of Yeager's solution, but only one larval bacteriome is necessary, taken in the last larval instar at its biggest size. Thereafter, the bacteria are counted in a Thoma cell under a phase contrast microscope ($\times 300$ magnification) and the number of SOPE determined in egg, ovary and larval bacteriome.

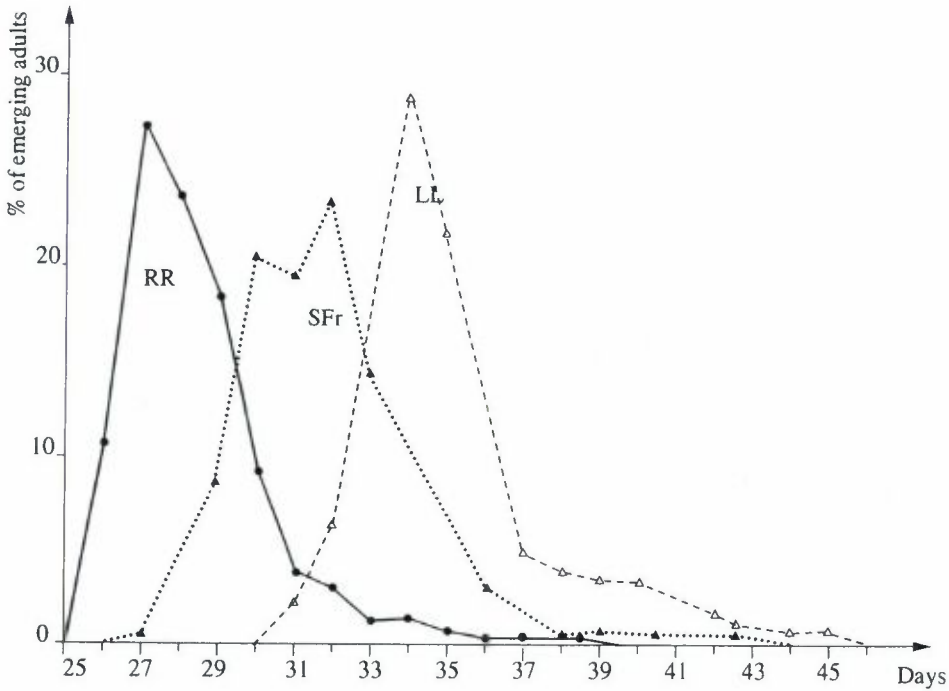


Figure 3. Curves of adult emergence from the grains of wheat in a non selected line (SFr), and in two lines, RR and LL, selected for a rapid and a slow development time, respectively.

Aposymbiotic (= deprived of symbiotes) insects can be obtained by rearing them one month at 35°C and 90% r.h. (Nardon, 1973).

Irradiations

All the irradiations have been made with a radiotherapy apparatus (Siemens Stabilipan). The conditions were 200 KV, 20 mA, with a 2 mm aluminium filter. 430 R/min were delivered, at room temperature. Insects were placed in plastic boxes, and the control insects were treated in the same manner, except for irradiation. Adult insects 3-weeks old, virgin or not, were irradiated at 4,500 R and 8,000 R. Four-days-old eggs were irradiated at 1,500 R. Eight days old larvae (2nd instar) and 17-days-old larvae (4th instar) were irradiated at 2,000 R, and 25-days-old pupae at 3,500 R.

3. Results and Discussion

Two types of experiments, selection and irradiation, have been conducted to demonstrate the control of symbiote number by the insect genome.

Crosses of selected strains

The mean of developmental time for SFr was 31.83 days with a coefficient of variation (cv) of 6.68% (n = 733). After 62 generations of selection for rapid development, we obtained a RR strain with \bar{x} = 28.56 days (cv = 6.32% and n = 809). After only 32 generations of selection for slow development we obtained a LL strain with \bar{x} = 35.19 days (cv = 5.92% and n = 706).

The important fact is the parallel evolution of the number of symbiotes. SFr strain had $57,000 \pm 3,000$ SOPE per ovary, while the selected RR and LL strains had $111,000 \pm 4,000$ and $42,000 \pm 3,000$, respectively. In Table 1 we compare 5 different strains. Two conclusions can be drawn: the evolution of the number of SOPE is similar in ovaries, eggs and larval bacteriome, and the more numerous the symbiotes are, the more rapid is the development. This has to be interpreted as a consequence of the supply of vitamins by the SOPE (Wicker, 1983) and of their interaction with the methionine metabolism (Gasnier-Fauchet and Nardon, 1987), allowing an economy of ATP.

The success of simultaneous selection for development time and number of symbiotes bears evidence of the role of SOPE and their possible control by a genetical mechanism. Another explanation could be the presence of two populations of SOPE in SFr strain, one population with a low metabolism, inducing the LL line, and one population with a high metabolism, inducing a faster development. In this case the selection would have separated the two types of bacteria, excluding the genetical control of the insect itself.

In order to know more and to elucidate this feature, we have crossed the two strains RR and LL.

Number of symbiotes per ovary :

Parents	RR female : $109,722 \pm 4,578$	n = 288
	LL female : $45,312 \pm 2,920$	n = 288
F1	Female RR \times male LL (= RL)	Female LL \times male RR (= LR)
	$76,530 \pm 3,439$ n = 384	$74,186 \pm 3,674$ n = 384

Table 1. Comparison of development time according to the number of symbiotes in *S. oryzae*. Counts in ovaries concern only previtellogenic and onset of vitellogenic stages.

Strains	RR1	RR2	SFr	LL	AA
Symbiote number per larval bacteriome		2,787,500 ($\pm 125,000$) n = 192	1,365,885 ($\pm 100,000$) n = 192	949,375 ($\pm 125,000$) n = 240	0
per ovary	111,328 ($\pm 6,500$) n = 96	88,800 ($\pm 4,900$) n = 240	56,510 ($\pm 2,850$) n = 96	41,930 ($\pm 3,000$) n = 240	0
per egg		23,090 ($\pm 2,700$) n = 96	18,576 ($\pm 3,000$) n = 96	14,930 ($\pm 2,250$) n = 96	0
Development time (days)	27.303 (± 0.153) i = 244	29.749 (± 0.034) i = 11,595	31.392 (± 0.120) i = 3,914	35.291 (± 0.071) i = 8,372	45.927 (± 0.164) i = 5,225

$\pm = ts/\sqrt{n}$ $p = 0.05$; n = number of counts; i = number of insects. To determine the development time, except for RR1, several generations have been grouped.

The number of symbiotes are identical in the two reciprocal F1, and the bacterial density is intermediate between that of RR and LL. That would not be the case if we had selected two populations of bacteria, since in this case we should have observed a maternal effect. Therefore, we must conclude that chromosomal factors with additive effect controlling the number of symbiotes do exist. This is confirmed by the study of backcrosses (Fig. 4). Despite some fluctuations, the introduction of RR genome in LL cytoplasm increases the number of SOPE, while the introduction of LL genome in RR cytoplasm decreases the density of bacteria. This is an additional proof that chromosomal factors of the host are involved in the control of symbiote density.

Study of irradiations

Another proof of the genetical control of the host upon symbiote number is given by studying the effect of irradiation.

An interesting observation appears when adult males are irradiated (5,000 R to 7,000 R) and crossed with non irradiated females. Then we obtain daughters

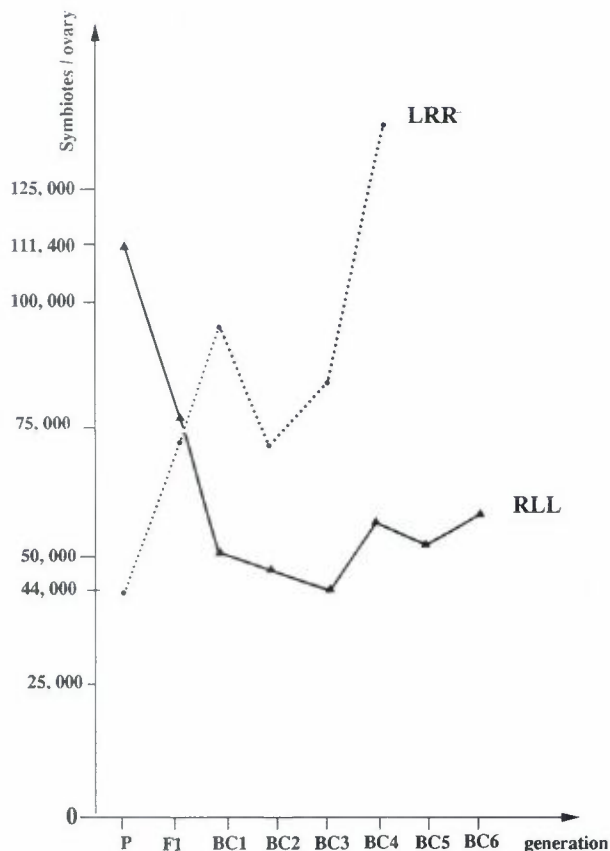


Figure 4. Evolution of the number of the ovarian symbiotes in successive reciprocal backcrosses. LRR: introduction of RR genome in LL cytoplasm; RLL: introduction of LL genome in RR cytoplasm.

completely deprived of SOPE. This can be interpreted by the presence of chromosomal factors affecting not only the number, but firstly the presence of symbiotes. A genetical mechanism necessary for the maintenance of SOPE probably exists, a mechanism inhibited or destroyed by irradiation.

In order to confirm the control hypothesis, we have crossed irradiated adult females (4,500 R) with non irradiated males and vice-versa (Table 2). Both reciprocal F1 crosses had about 32,500 symbiotes instead of 111,000 in the control female ovary. As mothers transmit bacteria to the progeny (these bacteria have been irradiated) the decreased bacterial number in offsprings seems to be due to genetical factors, since the irradiation of the father, which transmits no symbiotes, gave the same result.

Table 2. Influence of radiations (4,500 R on adult weevils) on the number of SOPE per ovary in different crosses: F1, F2, BC1, and F3.

		Female R × male R : Control			
		111,000±6,500	n = 96		
Female I × male R (IR)				Female R × male I (RI)	
32,500±3,600	n = 128	F1		32,400±4,000	n = 96
Female IR × male IR (IR.IR)				RI.RI	
40,000±4,500	n = 96	F2		65,000±5,500	n = 96
IR.RR				RI.RR	
58,000±5,000	n = 96	BC1		95,000±7,500	n = 96
(IR.IR) IR				(RI.RI) RI	
47,500±5,000	n = 96	F3		80,000±7,500	n = 96

I = irradiated parents; R = non-irradiated parents.

In the irradiated female line, the mean number of SOPE per ovary was 44,500 whereas it was 68,100 for the paternal line. Such a difference could be explained by an additional effect of radiation upon the symbiote itself. For instance we have IR.IR < RI.RI with $p < 0.001$. Nevertheless, since the effect of irradiation persists during three generations, even in the paternal line, it is another proof of the existence of a genetical mechanism controlling SOPE density.

A last interesting feature was observed after irradiations on eggs, on larvae or on pupae : we can obtain some females with ovaries deprived of oocytes and trophocytes. In this case the apical bacteriocytes of ovaries invade all the ovarioles, suggesting that the control of bacteriocytes inside the ovary is made by the germ cells.

4. General Discussion and Conclusion

Concerning the regulation of endocytobionts in general, Buchner (1965, p. 684) already noticed: "the host animals are in all respects master of the situation". Koch (1967, p. 51) also claimed "the complete control of the rate of reproduction of the symbionts by the host". This problem has been discussed by several authors (Nardon, 1988; Douglas, 1989; Ishikawa, 1989). The two main questions

are relative to the localisation and to the growth of the population of symbiotes. Integrated endocytobiontes are restricted to specialized cells (bacteriocytes or mycetocytes) or organs (bacteriomes or mycetomes). How the recognition occurs is currently unknown. It seems that inside bacteriocytes the symbiotes could be protected against hydrolytic activities. For instance, in aphids (Hinde, 1971), despite their migration through the body cavity, the symbiotes never infect other tissues, and those which are mislaid in hemolymph are inevitably phagocytised by hemocytes. Nevertheless, lytic digestion is not restricted to hemolymph or other non-harboring tissues (Nardon, 1971). The irradiation experiments have shown that elimination of symbiotes can be obtained by treatment of the male only. We can imagine that the radiations have disturbed or inhibited the genetical mechanism allowing the maintenance of SOPE. The presence of such a genetical mechanism is also supported by the fact that the bacteria are eliminated from the male germ line during embryogenesis while they proliferate in the female one. The physiological mechanism is still unknown.

The regulation of growth of the symbiote population has been recently studied on aphids (Whitehead and Douglas, 1993; Baumann and Baumann, 1994). It was shown that the density of the symbiote population changes according to the age of the insect. Indeed, in *Schizaphis graminum*, quantitative PCR was used to measure the number of copies of the gene coding for 16S rDNA (or *rrs* gene), since this gene is present in only one copy in the symbiote. A one-day-old aphid had 0.2×10^6 copies (= 200,000 bacteria), while a 9-day-old aphid had 5.6×10^6 copies (Baumann and Baumann, 1994). This implies only five division cycles. Similarly, it was shown in *Acyrtosiphon pisum* that "the growth and division of the bacteria in the maternal tissues varied with developmental age" (Whitehead and Douglas, 1993). Since the symbiotes, except in case of irradiation, never invade the whole organism, this implies a control. In *Sitophilus* we have shown that from 20,000 SOPE per egg we obtained about 3×10^6 SOPE in larval bacteriome after 3 weeks. This represents only seven division cycles. Like in aphids, this is very few and suggests a host control. Our experiments demonstrate for the first time the reality of such a control. An apparently polygenic system is present on the chromosomes of the host in *S. oryzae*, controlling the number of SOPE. All our results are convergent with such a concept. This control is necessary for the formation of the symbiocosme.

The subsequent question is the physiological nature of this control. In her excellent review, Douglas (1989) already pointed out that "the insect may destroy excess symbionts or suppress symbiont growth and division". This is the alternative.

An important feature is the fact that most of the obligatory intracellular

bacteria show a reduced genome size as compared to the free-living forms (Charles et al., 1997). *Buchnera aphidicola*, as previously seen, possesses only one *rrs* gene (in Baumann et al., 1995). In the tsetse fly the P-endosymbionts have also a single rDNA operon (Aksoy, 1995). In *S. oryzae*, the genome size is 3.0 Mb, versus 4.7 Mb for *E. coli*, a bacterium closely related to SOPE. Furthermore, SOPE has only two 23 S rDNA genes versus seven in *E. coli* (Charles et al., 1997). Such genomic deletions could explain a low metabolism of symbiotes, but do not explain the regulation itself. How the insect can modulate the division of symbiotes is unknown, perhaps by some proteins able to modify the expression of genes involved in the cell division. In a more speculative hypothesis we can also imagine that genes have been transferred from the bacterium to the host genome. If these genes are involved in bacterial division, it would explain why the symbiote is no longer able to grow *in vitro*, and how the insect can control its endocytobionts.

Another idea is brought from the fact that the number of SOPE is relatively stable in a given strain, but varies according to the diet. Indeed, a strain reared on wheat presented $54,200 \pm 3,600$ SOPE per ovary. Transferred on maize it had only $34,850 \pm 3,100$ SOPE. We conclude that the number of symbiotes is very sensitive to the nature of the diet. Thus, we can hypothesize a nutritional control at the cell level, since the endocytobionts exclusively depend on nutrition by the host. In the same way, according to Brooks (1960), the bacterioides of cockroaches are sensitive to the diet, particularly to mineral salts.

Another possible influence on symbiote number is the hormonal composition. In *Blattella germanica*, according to Tsang (1981) juvenile hormone could be necessary for symbiote growth. A similar conclusion is given by Philippe et al. (1988) for *Periplaneta americana*.

Suppression of symbiotes in excess, is another possibility of control, non contradictory with the control of division. Lytic digestion of endocytobionts has been described in *Sitophilus* (Musgrave and Grinyer, 1968; Nardon, 1971) and in other insects (Louis and Giannotti, 1974). One of the involved enzymes could be β -N-acetylglucosaminidase (Nardon et al., 1978). In *S. oryzae* lysis is mainly visible in apical bacteriomes of ovaries (Nardon, 1971) and in oocytes. But they are rare in larval bacteriome. According to their location, we may assume that the control is variable: control of divisions during the growth of the SOPE population in the larvae, and lytic mechanism in the adult, to maintain their density. Schwemmler and Muller (1986) also emphasized the role of lysozyme in leafhoppers.

Ishikawa (1989) put forwards the possible role of the antibacterial substances, such as cecropins, secreted by the insects. He also suggested "the presence of host mechanisms that regulate the growth of endosymbionts" and

he demonstrated "that the tissue extract of aphids contains substances that inhibit DNA, RNA and protein synthesis in their mycetocyte symbionts *in vitro*". This aspect must be further investigated.

We completely agree with Douglas (1989) when she noticed that symbiotic insects not only differ from aposymbiotic ones by the presence of symbiotes but also by an interaction between of the two partners. Their relation is not only additive, but interactive. That is the reason why we have proposed the concept of symbiocosme (Nardon and Grenier, 1993). The co-genomes interact and maintain the equilibrium in the symbiocosme. The genetic control of symbiote number is therefore an essential mechanism issued from the coevolution of partners.

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