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Julien Varaldi, M. Ravallec, C. Labrosse, M. Lopez-Ferber, M. Boulétreau, et al.. Artificial transfer and morphological description of virus particles associated with superparasitism behaviour in a parasitoid wasp.. *Journal of Insect Physiology*, 2006, 52 (11-12), pp.1202-1212. 10.1016/j.jinsphys.2006.09.002 . hal-00428031

**HAL Id: hal-00428031**

**<https://hal.science/hal-00428031>**

Submitted on 27 Jul 2023

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# Artificial transfer and morphological description of virus particles associated with superparasitism behaviour in a parasitoid wasp

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## Abstract

In parasitoids, the adaptive significance of superparasitism (laying of egg(s) in already parasitized hosts) has been the subject of strong controversy. The current view is to interpret this behaviour as an adaptation to increased competition for hosts, because the supernumerary egg still has a chance to win possession of the host. However, we recently discovered that in the solitary parasitoid *Leptopilina boulardi*, superparasitism is rather caused by an unknown infectious element: stable non superparasitizing lineages (NS) are transformed into stable superparasitizing lineages (S) after eggs from both lineages have competed inside the same host (superparasitism). In this report, we investigate the nature and location of the causative agent. Involvement of bacteria is unlikely because antibiotic treatments do not affect wasp phenotype and because bacterial 16S ribosomal DNA was not detected using PCR. We report successful injection experiments showing that the causative agents are located in wasp poison gland and ovaries and are stably inherited. Electron microscopic studies demonstrate that long filamentous virus particles located in wasp oviducts are strongly associated with superparasitism behaviour, leading to reconsider the adaptive significance of this behaviour in parasitoids. Interestingly, parasitoids are often infected with similar viruses for which no phenotypic effect has been documented. This raises the possibility that they could induce the same behavioural manipulation.

**Keywords:** Virus; Manipulation; Parasitoid; Superparasitism; Behaviour; *Leptopilina*; *Drosophila*; LbFV

## 1. Introduction

Many species of endoparasitoids contain viral particles in their reproductive apparatus. These particles reproduce in the oviducts, at the base of the ovaries (often called the calyx) and are transmitted to host larvae during egg laying (= oviposition). Some of these viruses are completely integrated into the wasp genome and have evolved toward a mutualistic relationship with the parasitoid since they inhibit the immune system of the parasitoid's host, thus allowing parasitoid development (polydnviruses found in Braconidae and Ichneumonidae, Schmidt et al., 2001;

Drezen et al., 2003; Federici and Bigot, 2003; Whitfield and Asgari, 2003; Espagne et al., 2004; Kroemer and Webb, 2004). In these cases, viral particles have lost their infectious capacities and the fitness of the viral particles is indistinguishable from that of the insect host.

Other viral particles including members of the Ascoviridae, Poxviridae, Reoviridae, picorna-like and filamentous viruses are also found in the reproductive apparatus of parasitoid (reviewed in Stoltz and Whitfield, 1992; Stoltz and Makkay, 2000; Federici and Bigot, 2003; Reineke and Asgari, 2005; Renault et al., 2005). Unlike polydnviruses, their infectiousness has been demonstrated or is suspected. Some members of each group have been proved able to replicate both in the parasitoid and its host (Renault et al., 2002; Lawrence, 2002; Rabouille et al., 1994; Stoltz and

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Makkay, 2000; Styer et al., 1987; Stoltz and Faulkner, 1978). Most of this type of viral particle are apparently not pathogenic to their parasitoid hosts and some parasitoid/virus associations appear to be partially specific, showing signs of coevolution (Bigot et al., 1997). Thus, for these viruses, transmission from one parasitoid generation to the next can be partly independent from that of parasitoid genes and the fitness of both partners is not completely linked as in the integrated systems described above. Consequently, the interests of both partners can, on some occasions, diverge. Note that this type of virus can coexist within a single female with polydnavirus in some species (Hamm et al., 1990; De Buron and Beckage, 1992; Stoltz and Makkay, 2000).

One situation where the interests of virus and parasitoid are predicted to diverge derives from the special life cycle of parasitoids. The unique free-living stage of parasitoids is the adult stage, whereas immature stages develop as parasites (of other arthropods and especially other immature stages of insects). Since parasitic development leads, in most parasitoid lifecycles, to the death of their host, parasitoids constitute a major agent of biological control of agricultural pests (Van Lenteren, 2003). Typically, after mating, adult females leave their natal patch and start to search for suitable hosts. During this foraging stage, females display a complex set of behaviours, whose efficiency is directly linked to the quantity of offspring produced. This tight link between behaviour and fitness has advanced parasitoids as ideal model organisms for the study of adaptation (see Godfray, 1994 for a review). When encountering a potential host, females have to decide whether or not to lay an egg (or a clutch of eggs) on or into it (in the following, we will focus on endoparasitoids that lay their eggs inside the host). This decision is under strong natural selection because (i) the offspring will not be able to change hosts during development, and (ii) the quality of resource that a host represents for parasitoid development is very variable from one host to another. In particular, female parasitoid wasps often encounter potential hosts that have already been parasitized by another female, or by the female wasp herself. These parasitized hosts represent poor quality resources, since one host can only support the development of a limited quantity of parasitoids (only one for solitary parasitoid species). However, a second clutch of egg(s) still has a chance to complete development, although the resident one is usually at a competitive advantage (Van Alphen and Visser, 1990). Thus, females of most parasitoid species are able to distinguish between parasitized and unparasitized hosts and usually refrain from ovipositing in the former (Van Lenteren, 1981). In contrast to the parasitoid, however, an infectious agent carried by the parasitoid could take a great advantage of superparasitism behaviour since that behaviour could allow its horizontal transmission from one immature parasitoid to another.

In a previous paper (Varaldi et al., 2003), we reported that a parasitoid (Hymenoptera: Figitidae: *Leptopilina*

*boulardi*) that attacks *Drosophila* larvae displays strong inter-female variability of superparasitism behaviour: when they forage alone on hosts, some female wasps always refuse to lay an additional egg into a previously parasitised host (henceforth called NS females), whereas other females readily accept such a host (= superparasitism behaviour, henceforth called S females). Differences in superparasitism behaviour between S and NS females are still observed whatever the number of females foraging on the same hosts, although the NS females will display superparasitism behaviour under strong competition conditions (Varaldi et al., 2005). Under superparasitism conditions, conflict and physiological interactions between parasitoid immatures inside the host always lead to the development of, at most, one parasitoid (solitary parasitoid species, Carton et al., 1986). Polymorphism of superparasitism behaviour is observed in the wild and is maternally inherited. Furthermore, we showed that during the interactions between parasitoid immatures for the possession of the host, the superparasitism phenotype could be horizontally transferred: daughters of non-superparasitising females that develop and win within-host competition against offspring of superparasitising females acquire the superparasitising phenotype on emergence (with a probability of approximately 55%) (Varaldi et al., 2003 and unpublished). Infection by this superparasitism-inducing element imposes an overall low cost on parasitoid physiology (no effect on adult survival and brood sex-ratio, adult size reduced by 2%, development duration increased by 3%, egg load increased by 10% and locomotor activity reduced by 46%, Varaldi et al., 2005) and specifically affects superparasitism behaviour (Varaldi et al., 2006). This behavioural modification can be shown theoretically to increase the fitness of the infectious behaviour-modifying agent at the expense of parasitoid fitness, in realistic ecological conditions (Gandon et al., 2006). Preliminary results suggested particles with viral morphology as potential candidates for inducing superparasitism behaviour (Varaldi et al., 2003).

Here, we report investigations that aimed at determining the nature and location of the particles involved in the modification of *L. boulardi* superparasitism behaviour. Since parasitic wasps are frequently infected with symbiotic bacteria (Vavre et al., 2000; Hunter et al., 2003; Zchori-Fein and Perlman, 2004) and because one bacterium is known to be associated with a change in the oviposition behaviour of the wasp (Zchori-Fein et al., 2001), we first test the hypothesis that a bacterium is responsible for this phenotype. We then report successful artificial horizontal transfer of the particles extracted from different tissues. Finally, transmission electron microscopy investigations of infected tissues are reported, revealing that the superparasitising phenotype is clearly associated with the presence of filamentous virus particles (LbFV) that are probably injected together with the eggs into the *Drosophila* host. We propose that modification of parasitoid

superparasitism behaviour could be a general pattern used by infectious parasitoid viruses.

## 2. Materials and methods

### 2.1. Strains and culture

The parasitic wasp *Leptopilina bouvardi* (Hymenoptera: Figitidae: *Leptopilina bouvardi*) develops as a solitary larval parasitoid primarily of *Drosophila melanogaster* and *D. simulans* (Carton et al., 1986). It is widely distributed in mediterranean climates. Starting from two homozygous strains (originating from Sienna, Italy and obtained through inbred crosses, described in Varaldi et al., 2003) displaying opposite superparasitism behaviour, called Sienna1 (non-superparasitising (NS) phenotype) and Sienna9 (superparasitising (S) phenotype), we created a new strain, called Sienna0, by repeatedly crossing Sienna1 females with Sienna9 males during five generations of back crosses. After these repeated backcrosses, the new Sienna0 strain bears most of the nuclear alleles of Sienna9 strain while the cytoplasmic compartment comes from the Sienna1 strain (the proportion of common nuclear alleles between Sienna9 and Sienna0 at a given generation  $t$  is given by the formula:  $\theta_{t+1} = \theta_t + 1/2(1-\theta_t)$ ). The introgressed strain (Sienna0), although harbouring 97% of the Sienna9 genotype, displayed a NS phenotype, thus confirming the maternal transmission of the trait (mean number of eggs per parasitized host  $\pm$ se, Sienna0 =  $1.00 \pm 0.00$ ,  $n = 15$ , Sienna9 =  $3.23 \pm 0.33$ ,  $n = 14$ ).

We also used two other strains that display opposite superparasitism behaviour: a French strain from Antibes (S phenotype) and a Portugese strain from the island of Madeira (NS phenotype). From the Antibes strain, we selected the few females that displayed the typical NS phenotype (no more than one egg per host, see next section for superparasitism measurement) to found a new strain. In each generation, the phenotype of a sample of females was checked as previously and only those exhibiting the NS phenotype were used to found the next generation. In only four generations, we obtained a stable NS strain (Antibes NS). Conversely, by superparasitism horizontal transmission, we obtained a S strain from the Madeira strain (Madeira S; Varaldi et al., 2003). The different strains, their phenotypes and the methods by which they were obtained, are summarized in Table 1. In the laboratory, parasitoids were reared at 26°C on a *D. melanogaster* strain originating from Ste Foy (France) fed with standard medium (David, 1962).

### 2.2. Estimation of superparasitism behaviour

As in Varaldi et al. (2003), isolated virgin females (1 or 2 days old) were placed from 5pm to 10am with ten first instar *Drosophila* larvae on an agar-filled Petri dish spotted with a small amount of yeast. After 24–48 h, five *Drosophila* larvae from each Petri dish were dissected and

the number of parasitic immatures (eggs and larvae) counted. Superparasitism behaviour of each female was estimated as the mean number of immatures per parasitized host that equals the mean number of eggs injected into the hosts by the female.

### 2.3. Antibiotic treatments and 16 S PCR amplification

#### 2.3.1. PCR amplifications

A PCR-based approach was used to detect for the possible presence of bacteria in *L. bouvardi*. Individuals used for DNA extractions were mechanically extracted from the pupae of their host in a droplet of 1% sodium hypochlorite to prevent external contamination. DNA extractions were then performed with Chelex 5% as described in Vavre et al. (2000). Primers were designed to amplify a 1180 bp fragment of 16S ribosomal DNA of all eubacteria (O'Neill et al., 1992). PCR conditions and primers are described in O'Neill et al. (1992). This setup was performed on 5 females from Sienna0 and Sienna9 strains. As a positive control, we used 6 females of a closely related species (*L. heterotoma*) which is, conversely to *L. bouvardi*, naturally infected with the endosymbiotic bacterium *Wolbachia* (Vavre et al., 1999, 2000).

#### 2.3.2. Antibiotic administration

Antibiotics were applied to parasitoids via the developing host larvae: parasitized *Drosophila* larvae were provided with standard medium mixed with the desired antibiotic, as described in Dedeine et al. (2001). This mode of administration proved effective for curing parasitoids of their bacterial symbionts in various parasitoid species (Vavre et al., 2000; Dedeine et al., 2001; Mouton et al., 2004; Pannebakker et al., 2004). We treated Sienna0 and Sienna9 strains with either Rifampicine (2 mg/g of *Drosophila* standard medium) for one generation, or Tetracycline (1 mg/g) for two successive generations. These concentrations are known to cure insects of their bacterial symbiont in a single generation (Vavre et al., 2000; Dedeine et al., 2001; Mouton et al., 2004; Pannebakker et al., 2004). Both rifampicine and tetracycline are among the most broad spectrum antibiotics known, active against both Gram negative and Gram positive bacteria (Campbell et al., 2001; Chopra and Roberts, 2001), and in the case of tetracycline even against some chlamydiae, mycoplasmas, and protozoan parasites (Chopra and Roberts, 2001). One generation after treatments ceased, the superparasitism behaviour of emerging females was estimated, as above.

### 2.4. Injection experiment

Injections were performed on second instar *Drosophila* larvae that had been parasitized by Sienna0 females (NS phenotype) during the 24 preceding hours. Different extracts from reproductive apparatus of donor females were prepared as follows: by dissection of females, we removed their long gland with its reservoir, which is known

Table 1

Name, phenotype (mean number of eggs/parasitized host  $\pm$  SE,  $n$ : number of females tested) and methods of obtainment of the different strains used in the experiments together with the presence of virus particles in wasp oviducts

Name	Phenotype	Method of obtainment	Virus presence
Sienna1	NS $1.00 \pm 0.00$ , $n = 5$	Sib-mating for 8 generations	—
Sienna9	S $3.28 \pm 0.37$ , $n = 20$	Sib-mating for 8 generations	1/1
Sienna0	NS $1.00 \pm 0.00$ , $n = 20$	Introgression of Sienna9 homozygous genotype into Sienna1 (NS) cytotype	0/1
Sienna0-PBS	NS $1.00 \pm 0.00$ , $n = 4$	Injection of PBS in Sienna0 strain	0/2
Sienna0-GI 0	NS $1.00 \pm 0.00$ , $n = 6$	Injection of long gland extracts from Sienna0 in Sienna0 strain	0/2
Sienna0-Ov 0	NS $1.00 \pm 0.00$ , $n = 5$	Injection of ovaries extracts from Sienna0 in Sienna0 strain	0/1
Sienna0-GI 9	S $3.69 \pm 0.58$ , $n = 23$	Injection of long gland extracts from Sienna9 in Sienna0 strain	4/5
Sienna0-Ov 9	S $3.55 \pm 0.32$ , $n = 11$	Injection of ovaries extracts from Sienna9 in Sienna0 strain	3/3
Antibes	S $3.25 \pm 0.47$ , $n = 16$	Natural	1/1
Antibes NS	NS $1.00 \pm 0.00$ , $n = 5$	Selected from Antibes	0/3
Madeira	NS $1.00 \pm 0.00$ , $n = 15$	Natural	0/2
Madeira S	S $2.65 \pm 0.47$ , $n = 15$	Horizontal transmission from Antibes through superparasitism	1/1

to contain particles that are injected into the host (Rizki and Rizki, 1990; Dupas et al., 1996; Labrosse et al., 2003), and their ovaries (including paired glands and part of the oviduct, see Labrosse et al., 2003 for a description of these organs). For each injection series, 25 long glands or 25 ovaries of either Sienna0 (NS) or Sienna9 (S) females were crushed in approximately 15  $\mu$ L of 1% PBS solution, centrifuged at 6000 g for 4 min, and kept on ice until injection. Injection of PBS solution alone was used as a control.

Injections (ca. 0.03  $\mu$ L extract/larva) were performed using an Eppendorf transjector (FemtoJet<sup>®</sup>) as in Labrosse et al. (2003). Success of fluid injection was checked by verifying slight dilatation of the injected larvae. We treated 100–250 host larvae in each combination (injections of PBS, long gland or ovaries from Sienna0 or Sienna9 females) since this technique can induce high mortality (Labrosse et al., 2003; 68% mortality in this study). Injected larvae were fed standard diet and kept at 25 °C until adult wasps emerged. Superparasitism behaviour of emerging females was then estimated for the different combinations as described above. A sample of females emerging from injected hosts were used to found isofemale lines in order to check for the transmission of phenotypes throughout generations.

### 2.5. Electron microscopy

After individual control of their superparasitism behaviour (using the setup described in the section Estimation of superparasitism behaviour), females were killed and their ovaries and long gland dissected. These organs were fixed for 2 h in a solution of 2% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.4) and post-fixed in 2% osmium tetroxide in the same buffer for 1 h (room temperature). Tissues were then dehydrated in a series of graded acetones and embedded in Epon's medium. Sections were cut on a LKB ultratome. Thin sections were double stained in uracyl acetate and lead citrate. Samples were examined with a Zeiss EM 10CR transmission microscope at 80 kV. Ovaries of a total of 11 females for each phenotype (S or NS) and long gland of one female for each phenotype (S or NS) were prepared in this way.

## 3. Results

### 3.1. Test for bacterial implication

A great number of insect parasitoid species harbour intra-cellular symbiotic bacteria like *Wolbachia*, that alter the reproduction (O'Neill et al., 1997), or even alter the

behaviour (Zchori-Fein et al., 2001) of the wasp. Thus, we first tested the hypothesis that the S phenotype could be due to a symbiotic bacterium in two ways: first, PCR amplification with primers designed to detect 16S ribosomal RNA genes from any prokaryote; and, second, by attempting to cure the wasps of any symbiotic bacteria by administering wide spectrum antibiotics.

We found that extracts from Sienna0 (NS) and Sienna9 (S) lineages all failed to detect prokaryotic 16S rDNA, whereas extracts from *L. heterotoma* which is naturally infected with the symbiotic bacteria *Wolbachia* (Vavre et al., 2000) were all positive (Fig. 1). Further, rifampicine and tetracycline antibiotic treatments did not influence the behaviour of Sienna0 and Sienna9 strains, that is, Sienna0 continued to lay 1 egg/host larvae (NS) whereas Sienna9 continued to be superparasitising (S, Table 2). Since the antibiotics used are efficient virtually against all eubacteria and that the PCR assay is designed to detect all eubacteria (O'Neill et al., 1992), these results strongly suggest that S phenotype is not caused by any eubacterial infection.

### 3.2. Identification of parasitoid tissues harbouring the superparasitism-inducing element

Our aim was to identify at least one parasitoid tissue harbouring the superparasitism-inducing element in order to further identify it using electron microscopy, and not to perform a comprehensive survey of all parasitoid tissues. The best candidates for tissues harbouring the superparasitism-inducing element are tissues that could explain the vertical transmission of the phenotype, as previously described

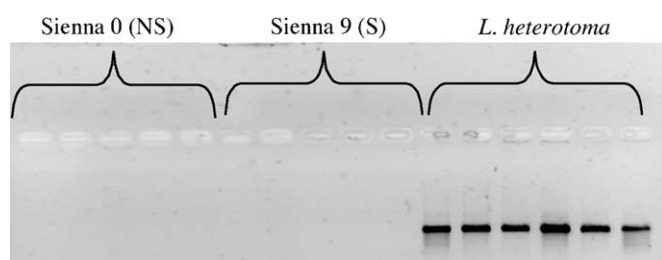


Fig. 1. Gel electrophoresis after PCR amplification using 16S generalist primers on non superparasitizing females *L. boulandi* (Sienna0), superparasitizing females *L. boulandi* (Sienna9) and females *L. heterotoma* naturally infected with the symbiotic bacteria *Wolbachia*.

(Varaldi et al., 2003). Thus we chose the long glands and the ovaries. To determine whether these tissues harbour the element, we performed injection experiments. *Drosophila* larvae parasitised by Sienna0 (NS) females were injected with freshly prepared extracts of long gland or ovaries dissected from Sienna0 (NS) or Sienna9 (S) females. The individual superparasitism behaviour of each emerging female was then tested in controlled conditions (see Section 2). First of all, control injection of PBS alone did not change the typical NS behaviour of emerging Sienna0 females (Fig. 2a). Second, injection of neither long gland nor ovary extracts from Sienna0 (NS) females changed emerging wasp behaviour (Fig. 2b). However, injection of extracts from Sienna9 (S) females did change the behaviour of emerging females: long gland extracts induced the S phenotype in 15/32 females (47%), and ovary extracts in 18/40 females (45%) (Fig. 2c). To test for the stability of transformed lines over generations, a sample of females that acquired the S phenotype after injection were mated (with males from the Sienna0 NS strain) and used to found lines ( $n = 11$  for each injection type). We also founded lines from the control injections (injections of PBS alone and of tissues from Sienna0 females). Each line that acquired S phenotype after injection proved stable for superparasitism behaviour in the next

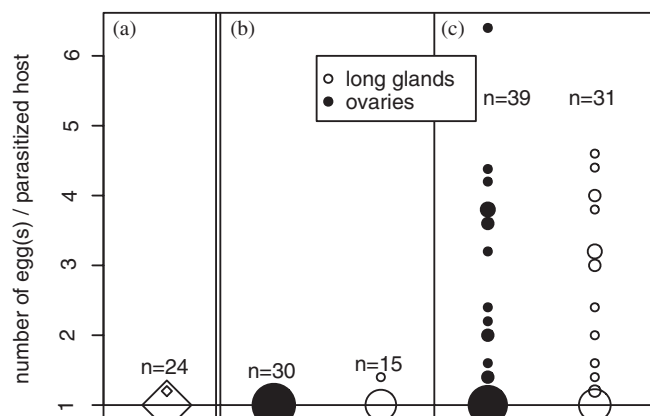


Fig. 2. Superparasitism behaviour of females emerging from hosts parasitised by Sienna0 (NS) females and injected with (a) PBS, (b) long glands or ovaries extracted from Sienna0 (NS) females or (c) long glands or ovaries extracted from Sienna9 (S) females. Area of symbols is proportional to number of individuals. The superparasitism behaviour of each female was estimated using the setup described in the method section (each female provided with 10 hosts).

Table 2

Effect of antibiotic treatments on the superparasitism behaviour of *L. boulandi*, measured as the mean number of eggs/parasitised host  $\pm$  SE

	Controls (no antibiotic)		Treatment		Statistics	P-value
	Sienna0 (NS)	Sienna9 (S)	Sienna0 (NS)	Sienna9 (S)		
Tetracyclin	1.00 $\pm$ 0.00 $n = 10$	3.68 $\pm$ 1.00 $n = 8$	1.05 $\pm$ 0.05 $n = 10$	3.46 $\pm$ 0.65 $n = 10$	F <sub>treatment</sub> = 0.02 F <sub>strain</sub> = 21.1	>0.8 <0.0001
Rifampicin	1.00 $\pm$ 0.00 $n = 8$	3.98 $\pm$ 0.70 $n = 10$	1.00 $\pm$ 0.00 $n = 10$	2.92 $\pm$ 0.40 $n = 10$	F <sub>treatment</sub> = 1.4 F <sub>strain</sub> = 31.2	>0.2 <0.0001

generations (Table 3), whereas control lines continued to display the NS phenotype. The infectious agent(s) responsible for superparasitism is thus present in the long gland and the ovaries of S females, absent in NS females, remains highly infective after tissue extraction and artificial injection, and is accurately inherited from one generation to the next.

This injection experiment thus provided us with five new strains sharing exactly the same homozygous genotype (from the initial inbred Sienna0 NS strain): two superparasitising strains (injected with long gland or ovaries extracts of Sienna9 S females), and three non superparasitising strains (injected with control PBS, long glands or ovaries of Sienna0 NS females). Table 1 gives a summary of the strains used in the experiments, their mode of obtainment and their respective phenotype.

### 3.3. Identification of the superparasitism-inducing element

In order to search for a putative agent responsible for the superparasitism behaviour, transmission electron micro-

Table 3  
Superparasitism phenotype of a sample of lines either injected with PBS, long gland from NS females (G1 NS), ovaries from NS females (Ov NS), long gland from S females (G1 S) or ovaries from S females (Ov S)

Injection	Line #	G0	G1	G4
PBS	21	1.00	1.00	1.00±0.00 (n = 2)
	23	1.00	1.00	1.00±0.00 (n = 2)
G1 NS	11	1.00	1.00	1.00±0.00 (n = 2)
	12	1.00	1.00	1.00±0.00 (n = 2)
	13	1.25	1.00	1.00±0.00 (n = 2)
Ov Ns	23	1.00	1.00	1.00±0.00 (n = 5)
G1 S	6	3.20	4.00	2.33±0.33 (n = 2)
	9	2.00	4.33	—
	13	3.75	3.33	—
	22	4.00	2.33	1.50±0.16 (n = 2)
	23	4.60	2.33	3.10±0.40 (n = 3)
	24	3.00	7.33	4.92±0.40 (n = 4)
	28	4.40	5.00	—
	29	3.20	3.67	4.25±0.25 (n = 4)
	30	3.20	3.67	5.00±1.00 (n = 2)
	31	1.60	3.33	5.16±1.16 (n = 2)
	32	1.40	5.00	3.00±0.33 (n = 2)
Ov S	4	1.60	9.66	—
	7	3.20	5.67	—
	9	2.25	2.67	—
	16	3.20	3.67	—
	24	1.40	1.33	—
	25	4.20	7.00	3.17±1.17 (n = 2)
	32	3.80	5.67	—
	34	2.20	2.00	5.00±0.67 (n = 2)
	36	6.40	3.67	2.67±1.35 (n = 3)
	39	2.00	1.67	—
	40	3.60	5.67	3.00±0.67 (n = 2)

The phenotype is given as the mean number of eggs/parasitized *Drosophila* larva and was measured on females emerging from the injected host (G0), and one generation later (G1). Part of these lines were maintained until the fourth generation (G4) and two to five females per line were then tested (mean ± SE, n = sample size).

scopy investigations were performed on the long gland and ovaries of all these strains, which both proved infectious. Preliminary non-exhaustive TEM studies on a single individual of each phenotype failed to reveal any putative agent in the long gland, despite its infectiousness. However, upon observing the classical symptoms of viral replication in nuclei at the base of the ovaries of a Sienna9 female (Fig. 3c and d) that were completely absent in the corresponding organs of Sienna0 females (Fig. 3a and b), we decided to focus our analysis on the oviducts of strains displaying opposite superparasitism behaviour. The presence of virus particles was clearly associated with superparasitising behaviour, whatever the origin of the strain (Table 1): No virus was found in females exhibiting the NS phenotype (11 females checked), whereas it was observed in 10 out of 11 females showing the S phenotype; the exception may have resulted from an inappropriate cutting level in the organ with the microtome. In effect, particles are observed only in a thin region of the oviduct located in its upper part, just after the ovarioles. There is a strong correlation between the presence of the virus and the superparasitism behaviour which is statistically highly significant (Fisher's Exact Test:  $P < 0.001$ ).

To study the apparent life cycle of this virus, thin sections of cells bordering the lumen of the oviducts of S females were analysed. Virogenic stromae were observed in the nuclei of most cells bordering the lumen of the oviduct, suggesting that particles replicate in the nucleus (Fig. 3c and d). Mature particles later move to the cytoplasm where they can reach high densities (Fig. 4a and b). Particles appear to concentrate at the border of the lumen (Fig. 4c). Nucleocapsids found in the nuclei are slightly flexuous and are often aggregated (Fig. 4d). The diameter of the nucleocapsid is about 45 nm (Fig. 4e) and length sometimes exceeds 1 µm. While moving from the nucleus to the cytoplasm, particles acquire an envelope (their diameter in the cytoplasm is 60 nm). We never observed particles within the eggs.

The apparent intranuclear replication of these particles makes them likely to be DNA viruses. Because of their long, rod-shaped and slightly flexuous capsids, we call them LbFV for *L. bouleardi* filamentous virus particles, based on the nomenclature used for previously described viruses of parasitoids (Krell, 1987; Styer et al., 1987; Stoltz et al., 1988; Hamm et al., 1990; Bigot et al., 1997; Gotham et al., 1998; Cheng et al., 2000; Stoltz and Makkay, 2000).

## 4. Discussion

We have demonstrated in a previous study the infectious transmission of the superparasitism phenotype in *L. bouleardi* strains (Varaldi et al., 2003). Using injections, we have here confirmed the high infectivity of this phenotype already demonstrated in natural superparasitism conditions. The failure of wide spectrum antibiotic treatment to affect superparasitism phenotype together with the failure of PCR using 16S general prokaryotic

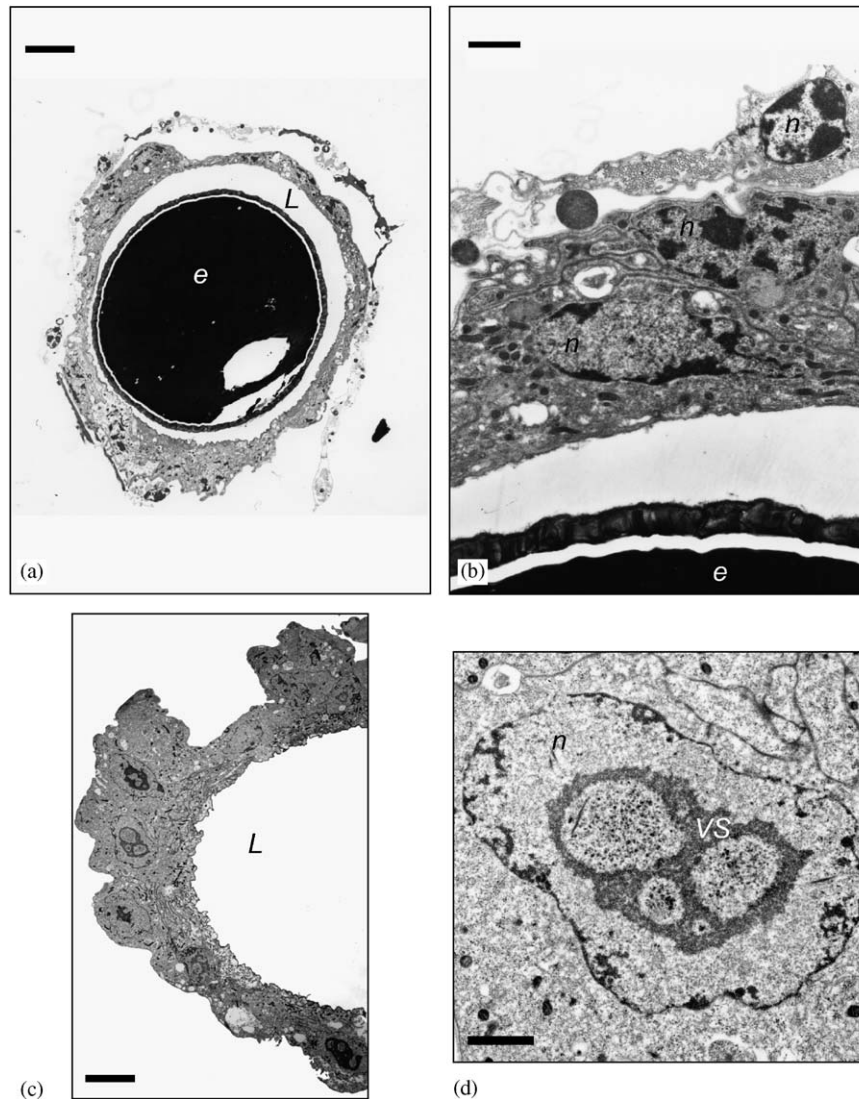


Fig. 3. Symptoms of virus infection in cells of the oviduct in “superparasitizing” females. Cross sections of NS (a and b) and S (c and d) oviducts as observed by transmission electron microscopy. (a) An egg is observed in the lumen of the oviduct (NS female). e = egg, L = lumen, bar = 6  $\mu$ m. (b) Nuclei show a normal morphology (NS female). n = nucleus, bar = 1  $\mu$ m. (c) Nuclei of the cells bordering the lumen show typical features of virus infection (S female). L = lumen, bar = 6  $\mu$ m. (d) Most nuclei of S females’ oviduct cells showed typical symptoms of virus infection with vacuoles probably corresponding to virogenic stromae where viral particles replicate. n = nucleus, VS = Virogenic Stroma, bar = 1  $\mu$ m.

primers designed to detect 16S rDNA genes of all eubacteria (O’Neill et al., 1992), make it unlikely that any eubacterial symbiont is involved. Injections indicate that the responsible agent is located in both the long gland and the ovaries, and TEM analysis revealed that the presence of virus particles is strongly correlated with superparasitism behaviour. Unfortunately, we have so far not succeeded in purifying these particles, and thus to definitively prove their responsibility by injecting them. However, we manipulated superparasitism behaviour through three independent ways: horizontal infectious transfer among parasitoid immatures within the host (Madeira strains, Varaldi et al., 2003), artificial transmission by injection of tissue extracts (Sienna homozygous strains), and loss by segregation (Antibes strains). With all methods, we observed a clear positive correlation between the presence

of LbFV in oviducts and superparasitism behaviour. Thus, our results very strongly suggest that the particles observed by TEM are responsible for superparasitism behaviour in *L. boulandi*. To our knowledge, this is the first time that virus have been shown to modify the behaviour of a parasitic wasp, and more generally one of the few documented examples of viral-induced modification of foraging behaviour (previously described effects include elevation seeking behaviour of virus-infected lepidopteran larvae and modification of feeding rates in mosquito–virus systems, see Moore, 2002 for a review).

#### 4.1. Mode of action of LbFV on superparasitism behaviour

The action of LbFV on superparasitism behaviour raises the question of the mechanisms underlying the manipulation



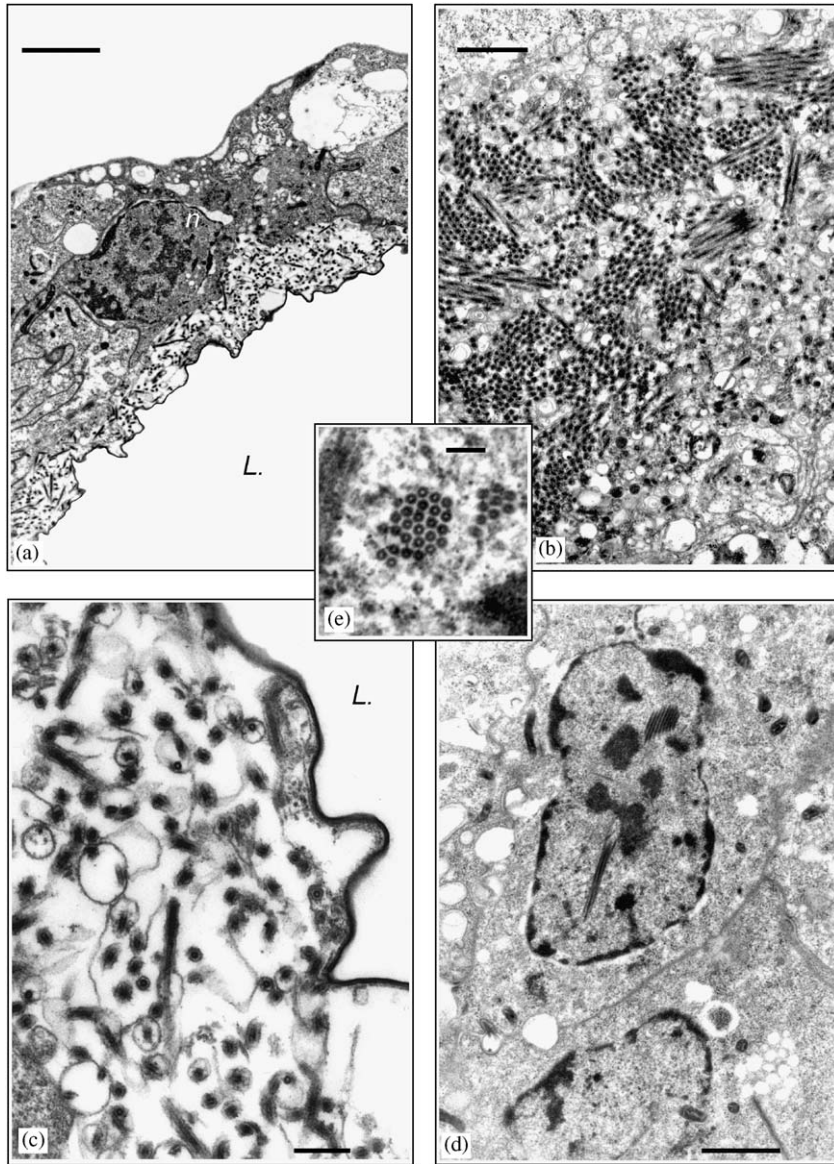


Fig. 4. Virus replication in superparasitising *L. boulandi*'s oviducts (TEM photographs). (a) Apparent intranuclear replication in cells bordering the lumen of the oviduct. n = nucleus, L = lumen, bar = 2.5  $\mu$ m; (b) high densities of virions in cell cytoplasm, bar = 1  $\mu$ m; (c) structure of virions accumulated close to the lumen, L = lumen, bar = 250 nm; (d) structure of viral particles within nucleus, bar = 1  $\mu$ m; (e) transverse section of nucleocapsids within nucleus, bar = 200 nm.

process. Little is known about the mechanisms used by parasites to alter their host behaviour, although some data are beginning to come forward, especially for macroparasites (Adamo, 2002; Thomas et al., 2003; Biron et al., 2005). In addition, the cytopathological effects of viruses responsible for behavioural disturbance have been studied from a medical perspective in mammals (Tomonaga, 2004). From these studies, it appears that these viruses cause neuronal disturbance in the central nervous system due to cell injury, to modification of the expression of host genes (Kramer et al., 2003; Solbrig et al., 1996) or because of a by-product of the host immune response (which indirectly harms infected cells, Furrer et al., 2001). Females *L. boulandi* need to pierce the skin of the host larvae with their ovipositor to detect chemical cues associated with a previous infestation: when an

uninfected female encounters an already parasitized host, she usually inserts her ovipositor but rejects the host quickly. The superparasitism behaviour modification induced by LbFV could thus result from a defect in the way the infected female perceives these cues (a reduction in discrimination abilities). One tentative hypothesis is that the virus particles injure the cells involved in signal perception (ovipositor sensillae) or disrupt the processing of the nervous signal in direction of the central nervous system. This can be the product of specific cell lysis or of modification of gene expression in the nerve cells.

However, superparasitism behaviour could also result from a modification of the female wasp's acceptance decision after a correct signal perception and integration in the central nervous system. Several theoretical models

predict that females should adjust their superparasitism decisions according to the quality of the environment in which they forage (for example increase superparasitism tendency when travel time between patches is high; Visser et al., 1992a). There is indeed some empirical evidence showing that females acquire information on their environment which they use to modulate their superparasitism tendency (Visser et al., 1992a, b). It is therefore possible that LbFV modify the female's superparasitism behaviour indirectly, by manipulating her perception of the overall quality of the environment.

Furthermore, processes of learning and memory strongly influence the superparasitism decisions of females (Van Lenteren, 1976; Ueno and Tanaka, 1996). Interestingly, in the closely related species *L. heterotoma*, if the first host encountered by a female upon emergence is parasitized, she will readily accept it, thus engaging in superparasitism whereas after an experience with unparasitized hosts she will start rejecting parasitized hosts (Van Lenteren, 1976). In addition, it has been shown that mimicking amnesia in the Ichneumonidae *Pimpla nipponica* results in a strong increase in self-superparasitism (Ueno and Tanaka, 1996). In this species, therefore, interfering with the learning abilities of females can result in changes in superparasitism decisions.

#### 4.2. Vertical transmission models for LbV

We previously showed that the superparasitism phenotype (i.e. virus particles) is vertically and horizontally transmitted. However, how does vertical transmission occur? Is vertical transmission effected through transovarian transmission or by re-infection of the parasitoid larval instar during consumption of the infected host haemocoel (pseudo-vertical)? Our results do not clearly answer this question but could suggest the possibility of pseudo-vertical transmission. First, even though we did not extensively search for virus within the egg, in the examinations we did carry out, we never found any in eggs. Moreover, successful contamination through injection clearly indicates that pseudo-vertical transmission is possible, an eventuality that previously had no support. Indeed, natural horizontal transmission previously demonstrated in superparasitism conditions (Varaldi et al., 2003) could be explained by contamination during conflicts between larvae, which frequently occur in this species (pers. obs.). Our results demonstrate that successful transmission does not require the occurrence of conflict or wounding, since newly infected females developed alone in their injected host. Furthermore, it is known that *L. bouleardi* females inject products of the long gland (which probably contains virus particles responsible for superparasitism) into its host during oviposition (Labrosse et al., 2003). Pseudo vertical transmission is thus possible, although it does not exclude transovarial transmission. The mean by which the viruses observed in the oviduct gain access to the oviduct is unknown. However, we can speculate that when an egg passes through the oviduct,

the mechanical disturbances provoked break the oviduct epithelium, thus releasing the virus. Indeed, infected cells often show a detachment of the epithelium from the adjacent cell membrane, which result in an electron-non-dense zone (see Figs. 4a and c). This structure is potentially more delicate than that observed in uninfected cells (see Fig. 3a and b). However, we never observed virions in the lumen, possibly because they are released only when an egg passes through. The question of whether this virus replicates within host larvae tissues remains to be investigated for the *L. bouleardi*/LbFV system, as it has been documented in some other parasitoid/virus systems (Stoltz and Faulkner, 1978; Styer et al., 1987; Rabouille et al., 1994; Lawrence, 2002; Bigot et al., 1997).

#### 4.3. Possible generality of the phenomenon

LbFV share some morphological traits with particles found in other parasitoid species (the braconids *Cotesia marginiventris*, *C. congregata* and the ichneumonids *Diadegma terebans*, Hamm et al., 1990; De Buron and Beckage, 1992; Krell, 1987; Stoltz and Vinson, 1977, 1979), which also all have long rod-shaped nucleocapsids (20–40 nm diameter, >600 nm length), and which also seem to be genetically independent from their parasitoid host. For instance, nudiviruses of *C. marginiventris* are capable of replicating within host larvae (Styer et al., 1987). They seem to replicate within the nuclei of the wall cells of oviducts (or so called calyx), suggesting that they are DNA viruses (attempts at purification and nucleic acid analysis failed). They also seem to be injected into host larvae together with parasitoid eggs. All of these viruses, as far as they have infectious capacities, can surely be horizontally transferred within a superparasitised hosts. Thus, it is likely that they can benefit from superparasitism behaviour, allowing them to colonise new parasitoid lines. Theoretical developments clearly indicate that increasing the females' tendency to superparasitise is beneficial for viral spread in most ecological conditions (Gandon et al., 2006). Until now, these parasitoid viruses could not be credited for any effect on their wasp hosts, and thus we wonder (i) whether they could also be horizontally transmitted in superparasitism conditions, and (ii) whether they could modify the superparasitism behaviour of females, as LbFV do. This hypothesis is supported by the occurrence of horizontal transmission among parasitoid larvae in some parasitoid/virus systems (Bigot et al., 1997; Gotham et al., 1998). Modifying superparasitism behaviour could be a general strategy that increases the chance of horizontal transmission of infectious viruses of parasitoids (reviewed in Federici and Bigot, 2003).

#### 4.4. Concluding remark

Some infectious viruses coexist with completely integrated polydnviruses that are integrated into and only replicate from the wasp genome (Stoltz et al., 1988; Stoltz

and Whitfield, 1992; Volkoff et al., 1995; Drezen et al., 2003; Federici and Bigot, 2003). Evolutionary relationships between infectious viruses and more integrated ones remain poorly understood despite preliminary molecular data that are now available (Federici and Bigot, 2003), and descriptions of new parasitoid/virus associations could be helpful. From this viewpoint, the discovery of LbFV is especially exciting, since they are possibly related to nudiviruses (as found in *C. marginiventris*) which have been considered a possible evolutionary source of polydnaviruses of braconid wasps (Federici and Bigot, 2003).

## Acknowledgements

We thank J. Wang, J.P. Rivals, N. Volkoff, M. Poirié, J.M. Drezen, P. Mavingui and V. Tran for helpful discussions, L. Humblot for technical help and D. McKey for English revisions. This work was supported by the CNRS (UMR 5558 and GDR 2153).

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