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Heritable variation in an extended phenotype: the case of a parasitoid manipulated by a virus

J. MARTINEZ, F. FLEURY & J. VARALDI

Laboratoire de Biométrie et Biologie Evolutive, CNRS, UMR5558, Université de Lyon, Villeurbanne, France

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Abstract

In host–symbiont interactions, the genes of both host and symbiont can influence phenotypic traits. In the context of a conflict of interest, fitness-related traits are subjected to opposing selective pressures in the genomes of the partners. In the *Drosophila* parasitoid *Leptopilina boulardi*, females usually avoid laying eggs into already parasitized larvae. However, when infected by the virus LbFV, they readily lay additional eggs into parasitized larvae. Inducing superparasitism allows the virus to colonize uninfected parasitoid lineages but is usually maladaptive for the parasitoid. We tested for the presence of resistance genes to this behavioural manipulation in the parasitoid genotype by sampling 30 lines from five populations with contrasting viral prevalence, after infecting them with a reference viral isolate. No geographical differentiation was observed although some genotypes underwent less manipulation than others, and these differences were heritable across generations. The viral titre was not correlated with these differences although fecundity differed between extreme lines.

Introduction

The genetic diversity underlying phenotypic variation provides the raw material on which selection works, provided that the variation is heritable. In addition to the nuclear and cytoplasmic genes of an individual, additional evolutionary potential may be provided by infection by various heritable symbionts, because symbionts can modify the host's phenotype by inducing diverse morphological, physiological or even behavioural changes (Poulin & Thomas, 1999; Stouthamer *et al.*, 1999; Zchori-Fein *et al.*, 2001; Moran, 2007; Douglas, 2009; Hurd, 2009). Because the symbiont's genes influence the host's phenotype, the resulting overall phenotype can be described as an 'extended phenotype' (Dawkins, 1982), although the original definition was much wider, including 'effects [of genes] on the world at large'.

In such a host–symbiont context, there are several potential genetic sources underlying phenotypic variation. First, in the absence of the symbiont, the genetic variation in the host may translate into phenotypic variation. Second, the infection status of the host, i.e. whether the host harbours the symbiont or not, may also lead to phenotypic differences. Third, when the host is infected, the observed extended phenotype results from the interplay between the genes of the host and those of the symbiont. Because the symbiont constitutes a genetic environment from the host's point of view, its contribution to the phenotype can be viewed as an indirect genetic effect (IGE) (Moore *et al.*, 1997; Shuster *et al.*, 2006). In this situation of infection, additional phenotypic variation can result both from variability in the host's genotype (Poinsot *et al.*, 1998; Jaenike, 2007; Kageyama *et al.*, 2009), which is equivalent to direct genetic effects in the IGE literature, and from variability in the symbiont's genotype (Oliver *et al.*, 2005; Dunbar *et al.*, 2007; Vorburger *et al.*, 2009), i.e. indirect genetic effects. Lastly, variation in the extended phenotype may partly be due to interactions between genotypes of the host and symbiont (James & Ballard, 2003; Lambrechts *et al.*, 2006; Heath, 2009; Lambrechts, 2010), in a manner equivalent to covariance between direct and indirect genetic effects.

In host–symbiont associations, the optimum values for a given phenotypic trait can be either similar or very

Correspondence: Julien Martinez, Laboratoire de Biométrie et Biologie Evolutive, CNRS, UMR5558, Université de Lyon, 69000 Lyon, Université Lyon 1, 69622 Villeurbanne, France.
Tel.: +33 4 7243 2908; fax: +33 4 7243 1388;
e-mail: julien.martinez@univ-lyon1.fr

different for the host and its symbiont, leading to an alignment or conflict of interest, respectively (Herre *et al.*, 1999; Smith, 2007). Alignment of interest is apparent for various traits in host–symbiont associations, for instance when the symbiont provides the host with nutrients (Moran & Degnan, 2006; Douglas, 2009) or with protection against natural enemies (Brownlie & Johnson, 2009; Oliver *et al.*, 2010; Xie *et al.*, 2010). Examples of traits subject to conflict of interest include the sex ratio in host–symbiont associations in which the symbiont is responsible for feminisation (Cordaux *et al.*, 2011) or the egg load in a host–parasite association in which the parasite castrates its host (Hurd *et al.*, 2001).

In the specific context of conflict of interest, the selective pressures acting on the host and symbiont genes are divergent and can lead to rapid co-evolutionary dynamics (Charlat *et al.*, 2007). However, the prerequisite for such a selective response is that sufficient heritable variation is present in the gene pools of both the host and the symbiont. In this paper, we will focus on a host–symbiont association where there is a strong conflict of interest for a behavioural trait. Our main aim will be to find out whether there is significant heritable variation in the parasitoid gene pool concerning the behaviour of interest, by controlling the symbiont genetic diversity.

The parasitoid wasp *Leptopilina boulardi* (Hymenoptera, Figitidae) exhibits considerable phenotypic variability in a type of behaviour known as superparasitism. Adult females specifically parasitize *Drosophila* larvae by laying their eggs in the body cavity of their host (Fleury *et al.*, 2009). The offspring then develop by consuming the *Drosophila* tissues, ultimately killing it. Only one adult parasitoid can emerge from a single *Drosophila* larva, whatever the number of eggs deposited (Carton *et al.*, 1986). Some females deposit a single egg inside each larva and usually reject already parasitized ones, thus avoiding competition during development. However, when infected by the maternally transmitted DNA virus known as LbFV (*Leptopilina boulardi* filamentous virus), females frequently deposit supernumerary eggs into previously parasitized larvae, leading to a situation of superparasitism (Varaldi *et al.*, 2003). Superparasitism is frequently observed in the field (Fleury *et al.*, 2004; Patot *et al.*, 2010). It allows the virus to colonize uninfected parasitoid lineages as the virus is horizontally transmitted between parasitoids sharing the same *Drosophila* larva. By a theoretical approach, Gandon *et al.* (2006) determined the optimum value for superparasitism (i.e. the rate of superparasitism acceptance) both for the parasitoid and for the virus. This model confirmed previous findings that in the absence of any virus, parasitoids may benefit from superparasitism under a high level of competition due to the rarity of unparasitized *Drosophila* larvae (van Alphen & Visser, 1990). However, the model indicates that the optimum value for superparasitism is always higher for the virus than for the parasitoid because of the opportunities for horizontal transmission under conditions of superparasitism, indicating a conflict of interest for this trait. Previous work showed that the virus is efficiently vertically transmitted (although <100%), has no detectable effect on other behavioural traits or female survival (Varaldi *et al.*, 2006a, 2009), but has a slightly positive effect on egg load and a weakly negative effect on tibia length and locomotor activity (Varaldi *et al.*, 2005a). In south-eastern France, the viral prevalence is spatially structured along a north–south axis, ranging from almost no infection in northern populations to nearly fixation in the south (Patot *et al.*, 2010).

In this paper, we sampled 30 parasitoid lines derived from five populations showing striking differences in viral prevalence. Using these lines, we addressed the following questions:

1. To what extent does the presence/absence of the virus explain the variation in superparasitism phenotype between different parasitoid lines?
2. Is there any genetic variation for superparasitism in the parasitoid gene pool in a context of infection (direct genetic effects)? If so, is there any sign that resistance evolved in heavily infected southern populations?
3. Does variation in the extended phenotype correlate with variation in virus titre or parasitoid egg load?

Materials and methods

Field sampling and rearing conditions of *L. boulardi*

Five populations of *L. boulardi* in south-eastern France were selected along the latitudinal gradient of viral prevalence (Fig. 1), ranging from populations with low prevalence in the north to heavily infected ones in the south (Patot *et al.*, 2010). In September 2007, the sampling procedure consisted of setting ten closed traps baited with split bananas in four orchards, except for site 5 where rotten apples were collected from the ground. Traps were exposed to colonization by *Drosophila* and parasitoids for 15 days and then brought back to the laboratory. Emerging *L. boulardi* females were then individually isolated to create isofemale lines. During subsequent generations, all parasitoids were reared with a 12-L/12-D photoperiod at 26 °C using a laboratory line of *Drosophila melanogaster* originating from Ste-Foy-lès-Lyon (site 1) fed with a standard diet (David, 1962).

Components of superparasitism variation within and between infection statuses

To assess the contribution of viral infection and parasitoid line to superparasitism variation, we included both uninfected and infected isofemale lines of each population (after PCR detection of LbFV) except at site 1, where no infected line was found. In total, six lines per population (6×5 populations = 30 lines) were maintained under laboratory conditions. To maximize

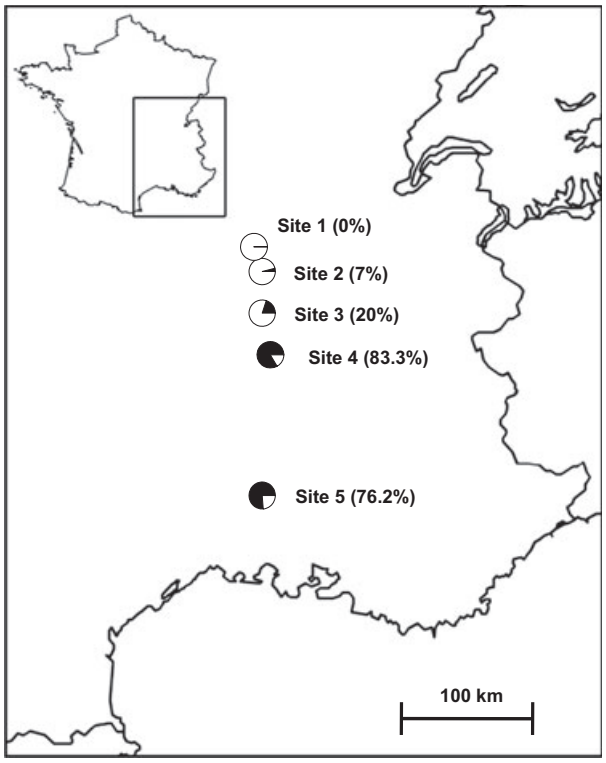

Fig. 1 Geographical locations of the sampling sites in south-eastern France. LbFV prevalence in 2007 is indicated in parentheses and by the black portion of pie charts (from Patot *et al.*, 2010).

the power of the analyses, brother–sister matings were performed for five successive generations of each isofemale line. This rearing method led to a minimum homozygosity of 67.2% for each line. The superparasitism phenotype of these inbred lines was then quantified in five females per line.

Contribution of the parasitoid genotype to the extended phenotype

To test whether nuclear genetic variation contributes to the expression of the behavioural manipulation, we partly introgressed genomes of the 30 inbred lines into a common infected cytoplasm. The common infected line, designated Sref (Varaldi *et al.*, 2006b), is a highly inbred line (eight generations of sib-mating, >82% homozygosity) originating from Sienna (Italy) that has been well characterized for several traits (Varaldi *et al.*, 2005a, 2006a).

Because the virus is maternally transmitted, the introgression was simply initiated by crossing males of the inbred lines with Sref females. Females from the first generation (G1) were then crossed again with males from their respective inbred lines to produce the second generation of introgression (G2). G1 and G2 females shared 50% and 75%, respectively, of their nuclear genome with their respective paternal inbred line. In order to test for possible maternal effects, common environmental effects or residual genetic variation in inbred lines, two independent introgression replicates per inbred line were performed.

Based on phenotypic data from G1 and G2, the introgression was continued for an additional generation (G3, 87.5% of the paternal inbred line genome introgressed on average) for four extreme lines (two high and two low superparasitizing lines). Each replicate was doubled to establish this third generation (four replicates at G3).

At each generation of introgression, five females from the uninfected counterpart of Sref (NSref) were phenotypically tested. The Sref and NSref lines share the same inbred genotype because they are both derived from the same highly inbred line. This NSref control was not included in the statistical analyses.

Quantification of superparasitism

Superparasitism measurements in emerging females were taken for inbred lines before introgression ($n = 5$ per line) and during the introgression procedure ($n = 5$ per replicate = 10 per line for G1 and G2, and 20 per line for G3), as described in the study of Varaldi *et al.* (2003). Briefly, 1- to 2-day-old mated females were individually isolated with ten 1st-instar larvae of our laboratory line of *D. melanogaster* in a 5-cm-diameter Petri dish containing a thin spot of yeast poured onto an agar layer. After one night at 26 °C (from 17.00 to 10.00), females were removed and individually frozen for subsequent PCR detection of LbFV. For each Petri dish, three to five *Drosophila* larvae were then dissected under a stereomicroscope to count parasitoid eggs. The superparasitism intensity of a given female was then determined as the mean number of parasitoid eggs laid per parasitized *Drosophila* larva. Although these experimental conditions probably enhanced the intensity of superparasitism that females would naturally express if they were not forced to stay on single patch, we must stress that superparasitism also occurs both in laboratory experiments without this constraint (Varaldi *et al.*, 2005b) and under field conditions (Fleury *et al.*, 2004; Patot *et al.*, 2010). Finally, although under these experimental conditions we measured self-superparasitism (i.e. laying of eggs in hosts previously parasitized by the same female), females display the same superparasitism intensity when facing hosts previously parasitized by conspecifics (Varaldi *et al.*, 2003; Appendix S1 in Supporting Information).

Genetic architecture underlying variation in the extended phenotype

To analyse the genetic determinism of the variability of behavioural manipulation in the parasitoid genome, we performed crosses between two infected introgressed lines that displayed contrasting superparasitism intensity

in the introgression experiment. Due to a severe dysfunction of our rearing incubator, most of the lines were lost after the introgression experiments. We therefore repeated the introgression for the surviving Av12 and Av3 inbred lines, in Sref cytoplasm, but for seven generations, leading to more than 99% of the respective inbred line genomes introgressed into the common cytotype.

These two lines were used to produce eight types of progeny (Table 1). Each type of progeny was produced by crossing independently ten females with males from the appropriate line. Superparasitism intensity was then quantified for two 1- to 2-day-old female offspring per family (n = 20 females per type of progeny). Based on the means and variances of progeny phenotypes, a joint-scaling analysis was performed to test for different genetic models incorporating a mean effect m, pooled additivity [a] and dominance [d] effects, as well as cytoplasmic maternal effects c (Cavalli, 1952; Gale *et al.*, 1977; Kearsey & Pooni, 1996; Lynch & Walsh, 1998). In *L. boulardi*, males are haploids, whereas females are diploids. There was, however, no need to take the haplodiploid mode of inheritance into account because we only worked with females, and the different types of progeny used were fully equivalent to progenies originating from strictly diploid species.

The adequacy of models was then tested by comparing the observed and expected means as described in the study of Lynch & Walsh (1998, Ch. 9, pp. 213–217), and the best model was selected according to the Akaike's information criterion (AIC) (Bieri & Kawecki, 2003).

PCR detection of LbFV

Although the DNA genome of LbFV has not been fully sequenced, previous studies led to the development of a molecular marker that can be used for PCR detection (Patot *et al.*, 2009). Using the protocol described in the study of Patot *et al.* (2009), we amplified a specific viral sequence (accession number: FM876312) with primers 102-F and 500-R in a multiplex reaction with insect primers RPS2-F and RPS2-R amplifying a parasitoid ribosomal protein gene to check for the quality of DNA extraction. The viral locus amplified is highly conserved because no genetic variation was observed among viral isolates from distant locations in the world (Patot *et al.*, 2010).

Table 1 Crossing design giving rise to eight types of progeny. *n*: number of tested females. For backcrosses (BC), the mothers originated from F1 hybrids and their two parental lines are indicated in parentheses.

Basic progenies	Crosses	*n*
P1	♀Av3 × ♂Av3	20
P2	♀Av12 × ♂Av12	20
F1.1	♀Av3 × ♂Av12	20
F1.21	♀Av12 × ♂Av3	20
BC1.1	♀(♀Av3 × ♂Av12) × ♂Av3	19
BC1.2	♀(♀Av3 × ♂Av12) × ♂Av12	20
BC2.1	♀(♀Av12 × ♂Av3) × ♂Av12	20
BC2.2	♀(♀Av12 × ♂Av3) × ♂Av3	18

Quantification of viral titre

To test for the correlation between the viral titre and the intensity of the behavioural manipulation, quantitative PCR was carried out on five 3- to 4-day-old G3 females per line that had not experienced oviposition. DNA extraction was performed as described in the study of Patot *et al.* (2009). For each sample, two independent reactions were carried out, one to amplify the specific viral sequence (accession number: FM876312) using primers I1CL1-F and I1CL1-R and one for an insect nuclear marker using 5′-GGACACGTGCAGACTCATCA-3′ and 5′-CCCACAGTTGTACTGTCATGC-3′ (unpublished, putative importin-beta gene). Reactions were carried out in 10 μL of a mixture containing 6 μL of distilled water, 1 μL of DNA template, 25 mM of $MgCl_2$, 500 nM of each primers and 1 μL of reaction mixture containing the Taq polymerase and the SYBRgreen (LightCycler® FastStart DNA Master SYBR Green I; Roche, Basel, Switzerland). The same cycling program was used for both markers: 10 s at 95°C, 10 s at 56°C, 20 s at 72°C for 35 cycles (LightCycler® 1.5 Instrument; Roche). After quantification, the virus gene copy number was divided by the insect gene copy number to obtain the viral titre per female.

Relationship between fecundity and behavioural manipulation

Because theoretical and empirical data suggest that the superparasitism phenotype may be influenced by egg load (Fletcher *et al.*, 1994; Sirot *et al.*, 1997), females of the two parental lines Av12 and Av3 that had not experienced oviposition were used to measure the fecundity. The egg load was measured on 5-day-old honey-fed females (n = 20 per line) under a microscope (Axio Imager, AxioCam, Software Axiovision LE; Zeiss, Thornwood, NY, USA). Tibia length was also measured to provide an estimate of the total body size of females.

Statistical analysis

All data sets were analysed using version 2.11.1 of *R* software. Given the nested structure of the designs, a hierarchical linear model was used for both the inbred line experiment (lines within infection status) and the introgression experiments (replicates within line, lines within population). Because we obtained unbalanced data due to losses of some lines or replicates across generations, we fitted a linear mixed model using the lme

function of the nlme package (Pinheiro & Bates, 2000). For generations G1 and G2, the population was treated as a fixed effect, because the five populations had been chosen on the basis of their viral prevalence, whereas the line (within population) and replicate (within line) effects were considered to be random. For G3 alone, the line effect was considered to be fixed because lines were chosen on the basis of their phenotype. The significance of fixed effects was tested on a model fitted by the restricted maximum likelihood method (REML), followed by conditional *F*-tests of the ANOVA method. After removing the nonsignificant fixed effects, variance components for random effects were also estimated by REML in association with 95% approximate confidence intervals (using the intervals function of the nlme package). The significance of random effects was evaluated by fitting models with the maximum likelihood method (ML). Models with and without the given random effect were then compared with a likelihood ratio test. Because there was only one random effect in G3 and lme cannot fit models without any random effect, we compared the full lme model to a model without a replicate effect fitted with the gls function (Pinheiro & Bates, 2000, Section 5.4, p. 250).

Results

Components of superparasitism variation within and between infection statuses

Thirty isofemale lines (22 uninfected/eight infected) derived from five original populations were established. After five generations of brother–sister mating, the inbred lines exhibited highly variable superparasitism intensity, ranging from 1.04 to 11.28 eggs on average per parasitized *Drosophila* larva (Fig. 2). The striking difference depending on the infection status of females is consistent with previous studies (Varaldi *et al.*, 2003; Patot *et al.*, 2010) and accounted for 77% of the total phenotypic variation (LR = 33.87; $P < 0.0001$). Clearly, uninfected females tended to avoid superparasitism and usually laid only one egg per *Drosophila* larva, whereas infected females superparasitized much more frequently, leading to very high levels of superparasitism intensity. There were however significant differences between lines with the same infection status, both for uninfected (LR = 19.09; $P < 0.0001$) and for infected lines (LR = 4.01; $P = 0.04$), accounting for 37 and 31%, respectively, of the within-infection status variation. Because in this first experiment each line consisted of only one replicate of full-sisters, we cannot rule out the possibility that maternal or common environmental effects may have underlain these differences. Nevertheless, this variation could also reflect parasitoid genotype effects both for uninfected and for infected lines, or viral genotype and/or genotype-by-genotype interactions effects between infected lines. In what follows, we tried to quantify and test for a contribution of the parasitoid genotype to the variation in superparasitism of infected females.

Are some lines totally refractory to infection by LbFV?

Both uninfected and infected inbred lines were introgressed into a standard infected cytotype (Sref). All of them were successfully infected by LbFV at generation G1 and transmitted the virus to G2 and G3 females.

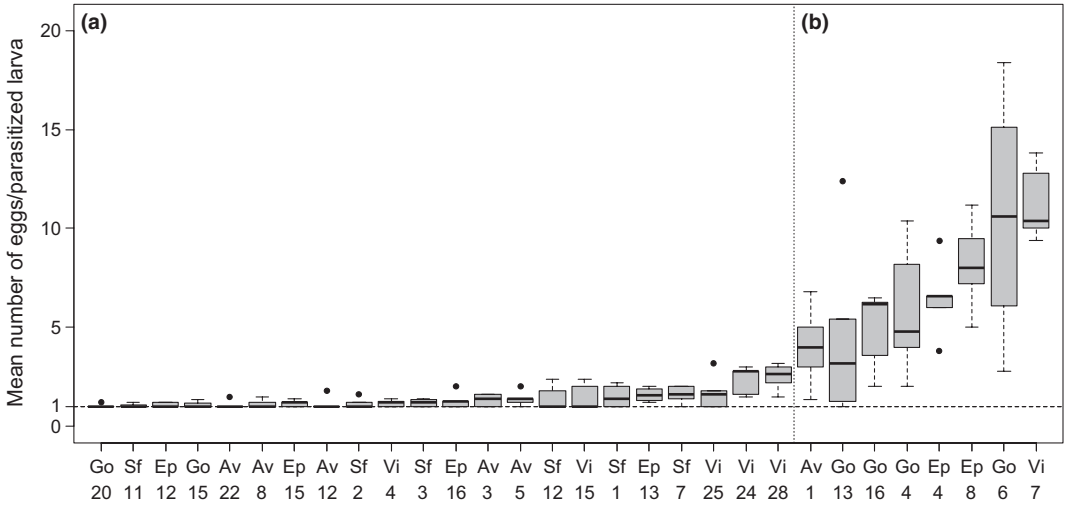

Fig. 2 Superparasitism intensity among inbred lines. (a) uninfected lines, (b) infected lines. The x-axis represents the inbred lines, which are designated by their population of origin: Sf = Sainte Foy-lès-Lyon (site 1); Vi = Villette-de-Vienne (site 2); Ep = Epinouze (site 3); Go = Gotheron (site 4); Av = Avignon (site 5).

The only exception was one of the two replicates of the Go20 line, in which no infection was detected at G1 and G2. Consequently, this replicate was not taken into account in the analyses. Note that because only two replicates per line were performed, we were not able to quantify the potential refractoriness of parasitoid lines to viral infection or the variation in vertical transmission rates between parasitoid lines. Nevertheless, none of the lines displayed complete refractoriness to infection.

Is there any genetic variation among introgressed lines regarding behavioural manipulation?

The superparasitism phenotype of each line introgressed into a common cytotype was quantified under standard conditions. For all generations, the introgressed lines showed higher superparasitism intensity compared with the uninfected control line, suggesting that behavioural manipulation occurs whatever the parasitoid genotype (Fig. 3). At G1 and G2, statistical analyses revealed almost the same pattern as there was no significant difference between populations or between lines for superparasitism intensity (Table 2). Despite the absence of line effect, the mean superparasitism intensity per line ranged from 3.2 to 12.9 eggs per parasitized larva in G1 and from 3.3 to 10.8 eggs in G2. The main difference between the generations concerned the replicate effect, which was significant in G2 and accounted for 15.2% of the total variance. Maternal effects, common environmental effects and genetic variation between replicates due to residual genetic diversity in the original inbred lines are potential explanations for this between-replicates effect. The line effect accounted for about 6% of the total variance for both generations, whereas residual variance represented 87.3% and 78.8% of the variation in G1 and G2, respectively. These levels of residual variance reflect huge differences between individual females of similar genotypes, which reduces the statistical power in detecting variation at higher levels, i.e. between replicates, between lines or between populations.

To further test for the presence of heritable variation in the superparasitism behaviour of infected females, we tested whether there was any correlation between G1 and G2 with regard to the mean superparasitism phenotype per line. Because the individuals phenotypically tested at G1 were not the mothers of the individuals

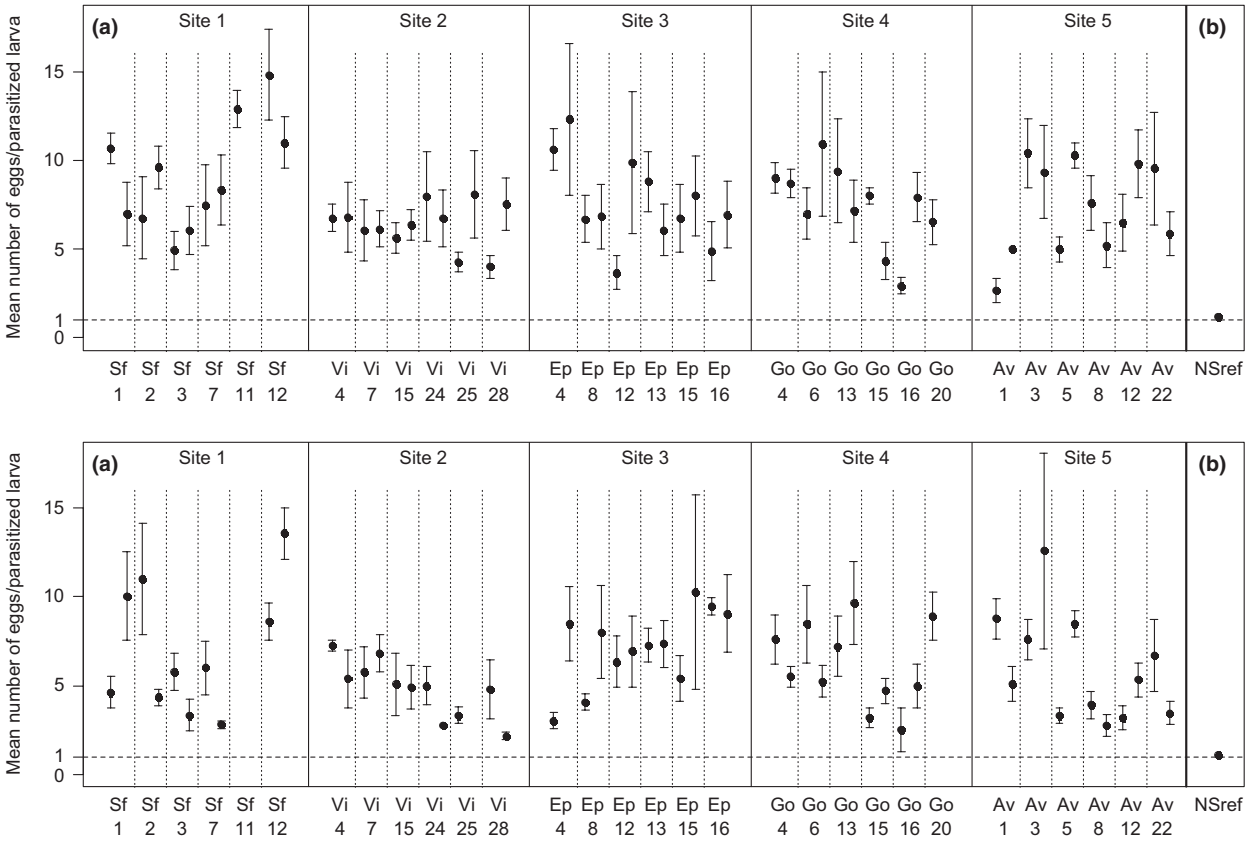

Fig. 3 Mean superparasitism intensity among introgressed lines at generations G1 (top) and G2 (bottom). (a) introgressed lines grouped according to their geographical origin (sites from north to south), (b) uninfected control line. Error bars are standard errors.

Table 2 Hierarchical linear mixed models of superparasitism intensity for the different generations of introgression. Fixed effects were tested using a conditional *F*-test. Random effects were tested by likelihood ratio tests after eliminating the nonsignificant fixed effects.

Generations	Source of variation	*F*-ratio for fixed effects/likelihood ratio for random effects	*P*	Variance component estimates with 95% confidence intervals (IC)	% of total variance
G1	Population	$F_{4,25} = 1.745$	0.17	–	–
	Line	LR = 0.8	0.37	1.10; IC = [0.37; 3.26]	6.2
	Replicate	LR = 1.23	0.27	1.12; IC = [0.36; 2.47]	6.4
	Residuals	–	–	4.13; IC = [3.77; 4.54]	87.3
G2	Population	$F_{4,24} = 1.203$	0.34	–	–
	Line	LR = 0.52	0.47	0.96; IC = [0.16; 5.82]	6
	Replicate	LR = 5.76	0.02	1.64; IC = [0.85; 3.18]	15.2
	Residuals	–	–	3.76; IC = [3.44; 4.14]	78.8
G3	Line	$F_{3,10} = 12.566$	0.001	–	–
	Replicate	LR < 0.0001	0.99	0.49; IC = [0.005; 49.3]	–
	Residuals	–	–	3.24; IC = [2.68; 3.91]	–

tested at G2 (it was their sisters that were used), we did not expect to find any correlation caused by maternal effects. Furthermore, because two independent replicates (two vials) were performed for each line, we did not expect to find any correlation caused by common environment. Yet, the mean superparasitism intensity per line showed a positive correlation between G1 and G2 ($r = 0.48$; d.f. = 27; $P = 0.009$), demonstrating that some of the variation in superparasitism behaviour is transmitted from one generation to another (Fig. 4). This strongly suggests that the phenotypic covariance between G1 and G2 results from genetic covariance between relatives and not from environmental covariance, indicating the presence of heritable variation of the extended phenotype.

Further introgression to establish G3 was performed with the extreme lines Sf12/Av3 and Go16/Av8, which displayed particularly high and low superparasitism intensity, respectively, at G1 and G2 (Fig. 4). In contrast to G2, there was no significant difference between the replicates of each line at G3. More interestingly, the results confirmed the line-specific differences observed in the previous generations (Table 2; Fig. 5). Indeed, an additional pairwise comparison showed that, as expected, the four lines can be assigned to two groups: the highly manipulated lines Av3 and Sf12 and the less manipulated lines Go16 and Av8.

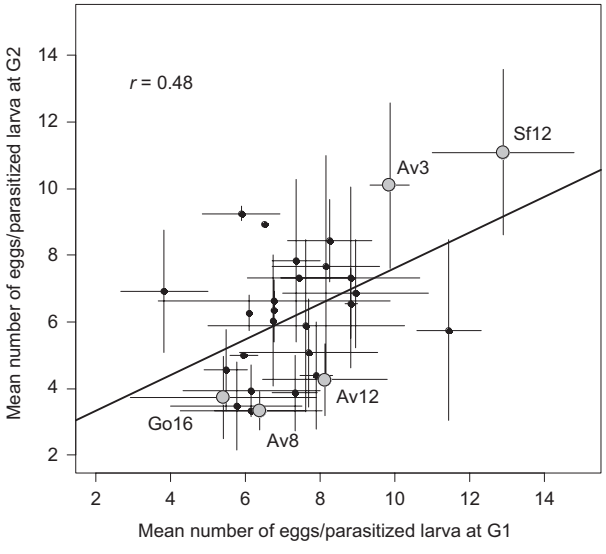

Fig. 4 Correlation of superparasitism intensity between generations G1 and G2. Dots represent the mean number of eggs/parasitized larva/female for each line calculated by averaging the means of the two replicates. Error bars are standard errors (no standard error when one replicate was missing). Grey dots correspond to the lines chosen for the establishment of generation G3 and for the crossing experiment between extreme lines.

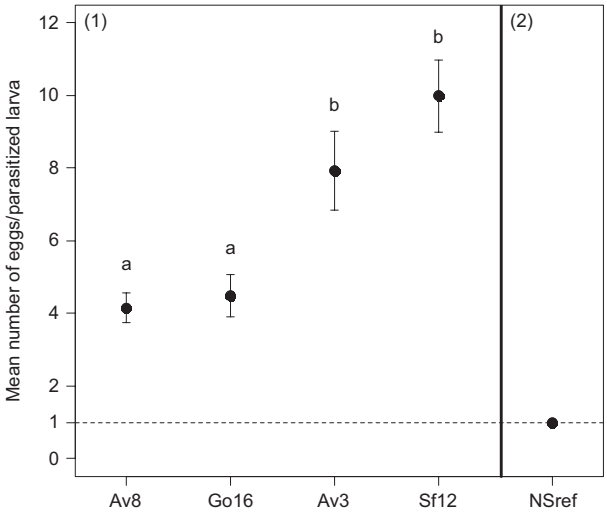

Fig. 5 Mean superparasitism intensity per line at generation G3. (1) four extreme introgressed lines; (2) uninfected control line. Different letters indicate significant differences based on a Tukey's honestly significant differences test. Error bars are standard errors.

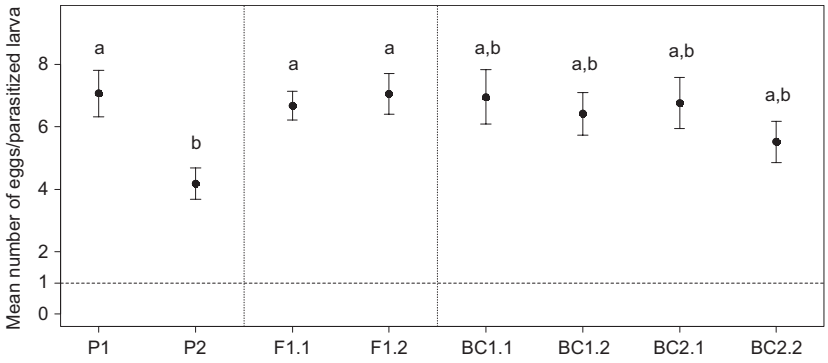

Fig. 6 Mean superparasitism intensity for the different progenies resulting from the crossing procedure between the low and high superparasitizing lines Av12 (P2) and Av3 (P1). Different letters indicate significant differences based on a Tukey's honest significant differences test. Error bars are standard errors.

Because most of the lines obtained from the previous experiments had been lost, we reconstructed extreme introgressed lines. The results obtained using these newly introgressed lines confirmed previous data because, as expected and in accordance with their initial phenotype (Fig. 4), the newly introgressed line Av12 superparasitized less than Av3 (data not shown). This pattern was stable across three generations of introgression ($F_{1,14} = 28.024$; $P < 0.0001$). The fact that the same result was observed in two independent experiments clearly supports the hypothesis that variation in the parasitoid genome is involved in the observed variation of superparasitism.

What is the genetic architecture of the determinants involved in the behavioural manipulation?

To address this question, extreme lines Av3 and Av12 were crossed (after seven generations of introgression). Analysis of their different types of progeny revealed significant differences between progenies ($F_{7,149} = 2.417$; $P = 0.02$), with the parental line Av12 (P2 in Fig. 6) showing, as expected, lower superparasitism intensity than Av3 (P1 in Fig. 6).

Three adequate models explained the segregation of superparasitism phenotype: m + [a] + [d], m + [d] + c and m + [a] + [d] + c (Fig. 6 and Table 3). Genetic effects [a] and [d] were significant when present in these models, but c did not differ from zero in the third model. Moreover, the AIC scores indicate that the most parsimonious model was m + [a] + [d], which means that there was no need to consider other effects, such as epistasis, to explain the phenotypic variation. The highly superparasitizing alleles (Av3 alleles) displayed strong dominance effect over poorly superparasitizing alleles (Av12 alleles). Thus, superparasitism intensity seems to be influenced by both additive and dominance effects, but not by cytoplasmic effects or epistatic interactions.

Table 3 Goodness-of-fit test of model adequacy, Student's t-tests of parameter estimates for adequate models and the corresponding Akaike's information criterion (AIC).

Model	d.f.*	χ^2_i statistic	Goodness-of-fit test (*P*)	Parameter estimate (*t*-test in parentheses)	AIC
m	7	22.26	0.002	–	–
m + [a]	6	12.74	0.004	–	–
m + [d]	6	13.38	0.037	–	–
m + [c]	6	16.72	0.01	–	–
m + [a] + [d]	5	7.32	0.198	m = 5.52 ($P < 0.0001$) [a] = 0.94 ($P = 0.007$) [d] = 1.35 ($P = 0.011$)	13.32
m + [a] + [c]	5	12.89	0.024	–	–
m + [d] + [c]	5	9.82	0.08	m = 5.41 ($P < 0.0001$) [d] = 1.37 ($P = 0.01$) [c] = 0.43 ($P = 0.031$)	15.82
m + [a] + [d] + [c]	4	6.18	0.186	m = 5.57 ($P < 0.0001$) [a] = 0.78 ($P = 0.029$) [d] = 1.22 ($P = 0.02$) [c] = 0.27 ($P = 0.144$)	14.18

*Degrees of freedom = number of progeny means – number of parameter estimates.

This supports the hypothesis that variation in superparasitism behaviour, in infected lines, is partly governed by variation in the nuclear genome.

Is there any correlation between behavioural manipulation and virus titre or egg load?

Viral density was measured on entire adult G3 females from extreme lines (Av8, Go16, Av3 and Sf12) as the ratio between a virus gene and an insect gene copy number. No significant difference in virus titre was found between the extreme lines ($F_{3,16} = 2.492$; $P = 0.1$; mean viral titre: 5.5 virus gene copies/100 insect gene copies) even if we pooled these lines according to their superparasitism phenotype (Av8 + Go16 vs. Av3 + Sf12, $F_{1,18} = 0.62$; $P = 0.44$), suggesting that the variation in superparasitism does not result from differences in the number of viral particles infecting the females.

Interestingly, the highly superparasitizing line Av3 had a higher egg load than Av12 ($F_{1,33} = 17.67$; $P = 0.0002$) although they had similar tibia lengths ($F_{1,35} = 0.07$; $P = 0.79$).

Discussion

Superparasitism can be expected to be subjected to high selective pressure because of the direct link between the decision-making process and the fitness of parasitoid females (van Alphen & Visser, 1990; Visser, 1993). In *L. boulardi*, the decision to superparasitize is targeted by manipulation induced by the heritable virus LbFV, which benefits from horizontal transmission under superparasitism conditions. Starting from a sample of *L. boulardi* lines originating from various localities, we found that the infection status of the parasitoids accounts for 77% of the total variation in superparasitism behaviour. We also found that the different parasitoid genotypes were not manipulated with the same intensity. Although the parasitoid line effect was not significant at the first two generations of introgression, the positive correlation of the mean line phenotypes between these generations supports the hypothesis of genetic line-specific differences. Taken together with what had been found after further introgression of extreme lines, these results showed that phenotypic variation is transmitted across generations. This line-specific variation is most probably due to nuclear genetic effects because parasitoid genotypes were introgressed into homogenous LbFV-infected cytoplasm. Although we cannot rule out the possibility that some other viral symbiont could be present in this cytotype, no symbiotic bacteria seem to infect *L. boulardi* (Varaldi *et al.*, 2006b). One could argue that the observed differences between introgressed lines result from the accumulation of mutations in LbFV or in other unknown cytoplasmic symbionts infecting the Sref line (and/or the loss of unknown cytoplasmic symbionts) during the course of the introgression experiment. However, such effects, which would be equivalent to maternal effects, were taken into account because several introgression replicates were performed per line. In addition, this hypothesis cannot explain why several independent introgression experiments always led to the expected phenotypes for both highly and poorly superparasitizing lines. Thus, we conclude that part of the variation was caused by genetic differences between parasitoid lines and was heritable with substantial additive and dominance effects. We did not find any complete resistance to manipulation, but parasitoid genotypes did exhibit differences in their sensitivity to viral manipulation. Reduced sensitivity can be viewed as partial resistance to the virus effect, which amounts to a kind of tolerance to the infection (Svensson & Raberg, 2010). Tolerance is a way the host can defend itself by alleviating the negative fitness consequences of an infection without preventing the infection or reducing the growth of the parasitic symbiont (Lefèvre *et al.*, 2011). Symbiont titre has been shown to vary according to the host genotype in several symbiotic associations (Kondo *et al.*, 2005; Mouton *et al.*, 2007). Under the hypothesis of a resistance to the infection, we would expect that variation in the extended phenotype would be correlated with variation in symbiont titre. In our study, however, the viral titre of the females did not differ between genotypes showing extreme and opposite behavioural phenotypes, which fits with a tolerance mechanism. Between-line differences in superparasitism intensity could be due to allelic variation in the parasitoid genes involved in functions that mediate superparasitism behaviour (tolerance) rather than in functions that control viral multiplication within parasitoid females (resistance). However, we must be cautious about this conclusion, because only four lines were tested, which reduces the statistical power of the viral titre analysis. Additional experiments will be required to test the two hypotheses.

Although there was heritable additive variation within the populations with regard to the superparasitism of infected females, on average the different populations displayed the same superparasitism intensity. This was rather unexpected in view of the contrasting ecological conditions experienced by the northern and southern populations. Indeed, the strength of selection exerted by LbFV is probably greater in the south given the much higher viral prevalence (Patot *et al.*, 2010; Fig. 1). Knowing that uninfected females rarely superparasitize, we can reasonably assume that the parasitoid optimum is lower than the superparasitism intensity expressed by infected females. Thus, given the existence of parasitoid genetic variability for manipulation intensity, we would have expected southern genotypes to be more resistant to manipulation than northern ones, because they are much more likely to be infected. A possible explanation for this absence of differentiation is the fact that the migration rate may be high in this area, thus preventing the development of geographical structuring of the

parasitoid gene pool. Alternatively, the virus could have invaded parasitoid populations only very recently, as has been observed for sigma viruses in *D. obscura* (invasion in England within the past 11 years, Longdon *et al.*, 2011) and *D. melanogaster* (Carpenter *et al.*, 2007). In this case, alleles conferring tolerance would not yet have had long enough time to spread through southern populations. Negative pleiotropy or genotype-by-environment interactions may also play a role in maintaining polymorphism (Gillespie & Turelli, 1989; Curtsinger *et al.*, 1994; Reinhold, 2000; Turelli & Barton, 2004; Byers, 2005; Hedrick, 2007). Our data suggest that a pleiotropic effect might indeed promote the coexistence of several parasitoid genotypes, because the poorly manipulated Av12 line had a lower egg load than the highly manipulated Av3 line. This constitutes a potential cost of tolerance. However, this correlation needs to be tested over a wider range of parasitoid and virus genotypes. Furthermore, in this system, superparasitism tends to decrease the efficiency of the *Drosophila* immune response, especially in *D. simulans* (J. Martinez, F. Fleury & J. Varaldi, unpublished data), thus constituting a potential fitness benefit for highly manipulated genotypes.

Finally, we can question whether selection can act efficiently on the parasitoid genetic variation involved in the expression of behavioural manipulation. This polymorphism, albeit phenotypically expressed, might be neutral with regard to local selective pressures. In this case, however, we would expect that uninfected females would also exhibit such a wide range of superparasitism intensity, which they do not. However, the large residual variance (around 80%) and the low between-line variance (around 6%) show that environmental heterogeneity has a strong influence on this phenotype, and so may preclude the action of selection.

The genetic architecture of superparasitism is clearly extremely complex. The phenotypic value of an individual is likely to depend on its infection status, its own genotype, the genotype of its symbiont, the environment and all the possible interactions. Furthermore, parasitoid and virus genes have different transmission routes and thus experience divergent selective pressures. The prevalence of the virus also differs considerably from one population to another, and so the strength of selection imposed on parasitoid populations might also be very variable. In this paper, we have tried to quantify the influence of one component (the genotype of the individual) infected by the same viral isolate on the extended phenotype and found that it made a significant but relatively small contribution. Further work should evaluate other contributions to the phenotypic variation of superparasitism, such as the viral genetic diversity (Wilfert & Jiggins, 2010), the genetic correlations between infection statuses or between fitness-related traits. This work should shed light on how selection acts on this complex extended phenotype. For instance, one interesting question is whether the differences that we observed among infected lines would also be observed among their uninfected counterparts (which could be done by introgressing the original inbred lines into the uninfected reference line). In this case, the differences observed between infected females would simply reflect baseline differences in the behaviour of uninfected females. In contrast, complex parasitoid genotype-by-infection status interactions may be found. As we pointed out in the introduction, the IGE theory (McGlothlin & Brodie, 2009) might help us to do this. IGE theory is used to study phenotypes of which the expression is influenced by genes present in other conspecifics. This theoretical framework could be extended to include interactions with genes present in a heritable symbiotic partner.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Comparison of NS (uninfected) and S (infected) parasitoid lines on conspecific superparasitism behaviour.

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