Parasitic success and venom composition evolve upon specialization of parasitoid wasps to different host species

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Abstract

Female endoparasitoid wasps usually inject venom into hosts to suppress their immune response and ensure offspring development. However, the parasitoid's ability to evolve towards increased success on a given host simultaneously with the evolution of the composition of its venom has never been demonstrated. Here, we designed an experimental evolution to address this question. We crossed two parasitoid lines of *Leptopilina boulardi* differing both in parasitic success on different *Drosophila* hosts and venom composition. F_2 descendants were reared on three different *Drosophila* species for nine generations. We tested for evolution of parasitic success over the generations and for the capacity of parasitoids selected on a given host to succeed on another host. We also tested whether the venom composition - ~~analyzed~~ based on a statistic analysis ~~the basis~~ of the variation in intensity of the venom protein bands on SDS-PAGE 1D - evolved in response to different host species. Results showed a specialization of the parasitoids on their selection host and a rapid and differential evolution of the venom composition according to the host. Overall, data suggest a high potential for parasitoids to adapt to a new host, which may have important consequences in the field as well in the context of biological control.

35 **Keywords:** parasitoid wasps, *Drosophila*, parasitic success, experimental evolution, venom
36 evolution, specialization

37 ## 1. Introduction

38 Endoparasitoid wasps are insects whose larvae develop inside the host, generally
39 leading to its death [1]. ~~and strong~~High selection pressures are therefore exerted to maximize
40 the parasitic success that has been ~~. Parasitic success was indeed~~ shown to evolve rapidly
41 according to host resistance [2–4] ~~but also~~and host species [5,6]. The ability to succeed ~~on~~ in
42 ~~several~~ multiple host species and adapt to a new host ~~are~~ is important for the abundance and
43 survival of parasitoids in ~~case~~ the event of environmental changes, such as the local extinction
44 of a host. The host range of parasitoids is generally not limited to a single species, even for
45 those considered specialists. For example, *Leptopilina boulardi,* considered a specialist of *D.*
46 *melanogaster* and *D. simulans,* can develop in other species of the *melanogaster* subgroup of
47 Drosophilidae, including *D. yakuba* [7,8].

48 Parasitoids have evolved different strategies to circumvent the host's immune defense,
49 which ~~generally~~ usually consists of the formation of a multicellular melanized capsule around
50 the parasitoid egg, together with the activation of the phenol~~-~~oxidase cascade which leads to
51 the production of melanin ~~production~~ and the release of toxic radicals [9–11]. The most
52 ~~prevalent~~ common strategy ~~relies on~~is the injection of venom ~~at oviposition~~with the egg, which
53 suppresses the encapsulation process [12–14]. To our knowledge, only two studies have
54 analyzed the ability of parasitoid venom ~~capacity~~ to evolve according to the host. Both
55 *Lysiphlebus fabarum* parasitic success and expression of venom genes ~~expression~~ rapidly and
56 differentially evolved depending on the ~~according to~~ strains of a defensive symbiont hosted by
57 the host *Aphis fabae* [4]. More recently, a rapid and differential evolution of the venom protein
58 composition of *L. boulardi* was described according to the susceptible/resistant phenotype of
59 two *D. melanogaster* host strains [15]. However, the relationship to parasitic success was not
60 analyzed. Since these studies involved a single host species, our goal ~~was~~ here was to

61 investigate whether the parasitic success and the venom protein composition ~~and parasitic~~

62 ~~success~~ could evolve according to different host species. We also sought to determine whether

63 the venom of a parasitoid wasp ~~succeeding on~~successful in several host species contains broad-

64 spectrum factors or a combination of specialized factors, each specific to a given host.

65 We used *L. boulardi* as a model because of its ~~shows~~ intraspecific variability ~~of~~ in both

66 venom composition and parasitic success on different hosts. ~~Indeed, t~~The ISm line always

67 succeeds on *D. melanogaster* but is consistently encapsulated by *D. yakuba* whereas the ISy

68 line can succeed on both *Drosophila* species but only on certain genotypes [16]. The venom of

69 these lines differs widely, mainly due to quantitative differences in the venomous proteins. As

70 an example, ~~as demonstrated for LbGAP. The amount of this~~a RhoGAP domain-containing

71 protein named LbGAP is in a ~~indeed~~ significantly higher amount in the venom of ISm than in

72 that of ISy [17,18]. LbGAP ~~seems required~~would be necessary for ISm parasitic success on an

73 ISy-resistant *D. melanogaster* strain ~~resistant to ISy parasitoids~~ through the induction of

74 morphological changes in the lamellocytes, host immune cells devoted to the encapsulation

75 [17,19–22]. LbSPN, a serine protease inhibitor of the serpin superfamily [18] illustrates the

76 qualitative variation of ~~in the~~ venom proteins between ISm and ISy. LbSPNy, which inhibits

77 the activation of the phenoloxidase cascade in *D. yakuba* ~~phenoloxidase cascade~~ [23] and

78 LbSPNm are one of the most abundant proteins ~~of~~ in the ISy and ISm venom, respectively.

79 Although they are encoded by allelic forms of the same gene, they differ in molecular weight

80 and the sequence of the active site, suggesting they have different targets and/or function [18].

81 Here, we report data from an experimental evolution designed to evaluate the

82 evolvability of *L. boulardi* (i) parasitic success and (ii) the venom protein composition

83 according to different *Drosophila* host strains and species. The experiment was designed to

84 characterize the venom allowing the parasitoid to develop on different hosts and to inform on

85 whether such venom contains rather broad-spectrum factors or a combination of factors specific to

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86 each host. For this, we crossed ~~Hybrids with new venom allelic compositions were obtained by~~

87 ~~crossing~~ *L. boulardi* ISm and ISy individuals ~~in both directions and the F_2 descendants were~~

88 ~~then~~and reared their descendants for nine generations independently on the ~~different~~ hosts

89 species and strains differing for their resistance to these wasps ~~for nine generations~~. We then

90 analyzed the parasitic success on the selection host and the venom composition in three

91 generations: after the first (F_3) and the last (F_{11}) generation under selection and after an

92 intermediate generation (F_7). ~~Females of the F_3, F_7 and F_{11} generations were analyzed for~~

93 ~~parasitic success and venom composition.~~ We also tested for a specialization of parasitoids by

94 measuring the parasitic success of individuals from the latest generation of selection on all the

95 different hosts. ~~The analysis of the venom protein composition was performed using a global~~

96 ~~approach, based on the analysis of the variation of intensity of venom protein bands among~~

97 ~~individual wasps on 1D SDS-PAGE coupled with further statistical analysis of the protein~~

98 ~~bands intensity [24]. This approach identified many venom protein bands whose intensity has~~

99 ~~changed on a given host. We then used "omics" data previously obtained on the ISm and ISy~~

100 ~~venoms [18] to tentatively identify the most abundant protein in these bands. We also checked~~

101 ~~the global approach accuracy using a Western Blot analysis designed to analyse the evolution~~

102 ~~of the amounts of three venom proteins, LbSPN, LbGAP and LbGAP2, another RhoGAP~~

103 ~~present in the *L. boulardi* venom.~~ Overall, parasitoids have shown ~~we observed a~~ specialization

104 ~~of parasitoids~~ to their selection host as well as a rapid and differential evolution of the venom

105 composition. ~~Most c~~Changes in the intensity of the venom protein bands were mainly observed

106 in response to selection on a single host, although some changes occurred under selection by

107 ~~multiple~~ several hosts. This suggests that most of the venom factors of this wasp are host-

108 specific while a few may have a ~~broader~~ wider spectrum.

109

110 **2. Material and methods**

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2.1. Biological material

L. boulardi ISm (Gif stock number 431) and ISy (Gif stock number 486) isofemale lines originate from populations collected in Nasrallah (Tunisia) and Brazzaville (Congo), respectively [24]. Both lines were reared on their susceptible *Drosophila melanogaster* maintenance strain (Nasrallah from Tunisia, Gif stock number 1333, here called S_{Nasr}) at 25°C. Emerged adults were kept at 20°C on agar medium and fed with honey.

Five *Drosophila* host strains from three different species differing in their resistant/susceptible phenotype to ISm and ISy were used (Figure ~~S~~1A). The *D. melanogaster* R strain (Gif stock number 1088), originating from isofemale lines obtained from a population of Brazzaville, Congo [25] through subsequent genetic approaches [26,27], is resistant to ISy and susceptible to ISm [28,29]. The *D. simulans* Japan strain, from a population collected in Japan, is susceptible to ISm and ISy. *D. yakuba* 1907 (Gif stock number 1907) and 307 (Gif stock number 307.14), originate from Tanzania and from the São Tomé island, respectively. *D. yakuba* 1907 is resistant to both parasitoid lines while 307 is resistant to ISm and susceptible to ISy.

2.2. Experimental evolution protocol

Three wasp mass rearings were created from (♀ISm x ♂ISy and ♀ISy x ♂ISm) crosses of 12 virgin females and six males on the *D. melanogaster* S_{Nasr} host (Figure ~~S~~1B). A total of 15 replicates were then created from these mass rearings using six F_1 hybrid females and three F_1 males from each direction of crossing, still on *D. melanogaster* S_{Nasr}. Finally, F_2 individuals from each of the 15 replicates were used to produce five groups that were then reared separately on the five different hosts until the F_{11} generation (F_6 only for *D. yakuba* 1907, therefore excluded for further analyses; Figure ~~S~~1B). For each of the 75 populations (15 replicates x five hosts), up to 10 females and five males (mean: 9.51 ♀, 4.31 ♂) were randomly chosen to

produce the F_3 generation, and up to 20 females and 10 males (mean: 18.48 ♀, 8.99 ♂) to produce F_4 to F_{11} generations.

The parasitic success and venom composition were analyzed for females of three generations: the first generation of selection on different hosts (F_3), an intermediate generation (F_7), and the last generation (F_{11}). Since the venom composition and the parasitoid behavior could vary between females depending of the number of eggs previously laid, only females never allowed to ~~parasite~~ oviposit were used for the analyses.

2.3. Analysis of the outcome of the Drosophila – L. boulardi *interaction*

Parasitic tests and dissection:

Twenty second-instar larvae of the investigated host species were deposited in small medium-containing dishes with one parasitoid female. The parasitoid was removed after 4h at 25°C and the dishes kept at 25°C for 48h until dissection of the *Drosophila* larvae ~~since the peak of lamellocytes released for the encapsulation process occurs 24h after parasitism~~. They were then categorized as (i) non-parasitized, (ii) mono-parasitized and (iii) multi-parasitized (see Figure S~~2~~1 for proportions of each). Only mono-parasitized host larvae were considered for the analysis to avoid unpredictable effects of the presence of several parasitoid larvae in a single host.

Three possible outcomes were then recorded: i) free parasitoid larva alone, ii) free parasitoid larva together with an open capsule and iii) complete closed capsule (Figure 2~~1~~A). Among these, the percentage of outcomes for i) and iii) we~~a~~re generally used to ~~evaluate respectively~~assess the parasitoid's immune suppression ~~capacity~~ ability ~~of the parasitoid~~ and

161 the host encapsulation capacity ~~of the host~~ [5,25,30]. In this work, we added the outcome ii) to
162 ~~investigate~~ evaluate the parasitoid's ability ~~of the parasitoid~~ to escape the encapsulation process
163 initiated by the host after recognition of the parasitoid egg. Since the escaped parasitoid larva
164 was alive, we considered this outcome to be~~as~~ a parasitic success, similar ~~as~~ to a free parasitoid.

165 *Statistical analysis:*

166 We used a generalized linear mixed model (GLMM) to analyze each parameter with the
167 continuous generation or the selection host as a fixed effect, the replicates as a random effect
168 and a binomial error distribution. The GLMMs were fitted with the *glmer* function in "lme4" R
169 package [31], except for unbalanced data for which they were fitted with the *bglmer* function
170 implemented in the "blme" R package [32]. As for all the binomial GLMMs performed,
171 overdispersion was tested with the *overdisp_fun* function [33] and accounted for when
172 necessary ($p < 0.05$) by adding a random factor corresponding to the observations number [34].
173 GLMMs were followed by post-hoc Tukey tests with the "multcomp" R package [35] to
174 compare hosts two by two.

175

176 *2.4. Venom separation, western blot and data acquisition*

177 *L. boulardi* venom reservoirs were dissected individually in 15 µl of insect Ringer
178 supplemented with protease inhibitors cocktail (PI; Roche), mixed with an equivalent volume
179 of Laemmli reducing buffer and heated (95°C, 10 min). The individual protein samples were
180 then split in two, half being used for the global analysis, half for the specific analysis (see the
181 two next paragraphs). Protein separation was done by 1D SDS-PAGE electrophoresis using
182 commercial gels to ensure reproducibility (8–16% Criterion™ TGX™ Precast Midi Protein
183 Gel, Bio-Rad). A sample of venom of ISm and ISy lines, equivalent to half a female reservoir
184 (from a pool of venom from 60 individuals collected in 1.8 ml) was also loaded on each gel and
185 served as a reference.

The global analysis was performed as described in [36] and used in [15]. Briefly, the gels were silver-stained and their digital pictures analyzed with the Phoretix 1D software (now CLIQS, TotalLab, UK) to extract the intensity profile of each lane (individual sample). We then used R functions to obtain for each lane the intensities of a set of “reference bands” of known molecular weight. These intensities were normalized and estimated with the following combination of parameters: without background – peak height – quantile normalization (more details in [36]). The normalized intensities of the reference bands are the variables characterising the venom composition.

The specific analysis was performed using Western blots and antibodies targeting previously characterized *L. boulardi* venom proteins as described in [15]. The LbSPN, LbGAP and LbGAP2 polyclonal antibodies are described in [19,20,37,38]. The secondary was a goat anti-rabbit IgG horseradish peroxidase conjugate (1:10,000; Sigma). Western blots were revealed with a luminescent substrate (Luminata Crescendo; Millipore) and digitalized. Data were then recorded as follows for each individual:

i) LbSPN: the genotype at the *lbspn* locus was determined by the presence/absence of the LbSPNy (54 kDa) and LbSPNm (45 kDa) bands.

(ii) LbGAP: a strong signal was observed in the venom of ISm or (ISm × ISy) F_1 only. The presence/absence of a signal at the expected size was then used to distinguish *lbgap* homozygotes and *lbgap*/$lbgap_y$ heterozygotes from $lbgap_y$ homozygotes.

(iii) LbGAP2: the variation in quantity was more continuous. The normalized quantity of LbGAP2 was estimated as the ratio between the signal intensity on the Western blot without background and the sum of the intensities of the reference bands (used as a proxy for the amount of protein in the venom samples) in the corresponding lane on silver stained gels.

2.5. Statistical analysis for the global analysis of the venom

211 Evolution of the venom composition. We analyzed the evolution of the venom
212 composition using permutational PERMANOVAs (permutational MANOVAs; "vegan" R
213 package; function *adonis2*; [39,40]). PERMANOVAs mesure the association between the
214 multidimensional variation of some explained variables (venom) and some explanatory
215 variables, but at the difference of MANOVAs, they don't compare the correlation structure
216 among different groups. For this analysis, we measured the multidimensional variation through
217 the Euclidean distance, and we tested the significance of explanatory variables' marginal effects
218 with 5,000 permutations to assess how Euclidean distances between the venom composition of
219 individuals were explained by the marginal effect of the explanatory variables. To determine
220 whether the venom composition has evolved on each host separately, PERMANOVAs were
221 fitted with the generation (F_3, F_7 or F_{11}; continuous variable) and the replicates population (same
222 as replicates in this situation) as explanatory variables. For *D. yakuba* 307, we fitted an
223 additional PERMANOVA without F_3 individuals due to their small number. We then
224 determined whether the venom composition has evolved differently depending on the host by
225 fitting a PERMANOVA with permutations within replicates and the following explanatory
226 variables: the generation, the host, the interaction of both and the 44 experimental populations
227 (sum of replicates maintained on each host until the end of the experiment) to account for the
228 effect of genetic drift. Then, PERMANOVAs were performed to compare the venom
229 composition two by two between hosts to determine whether a venom composition evolved on
230 one host differed from that evolved on another.

231 To characterize the evolutions detected by the PERMANOVAs, we used linear
232 discriminant analyses (LDA). Specifically, wWe performed linear discriminant analyses (LDA)
233 to characterize (i) the evolution of the venom composition on each host separately (LDAs
234 performed with the three groups of individuals i.e. from the three analyzed generations) and (ii)
235 the differential evolution of venom composition depending on the host (LDAs performed with

236 the 12 groups of individuals (three generations × four hosts) and each two by two comparison
237 of venom composition between hosts. We used the “ade4” R package [41] with the individual
238 venom compositions as continuous variables and the generation or the “host × generation”
239 interaction as a factor. Since LDA does not account for the variation between replicates, they
240 were centered before the analysis using the *wca* function (within class analysis) in the “ade4”
241 R package. With this additional step, all replicates had the same mean for each variable and
242 thus the variation among generations and hosts cannot result from the variation from replicates
243 andunbalanced data. The ~~biological meaning of the~~ LDA axes were labeled with the biological
244 meaning ~~was~~ determined ~~based on~~from the position of the 12 groups of individuals (generations
245 × hosts) on these axes. To describe the venom evolution trend, we plotted for each host in each
246 LDA an arrow representing the linear regression ~~calculated from~~fitted to coordinates of the
247 three centroids ~~points~~ corresponding to the F_3, F_7 and F_{11} generations and weighted by the
248 number of individuals per generation and host. The only exception was the LDA on *D. yakuba*
249 307 for which the linear regression was calculated from centroid points of the F_7 and F_{11}
250 generations only.

251 Evolution of venom protein bands. Non-parametric Spearman rank correlation tests
252 were performed for each LDA done for each host separately to identify the protein bands that
253 correlated with the regression describing the trend of venom evolution. P-values were
254 Bonferroni corrected using the *p.adjust* R function.

255 As some protein bands were probably indirectly selected due to their correlation with
256 other bands, a combination of clustering and partial correlation analyses was used to identify
257 bands undergoing direct selection. We first performed an UPGMA clustering analysis (“hclust”
258 R package) with~~-~~ 1 - |ρ| as the metric distance, where ρ is the spearman correlation. Then, we
259 used a threshold correlation of 0.4 to construct band clusters for which false detection of certain
260 bands as “correlated” could have occurred. For each cluster with at least two protein bands

261 correlated to the regression (represented by an arrow), we performed partial correlations with
262 the *pcor* function in the "ppcor" R package to determine if the residual variation in the intensity
263 of each band, independent from the other bands of the cluster, was still correlated to the
264 regression describing the evolution. P-values were also Bonferroni corrected using the *p.adjust*
265 R function.

266 Comparison of the venom composition evolved on each host to that of the parental
267 lines. To make this comparison, we computed the averages of venom composition evolved on
268 each host separately for each replicate. Each of the 44 populations from the first and last
269 generation of selection (F_3 and F_{11}) was scaled between 0 and 1 depending on its distance to the
270 ISm or ISy venom using the formula $D_{ISy}/(D_{ISm} + D_{ISy})$, D_{ISy} and D_{ISm} being the Euclidean
271 distances between the average venom composition of the population and the venom
272 composition of ISm and ISy. Then, we tested whether the venom composition of parasitoids
273 reared on each host evolved toward a parental line by comparing the scaled distances between
274 F_3 and F_{11} generations with paired Wilcoxon rank tests. Finally, to determine whether a protein
275 band selected on a given host corresponded to an ISm or ISy band, we assigned it a value from
276 0 to 1 in relation to a higher intensity in ISy or ISm, respectively. This value is the intensity of
277 the band in the venom of ISm divided by the sum of its intensities in the venom of ISm and ISy.
278

279 *2.6. Statistical analysis for the specific analysis of venom*

280 The three variables describing LbSPN, LbGAP and LbGAP2 are of different type
281 (categorical for LbSPN with two different alleles, presence/absence for LbGAP, continuous for
282 LbGAP2 with the relative intensity). We therefore used different approaches to analyse them.

283 LbSPN is a codominant marker with two alleles (*lbspnm* and *lbspny*) encoding proteins
284 of distinct molecular weight. To determine if the frequency of these alleles had evolved on each
285 host, we fitted one generalized linear mixed model (GLMM) per host with generation as a fixed

286 continuous effect and replicates as a random effect with a binomial distribution using the
287 "lme4" R package [31].

288 LbGAP is a dominant marker with two alleles (*lbgap* and *lbgap*$_y$) determining the
289 presence in the venom of the LbGAP protein in detectable quantity (*lbgap* homozygotes and
290 *lbgap*/*lbgapy* heterozygotes) or not (*lbgapy* homozygotes). The evolution of the
291 presence/absence of the LbGAP/LbGAPy proteins independently on each host was tested using
292 the same procedure as for LbSPN.

293 For LbGAP2 (continuous variation), we used ~~some~~ linear mixed models (LMM) with
294 generation as a fixed continuous effect and replicates as a random effect to determine ~~separately~~
295 ~~for each host~~ if the quantity of LbGAP2 had evolved, separately for each host. The models were
296 fitted with the "nlme" R package [42] on the box cox-transformed (lambda = 0.25) standardized
297 intensity of LbGAP2 to normalize the residues.

298

299 **3. Results**

300 *3.1. Experimental evolution protocol*

301 The variability on which the selection took place was generated by reciprocal crosses
302 between the ISm and ISy parasitoid lines. F_1 hybrids were then used to produce the 15 F_2
303 replicates from which five groups were formed and reared independently on *D. melanogaster*
304 S_{Nasr}, *D. melanogaster* R, *D. yakuba* 307, *D. yakuba* 1907 and *D. simulans* (Figure ~~S~~1B). Of
305 these 75 starting F_2 experimental wasp populations, 44 were successfully maintained until the
306 F_{11} generation. Among these, none of the *D. yakuba* 1907 replicates survived after the F_6,
307 preventing further analyses for this host. 12 and 15 replicates survived on *D. melanogaster* S_{Nasr}
308 and *D. simulans*, respectively (Table S1), and 13 on *D. melanogaster* R, whereas only four
309 could be maintained on *D. yakuba* 307 until the F_{11}, the other replicates mainly becoming
310 extinct at the first generation of selection (F_3) (Table S1).

3.2. Evolution of the interaction outcome according to the selection host

We tested the evolution of the parasitoids capacity to bypass the encapsulation response of their selection host after five and nine generations of selection (i.e. F_7 and F_{11}), using two females per experimental population, except for *D. yakuba* 307 for which four females were tested since fewer replicates were available. Three parameters were analyzed: (i) the parasitic success, i.e. among mono-parasitized host the proportion of hosts containing a free parasitoid larva alone or together with an open capsule,~~-~~ (ii) the capacity of the parasitoid to inhibit the capsule formation, i.e. ~~the proportion~~among mono-parasitized host the proportion of ~~hosts~~ *Drosophila* larvae containing a free parasitoid larva alone and (iii) the capacity of the parasitoid to escape from the capsule, i.e. among mono-parasitized hosts showing an encapsulation response, the proportion of hosts containing a free parasitoid larva together with an open capsule (Figure 2~~1~~A).

The parasitic success of parasitoids reared on *D. melanogaster* S_{Nasr} or *D. simulans* on this same host remained close to 100% throughout the experimental evolution (Figure 2~~1~~B, Table S2, GLMM, p > 0.~~05~~30 for both hosts). In contrast, it increased with the generation on *D. melanogaster* R (from about 80% at F_3 to 90% at F_{11}) and *D. yakuba* 307 (from about 20% at F_3 to 65% at F_{11}) (Figure 2~~1~~B, Table S2, GLMM, p = 0.002 for *D. melanogaster* R and p < 0.001for *D. yakuba* 307). For *D. melanogaster* R ~~T~~this increase seemed to result solely from the increased capacity to escape from the capsule (Figure 2~~1~~B, Table S2, GLMM, p = 0.001) ~~for *D. melanogaster* R~~ since no significant change was observed for ~~the~~ its ability to inhibit encapsulation (Figure 2~~1~~B, Table S2, GLMM, p = 0.33~~> 0.05~~). For *D. yakuba* 307, the successi~~s~~ increase seemed to result mainly from a higher capacity to escape from the capsule (Figure 2~~1~~B, Table S2, GLMM, p <0.001) but also, to a lesser extent, to inhibit encapsulation at F_{11} (Figure 2~~1~~B, Table S2, GLMM, p = 0.003). Finally, a much lower parasitic success was observed at F_3

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for *D. yakuba* 307 (about 20%) than for the other hosts (about 80% to 100% depending on the host) (Figure 21B).

3.3. Specialization of parasitoids to their selection host

To determine whether the change in parasitoid ability to bypass host encapsulation was specific to the selection host, we compared the success of F_{11} parasitoids on their own selection host *vs.* the three other hosts. The parasitic success on *D. melanogaster* S_{Nasr} and *D. simulans* was close to 100% regardless of the selection host (Figure 21C, Table S3, GLMM, Tukey post hoc-test, $p > 0.0570$ for both hosts). In contrast, parasitoids reared on *D. melanogaster* R were more successful on this same host than those reared on *D. yakuba* 307 (Figure 21C, Table S3, GLMM, Tukey post hoc-test, $p < 0.001$). This can be explained by a higher capacity of parasitoids selected on *D. melanogaster* R to both inhibit encapsulation and escape from a capsule compared to those selected on *D. yakuba* 307 (Figure 21C, Table S3, GLMM, Tukey post hoc-test, $p = 0.015$ for encapsulation inhibition and $p < 0.001$ for escape capacity). Finally, parasitoids reared selected on *D. melanogaster* R also had a higher capacity to escape from a capsule of this same host compared to parasitoids reared selected on *D. melanogaster* S_{Nasr} or *D. simulans*, despite a similar parasitic success (Figure 21C, Table S3, GLMM, Tukey post hoc-test, $p < 0.001$for the escape capacity between *D. melanogaster* R and the two other hosts; $p = 0.09$ and $p = 0.30$ and for the parasitic success between *D. melanogaster* R vs *D. melanogaster* S_{Nasr} or vs *D. simulans*, respectively).

Parasitoids reared on *D. yakuba* 307 had a higher parasitic success on this same host than parasitoids reared on all the other hosts (Figure 21C, Table S3, GLMM, Tukey post hoc-test, $p < 0.005$ for all comparisons involving *D. yakuba* 307). The parasitoids capacity to inhibit encapsulation by *D. yakuba* 307 was very low, with no significant difference between the selection hosts (Figure 21C, Table S3, GLMM, Tukey post hoc-test, $p > 0.0560$). Therefore,

361 the difference in parasitic success between selection hosts was most probably explained by ~~the~~

362 ~~higher~~an increase capacity to escape from the capsule (Figure 2~~1~~C, Table S3, GLMM, Tukey

363 post hoc-test, $p < 0.05$ for each pairwise comparison except for *D. melanogaster* R and *D.*

364 *simulans* for which $p = 0.76$).

365

366 *3.4. Differential evolution of venom composition according to the host*

367 The venom analysis was performed on 50 individuals per host and generation distributed

368 over all replicates, except for *D. melanogaster* S_{Nasr} at F_3 (47 ♀), R at F_{11} (49 ♀) and *D. yakuba*

369 307 (10, 39 and 40 ♀ at F_3, F_7 and F_{11}, respectively). In total, the venom protein content was

370 analysed for 535 females and 36 reference protein bands were identified whose intensities

371 represent the variables describing the venom composition (Figure S2~~3~~).

372 The PERMANOVAs performed for each host separately revealed a strong generation

373 effect for *D. melanogaster* S_{Nasr} and R, and *D. simulans* (Table S4, $p < 0.01$), suggesting that

374 the venom composition evolved in response to each of these hosts. This was confirmed by a

375 linear discriminant analysis (LDA) discriminating the groups of individuals based on generation

376 for the three hosts (Figure 3~~2A to 2C~~). Of note, the generation effect revealed by the

377 PERMANOVA was only significant for *D. yakuba* 307 when removing F_3 individuals from the

378 analysis (Table S4, $p = 0.042$), likely because only few females were available at F_3. LDA may

379 overfit groups composed of few individuals [43,44] and was therefore performed for *D. yakuba*

380 307 only between F_7 and F_{11}. LDA discriminated these two groups, therefore confirming the

381 generation effect revealed by the PERMANOVA (Figure ~~2~~3~~D~~).

382 A PERMANOVA fitted to all hosts together evidenced a significant effect of the “host”

383 ($p < 0.001$), the “generation” ($p < 0.001$) and their interaction ($p < 0.001$) on the variation of

384 bands intensity, demonstrating that the venom composition evolved differently on the different

385 hosts (Table S5). Yet, although significant, the combined effects of the “host”, “the generation”,

and the interaction of the two only accounted for a small part of the variance of the venom composition (R^2 = 8.7%; Table S5) compared to that explained by the experimental "population" (R^2 = 26.6%; Table S5). The LDA confirmed the differential venom evolution according to the host (Figures 4A and 4B, $p < 0.001$) but the 12 groups (four hosts x three generations) largely overlapped, which confirmed the small part of the variance explained by the host and the generation. The first four out of the 11 discriminant axes were the only ones to be biologically meaningful (Figures 4A and 4B). The first two axes discriminated the F_7 and F_{11} generation for parasitoids reared on the different hosts, except for the two *D. melanogaster* strains (Figure 4A), evidencing a rapid and differential evolution of the venom composition according to the host species. This was confirmed by the arrows describing the venom evolution that were orthogonal or in opposite direction for the different host species except for the two *D. melanogaster* strains (Figure 4A). Axis 3 discriminated the three generations for parasitoids reared on *D. melanogaster* strains and *D. simulans* while individuals reared on *D. yakuba* 307 were discriminated by axis 4 (Figure 4B). This suggests that the venom of parasitoids reared on *D. yakuba* 307 evolved differently than that of parasitoids reared on the other hosts as confirmed by the direction of the arrow describing the venom evolution for individuals raised on this species (Figure 4B).

To better identify the hosts on which the venom composition has evolved differentially, PERMANOVAs and LDAs were performed to compare them two by two. There was a strong effect of the "generation × host" interaction for all comparisons involving *D. yakuba* 307 (Table S5, $p < 0.01$) and the comparison between *D. melanogaster* S_{Nasr} and *D. simulans* (Table S5, $p = 0.004$). This was confirmed by the LDA since groups of individuals were separated at F_7 and F_{11} and arrows representing the direction of evolution were almost orthogonal (Figures 4C to 4F). No effect of the "generation × host" interaction was observed in the two PERMANOVAs comparing evolution on *D. melanogaster* R to either *D. melanogaster* S_{Nasr} or

411 *D. simulans* (Table S5, p =~~>~~ 0.0~~5~~6 for the comparison with *D. melanogaster* S_{Nasr}; p = 0.35 for
412 the comparison with *D. simulans*). The LDAs nevertheless suggested a trend of differential
413 evolution although the separation of groups at generations F_7 and F_{11} was lesser between the
414 two strains of *D. melanogaster* than between *D. melanogaster* R and *D. simulans* (Figures 4~~3~~G
415 and 4~~3~~H).
416
417

418 *3.5. Identification of protein bands that evolved on each host*

419 Selected protein bands were identified based on their correlation to the linear regressions
420 (calculated from centroid points of generation groups and represented by arrows) describing
421 the venom evolution in the LDAs performed for each host separately (Figure 3~~2~~). However,
422 some of ~~the identified~~these protein bands could have been selected only indirectly because of
423 their correlation with other directly selected bands, either due to their migration proximity on
424 the gel or to a linkage disequilibrium. To disentangle them, we used a combination of clustering
425 (Figure S3~~4~~) and partial correlation analyses (Table S6) as described in Cavigliasso et al.
426 (2019). The analysis revealed that (i) seven protein bands had evolved on *D. melanogaster* S_{Nasr}
427 (four selected, three counter-selected), (ii) six on *D. melanogaster* R (three selected, three
428 counter-selected), (iii) seven on *D. simulans* (five selected, two counter-selected) and (iv) two
429 on *D. yakuba* 307 (one selected, one counter-selected) (Table 1, Table S6 and Figure S5).

430 Of the 17 protein bands that evolved in response to at least one host, only three evolved
431 in the same direction on several hosts. Indeed, bands #10 and #19 were respectively selected
432 and counter-selected on *D. melanogaster* S_{Nasr}, *D. melanogaster* R and *D. simulans* (Table 1)
433 and band #27 was selected on *D. simulans* and *D. yakuba* 307. Overall, this confirmed that the
434 venom composition evolved rapidly and differentially between hosts.
435

3.6. Putative identification of proteins that evolved on each host

The tentative identification of the proteins contained in the bands under selection was performed by matching these bands with those in 1D electrophoresis gels used for *L. boulardi* ISm and ISy venom proteomics [18]. The level of intensity of a band can result from that of several proteins having migrated at the same position. However, since the initial composition of the venom resulted from crosses between the ISm and ISy lines, the proteins responsible for a high intensity of a band were likely to be the most abundant proteins in the corresponding protein band of ISm or ISy venom. We therefore used the number of peptides matches from a previous mass spectrometry [18] to classify the proteins in the bands as abundant or not (Table 2). We could identify at least one abundant protein for 12 out of the 17 bands under selection, which are most likely responsible for the observed changes in the overall band intensity.

Although their coding sequence has been previously determined[18], the most abundant proteins in five out of these 12 bands had no similarity to known proteins (Table 2). Their biochemical function thus remains to be determined- as for a majority of the proteins contained in the ISm and ISy venoms[18]. Among the bands that evolved on one host only, a Glucose-Methanol-Choline (GMC) oxidoreductase was the most abundant protein in band #12 selected on *D. melanogaster* R (Table 2), and a RhoGAP, LbGAP, was the most abundant protein in band #23 counter-selected on *D. melanogaster* S_{Nasr}. We performed a specific analysis for LbGAP that confirmed the counter-selection on *D. melanogaster* S_{Nasr} but also on *D. melanogaster* R (Figure 54A, GLMM, p = 0.009 for *D. melanogaster* S_{Nasr}, p = 0.021 for *D. melanogaster* R). Without a good specific antibody effective against the GMC oxidoreductase, no specific analysis could be performed for this protein. The most abundant protein in band #25, counter-selected on *D. simulans*, was another RhoGAP, LbGAPy4. A third RhoGAP, LbGAP2, was found as the most abundant protein in bands #31, selected on *D. melanogaster* R and #32, selected on *D. simulans*, as well as in band #27, selected on *D. simulans* and *D.*

yakuba 307 (Table 2). However, the selection/counter-selection of LbGAP2 was not detected in the specific analysis (Figure 5~~4~~B, LMM, p =~~>~~ 0.~~05~~14 for *D. melanogaster* R and *D. simulans*, p = 0.20 for *D. melanogaster* S_{Nasr} and p = 0.44 for *D. yakuba* 307).

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Regarding the two other bands that evolved on more than one host, a Sushi/SCR/CCP domain-containing protein (SCR: Short Consensus Repeat; CCP: Complement Control Protein) was the most abundant protein in band #10, selected on *D. melanogaster* S_{Nasr}, *D. melanogaster* R and *D. simulans* (Table 2). Finally, the most abundant protein in band #19, counter-selected on all hosts except *D. yakuba* 307, was the serpin LbSPNy. The counter-selection of the *lbspny* allele encoding LbSPNy was confirmed by the specific analysis on *D. melanogaster* S_{Nasr}, R and *D. simulans* (Figure 5~~4~~C, GLMM, p = 0.022 for *D. melanogaster* S_{Nasr}, p = 0.018 for *D. melanogaster* R, p = 0.015 for *D. simulans*). It also revealed a selection of *lbspny* on *D. yakuba* 307 (Figure 5~~4~~C, GLMM, p = 0.005) although not detected by the global approach.

3.7.Trends of venom evolution

The average venom composition of each of the 44 experimental populations was scaled between 0 and 1 according to their distance to the venom composition of ISm and ISy (Figure 6~~5~~). When comparing the venom composition in F_3 and F_{11} generations, we observed a trend for evolution towards the venom composition of ISm for all hosts, except for *D. yakuba* 307 for which the evolution of the venom did not change the relative distance to parental strains (Figure 6~~5~~, paired Wilcoxon test, p = 0.011 for *D. melanogaster* S_{Nasr}, p = 0.010 for *D. melanogaster* R, p = 0.045 for *D. simulans*, p = 0.60 for *D. yakuba* 307).~~p < 0.05~~).

We then assigned a value between 0 and 1 to each protein band: 0 when a band was present in ISy but absent in ISm, 1 for the opposite (Table 1). In agreement with the above results, most

selected protein bands were of the ISm type, whereas most of the counter-selected bands were of the ISy type (Table 2).

4. Discussion

This study aimed at testing simultaneously the evolution of (i) the parasitic success and (ii) the venom composition of *L. boulardi* on *Drosophila* hosts from three different species. It was also expected to be informative on whether a venom allowing to develop on different hosts would rather contain broad-spectrum factors or a combination of factors specific to each host. We used an experimental evolution in which selection acted on the standing genetic variation, not on new mutations appeared during the experiment, as is usually done when using fast-growing organisms [45]. The experimental evolution was initiated by crossing *L. boulardi* ISm and ISy parasitoid lines, which differ in their parasitic success on *D. melanogaster* and *D. yakuba* species [8] and their venom composition [18]. Since both lines have spent many generations in the laboratory, the initial variation on which natural selection could act during the experimental evolution resulted mainly from the variation between them. The F_2 female offsprings, whose venom contains new combinations of proteins, were separated in independent populations and reared ~~until~~ up to the F_{11} generation on the four different host strains / species on which ISm and/or ISy are ~~able to succeed~~successful.

Among these four hosts, *D. melanogaster* S_{Nasr} and *D. simulans* are both susceptible to ISm and ISy. We previously evidenced an evolution of the venom composition of *L. boulardi* not only on a resistant host strain but also on a susceptible one [15]. *D. melanogaster* S_{Nasr} and *D. simulans* were ~~thus~~ therefore used to determine whether two hosts could exert a different selection pressure on the venom composition of *L. boulardi* despite their susceptibility. Our objective was also i) to confirm that the creation of new combinations of venom factors ~~(by the reciprocal ISm x ISy crosses)~~ still allowed parasitic success on these susceptible host strains

510 and ii) to assess whether the venom composition could nevertheless evolve, possibly by
511 selecting still effective but less expensive factors.

512 A fifth host was used in the experimental evolution, *D. yakuba* 1907 on which neither
513 ISm nor ISy are successful. Interestingly, parasitoids managed to develop on this host until the
514 F_6 generation suggesting that the creation of new combinations of venom factors at F_2 has
515 allowed them to develop in a clearly refractory strain for a certain time. However, these new
516 combinations were not successfully selected in any of the replicates since all populations raised
517 on this host ended up being extinct. This could may suggest that the virulence factors
518 responsible for this temporary parasitic success are encoded by the same loci leading to a higher
519 fitness of heterozygotes (overdominance), such alleles having been lost due to the small
520 population size.

521

522

523 *4.1. Parasitic success and venom evolution according to the host*

524 Although Tthe parasitic success remained close to 100% on the two hosts on which the
525 two *L. boulardi* lines always succeed, *D. melanogaster* S_{Nasr} and *D. simulans*, . Tthe
526 composition of the parasitoid venom has nevertheless evolved on these susceptible hosts.
527 Although genetic drift was expected to impact venom composition due to the small number of
528 individuals per replicate necessarily impacted the venom composition, the observed changes
529 observed most likely occurred under selection. Indeed, gGenetic drift is a random phenomenon
530 that differently affects each population whereas changes in venom composition were common
531 to most of the replicates suggesting a selection strong enough not to be masked by the drift. The
532 evolution of venom in response to susceptible hosts suggests a selection of some venom
533 components over others, potentially less costly to produce. The venom is not only important to
534 overcome host immune defenses but also to ensure the quality of offspring developement [46].

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535 The differential venom evolution on the two susceptible hosts could therefore also result from
536 a selection to increase the fitness of the developing offspring that are facing different susceptible
537 host strains. This scenario would suggest a fine tuning of the host physiology to ensure the best
538 match with the parasitoid larvae requirements.

539 In contrast, the success of parasitoids ~~reared~~ selected on ~~hosts on which ISm and ISy~~
540 ~~differ in virulence,~~ *D. melanogaster* R and *D. yakuba* 307 significantly increased with the
541 generation. This rapid increase was not surprising since the selection pressures on parasitoids
542 are very high and it has previously been documented for other host – parasitoid interactions.
543 Accordingly, the parasitic success of *Asobara tabida* increased after seven generations of
544 selection on *D. melanogaster* [47] and that of two aphid parasitoids increased in response to the
545 symbiont-associated resistance of the host [2,3]. A suprising result was the ~~This increase was~~
546 much higher increase in parasitic success for the parasitoids reared on *D. yakuba* 307 than on
547 *D. melanogaster* R, probably because the former was much less successful at F_3 (about 20%
548 versus 80%). This difference in parasitic success at F_3 is quite surprising since the ISm line
549 always succeeds on *D. melanogaster* R and ISy on *D. yakuba* 307. It was however consistent
550 with the high extinction rate of F_3 replicates on *D. yakuba* 307, suggesting a lower virulence of
551 F_2 parasitoids on this host than on the others. Accordingly, [5,24] identified the virulence of F_1
552 (ISm x ISy) hybrids as semi-dominant on *D. melanogaster* and recessive on *D. yakuba*,
553 although they used different strains of *D. melanogaster* and *D. yakuba* than ours.

554

555 *4.2. Specialization of parasitoids to their selection host and differential venom evolution*

556 The ~~increase in~~greater parasitic success on resistant hosts (*D. yakuba* and *D.*
557 *melanogaster* R) proved to be specific to the selection host (Figure 2C). ~~Indeed, p~~Parasitoids
558 maintained on a given resistant host were more successful on this host than those reared on
559 another host. The~~This~~ differential evolution of ~~the~~ both parasitic success and venom evolution

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560 suggests a specialization of parasitoids on their selection host, as previously reported
561 experimentaly in relation to ~~the~~ host resistance ~~of the host~~ [3,4] and predicted by simulation
562 results [48]. Such a differential evolution of venom has also been observed in a vertebrate model
563 – the snake of genus *Echis* – in response to diet [49]. ~~We also evidenced a rapid and differential~~
564 ~~evolution of the venom composition specific to the selection host. In addition, we observed~~
565 ~~t~~The selection of certain protein bands for each host, ~~suggesting~~ suggests a role in parasitic
566 success, while ~~others were~~the counter-selectioned~~,~~ ~~suggesting~~ suggests a cost associated with
567 their production or function on a given host. These differential selection and counter-selection
568 of the venom content could explain the lesser success of a parasitoid on a resistant host (such
569 as *D. melanogaster* R or *D. yakuba* 307) after a selection on another host strain. For example,
570 the counter-selection of the protein band #19 in the venom of parasitoids selected on *D.*
571 *melanogaster* S_{Nasr}, R, and *D. simulans* may have reduced their success on *D. yakuba* 307.
572 Consistently, another study with other host strains showed that F_1 (ISm x ISy) hybrids
573 experienced a decrease ~~of~~ in virulence against *D. yakuba* after being reared on *D. melanogaster*,
574 suggesting that virulence factors selected on *D. melanogaster* were costly for parasitism on ~~a~~
575 ~~cost for virulence factors specific to~~ *D. yakuba* [5,50].

576 A possible hypothesis to explain the differential evolution of venom between parasitoids
577 selected on the two susceptible hosts, *D. melanogaster* S_{Nasr} and *D. simulans* is the occurence
578 of a host-specific cost to produce certain venom components, resulting in the counter-selection
579 of different proteins. Interestingly, the success on *D. yakuba* of parasitoids selected on *D.*
580 *melanogaster* S_{Nasr} is lower than those selected on *D. simulans*, confirming the selection of
581 specific venom components to bypass host defenses. It also suggests a difference in immune
582 defenses between each host. As an example, the prophenol-oxidase (PPO) sequences, essential
583 proteins for the melanization process, differ between host species and might be targeted by
584 different venom proteins [51–53].

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4.3. A combination of mostly host-specific proteins and a few broad-spectrum proteins in venom to succeed on several hosts

~~Overall, the specificity of the evolution of parasitic success we observed suggests a selection of different virulence factors depending on the host. Indeed, t~~The change in intensity observed for most of the 17 venom protein bands which evolved~~,~~ occurred in response to selection by a single host only. This is in accordance with the study of [4] in which most of the differentially expressed genes of selected parasitoids were lineage-specific. As a counter-example, two protein bands evolved in the same direction for *D. melanogaster* S_{Nasr} and R, and *D. simulans* and one for *D. simulans* and *D. yakuba* 307. Therefore, although the number of evolving protein bands may have been underestimated due to a lack of power of the global approach or because they contain several proteins of opposite evolution, our data suggest that the venom of the selected parasitoids contain a combination of mostly host-specific proteins and a few broad-spectrum proteins to succeed on these hosts.

4.4. The venom of parasitoids selected on D. yakuba 307 evolved more specifically

All PERMANOVAs involving *D. yakuba* 307 showed a significant effect of the “generation × host” interaction suggesting that the venom composition of parasitoids reared on this host evolved more differently than that of those reared on the other hosts. Moreover, parasitoids selected on *D. melanogaster* strains and *D. simulans* showed a trend for evolution towards the venom composition of ISm between F_3 and F_{11} unlike those selected on *D. yakuba* 307. Likewise, the protein bands #10 and 19, selected and counter-selected on all hosts except *D. yakuba* 307 are of ISm and ISy origin, respectively. In addition, the specific analysis revealed a selection of~~-~~ the *lbspny* allele on *D. yakuba* 307 and a counter-selection on all other hosts. Since *lbspnm* and *lbspny* are two alleles of a co-dominant marker, LbSPN, this could also be

610 interpreted as a selection of *lbspnm* on *D. melanogaster* and *D. simulans* although its role in
611 parasitic success is yet to be demonstrated. These differential trends in the venom composition
612 towards ISm and ISy types may reflect the geographic distribution of parasitoids and hosts - the
613 ISm type of *L. boulardi* being Mediterranean and therefore not encountering *D. yakuba*, mainly
614 present in tropical regions of Africa [5,16,50], as well as the host preferences of the *L. boulardi*
615 lines. ISy was ~~indeed~~ previously shown to oviposit preferentially in *D. yakuba* while ISm
616 preferred *D. melanogaster* [54].

617

618 *4.5. Proteins whose quantity evolved*

619 For many protein bands identified as evolving, the most abundant protein – which is
620 likely responsible for changes in the band intensity – had no predicted function although its
621 coding sequence has been previously determined [18]. These proteins have thus been little or
622 not studied so far but could nevertheless play an essential role in parasitic success and would
623 deserve more attention. Our method without *a priori* seems therefore relevant to identify new
624 candidate proteins, possibly involved in the parasitic success.

625 Among the proteins with a predicted function and identified as the most abundant one
626 in their evolving band, we found the RhoGAPs LbGAP and LbGAP2 and the serpin LbSPN for
627 which a Western blot analysis with specific antibodies was also performed. The global approach
628 and the specific analysis agreed on the counter-selection of LbGAP on *D. melanogaster* S_{Nasr}.
629 This was also evidenced on the resistant strain of *D. melanogaster* R but with the specific
630 analysis only, probably because of the lower power of the global approach. This was surprising
631 since a selection of LbGAP on *D. melanogaster* R was observed in a previous work [15]~~, in~~
632 ~~agreement with its possible role in virulence on this host [17,19–22]~~. However, in this current
633 study, the proportion of LbGAP individuals at F_3 on *D. melanogaster* hosts was much higher
634 than expected under Hardy-Weinberg equilibrium (90% vs 75%). We therefore cannot exclude

635 a decrease of the frequency of LbGAP individuals to reach equilibrium at F_{11} instead of a
636 counter-selection. Moreover, the initial crosses were done in both directions, instead of only
637 one in the previous study, leading to more balanced frequencies for the overall ISm / ISy alleles.
638 The selection may therefore have acted differently in the two experiments and other alleles than
639 *lbgap* been selected on *D. melanogaster* R. LbGAP is involved in changes of the morphological
640 shape of *D. melanogaster* R lamellocytes, possibly preventing the encapsulation by the host
641 [17,19–22]. We could hypothesize that LbGAP is involved in a strategy to suppress
642 encapsulation. However, we did not observe the increase in parasitoid ability to suppress
643 encapsulation that would therefore be expected under this assumption, but rather an increase of
644 the parasitoid ability to escape from a capsule (Figure 2B). Besides, tThe venom contains
645 several other RhoGAPs, including LbGAP2, and, although they are all mutated on their
646 catalytic site, they which might "somehow" compensate for the reduced LbGAP quantity by
647 acting in parasitism success. The selection of LbGAP2 on *D. melanogaster* R, *D. simulans* and
648 *D. yakuba* 307 based on the global approach was however not supported by the specific
649 analysis. This suggests that other proteins in the LbGAP2-containing bands are responsible of
650 the changes in intensity detected by the global approach.

651 Interestingly, we evidenced the selection of LbSPNy on *D. yakuba* 307, although with
652 the specific analysis only, probably because of the small number of individuals at F_3 on that
653 host. This is in line with the demonstrated involvement of LbSPNy in the inhibition of the
654 phenoloxidase cascade activation of this same species of *Drosophila* [23]. The phenoloxidase
655 cascade leads to the melanization of the capsule and the release of cytotoxic radicals to kill the
656 parasitoid [23,51–53]. The melanisation process being involved in the strengthening of the
657 capsule, this selection would be rather consistent with the increase of the escape ability of the
658 formed capsule in parasitoids selected on *D. yakuba* associated with the increase in parasitic
659 success (Figure 2B). The global approach and the specific analysis also showed the counter-

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660 selection of LbSPNy on *D. melanogaster* S_{Nasr} and R and *D. simulans* in agreement with
661 previous results for *D. melanogaster* hosts [15]. This suggests a possible cost of either the
662 production or the presence of LbSPNy in their venom.

663 In conclusion, the parasitoid model is very relevant for an experimental evolutionary
664 approach compared to other models of venomous animals such as scorpions or snakes although
665 promising advances were obtained from studies of venomics and virulence on such models
666 [49,55]. Our results have highlighted a specialization of parasitoids on their selection host, in
667 link with a rapid differential evolution of the composition of the venom according to the host.
668 Most of the evolving venom proteins evolved in response to selection by a single host,
669 suggesting that parasitoids use at least partially different mechanisms to bypass the defenses of
670 different hosts, and therefore that these host species may also implement partly different defense
671 mechanisms. *D. melanogaster* and *D. simulans* might share some of them so that part of the
672 venom proteins to succeed on these host species would be common. *D. yakuba* is more apart,
673 the maintenance of parasitoids on this species resulting in the selection of more specific venom
674 factors. From these data, we end up with no universal answer to the question of the venom
675 content of a "generalist" parasitoid in terms of broad-spectrum or host-specific factors. The
676 venom of ISm may contain a mixture of both, allowing success on *D. melanogaster* and *D.*
677 *simulans*, species usually found in sympatry, while the question remains more open for ISy
678 venom. Finally, in a more general context, the rapid evolution of the venom highlights the
679 strong capacity of parasitoids to possibly adapt to their environment [56]. It is notably striking
680 that crosses between parasitoids creating new combinations of venomous proteins could help
681 them succeed on initially refractory hosts.
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Data Accessibility: The data sets supporting the results are available in a figShare repository (https://doi.org/10.6084/m9.figshare.13139090.v1). Drosophila strains/species and parasitoid lines are maintained in the laboratory and available upon request.

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Table 1. Summary of protein bands evolution in response to the host. Only ~~P~~protein bands correlated to regressions (arrows) in Figure 3~~2~~ and S5~~4~~ in response to at least one host are represented. A ~~by~~ "↗" corresponds to ~~for~~ a selection, a ~~or~~ "↘" ~~for~~ to a counter-selection in response to the host. ~~Bands in bold are those selected/counter selected in response to at least one host.~~ The origin of the band was estimated by dividing the band intensity in ISm venom by the sum of its intensity in ISm and ISy venoms; 0 < ISy <0.5; 0.5 < ISm < 1. For the complete data, see Table S6.

Band Number	Band Origin	Protein bands evolution in response to the host			
		D. melanogaster S_{Nasr}	*D. melanogaster* R	*D. yakuba* 307 (F_7 *vs* F_{11})	*D. simulans*
2	0.60	↗			
4	0.26			↘	
7	0.44	↗			
10	0.51	↗	↗		↗
12	0.62		↗		
17	0.36		↘		
18	0.23		↘		
19	0.18	↘	↘		↘
22	0.28	↘			
23	0.58	↘			
25	0.32				↘
27	0.64			↗	↗
31	0.66		↗		
32	0.63				↗
34	0.54				↗
35	0.54				↗
36	0.63	↗			

Table 2. Correspondence between evolving bands and their putative protein content determined from the comparison with data from Colinet et al. (2013a). Only proteins for which at least 10 peptide matches were found in Mascot searches to unisequences identified in transcriptomics of the venom apparatus [18] were considered as abundant and therefore listed. The number of proteins found in the band, their predicted function and the number of peptide matches for each unisequence are provided. Data on the band origin and direction of evolution are from Table 1. Up ("↗") and down ("↘") arrows indicate a selection or a counter-selection, respectively. ~~Simu: *D. simulans*;~~*D. mel.* S_{Nasr}: *D. melanogaster* S_{Nasr}; *D. mel.* R: *D. melanogaster* R~~; yak: *D. yakuba* 307~~. NA: data not available. On *D. yakuba* 307, the evolution reflects changes between F_7 and F_{11} only.

Reference band	**Number of proteins in the band**	**Putative function**	**Number of peptide matches**	**Band origin**	**Band evolution on**			
					***D. mel.* S_{Nasr}**	***D. mel.* R**	***D. yakuba* 307 (F_7 *vs* F_{11})**	***D. simulans***
2	1	Unknown	12	ISm	↗			
4	3	Unknown Sushi/SCR/CCP domain containing protein Unknown	55 11 10	ISy			↘	
7 (no abundant protein found)	NA	NA	NA	ISy	↗			
10	2	Sushi/SCR/CCP domain containing protein Unknown	10 10	ISm	↗	↗		↗
12	1	GMC oxidoreductase	40	ISm		↗		
17	1	Unknown	20	ISy		↘		
18 (not sequenced in [18])	NA	NA	NA	ISy		↘		

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Table 2. (continued)

Reference band	Number of proteins in the band	Putative function	Number of peptide matches	Band origin	Band evolution on			
					D. mel. S_{Nasr}	*D. mel.*R	*D. yakuba* 307 (F_7 *vs* F_{11})	*D. simulans*
19	1	Serpin (LbSPNy)	81	ISy	↘	↘		↘
22	2	Unknown Serpin (LbSPNy)	19 10	ISy	↘			
23	5	RhoGAP (LbGAP) Unknown Serpin (LbSPNm) Unknown Unknown	52 21 17 12 11	ISm	↘			
25	1	RhoGAP (LbGAPy4)	24	ISy				↘
27	3	RhoGAP (LbGAP2) RhoGAP (LbGAP1) Serpin (LbSPNm)	43 23 15	ISm			↗	↗
31 or 32 (not separated in [18])	2	RhoGAP (LbGAP2) Unknown	20 18	ISm		↗ (#31)		↗ (#32)
34 (no abundant protein found)	NA	NA	NA	ISm				↗
35	1	Unknown	18	ISm				↗
36 (no abundant protein found)	NA	NA	NA	ISm	↗			

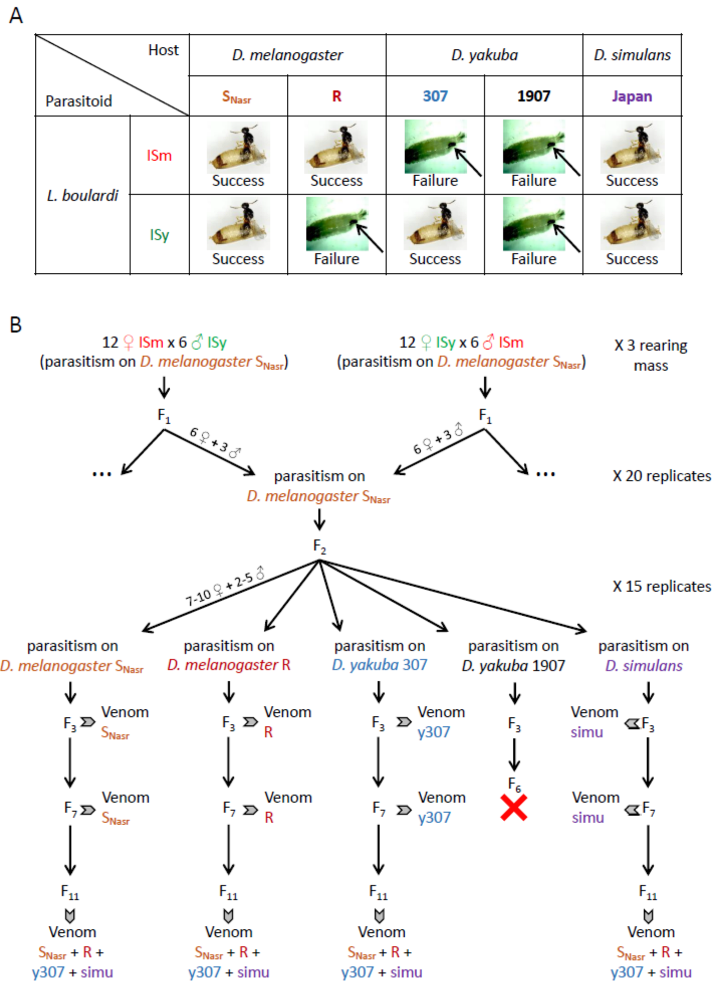

Figure 1. Biological model and experimental evolution protocol. A. Outcome of interaction between the five host strains (*D. melanogaster* S_{Nasr}, *D. melanogaster* R, *D. yakuba* 307, *D. yakuba* 1907 and *D. simulans*) and the two *L. boulardi* lines (ISm and ISy). The black arrow shows an encapsulated parasitoid egg inside a *Drosophila* larva. B. Design of the experimental evolution. ISm and ISy: ISm and ISy lines of *L. boulardi*; S_{Nasr}: *D. melanogaster* S_{Nasr}; R: *D. melanogaster* R; y307: *D. yakuba* 307; simu: *D. simulans*. The red cross indicates the extinction of parasitoids reared on *D. yakuba* 1907, thus no analysis was performed on individuals from this host. F_3, F_7 and F_{11}: the three generations of *L. boulardi* that were analyzed for venom composition and parasitic success. The F_3 is the first generation under selection.

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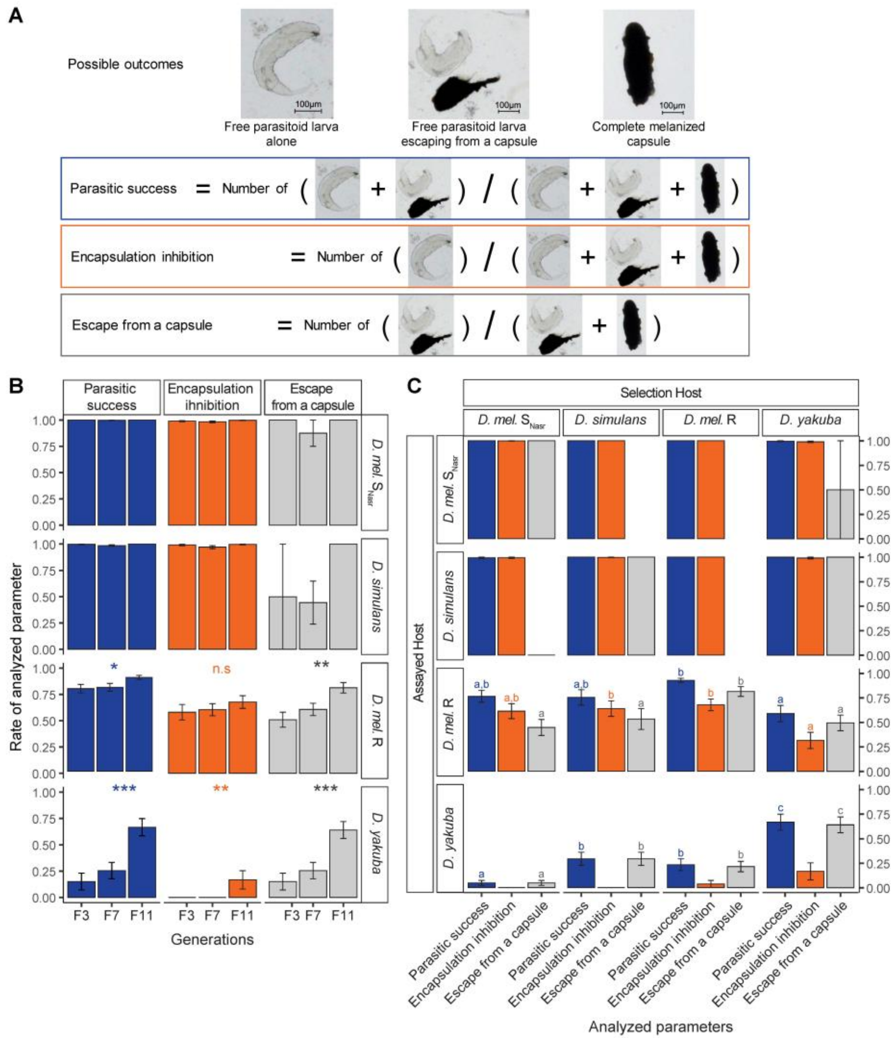

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911 **Figure 2~~1~~.** Evolution of the parasitoid's ability to bypass the encapsulation response of the

912 host. A. ~~Responses~~ Possible outcomes observed in *Drosophila* larvae 48 hours after

913 parasitism and details of the analyzed parameters: ~~free parasitoid larva alone (a), free~~

914 ~~parasitoid larva escaping from a capsule (b), complete melanized capsule surrounding the~~

915 ~~parasitoid egg (c). P~~parasitic success, ~~= (a + b) / (a + b + c); P~~parasitoid capacity to inhibit

916 encapsulation and ~~= a / (a + b + c); P~~parasitoid capacity to escape from a capsule ~~= b / (b +~~

917 ~~c)~~. B. Evolution of (i) parasitic success, (ii) parasitoid capacity ~~of the parasitoid~~ to inhibit the

918 egg encapsulation by the host or (iii) parasitoid capacity to escape from a capsule, depending
919 on of the selection host. , *D. melanogaster* S_{Nasr}, *D. simulans*, *D. melanogaster* R and *D.*
920 *yakuba* 307. C. Capacity of parasitoids of from the F_{11} generation to bypass encapsulation by
921 four different hosts (*D. melanogaster* S_{Nasr}, *D. simulans*, *D. melanogaster* R and *D. yakuba*
922 307). The hosts listed to the right on the topof each bar plot isare the "selection hosts", those
923 listed down on the left below aare the "tested assayed hosts", used for parasitism assays. S_{Nasr}:
924 *D. mel*. Nasr; *D. mel*. R: *D. melanogaster* R; *D. sim*.: *D. simulans*; *D. yak*.: *D. yakuba* 307.
925 Letters above bars indicates the significance of the difference. Different colors of letters
926 indicate different statistical tests. Error bars indicate standard errors.
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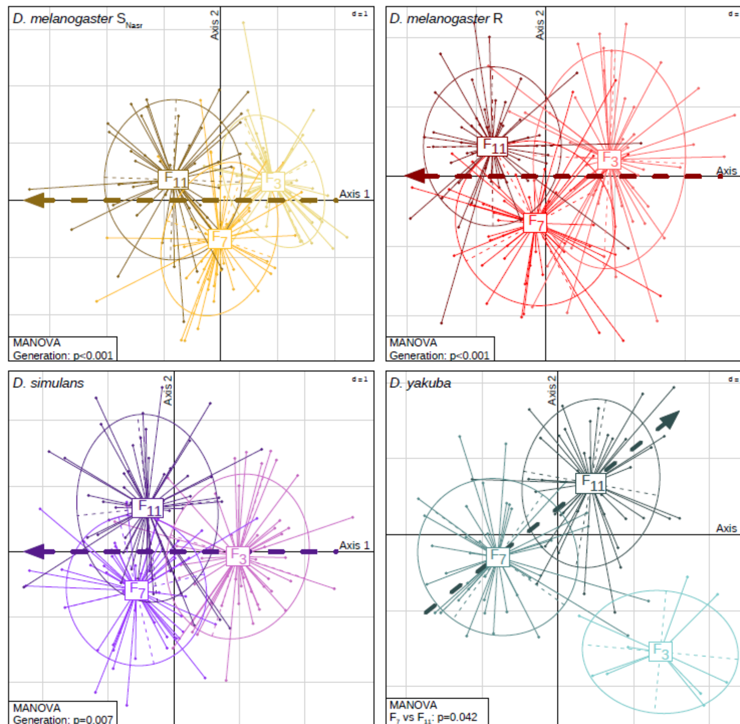

Figure 3~~2~~. Evolution of the venom composition according to the selection host. Position of the individuals (shown as dots) on the two discriminant axes for each selection host. Individuals are grouped and coloured according to the generation (F_3, F_7 and F_{11}). The arrows represent the trend of the venom evolution. The dotted arrows represent the linear regressions calculated from coordinates of the three centroid points corresponding to the F_3, F_7 and F_{11} generations and weighted by the number of individuals per generation. For *D. yakuba* 307, the linear regression was calculated from centroid points of F_7 and F_{11} only due to the low number of females available in F_3. P-values obtained from the PERMANOVA for the "generation" effect are provided at the bottom right of the LDA (see Table S4). ~~d (top right) represents the scale between two lines.~~

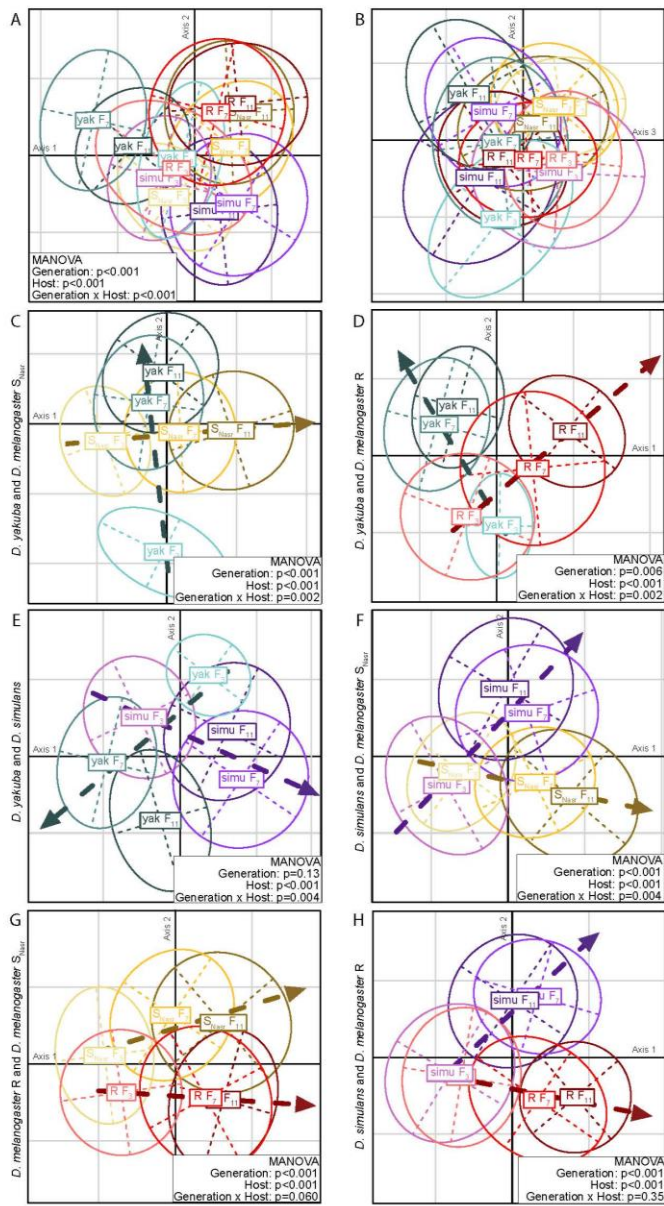

Figure 4~~3~~. Differential evolution of venom composition. Discriminante analysis showing elipses and centroids (intersection of dashed lines) of each group formed by the host and the generation are represented (see Figure S4 for more details on the position of each individual). The name of each group is written on the centroid. S_{Nasr}: *D. melanogaster* S_{Nasr}; simu: *D.*

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960 *simulans*; R: *D. melanogaster* R; yak: *D. yakuba* 307. -A-B. Venom evolution in response to

961 Position of all the individuals (shown as dots; selected on the four hosts at F_3, F_7 and F_{11}

962 generations) on discriminant axes 1 and 2 (A) and discriminant axes 3 and 4 (B). C-H. Position

963 of the individualsDiscriminant analysis performed on the two first discriminant axes for each

964 two-by-two comparison of venom composition between parasitoids selected on different hosts.

965 For each of these LDAs, the pair of host considered is indicated at the bottom left. Individuals

966 are grouped and coloured according to their selection host and generation. The dotted arrows

967 representing the direction of the venom evolution are the linear regressions fitted to the

968 coordinates of the three centroid points (F_3, F_7 and F_{11}). P-values obtained from the

969 PERMANOVA for effects of the "generation", "host" and "generation × host" interaction are

970 provided at the bottom right of the LDA (see Table S5 for more details). d (top right) represents

971 the scale between two lines.

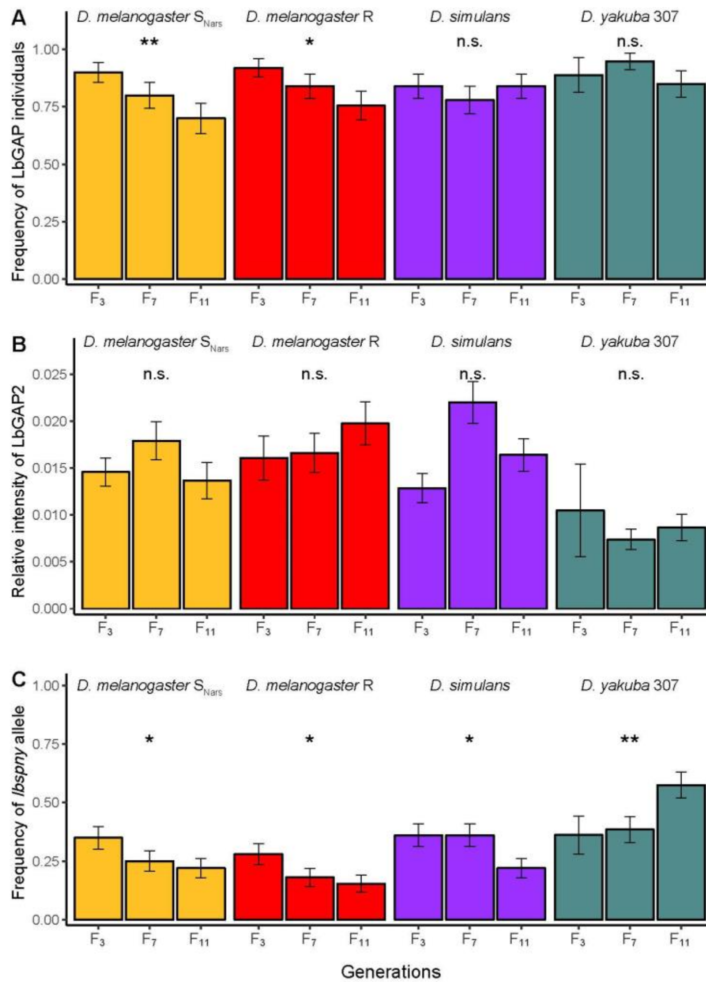

Figure 54. Specific analysis of the evolution of LbGAP, LbGAP2 and LbSPN proteins of parasitoids selected on *D. melanogaster* S_{Nasr} (in yellow), *D. melanogaster* R (in red), *D. simulans* (in purple) and *D. yakuba* 307 (in grey-green). A. Evolution of the frequency of individuals harbouring a high quantity of the LbGAP protein. B. Evolution of the LbGAP2 protein amount, relative to the total amount of proteins in the venom samples. C. Evolution of the frequency of the *lbspny* allele.

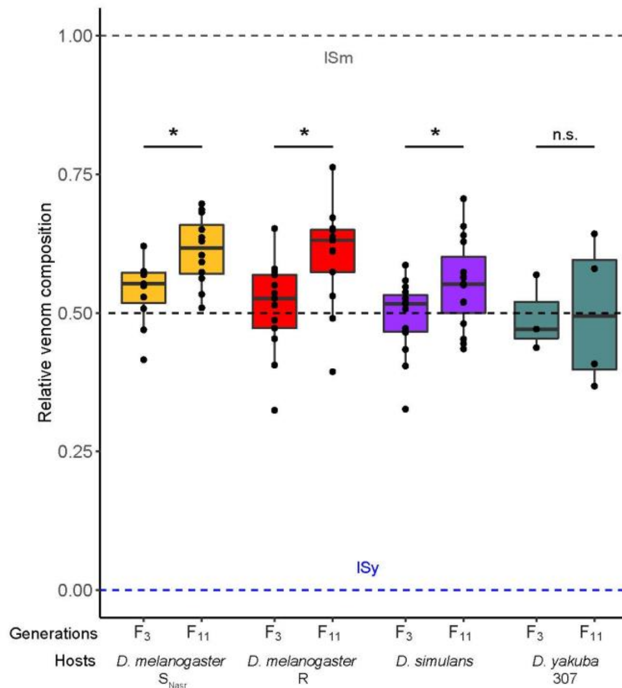

Figure 6~~5~~. Relative distance between the venom composition of parasitoids selected on *D. melanogaster* S_{Nasr} (in yellow), *D. melanogaster* R (in red), *D. simulans* (in purple) and *D. yakuba* 307 (in grey-green) in F_3 and F_{11} and that of *L. boulardi* ISm (1) and ISy (0) lines. The horizontal dashed black line indicates the proportion of ISm alleles after the crossing between ISm and ISy lines (~~so~~ 0.5 at the beginning of the experiment~~; 0.5~~). In *D. yakuba* 307, one replicate is missing in F_3 because not enough individuals were available to produce the next generation, perform parasitic tests and do the venom analysis.