Prevalence of a virus inducing behavioural manipulation near species range border

S. PATOT,*‡ J. MARTINEZ,* R. ALLEMAND,* S. GANDON,† J. VARALDI* and F. FLEURY*

* Université de Lyon; F-69000, Lyon; Université Lyon 1; CNRS, UMR5558; Laboratoire de Biométrie et Biologie Evolutive; F-69622, Villeurbanne, France, †Centre d'Ecologie Fonctionnelle et Evolutive (CEFE); UMR 5175; 1919 route de Mende; F-34293 Montpellier, France, ‡Present address: Instituto Gulbenkian de Ciência; Rua da Quinta Grande 6; P-2780-156 Oeiras, Portugal.

Abstract

The densities of conspecific individuals may vary through space, especially at the edge of species range. This variation in density is predicted to influence the diffusion of species-specific horizontally transmitted symbionts. However, to date there is very little data on how parasite prevalence varies around the border of a host species. Using a molecular epidemiology approach, we studied the prevalence of a vertically and horizontally transmitted virus at the edge of the geographic range of its insect host, the *Drosophila* parasitoid wasp *Leptopilina boulardi*. *L. boulardi* is a Mediterranean parasitoid species showing a recent range expansion to the north (in France). The LbFV virus manipulates the behaviour of females, increasing their tendency to lay additional eggs in already parasitized *Drosophila* larvae (superparasitism). This is beneficial for the virus because it allows the virus to be horizontally transferred during superparasitism. We show that LbFV prevalence is very high in central populations, intermediate in marginal populations and almost absent from newly established peripheral populations of *L. boulardi*. We failed to detect any influence of temperature and diapause on viral transmission efficiency but we observed a clear relationship between prevalence and parasitoid density, and between parasitoid density and the occurrence of superparasitism, as predicted by our epidemiological model. Viral strains were all efficient at inducing the behavioural manipulation and viral gene sequencing revealed very low sequence variation. We conclude that the prevalence reached by the virus critically depends on density-dependent factors, i.e. superparasitism, underlying the selective pressures acting on the virus to manipulate the behaviour of the parasitoid.

Keywords: behavioural manipulation, *Drosophila* parasitoids, epidemiology, *Leptopilina,* species range border, vertically transmitted virus

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Introduction

All species have a limited distribution and understanding the ecological processes that shape their geographical ranges is an important issue in ecology and biogeography (see Gaston 2009 for a review). Although species have heterogeneous distribution at large spatial scale, a widespread biogeographical assumption is that local abundance and population size declines from the centre towards the range edges of species (Sagarin & Gaines 2002; Sagarin *et al.* 2006). Despite the fact that this general rule suffers exceptions (see Sagarin *et al.* 2006), many empirical studies are consistent with this 'abundant-center hypothesis' (Sagarin & Gaines 2002; Angert 2009; Gaston 2009; Sagarin *et al.* 2006 for a review).

Variation in abundance towards the range edges is expected to have particular consequences in the context of host–parasite interactions where density-dependent transmission takes place. In particular, classical epidemiological model predicts that specialist parasites are expected to go to extinction at low host density

Correspondence: Sabine Patot; E-mail: spatot@igc.gulbenkian.pt

(Anderson & May 1981; but see Antonovics 2009). Consequently, it is expected that the decrease of density around host species margins should lead to reduced prevalence or to the absence of specialized parasites. However, very few empirical investigations focused on the epidemiology of parasites at the range limit of their hosts (Briers 2003; Alexander *et al.* 2007; references in Gaston 2009).

Symbioses (defined here in the general sense: intimate associations between species that can be mutualistic or parasitic) are extremely frequent in insects, and may have strong effects on host phenotype (Varaldi *et al.* 2003; Tsuchida *et al.* 2004; Varaldi *et al.* 2006a; Teixeira *et al.* 2008; Oliver *et al.* 2009). Symbionts are diverse (viruses, bacteria, fungi), their transmission mode ranges from strictly vertical to strictly horizontal and the interactions with their host range from parasitism to mutualism. Decrease in the density of the host in marginal populations is expected to have consequences on the persistence and spread of symbiotic partners (Anderson & May 1981). Clearly, both the transmission modes of the symbiont (Altizer & Augustine 1997; Rudgers *et al.* 2009) and its host range are key factors determining the expected consequences of a reduction in host density. The prevalence of horizontally transmitted symbionts with density-dependent transmission (see de Castro & Bolker 2005) should decline with a decrease in host density (see, however, the special case of sexually transmitted diseases whose transmission is expected to be frequency dependent, Ryder *et al.* 2005). However, when symbionts are strictly vertically transmitted to host offspring, the frequency of the symbiont should be less affected by variations in host density. Their dynamics should mainly depend on the fitness of infected hosts. Thus, in a given environment, they should provide a fitness benefit to their host to spread (at the exception of manipulative parasites, Vavre *et al.* 2009), as observed with protective symbionts in aphids (Oliver *et al.* 2003, 2009). Lastly, symbionts may benefit from mixed horizontal and vertical transmission. In this case, the transmission of the symbiont is partly linked to the fitness of the host and partly linked to density-dependent processes defining the opportunities of horizontal transmission. Depending on the relative contribution of each transmission mode to the fitness of the symbiont, the consequences of variations in host density may be more or less dramatic.

In this article, we studied a fly-parasitoid-virus community to test whether a decrease in the density of the parasitoid in marginal populations will affect the epidemiology of a virus that benefits from mixed vertical and horizontal transmission. In the south-east of France, *Drosophila melanogaster* and *Drosophila simulans* larvae are mainly attacked by two related endoparasitic wasps,

Leptopilina heterotoma and *Leptopilina boulardi* (Hymenoptera: Figitidae). Both species are solitary parasitoids, i.e. only one parasite per host can complete development to the adult stage. Accordingly, female parasitoids lay a single egg per oviposition. *L. heterotoma* has a holarctic distribution, whereas the distribution of *L. boulardi* is restricted to tropical and Mediterranean climates. In the south of France, where both species coexist, *L. boulardi* is by far the dominant species, outcompeting *L. heterotoma* (Fleury *et al.* 2004, 2009). More to the north, the abundance of *L. boulardi* strongly decreases with a clear-cut species border situated around Valence (45°N, 100 km south of Lyon, Allemand *et al.* 1999 and Fig. 1). Thirty kilometres above this latitude, *L. boulardi* is completely absent and *L. heterotoma* dominates the whole parasitoid community. This situation observed in 1998 has drastically changed during the last decade with a progression of *L. boulardi* of about 60 km to the north (Allemand, unpublished data). This expansion of *L. boulardi* geographic range could be explained by climate modification combined with the high competitive ability of this species. Moreover, some females of *L. boulardi* are sometimes infected with a heritable virus that manipulates their egg laying strategy: infected females are more willing to oviposit in hosts previously parasitized by conspecifics than uninfected females, a behaviour called superparasitism (Varaldi *et al.* 2003, 2006c). This virus, named LbFV (for *Leptopilina boulardi* Filamentous Virus), takes advantage of this behavioural alteration since it can be horizontally transferred during the coexistence of uninfected and infected parasitoid larvae inside the superparasitized *Drosophila* larva (Varaldi *et al.* 2003, 2006a). On the contrary, the sympatric species *L. heterotoma* is not infected by the virus, because of virus specialization or refractoriness of *L. heterotoma* to virus
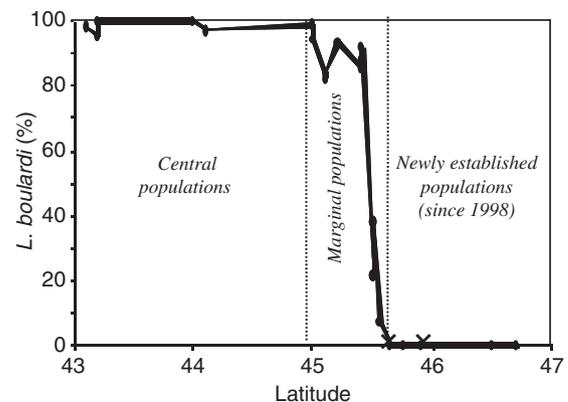

Fig. 1 Relative abundance of *L. boulardi* among *Leptopilina* species (*L. heterotoma* and *L. boulardi*) attacking *Drosophila* larvae in the Rhône valley in 1998 (modified from Allemand *et al.* 1999). The three population groups described in this study are separated on the figure by dotted lines.

infection (Patot *et al.* in prep). Furthermore, to date, all studies failed to detect the virus in *Drosophila* hosts (personal observation). Laboratory experiments showed that LbFV imposes an overall low cost on parasitoid physiology (Varaldi *et al.* 2005) and its effects are restricted to superparasitism behaviour (Varaldi *et al.* 2006b, 2009). Theoretical developments show that horizontal transmission can complement the imperfect vertical transmission allowing it to persist at high frequencies in wasp populations (Gandon *et al.* 2006). However, the model also indicates that the frequency of horizontal transmission is a critical parameter for the epidemiology of the virus (Varaldi *et al.* 2009) and may be impacted by variation in superparasitism phenotype or by variation in the density of *L. boulardi* (which is expected to be directly linked to superparasitism).

In this article, we tested whether the prevalence of LbFV varies among 15 *L. boulardi* populations distributed along the north-south axis of the Rhône valley in France. This sampling includes central, marginal and newly established populations. Because the ability of the virus to manipulate the behaviour of the parasitoid is a critical parameter for virus epidemiology, we also tested whether viruses from different localities were able to manipulate the behaviour of the parasitoid. We

further tested whether the differences in temperature experienced by northern and southern populations may impact the transmission of the virus. Finally, we explored whether variation in *L. boulardi* density were observed at this geographical scale and hence may impact transmission of the virus.

Materials and methods

Sample sites and collection of L. boulardi parasitoids

Field populations of *L. boulardi* were sampled during 2004 and/or 2007 in 15 orchards of the south-eastern France, distributed from north to south along the Rhône valley axis (Table 1). We chose sampling sites according to the ecological situation initially observed in 1998 (Fig. 1). We thus defined as central populations (sites 11:15) those that were always included in the geographic range of the species with very high levels of abundance, marginal populations (sites 6:10) those where *L. boulardi* was previously detected with intermediate level of abundance corresponding to the ancient border of the species range and newly established populations (sites 1:5) where *L. boulardi* was never observed before 1998 (Table 1).

Table 1 Collections of *L. boulardi* populations and prevalence of LbFV

Site	Locality	Geographic coordinates	Sampling year	n*	LbFV prevalence	Sequencing
1	Chasselay	45°52′ N-4°46′ E	2007	11	9 %	x
2	Cailloux-sur-Fontaine	45°51′ N-4°52′ E	2007	29	7 %	x
3	St Maurice-de-Beynost	45°49′ N-4°58′ E	2007	30	3 %	x
4	Ste Foy-lès-Lyon	45°44′ N-4°48′ E	2007	58	0 %	
			2009	14	0 %	
5	St Laurent-d'Agny	45°38′ N-4°41′ E	2007	12	0 %	
6	Villette-de-Vienne	45°35′ N-4°54′ E	2007	25	4 %	x
			2009	10	0 %	
7	Sonnay	45°21′ N-4°54′E	2007	120	29 %	x
			2009	21	24 %	
8	Epinouze	45°18′ N-4°55′ E	2007	15	20 %	x
9	Lens-Lestang	45°17′ N-5°20′ E	2004	59	10 %	x
10	Annonay	45°14′ N-4°40′ E	2004	40	65 %	
			2007	30	67 %	
11	Gotheron	44°56′ N-4°56′ E	2004	40	90 %	x
			2007	18	83 %	
			2009	24	71 %	
12	Avignon	43°54′ N-4°52′ E	2004	40	95 %	x
			2007	23	70 %	
13	Antibes	43°34′ N-7°07′ E	2004	40	80 %	x
14	Bras	43°28′ N-5°57′ E	2004	40	83 %	x
15	Pierrefeu-du-Var	43°13′ N-6°08′E	2004	40	55 %	x

Samples represent newly colonized (1–5), marginal (6–10) and central (11–15) populations.
*Number of independent females or isofemale lines tested by PCR in order to estimate LbFV prevalence.

Among the 15 wild-caught *L. boulardi* populations, four were collected during the autumn 2004 (September or October), eight during the autumn 2007 and three in both periods (Table 1). All sampled orchards are planted with apple trees and thus make resources available to *Drosophila* from the end of May to October. Temperature measured each hour from June to September 2009 revealed a mean of 21.5 °C in a newly established population (site 4) and of 22.2 °C in a central population (site 12). Two sampling procedures were used for wasp collection. In 11 localities, we placed in each orchard 10–12 closed traps baited with split bananas. They were exposed to natural colonization for 15 days. In the four others sites (7, 8, 10, and 12), we collected 25–30 rotten apples on the ground. Traps and apples were then brought back to the laboratory, and after incubation at approximately 23 °C, all emerging *L. boulardi* and *L. heterotoma* were collected daily. For 13 populations, individuals were kept in alcohol (100% ethanol) at −80 °C before DNA extraction. For the other two populations (sites 11, 12; samples 2007), a number of isofemale lines (one foundress per line) were established and reared under laboratory conditions. Molecular tests were performed on several emerging females of the first generation (F1) and the lines were declared positive if at least one daughter was infected.

As a polymerase chain reaction (PCR) positive control, we used a laboratory line of *L. boulardi* infected by LbFV (named S_{ref}). This line derives from an uninfected line (obtained through brother sister mating yielding to a homozygosity exceeding 82%) (named NS_{ref}) originating from Sienna (Italy), after injection of viral particles. This artificially infected line proved stable over generations for virus infection (Varaldi *et al.* 2006c).

In the laboratory, parasitoids were reared under a 12L:12D photoperiod at 25 °C, using *D. melanogaster* originating from Ste Foy-lès-Lyon (Rhône, France) as host. *Drosophila* larvae were fed with a standard diet (David 1962).

PCR detection of LbFV in L. boulardi *populations*

LbFV has at least a DNA stage during its replication and has probably a DNA genome (Patot *et al.* 2009). Total DNA was extracted from parasitoid females by crushing individuals individually in 150 μL 5% (w/v) Chelex solution (Bio-Rad, USA) and 5 μL of proteinase K (20 mg/mL; Eurobio, France). Extracts were incubated overnight at 56 °C. After 20 min at 95 °C, samples were centrifuged and 2 μL of the supernatant were used for PCR reactions.

We used a recently developed molecular marker to detect the presence of LbFV in *L. boulardi* as described in Patot *et al.* (2009). Briefly, a specific viral sequence

(accession no: FM876312) was amplified using the primers I1CL1-F and I1CL1-R in uniplex PCR reaction, or using viral primers 102-F and 500-R in combination with insect primers RPS2-F and RPS2-R (amplifying a ribosomal protein gene) in multiplex reaction. This multiplex reaction allowed to control for the quality of the DNA extraction. PCR reactions were performed in a 25 μL final volume reaction using 2 μL of DNA template, 1.5 mM $MgCl_2$, 50 μM of each dNTP, 200 nM of each primer, 0.5 U of *Taq* polymerase (EuroBlueTaq, Eurobio, France). The following cycling program was used for both uniplex and multiplex PCR reactions: 1 min at 95 °C, 30 s at 94 °C, 30 s at 56 °C, 30 s at 72 °C, 30 cycles and 10 min elongation time at 72 °C (PTC-100, MJ Research, USA). PCR products were then analysed on 2% agarose gel electrophoresis. The quality of DNA extracts has been checked using insect primers, either in a multiplex reaction (populations 1, 2, 3, 4, 5, 9) or in a separate uniplex PCR reaction (other populations) (Table 1).

Viral sequences from various populations of L. boulardi

To assess viral variability and possible divergence of the virus among host populations, we sequenced PCR products obtained with the viral primers 102-F/500-R. Viral samples included 12 of the 15 populations studied from south-eastern France (Table 1). We also added samples originating from Italy (Sienna), from Spain (Palma de Mallorca), from Africa (Ivory Coast, Lamto, G495) and from Brazil (Rio de Janeiro). The sequences obtained were aligned with the program CLUSTAL X (Thompson *et al.* 1997).

Behavioural manipulation induced by LbFV strains

The effect of LbFV on superparasitism behaviour was studied for six populations of *L. boulardi* using sympatric parasitoid–virus associations. Five of them correspond to populations previously described: two from the central area (sites 11 and 12; samples 2007), two from the marginal sites (sites 6 and 8) and one in the newly colonized area (site 2). In order to obtain two replicates for the last area, we also included the superparasitism behaviour of *L. boulardi* individuals collected in October 2008 at Ville-Sollier (46°80′N, 4°52′E). This location is situated 30 km to the north from site 1 and is the most northerly record of *L. boulardi*. Because very few individuals emerged from the eight traps, LbFV prevalence was not estimated in this site. For all populations, several isofemale lines founded by single infected or uninfected females were established.

For each of these six populations, 25 to 30 females from infected (I) and uninfected (UI) isofemale lines

were tested after one to five generations performed under laboratory conditions: Ville-Sollier: 4 UI and 4 I lines, Cailloux-sur-Fontaine: 4 UI and 4 I lines, Villette-de-Vienne: 4 UI and 1 I lines, Epinouze: 4 UI and 2 I lines, Gotheron: 2 UI and 4 I lines and Avignon: 4 UI and 1 I lines. The superparasitism phenotype was estimated using the same protocol previously described in Varaldi *et al.* (2003). Briefly, individual females (1 or 2 days old) were placed according to their daily rhythm (Fleury *et al.* 2000) from 5 pm to 10 am on 10 first instar *D. melanogaster* larvae in a petri dish containing a layer of agar and a small amount of live yeast. Forty-eight hours later, three to five larvae from each petri dish were dissected and the number of parasites (eggs and larvae) was counted. Superparasitism behaviour of each female was estimated as the mean number of parasitoids per parasitized *Drosophila* host.

Effect of temperature on viral transmission

In order to test for a potential effect of the temperature on viral prevalence, we tested whether temperature may affect viral transmission from one generation to the next. Three temperatures were chosen (18 °C, 20 °C and 26 °C) according to the diapause phenomenon, which is a physiological state of dormancy allowing the parasitoid to survive under low temperatures. In *L. boulardi*, diapause occurs at the end of the larval development. Claret & Carton (1980) showed that *L. boulardi* exhibits 97.4% diapausing larvae at 17.5 °C, 52.7% at 20 °C and 0% at 25 °C, which allowed us to test in the same experiment for the combined effect of temperature and diapause on viral transmission.

For each temperature, 15 replicates were performed by allowing one infected female (S_{ref} line) to oviposit for 24 hours at 26 °C under a 12L:12D photoperiod. Each adult female was placed in a rearing plastic tube (95 mm × 23 mm) containing 15 mL of standard medium and 1-day-old *D. melanogaster* larvae hatched from 125 eggs. In these conditions, superparasitism events are negligible. Consequently, the transmission of the virus is mainly vertical. After the 24 h egg-laying period at 26 °C, tubes were transferred to their respective temperature (18 °C, 20 °C and 26 °C) for development. Ten days after the emergence of the nondiapausing parasitoids, rearing tubes were placed at 26 °C to induce the emergence of diapausing parasitoids. All emerging adult wasps were daily collected and counted. As expected, no diapause was observed at 26 °C, almost all parasitoids entered diapause at 18 °C (except one individual), and intermediate levels of diapause were observed at 20 °C. Thus, the effect of diapause on vertical transmission could only be tested at this last temperature. In total, 50 diapausing *L. boulardi* females and one nondiapausing female were tested for viral infection at 18 °C, 50 diapausing and 50 nondiapausing females at 20 °C and 50 nondiapausing females at 26 °C, using multiplex-PCR (see above).

Relative density of L. boulardi and superparasitism in the field

The relative density of *L. boulardi* has been studied in four populations distributed along the north-south axis: one in central area (site 11), two in marginal area (sites 6 and 7) and one in the newly established populations' area (site 4). In September 2009, 12 traps were placed in each orchard during 10 days using the same protocol as previously described. However, in this case, the traps were open, allowing the insects to enter the traps, lay their eggs and leave the traps freely. This provides a reliable estimation of the abundance of each insect species of the community. In the laboratory, all *Drosophila* and parasitoids emerging from traps were collected daily. Based on these counts, we defined a proxy for the density of *L. boulardi* by calculating the ratio between emerging *L. boulardi* and an estimation of the initial number of potential host flies present in the trap. The number of potential host flies was estimated by summing the number of emerging *D. melanogaster*, *D. simulans*, *L. heterotoma* and *L. boulardi* (because each parasitoid emerged from one *Drosophila* host only). This index is a rough estimate of the proportion of potential hosts that were parasitized by *L. boulardi* and will be referred to as the relative density of *L. boulardi* in the following (on average 975 insects per trap were used for this calculation). Note that *D. melanogaster* and *D. simulans* were by far the most abundant *Drosophila* species (mean frequency = 0.98) in the traps. On a sample of *L. boulardi* females, we also studied LbFV prevalence using the multiplex PCR diagnostic as previously described (Table 1). The link between the relative density of *L. boulardi* and the occurrence of superparasitism was studied using data collected in Gotheron (site 11) using similar open traps ($n = 10$). Traps were exposed in the field for few days (before the pupation of *Drosophila* larvae) and a sample of 30 *Drosophila* larvae per trap were dissected in order to count the number of parasitoid eggs per host. This gives a measurement of the intensity of superparasitism. Traps were then incubated in the laboratory at 23 °C until the emergence of all insects in order to calculate the relative density of *L. boulardi* as previously described. They were exposed in June, July, August and September 2001. During this period, the dynamics of *L. boulardi* varies greatly, allowing a test of the effect of density variation on superparasitism intensity.

Modelisation of the epidemiology of the virus in relation to parasitoid density

We used the model of Gandon *et al.* (2006) developed to study the epidemiology of LbFV in populations of *L. boulardi*. In this model, the virus is both vertically and horizontally transmitted and it manipulates the behaviour of the parasitoid. We used this model to generate predictions regarding the expected relationship between different variables of the model when the system is assumed to reach an epidemiological equilibrium (see Supporting information 1). First, we focused on the relationship between the expected number of parasitoid eggs per parasitized hosts and the number of parasitoids as this informs our interpretation of the observed data presented in Fig. 5. Second, we used this model to study the relationship between the relative density of the parasitoids (measured as the proportion of parasitized larvae) and the prevalence of the virus among adult parasitoids. To do this, we simulated variations in the density of parasitoids by varying the mortality rate of adult females. We present numerical simulations for various parameter values of the mortality rate of adult females and various rates of horizontal transmission

(see Supporting information 1 for additional information on the model).

Statistical analysis

Data were analysed using R version 2.7.0. To analyse prevalence and relative density data, we used a generalized linear model (GLM). Given the binary nature of the data (presence/absence of infection), we used a binomial error structure (logit link function) and chi-square tests of significance.

Results

Prevalence of LbFV among L. boulardi *populations*

Infections by LbFV vary according to the geographic origin of *L. boulardi* populations (Fig. 2). Analysis performed separately on 2004 and 2007 samples both showed a significant negative correlation between latitude and LbFV prevalence (2004: dev = 30.03, ddl = 1, $P < 0.0001$; 2007: dev = 100.15, ddl = 1, $P < 0.0001$). Clearly, infections were very high in central populations with a mean of 77.5%. In marginal populations, preva-
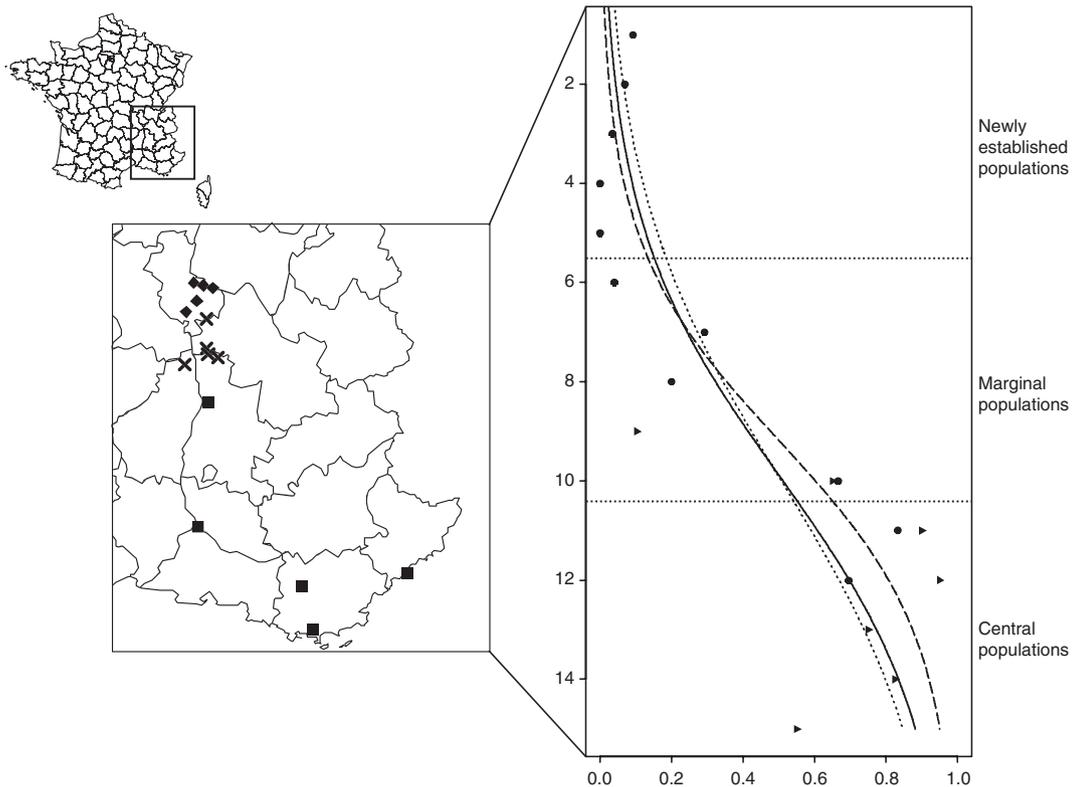

Fig. 2 Prevalence of LbFV across the *L. boulardi* populations in south-eastern France and predictions of the generalized linear model. The populations were sampled in 2004 (triangles) or 2007 (dots). Continuous line: model prediction for all data (2004 and 2007); dotted line: model prediction for 2004; dashed line: model prediction for 2007.

lence of the virus sharply decreased reaching an average of 30% whereas in newly established *L. boulardi* populations, the prevalence dropped to 3.3% with two populations in this area apparently virus free (sites 4 and 5). Overall, these results show that virus infection is very common since LbFV was detected in 13 out of 15 populations with a progressive decrease of LbFV prevalence in direction of the limit of the *L. boulardi* geographic range.

Three populations sampled both in 2004 and 2007 allowed to test for the temporal stability of viral infection (Table 1). In these three populations, there was no strong variation of the LbFV prevalence between these two sampling years, although this effect was significant ($\chi^2 = 8.59$, ddl = 1, $P = 0.0034$). The infection proved stable in site 10 (Annonay) and a slight decrease was observed in 2007 for sites 11 and 12 (Gotheron and Avignon). This may be explained by the fact that molecular test was performed on F1 females (after one generation in the laboratory) in 2007 whereas it was done on individuals emerging from field traps in 2004 (see 'Materials and methods'). Under the hypothesis that some imperfect vertical transmission occurred, a lower prevalence is expected when tests were performed on F1 generation.

Viral sequence diversity

Twelve viral sequences of 399 pb were obtained from the French populations (one per locality) (accession no.: FN691999, FN692010 : FN692020) together with four other viral sequences from the south of Europe (Italy and

Spain), Africa (Ivory Coast) and South America (Brazil) (accession no.: FN692021 : FN692024). The alignment of the 16 sequences revealed that only one originating from the locality Gotheron (site 11), differed from the others by 1 bp only (site 224). This difference was found with two independent sequencing reactions, which ruled out the hypothesis of a sequencing error. However, the sequencing of nine additional viral samples from Gotheron (accession no.: FN692000 : FN692009) did not reveal the 1 bp difference found previously and appeared identical to the 15 other sequences. Apart from this difference, all sequences were identical to the previously described sequence (Patot *et al.* 2009).

Behavioural manipulation induced by LbFV strains

The effect of viral infection on superparasitism behaviour of parasitoid females was tested in two populations of each of the three modalities, i.e. central, marginal and newly colonized sites (Ville-Sollier and sites 2, 6, 8, 11, 12). For these six sites, we compared superparasitism levels of uninfected and infected lines, but since we analysed sympatric parasitoid–virus associations where both the virus and host genotypes may vary, we did not test the site effect. Moreover, this comparison is limited because behavioural assays were not performed at the same time among sites. In all parasitoid–virus associations tested, LbFV induces a very high level of superparasitism whereas uninfected lines did not show a significant level of superparasitism (one parasitoid per *Drosophila* larva) (Fig. 3). Thus in all sites,
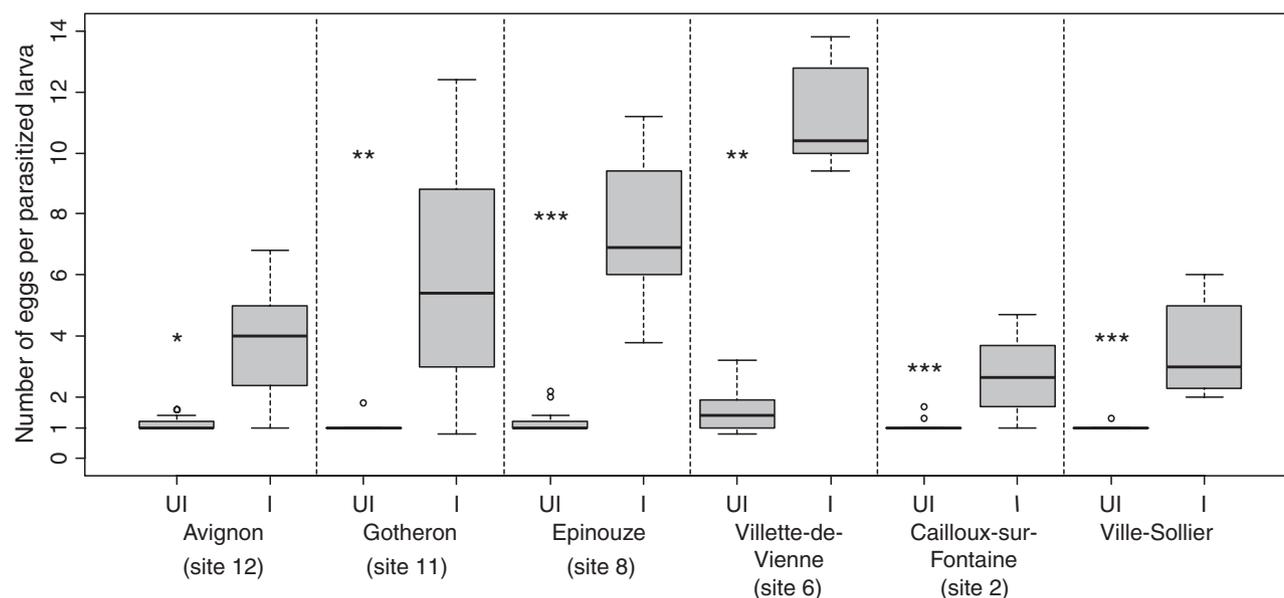

Fig. 3 Superparasitism behaviour of uninfected (UI) and infected (I) isofemale lines from six *L. boulardi* populations of south-eastern France. Wilcoxon test: *$P < 0.01$, **$P < 0.001$, ***$P < 0.0001$.
Sites 11 and 12: central populations; sites 6 and 8: marginal populations; sites 2 and Ville-Sollier: newly established populations.

LbFV is able to manipulate egg laying behaviour of the parasitoids by inducing superparasitism.

Effect of temperature on virus transmission

As expected, temperature had a drastic effect on the probability of a parasitoid larva entering diapause ($P = 6 \times 10^{-9}$). However, there was no effect of temperature on viral transmission, whether diapause occurred or not. Indeed, results show very little variation since the frequency of infection after development was 0.98 (49/50) at 18 °C (diapausing), 0.98 (49/50) at 26 °C (nondiapausing) and 1.0 (50/50) at 20 °C both for diapausing and nondiapausing females. Furthermore, the only individual that did not enter diapause at 18 °C was infected. Thus, there is no evidence for an effect of temperature or diapause on viral vertical transmission (all P values > 0.99).

Relative density of L. boulardi and viral prevalence

Sampling conducted in 2009 permitted to estimate the density of *L. boulardi* in the *Drosophila*-parasitoids community in four sites distributed along the north-south axis. The relative density of *L. boulardi* was clearly correlated with the position of populations along the north to south axis ($\chi^2 = 7244.5$, d.f. = 1, $P < 0.0001$) (Fig. 4). In the two most northern populations, the relative densities of *L. boulardi* (measured as the proportion of potential hosts that are parasitized by *L. boulardi*) were only 0.006 at Ste Foy-lès-Lyon (site 4) and 0.015 at Villette-de-Vienne (site 6). More to the South, the relative density increased (0.107 at Sonnay, site 7), and finally
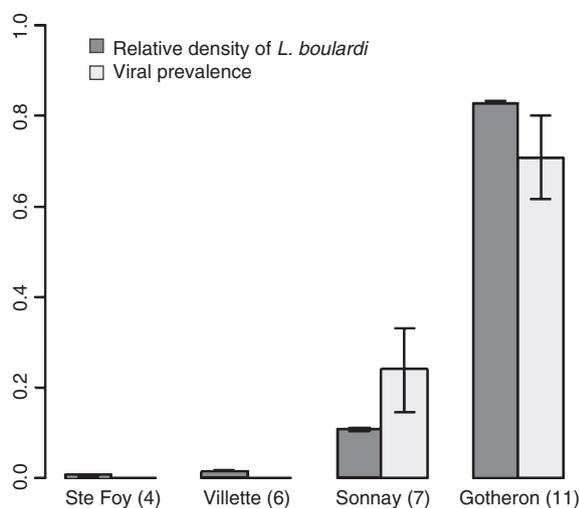

Fig. 5 Mean number of eggs per parasitized host as a function of the relative density of *L. boulardi*. Points correspond to traps exposed from June to September 2001 in Gotheron (site 11). The relative density of *L. boulardi* was transformed using $p/(1-p)$ transformation in order to achieve linearity for regression analysis.

reached 0.83 in the most southern population (Gotheron, site 11).

We also studied the LbFV prevalence in these four locations. Interestingly, the prevalence obtained in 2009 sampling were consistent with data obtained in 2004 and 2007 (see Table 1). Hence, this 2009 sampling revealed a similar cline to that observed in 2007 and 2004. Importantly, there is a clear positive correlation between the relative density of *L. boulardi* and viral prevalence ($t = 6.1$, d.f. = 2, $P = 0.026$) (Fig. 4). Furthermore dissections of *Drosophila* larvae in the field indicated a clear positive correlation between the density of *L. boulardi* and the intensity of superparasitism ($F(1,35) = 56.46$, $P < 10^{-8}$; Fig. 5). This data is consistent with the prediction of our epidemiological model (see Fig. S1).

Modelisation of the epidemiology of the virus in relation to parasitoid density

By varying the mortality rate of the parasitoid, we obtained variations in the proportion of parasitized hosts (similar to the 'relative density' in previous sections). The model predicts that below a critical value of the proportion of parasitized host, the virus cannot maintain in the populations (when m is around 0.1 with this parameter set). Above this threshold, the virus prevalence increases with an increase in the proportion of parasitized hosts (Fig. 6). Note, however, that for very low values of mortality rates the model predicts a

Fig. 4 Relative density of *L. boulardi* and LbFV prevalence in four populations of south-eastern France sampled in September 2009. Bars indicate standard deviation.
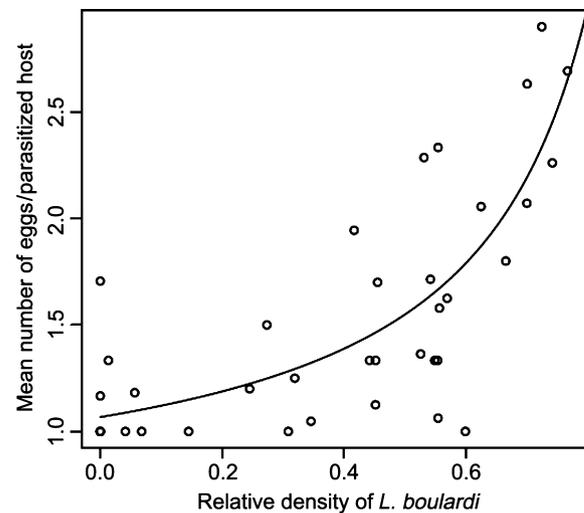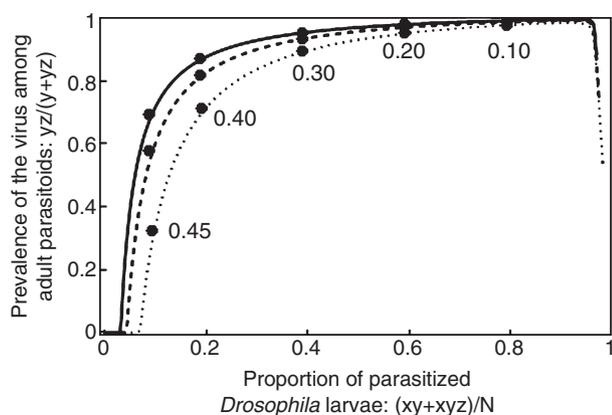
Fig. 6 Predictions for the relation between the equilibrium frequency of virus infection in *L. boulardi* and the rate of parasitism of *Drosophila* larvae using the epidemiological model presented in Gandon *et al.* (2006). Variations in the rate of parasitism were obtained by varying the mortality of the parasitoid (*m* varies between 0.001 and 0.5) and the probability of horizontal transmission: τh takes the values 0.25 (dotted line), 0.5 (dashed line) and 0.75 (full line). Some values of *m* are indicated on the figure. Parameter values are: $N = 100$, $d = e = 0.2$, $\phi = 0.5$, $a = 0.01$, $t_1 = 0$, $t_2 = 0.1$, $E = 100$, $\tau_v = 0.98$.

decrease of the prevalence. This is due to the effect of egg limitation. In nature, such low levels of mortality are probably unrealistic. Besides, several studies report very low values of egg limitations in parasitoids (Sevenster *et al.* 1998; Rosenheim 1999). Thus, overall, the model predicts a positive correlation between the proportion of parasitized hosts and the prevalence of the virus. In these numerical simulations, we only varied the extrinsic mortality rates of adult parasitoids. This is a parameter likely to vary between northern and southern populations of *L. boulardi* but we do not yet have any data supporting this variation.

Discussion

Although it is expected that variations in host density should affect the dynamics of parasites with frequency-dependent transmission (Anderson & May 1981), and that species often show reduced densities in their range edge (Sagarin & Gaines 2002), marginal populations have rarely been used to test this prediction. In this article, we analysed the prevalence of a virus that manipulates the behaviour of a parasitoid at the border of its geographic range. The results showed that the virus LbFV is widespread in *L. boulardi* populations, especially in more central populations, and that its prevalence dramatically decreases in marginal populations. This cline of prevalence was repeatedly observed in 2004, 2007 and 2009 suggesting that other forces than simple stochastic effects are involved in this pattern.

A widespread virus with low sequence diversity

LbFV was detected in most *L. boulardi* populations in south-eastern France, in strains originating from Italy, Spain, Ivory Coast and Brazil indicating a wide distribution of this virus. LbFV gene sequencing revealed almost no sequence diversity in all samples (only one nucleotide difference was observed in one sequence). This absence of variation in viral sequences could have several nonexclusive explanations. LbFV may have recently invaded *L. boulardi* populations, possibly after an event of cross-species transmission. This phenomenon has been suggested to explain the low sequence diversity observed for a vertically transmitted virus infecting *D. melanogaster* (Carpenter *et al.* 2007). Alternatively, an LbFV mutant may have recently replaced the resident LbFV genotype. Finally, the low viral diversity may be the consequence of strong stabilizing selection acting on the gene. Indeed, we suspect that this is an essential gene for the virus because the cDNA of this gene, obtained from a suppressive subtracted hybridization on *L. boulardi* ovaries, was by far the most frequently observed sequence in the subtracted library (Patot *et al.* 2009).

Low viral prevalence in marginal populations

Our study revealed that LbFV prevalence in natural *L. boulardi* populations is correlated with the latitude. Whereas LbFV is well established and reached very high prevalence in central southern populations, virus infection decreases in marginal populations and reaches a very low frequency (close to zero) in newly established peripheral populations. In the literature, colonization of new habitats has been linked to the loss of symbiont or parasite. For instance the intracellular bacteria *Wolbachia* infecting the Argentine ants (*Linepithema humile*) and fire ants (*Solenopsis invictae*) is almost absent from worldwide introduced populations whereas it is highly prevalent in native South American populations (Shoemaker *et al.* 2000; Reuter *et al.* 2005). Also, in a meta-analysis, Mitchell & Power (2003) found that plants introduced in the USA from Europe are less infected by viral and fungal pathogens than their native counterparts. Very few articles have addressed the question of the prevalence of symbionts in the context of marginal populations. Furthermore, contrasting results have been obtained. For instance, Briers (2003) found that a freshwater gastropod snail suffered more from trematode parasitism at the border of its geographical range than in its central area. Similarly, but on plant models, Garcia *et al.* (2000) found that the intensity of predation targeting *Juniperus* was much higher in its marginal area than in its central area. On

the contrary, Alexander *et al.* (2007) found the opposite pattern for the forest sedge *Carex blanda* and its pathogens and predators. Depending on the system, variations in host densities across the geographical range were or were not observed and various alternative factors may explain the observed patterns. Here, we will discuss several hypotheses that may explain the observed cline in viral frequency among *L. boulardi* populations.

Cost of infection

Laboratory experiments showed that viral infection in *L. boulardi* females induces no cost on adult survival but slightly reduced developmental rate and adult body size, and leads to a significant decrease in locomotor activity (Varaldi *et al.* 2005). However, the virus does not affect the behaviour of the wasp, except superparasitism (Varaldi *et al.* 2006b). Because we do not know how these costs are expressed in the wild especially under unusual ecological conditions such as in newly colonized sites, we cannot rule out the possibility that infection induces a higher cost at the limit of the geographic range of the species. Under this hypothesis, virus free individuals should have a higher fitness, thus limiting the spread of the virus at the wasp's range edge. Another hypothesis would be that the cost of infection concerns the dispersal ability of the wasp. Dispersion would then be mainly realized by uninfected individuals, which colonize new sites. Under this hypothesis, the spread of the virus would not have yet occurred and thus newly colonized sites would show lower viral prevalence. However, under this hypothesis, we would expect an increase in virus frequency through successive seasons, because infected individuals would eventually arrive in these newly established populations. The data did not reveal such an increase in virus frequency but instead a great stability from 2004 to 2009.

No evidence of a temperature effect

Some studies reported that the transmission of numerous symbionts of insects is dependent on temperature (Hurst *et al.* 2001; Kilpatrick *et al.* 2008; Osaka *et al.* 2008). For viruses, several authors observed that larval host susceptibility to nucleopolyhedrosis virus (NPV) depends on temperature that controls the kinetics of NPV replication (Kobayashi *et al.* 1981; Johnson *et al.* 1982; Frid & Myers 2002). Because in our study a north-south gradient of temperature (lower temperature to the north) correlated with the cline in virus frequency, we tested whether variations in temperature may impair virus transmission across generations, possibly

explaining the pattern. We thus studied the virus transmission efficiency from mother to offspring (vertical transmission) under three temperatures (18 °C, 20 °C and 26 °C) in the laboratory. Consistently with previous data (Varaldi *et al.* 2006a), we found an overall very high transmission fidelity (only 2 out of 150 offspring of infected females were PCR-negative) and no effect of the temperature was detected. Furthermore, part of the individuals of this study entered into the typical overwintering stage (larval diapause), and we found no influence of the diapause on viral transmission. Because diapause probably lasts for months under natural conditions, we cannot completely rule out the possibility that more drastic conditions could impair viral transmission. Also we cannot exclude that temperature impacts the efficiency of horizontal transmission. Previous studies on the symbiotic bacteria *Wolbachia* showed that diapause could lead to the loss of infection in the parasitoid wasp *Nasonia vitripennis* (Perrot-Minnot *et al.* 1996), and to decreased bacterial density in the mosquito *Aedes albopictus* (Ruang-areerate *et al.* 2004). Several heritable insect viruses, especially arboviruses of mosquitos, have also been the subject of investigations regarding diapause, and the results indicate that viruses are less transcribed in diapausing versus nondiapausing eggs but successfully reach the adult stage in both conditions (Woodring *et al.* 1998; Reisen *et al.* 2002; Guo *et al.* 2007). However, on these mosquito/virus systems, no quantitative test of the influence of diapause on the fidelity of the viral transmission is available. In conclusion, our data on *L. boulardi* suggest that in our experimental conditions neither temperature nor diapause affect vertical transmission of LbFV and consequently that other factors are probably involved in the observed decrease of LbFV prevalence in northern populations.

Variations of parasitoid densities and superparasitism opportunities

Depending on ecological conditions such as the parasitoids-*Drosophila* ratio, the opportunities of horizontal transmission of LbFV among *L. boulardi* individuals may vary. Indeed, if this ratio is low (numerous *Drosophila* for few parasitoids), there will be only a few cases of superparasitism and consequently few horizontal transmission opportunities. On the contrary, if this ratio is high (numerous parasitoids compared to *Drosophila*), opportunities for horizontal transfer may be frequent. Assuming an incomplete vertical transmission, we showed theoretically that the relative density of the parasitoid (or parasitism rate of *Drosophila* larvae) must exceed a threshold for the virus to persist in the parasitoid populations (Fig. 6 of this article and Varaldi *et al.* 2009). Below this threshold, opportunities for horizontal

transmission are not sufficient to compensate for the imperfect vertical transmission of the virus. Above this threshold, the virus is able to persist in the population but its equilibrium frequency will directly depend on the relative density of the parasitoid (Fig. 6 of this article and Fig. 13.3 in Varaldi *et al.* 2009). Thus, from a theoretical point of view, variation in the parasitoid-*Drosophila* ratio should induce variation in opportunities for horizontal transmission and finally variation in virus prevalence.

By using open traps, we were able to calculate a parasitoid-*Drosophila* ratio, which constitutes a good index of the density of *L. boulardi* in newly established, marginal and central populations. This index is a rough parasitization rate of *Drosophila* larvae by *L. boulardi* and thus measures the relative density of the parasitoid among *Drosophila* larvae. This index decreased from the South (central populations) to the North (newly established populations). Furthermore, dissections of larvae collected in the wild revealed the occurrence of frequent superparasitism and a clear correlation between the density index and the intensity of superparasitism. Taken together, these data suggest that the competition for hosts among *L. boulardi* females is very high in the South with frequent superparasitism events and very low in the North with much less superparasitism, consistently with previous data (Fleury *et al.* 2004). Thus, the opportunities for horizontal transmission are much higher in the central than in marginal or in newly established populations. As predicted by our epidemiological model, this pattern of variations in the density of *L. boulardi* should translate into variations in the viral prevalence which is consistent with observations.

Conclusion

This study aimed at investigating whether variations in the density of a species in its range edge may induce variations in the prevalence of a symbiont showing mixed vertical and horizontal transmission. Our results clearly showed a cline in prevalence of the virus LbFV among newly established, marginal and central populations of its parasitoid host *L. boulardi.* This pattern is unlikely to result from stochastic factors since it was repeatedly observed in 2004, 2007 and 2009. We were unable to induce variation in vertical transmission rates by altering the temperature. However, our data indicate that opportunities for horizontal transmission (superparasitism) vary monotonically among populations and theoretical developments show that this may explain the cline in viral prevalence. Although alternative hypothesis may be evoked (as discussed above), this provides a satisfying model explaining the pattern. If this scenario is true, we can draw two conclusions.

First, the predictions rely on the fact that vertical transmission is incomplete. Indeed, if vertical transmission was perfect, the virus should either invade the entire population or should be eliminated from the populations depending on the fitness cost and horizontal transmission opportunities (Lipsitch *et al.* 1995). Here, the data indicate intermediate viral prevalence, suggesting that vertical transmission is incomplete in the field. Second, our results underline the critical role played by horizontal transmission in the propagation and maintainance of the virus. Because horizontal transmission opportunities are obtained from superparasitism, this suggests that superparasitism manipulation induced by the virus is under strong selective pressure, as has been shown theoretically (Gandon *et al.* 2006). This is consistent with the fact that viruses from all populations are able to manipulate the behaviour of the wasp.

In turn, these results raise new questions about the possible consequences of the presence of LbFV on the community. The presence of the virus strongly reduces the competitive ability of *L. boulardi* against *L. heterotoma*, another parasitoid of the community uninfected by LbFV (Patot *et al.* in prep). In central area of *L. boulardi* distribution, high infection rate may thus participate in maintaining parasitoids diversity, in particular the coexistence between *L. boulardi* and *L. heterotoma* (Fleury *et al.* 2004, 2009). By inducing detrimental effects on *L. boulardi* (due to egg-wastage induced by superparasitism), LbFV would reduce the superiority of *L. boulardi*, thus balancing the asymmetry in competitive abilities among *Leptopilina* species. The intensity of the inter-specific competition in these situations and the precise role of this widespread heritable virus on the *Drosophila*-parasitoid community have yet to be evaluated.

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Sabine Patot is interested in symbiont influence on the structure of host's populations and in symbionts interactions. Julien Martinez is a PhD student working on evolutionary issues on the *L.boulardi*/LbFV interaction. Roland Allemand is involved in researches on the structure and dynamics of host-parasitoids populations in frugivorous *Drosophila* communities. Sylvain Gandon is a theoretical biologist interested in evolutionary epidemiology. Julien Varaldi is working on the influence of symbiont in host-parasitoid interactions. Finally, Frédéric Fleury's work focuses on the genetics of adaptation in insects.

Supporting Information

Additional supporting information may be found in the online version of this article.

Supporting information 1: an epidemiological model

Table S1 Main parameters and variables of the model.

Fig. S1 Predictions for the relation between the expected number of parasitoid eggs per host and the proportion of parasitized larvae using the epidemiological model presented in Gandon *et al.* (2006) at equilibrium.

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