

1 **Plant responses to butterfly oviposition partly explain preference-performance**
2 **relationships on different brassicaceous species**

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17

18 **Abstract**

19 According to the preference-performance hypothesis (PPH), also known as ‘mother-knows-best
20 hypothesis’, herbivorous insects prefer those plants for oviposition, which yield the best offspring
21 performance. Yet, most studies testing the PPH neglect the possibility that plant responses to insect
22 eggs may affect both egg survival and larval performance. Here, we tested the PPH by studying
23 responses of seven Brassicaceae plant species to oviposition by two cabbage white species. When
24 including the egg phase, our study supports the ‘mother-knows-best hypothesis’: larvae of *Pieris*
25 *rapae* (solitary) or *P. brassicae* (gregarious) gained most weight on those plant species which had
26 received most eggs (*B. nigra* or *B. montana*, respectively). However, our experiments did not reveal
27 any relationship between oviposition preference and egg survival. Brassicaceous species are known
28 to respond to these butterfly eggs with a hypersensitive response (HR)-like necrosis, which can
29 lower egg survival. *Pieris* eggs frequently induced necrosis in five of the tested plant species.
30 Survival of clustered *P. brassicae* eggs was unaffected by HR-like in four of the five species.
31 Therefore, our experiments did not reveal any relationship between *P. brassicae* egg survival and
32 oviposition preference. Females of *P. rapae* preferred oviposition on plant species which most
33 frequently showed HR-like necrosis. Remarkably, although egg survival was lower on HR-like
34 plants, larval biomass was higher compared to plants without a necrosis. We conclude that egg
35 survival does not seem to be a deciding factor for oviposition choices. However, egg-mediated plant
36 responses might be important to explain the PPH of the two *Pieris* species.

37

38 **Keywords:** Preference-performance hypothesis, Hypersensitive response, Brassicaceae, Pieridae,
39 egg-killing, priming

40

41 **Lay summary:**

42 Egg-laying preferences of herbivorous insects can often be linked to offspring performance.
43 Commonly, the fate of insect eggs and the plant responses to the eggs have been ignored when

44 studying the link between preference and performance. By including the egg phase, our study
45 supports the ‘mother-knows-best hypothesis’, explained by butterfly oviposition and associated egg
46 and larval performances on different plant species. We especially found that egg-mediated
47 responses seem a deciding factor for butterfly oviposition choices.

48

49

50 **Introduction**

51 Host-plant selection for oviposition by insect females is a decisive step in establishing a new
52 herbivore generation (Gripengberg et al., 2010; Thompson, 1988a, b; Thompson and Pellmyr, 1991).
53 The preference-performance hypothesis (PPH) or ‘mother-knows-best’ hypothesis states that
54 natural selection favors those insect females which prefer host plants where the offspring performs
55 best, especially when immature stages are less mobile than adults. A good host plant is usually
56 characterized either by high food quality and/or by enemy-free space (Craig and Ohgushi, 2002;
57 Gripengberg et al., 2010; Jaenike, 1990; Mayhew, 1997, 2001). Indeed, the PPH is supported by
58 numerous studies of butterflies and moths (Forister, 2004; Forister et al., 2009; Harris et al., 2001;
59 Thompson, 1988a, b; Thompson and Pellmyr, 1991). However, there are also numerous studies of
60 plant - Lepidoptera interactions, where no support was found for the PPH (Gripengberg et al., 2010;
61 Jaenike, 1990; König et al., 2016; Mayhew, 1997; Scheirs et al., 2000; Thompson, 1988a). In
62 addition to host plant quality and presence of natural enemies, various factors such as local host
63 plant abundance or distribution patterns of host plants shape oviposition preferences and larval
64 performance (Friberg et al., 2015; Wiklund and Friberg, 2008, 2009).

65

66 Yet, the vast majority of innumerable laboratory studies testing the PPH did not consider that plants
67 can activate defenses in response to egg deposition. Research has provided evidence that numerous
68 plant species across highly diverse taxa defend against egg depositions of various insect species
69 (Hilker and Fatouros, 2015). Plants are capable of killing eggs (Fatouros et al., 2016). For example,
70 egg-induced formation of a neoplasm (Petzold-Maxwell et al., 2011) or by hypersensitive response
71 (HR)-like necrosis at the oviposition site (Fatouros et al., 2014; Griese et al., 2017; Shapiro and
72 DeVay, 1987) may result in egg detachment from the plant or egg dehydration. Furthermore, a plant
73 can kill insect eggs by growing tissue that is crushing the eggs (Aluja et al., 2004; Desurmont and
74 Weston, 2011; Karban, 1983; Mazanec, 1985). In addition, plants receive insect eggs as early
75 ‘warning cues’ of impending herbivory and reinforce or prime their defenses against the

76 subsequently feeding larvae (Austel et al., 2016; Bandoly et al., 2015; Hilker and Fatouros, 2015,
77 2016; Hilker et al., 2016; Pashalidou et al., 2015c; Pashalidou et al., 2013). As a consequence,
78 larval performance on initially egg-infested plants may be worse than on egg-free plants (Hilker and
79 Fatouros, 2016). Additionally, but less frequently shown so far, egg deposition can suppress plant
80 defenses against larvae (Reymond, 2013). The effects of egg deposition on subsequent plant
81 defenses against larvae that hatch from the eggs have been extensively overlooked until recently,
82 with most studies on larval performance being conducted by placing larvae onto an egg-free host
83 plant (Hilker and Fatouros, 2015, 2016).

84

85 The insect oviposition mode can have a significant impact on egg survival and larval performance.
86 When eggs are laid in clusters, neonate larvae often show gregarious feeding behavior, which
87 benefits offspring performance in some insect species (Allen, 2010; Clark and Faeth, 1997; Clark
88 and Faeth, 1998; Denno and Benrey, 1997; Desurmont and Weston, 2011; Desurmont et al., 2014;
89 Fordyce, 2003; Martínez et al., 2017; Wertheim et al., 2005). On the other hand, many herbivorous
90 insects lay single eggs, spreading them over a larger area, possibly as a means of reducing predation
91 risk and competition (Nufio and Papaj, 2001; Root and Kareiva, 1984). Egg-induced plant defense
92 affecting larval performance is especially known for insect species laying eggs in clutches (Hilker
93 and Fatouros, 2015). However, also the plant's response to singly laid eggs of *Manduca sexta*
94 reinforces the defense against *M. sexta* larvae (Bandoly et al., 2016). It remains to be elucidated
95 whether the egg laying mode (single eggs vs. egg clutches) affects egg-induced plant defense
96 targeting the eggs and how this in turn depends on the plant species receiving the eggs.

97

98 The aim of this study is to elucidate whether the PPH still holds when not only considering
99 relationships between oviposition preference and larval performance, but also when including egg
100 survival rates and egg-induced changes in plant suitability for feeding caterpillars. Therefore, we
101 investigated oviposition preference, egg survival and larval performance of *Pieris brassicae* and *P.*

102 *rapae* on eight Brassicaceae species. Pierid butterflies have co-evolved since 90 million years ago
103 with their host plants in the order Brassicales (Edger et al., 2015; Wheat et al., 2007). Both butterfly
104 species are known to use various wild and cultivated brassicaceous plants as hosts (Chew and
105 Renwick, 1995; Feltwell, 1982; Gols et al., 2011), whereby *P. rapae* can include also non-
106 brassicaceous plants in their diet (Friberg et al., 2015). *Pieris* caterpillars are well adapted to
107 Brassicaceae by their ability to detoxify glucosinolates, plant secondary metabolites characteristic
108 for this plant taxon (Hopkins et al., 2009). While *P. rapae* lays single eggs on plants, *P. brassicae*
109 lays egg clutches containing up to 200 eggs (Feltwell, 1982). Egg deposition by these pierid species
110 is known to induce an HR-like leaf necrosis in several host plant species (Fatouros et al., 2016).
111 Additionally, previous egg deposition by *P. brassicae* on brassicaceous plant species was shown to
112 negatively affect larval performance (Bonnet et al., 2017; Geiselhardt et al., 2013; Lortzing et al.,
113 2018; Pashalidou et al., 2015a; Pashalidou et al., 2015b; Pashalidou et al., 2015c; Pashalidou et al.,
114 2013). It remains unknown so far how egg deposition of the conspecific solitary species *P. rapae*
115 affects subsequently feeding larvae through egg-mediated plant responses.

116

117 We specifically addressed the following questions: (1) Do females of the two pierid species prefer
118 to oviposit on plants on which their eggs show highest survival rates and larvae perform best? (2) Is
119 this oviposition choice affected by the plant species' capability to activate an egg-killing response
120 (i.e. HR-like necrosis)? (3) Is the butterflies' oviposition choice affected by plant responses to
121 oviposition that subsequently affect feeding larvae, e.g. egg-mediated priming of defenses? (4)
122 Does the egg-laying mode of the two pierid species affect their oviposition choice?

123

124 **Material and Methods**

125 *Insects and plants*

126 *Pieris brassicae* L. and *P. rapae* L. (Lepidoptera: Pieridae) were reared in a greenhouse
127 compartment (21±1°C, 50 – 70% RH, L16:D8) on *B. oleracea* var. *gemmifera* L. plants. Female

128 butterflies mated two to three days after eclosion. Their oviposition preferences were tested two
129 days after mating. The females have a high egg load at this age and mating status (David and
130 Gardiner 1962).

131

132 Eight different brassicaceous species were used in a preference experiment, seven in a performance
133 experiment. Apart from *Raphanus sativus* L., all plant species were non-domesticated species. We
134 obtained *R. sativus* from De Bolster seed company (The Netherlands), *Hirschfeldia incana* L. Lagr.-
135 Foss. from the U.S., California, *Brassica nigra* L. from the Centre of Genetic Resources (CGN,
136 Wageningen, the Netherlands) from an early flowering accession (CGN06619), *Sinapis arvensis* L.
137 from Vlieland, in the north of The Netherlands, *B. montana* Pourr. from CGN (CGN18472
138 accession from Italy), *B. rapa* L. from Binnenveld, west of Wageningen (The Netherlands) and *B.*
139 *oleracea* L. ‘Kimmeridge’ from the south coast of England. *Arabidopsis thaliana* (Col-0) was used
140 only for the preference tests. Because of its small size, it was excluded from performance studies, as
141 more than just the focal egg-induced plant would be needed to feed the caterpillars. All plants were
142 in the non-flowering stage when tested, except *A. thaliana*, which already flowered. All plants were
143 cultivated in pots filled with potting soil; they grew in a climate room (18 ± 4 °C, 60 – 80% RH,
144 L16:D8). To use plants of similar biomass in the bioassays, *B. oleracea* was four weeks old, and all
145 other plants were three weeks old at the time of infestation.

146

147 *Butterfly oviposition preference*

148 To determine which plant species is preferred for oviposition, we simultaneously offered the above-
149 mentioned eight plant species to a mated female butterfly. One individual of each plant species was
150 placed into a mesh cage (75 x 75 x 115 cm). The plants were set up in a circle with the leaves not
151 touching each other. The design was a randomized block with 18 replicates per butterfly species.
152 The two butterfly species were tested in separate cages at different time points in a greenhouse
153 compartment (23 ± 5 °C, 50-70% RH, L16:D8). After placing the plants inside a cage, one mated
154 female butterfly was released. The number of *P. rapae* eggs or *P. brassicae* egg clutches,

155 respectively, was counted on each plant three hours after release of the butterfly. Preliminary
156 experiments showed that most butterfly females will make an oviposition choice within this time
157 period.

158

159 *Plant treatments for performance tests*

160 To assess egg survival rates and performance of larvae on previously egg-deposited plants, a plant
161 individual of each species was infested with either *P. rapae* or *P. brassicae* eggs. Each plant was
162 placed in a cage, which was located in a climate room ($21 \pm 1^\circ\text{C}$, 50-70% RH, L16:D8). The first
163 fully developed leaf of each plant (fourth or fifth from the top) was exposed to either *P. brassicae*
164 or *P. rapae* butterflies for egg deposition, while the rest of the plant was covered with a fine mesh.
165 We limited the number of eggs deposited onto a plant to 20 eggs of *P. brassicae* (laid in a clutch)
166 and to eight single *P. rapae* eggs per plant. Limiting egg deposition was done by observing the
167 butterflies after introduction into the cages and removing them as soon as they had deposited the
168 mentioned number of eggs. Those numbers were chosen to mimic naturally occurring egg numbers
169 per plant (Fatouros et al., 2014; Feltwell, 1982). Occasionally extra laid eggs were immediately
170 removed using a fine brush (see Pashalidou et al. (2013) for details). In total, seven to nine plant
171 individuals per species were infested with *P. brassicae* eggs, and six to seven plant individuals per
172 species received *P. rapae* eggs.

173

174 *Plant response to egg deposition, egg mortality and larval performance*

175 To determine egg survival, we counted the number of larvae hatching from the twenty (*P.*
176 *brassicae*) or eight (*P. rapae*) eggs deposited on a plant. To assess larval performance and the
177 impact of the plant's response to previous egg deposition on larval performance, we divided the
178 neonate larvae hatching from egg-deposited plants into two groups. Half of them were placed back
179 onto the previously egg-infested plant (labeled 'egg and feeding', EF) (on the adaxial side of the
180 leaf where they hatched), and the other half was transferred to an egg-free plant (labeled 'feeding',
181 F) plant of the same species and placed onto the adaxial side of the leaf as well. Three and seven

182 days after hatching, caterpillar weight was measured on a microbalance (accuracy = 1 µg; Sartorius
183 AG, Göttingen, Germany). We weighed each caterpillar individually, and afterwards the caterpillars
184 were transferred back to their original position, on EF or F plants. Every EF and F plant was
185 considered one replicate.

186

187 *Statistical analysis*

188 Data on *P. rapae* oviposition preferences for host plants were analyzed by a generalized linear
189 model (GLM) (poisson family), with plant species as fixed factor and the number of eggs per plant
190 as response variable. The post-hoc analysis was performed using a linear hypothesis test (multcomp
191 package). Because *P. brassicae* laid most eggs in a single clutch each time, only oviposition ‘yes’
192 or ‘no’ was scored when determining oviposition preferences. These data were analyzed by a GLM
193 (binomial family) with the plant species as fixed factor, and the presence/absence of oviposition as
194 response variable (post hoc test: linear hypothesis test).

195

196 Data on egg survival of each butterfly species were analyzed by a generalized linear mixed effect
197 model (GLMM, lme4 package) with binomial distribution. The model included egg survival as
198 response variable, and plant species, presence/absence of HR as well as the interaction between
199 both variables were used as fixed factors. Date of infestation was used as random factor. A post-hoc
200 analysis was conducted using linear hypothesis tests for plant species and interaction terms.

201

202 To evaluate whether oviposition preferences of a plant species can be linked to egg survival, we ran
203 a correlation analysis by using Spearman correlation as well as linear regression to generate
204 regression lines. We conducted this analysis first by relating the fraction of eggs (or egg clutches)
205 laid on each plant species with the fraction of eggs surviving on each plant species. To elucidate the
206 relationship between the plant’s ability to express HR-like necrosis and egg survival, we correlated
207 the fraction of eggs laid on each plant species to the fraction of eggs surviving on those plants,

208 which expressed HR-like necrosis in response to the eggs. To elucidate the relationship between the
209 plant's ability to express HR-like necrosis and oviposition preference, we linked the fraction of eggs
210 or egg clusters laid to the fraction of plants expressing HR.

211

212 Data on caterpillar weight obtained on all plant species (subjected to prior egg deposition or not)
213 were analyzed by using linear mixed effect models (LMM). We calculated the average caterpillar
214 weight per plant. The logarithm of the mean caterpillar weight three or seven days after hatching
215 was used as dependent variable, plant species and egg infestation as well as the interaction between
216 them were used as independent variables, the random factor was the date of egg infestation. A post-
217 hoc analysis was conducted using linear hypothesis tests for plant species and interaction terms. The
218 effect of HR-like necrosis on caterpillar weight was tested by using the subset of plants infested
219 with eggs and performing LMM on the logarithmic data of the mean caterpillar weight. Expression
220 of HR-like necrosis, plant species as well as the interaction between both factors were included into
221 the model. A post-hoc analysis was conducted by using linear hypothesis tests for plant species and
222 interaction terms.

223

224 To detect possible links between butterfly oviposition preference and performance of three or
225 seven-day-old caterpillars, a linear regression analysis was conducted. We conducted this analysis
226 first by relating the fraction of eggs laid on each plant species with the weight of caterpillars on
227 each plant species. Furthermore, we ran an analysis by relating the fraction of eggs laid on each
228 plant species to the weight of caterpillars on (i) plants which received eggs prior to larval feeding
229 (EF) and (ii) plants which were left without any eggs (F); thus, we aimed to test the hypothesis that
230 females prefer to oviposit on plants with most modest (for *P. rapae*: putative) egg-mediated
231 reinforcement of defense against the larvae. In addition, we analyzed the relationship between
232 caterpillar weight and the fraction of eggs laid on plants expressing HR-like necrosis; thus, we
233 aimed to test the hypothesis that females prefer to oviposit on plants whose HR-like necrosis has the

234 most modest effect on the performance of their offspring. Finally, we tested the relationship
235 between the fraction of plants expressing HR-like necrosis and caterpillar weight. Thus, we aimed
236 to gain insight in whether the frequency of HR-like necrosis in response to the eggs relates to
237 caterpillar weight.

238 All analyses were performed using R 3.3.2 (R Core Team, 2016).

239

240 **Results**

241 *Oviposition preference*

242 *Gregarious species.* Even though, there is a marginal effect of egg distribution for *P. brassicae*
243 among the plants ($\chi^2 = 19.65$, $df = 7$, $P = 0.04$, GLM, Figure 2A), the post-hoc test did not reveal
244 any significant differences (Supplementary Table 1). *Arabidopsis thaliana*, which was in the
245 flowering stage (in contrast to all other plant species), did not receive any egg clutch by
246 *P. brassicae* in this setup. Oviposition choices of *P. brassicae* were not correlated to plant fresh
247 weight ($S = 1720.7$, $\rho = 0.25$, $P = 0.24$, Spearman correlation, Supplementary Figure S1).

248

249 *Solitary species.* *Pieris rapae* females significantly preferred to oviposit on *B. nigra* over all other
250 seven simultaneously offered plant species (Supplementary Table 1). The plant species chosen least
251 frequently for oviposition were *B. oleracea* and *A. thaliana* ($\chi^2 = 292.67$, $df = 7$, $P = <0.001$, GLM,
252 Figure 2B). The oviposition preference of *P. rapae* was not correlated to plant fresh weight ($S =$
253 1897.2 , $\rho = 0.18$, $P = 0.41$, Spearman correlation, Supplementary Figure S1).

254

255 *Egg survival and effect of plant species and HR-like necrosis*

256 *Gregarious species.* Egg survival was significantly affected by the plant species chosen by
257 *P. brassicae* for egg deposition ($\chi^2 = 20.39$, $df = 6$, $P = 0.002$, GLMM, Figure 3A). Five out of the
258 seven tested plant species expressed an HR-like necrosis in response to *P. brassicae* eggs (Figure
259 3A, Table 1). We observed the highest egg survival rates (almost 100%) when deposited on the
260 plant species that were chosen most for oviposition (*B. montana*, *B. nigra*). Egg survival was

261 significantly lower on *H. incana* and *R. sativus* compared to all other plants (apart from *B. nigra*)
262 (Supplementary Table S2). Overall, induction of HR-like necrosis did not significantly affect egg
263 survival ($\chi^2 = 0.41$, $df = 1$, $P = 0.52$, GLMM), while the interaction between the factors 'HR' and
264 'plant species' significantly affected survival of *P. brassicae* eggs ($\chi^2 = 30.83$, $df = 4$, $P < 0.001$,
265 GLMM). On *B. montana*, egg survival was much lower on the two plants expressing HR than on
266 the seven non-HR-plants (Figure 3A, Supplementary Table S3).

267

268 *Solitary species*. The plant species selected by *P. rapae* females did not significantly affect egg
269 survival ($\chi^2 = 11.19$, $df = 6$, $P = 0.08$, GLMM, Figure 3B). Six out of the seven tested plant species
270 expressed HR-like necrosis induced by *P. rapae* eggs (Figure 3B, Table 1). A significantly higher
271 fraction of *P. rapae* eggs survived on non-HR plants compared to plants expressing HR-like ($\chi^2 =$
272 13.58 , $df = 1$, $P < 0.001$, GLMM, Figure 3B). This effect of *P. rapae* egg-induced HR-like necrosis
273 on egg survival was – in contrast to the *P. brassicae* egg-induced response – independent of the
274 plant species ($\chi^2 = 4.43$, $df = 4$, $P = 0.35$, GLMM).

275

276 *Correlation between oviposition preference and egg survival*

277 When ignoring the expression of HR-like necrosis, we did not detect a significant correlation
278 between oviposition preference and survival of the eggs for either of the two butterfly species
279 (fraction of eggs laid related to egg survival; Spearman correlation; for *P. brassicae*: $S = 32335$, $\rho =$
280 0.10 , $P = 0.44$, for *P. rapae*: $S = 9086.3$, $\rho = 0.01$, $P = 0.97$, Figure 4A). When looking at HR-like
281 necrosis specifically, the fraction of *P. rapae* eggs laid was positively correlated with the fraction of
282 plants expressing HR-like necrosis against those eggs ($S = 4.57$, $\rho = 0.96$, $P < 0.001$, Spearman
283 correlation, see Figure 4B). For *P. brassicae*, this correlation was not significant ($S = 67.72$, $\rho =$
284 0.19 , $P = 0.64$, Spearman correlation, see Figure 4B).

285

286 *Effect of plant species, egg infestation and HR on larval performance*

287 *Gregarious species*. The weight of seven-day-old *P. brassicae* caterpillars did not vary significantly

288 depending on the plant species they were feeding on ($\chi^2 = 12.44$, $df = 6$, $P = 0.05$, LMM, Figure
289 5A). However, the plants' response to prior egg deposition significantly affected performance of *P.*
290 *brassicae* larvae. Seven-day-old larvae developing on plants that previously had received eggs (EF)
291 performed significantly worse than those on plants that had not received eggs (F) ($\chi^2 = 5.27$, $df = 1$,
292 $P = 0.02$, LMM, Figure 5B). This egg-mediated effect on anti-herbivore plant defense was
293 independent of the plant species (no interactive effect between plant species and egg infestation on
294 larval weight; $\chi^2 = 2.51$, $df = 6$, $P = 0.87$, LMM). HR-like necrosis induced by previously laid eggs
295 did not affect the weight of caterpillars ($\chi^2 = 0.72$, $df = 1$, $P = 0.40$, LMM, see Figure 5C), and
296 neither did plant species nor did the interaction between plant species and HR-like necrosis ($\chi^2 =$
297 5.76 , $df = 6$, $P = 0.45$ and $\chi^2 = 5.46$, $df = 4$, $P = 0.24$, LMM).

298

299 *Solitary species.* When considering seven-day-old *P. rapae* caterpillars on both egg-free and
300 previously egg-deposited plants, their weight was not affected by the plant species they were
301 feeding on ($\chi^2 = 5.04$, $df = 6$, $P = 0.54$; LMM, Figure 5A). When excluding the occurrence of HR-
302 like necrosis induced by egg deposition, egg infestation preceding larval feeding did not affect
303 larval weight ($\chi^2 = 0.001$, $df = 1$, $P = 0.97$; LMM, Figure 5B). Neither did the interaction between
304 egg infestation and plant species affect larval weight ($\chi^2 = 1.09$, $df = 6$, $P = 0.98$, LMM, Figure 5B).
305 Yet, larvae feeding on EF plants expressing an HR-like necrosis were significantly heavier than
306 those feeding on EF plants that did not show HR-like necrosis ($\chi^2 = 4.14$, $df = 1$, $P = 0.04$, LMM,
307 Figure 5C). Neither plant species nor the interaction between plant species and HR-like necrosis
308 affected caterpillar weight on previously egg-infested plants ($\chi^2 = 3.73$, $df = 6$, $P = 0.71$ and $\chi^2 =$
309 3.93 , $df = 3$, $P = 0.27$, LMM).

310

311 *Correlation between oviposition preference and larval performance*

312 To assess whether there was a correlation between adult oviposition preference and larval
313 performance, we first analyzed the relationship between the fraction of eggs laid and the weight of

314 three or seven-day-old caterpillars feeding on previously oviposited EF plants and egg-free F plants
315 for each plant species.

316

317 *Gregarious species*. Weight of seven-day-old *P. brassicae* larvae significantly and positively
318 correlated with the number of eggs laid. Seven-day-old *P. brassicae* larvae were the heaviest on
319 those plant species that received most egg clusters ($S = 15964000$, $\rho = 0.17$, $P < 0.001$, Spearman
320 correlation, Figure 6A). The fraction of egg clusters laid did not correlate with the weight of
321 caterpillars feeding on egg-free plants ($S = 34$, $\rho = 0.39$, $P = 0.40$, Spearman correlation,
322 Supplementary Figure S2A).

323

324 When considering the weight of seven-day-old *P. brassicae* caterpillars with respect to the plant's
325 capability to express HR in response to the eggs, weight of caterpillars feeding on previously egg-
326 deposited HR+ plants did not correlate with the fraction of egg clutches per plant ($S = 22$, $\rho = -0.1$,
327 $P = 0.95$, Spearman correlation). Neither was a correlation found between the fraction of plants
328 expressing HR-like necrosis in response to oviposition and the caterpillar weight ($S = 36.65$, $\rho =$
329 0.35 , $P = 0.45$, Spearman correlation).

330

331 *Solitary species*. In contrast to *P. brassicae*, the weight of seven-day-old *P. rapae* larvae feeding on
332 previously egg-infested plants did neither correlate with the fraction of eggs laid ($S = 340680$, $\rho =$
333 0.13 , $P = 0.17$, Spearman correlation, Figure 6B) nor did larval weight correlate with egg load when
334 larvae were feeding on egg-free plants ($S = 50$, $\rho = 0.11$, $P = 0.84$, Spearman correlation,
335 Supplementary Figure S2B). Weight of seven-day-old caterpillars feeding on HR+ plants did not
336 correlate with the fraction of eggs laid ($S = 32$, $\rho = -0.6$, $P = 0.35$, Spearman correlation).
337 Furthermore, the weight of seven-day-old larvae was not correlated with the fraction of plants
338 expressing HR-like necrosis ($S = 29.52$, $\rho = 0.47$, $P = 0.28$, Spearman correlation).

339

340 In addition to the weight of seven-day-old larvae, we also analyzed how the weight of three-day-old

341 larvae is related to oviposition preference, to effects of prior egg deposition and expression of HR-
342 like symptoms (compare Supplementary Figures S3, S4, and supplementary description of results).
343 Two major differences were found when comparing the relationships of seven- and three-day-old
344 larvae with the above-mentioned parameters. First, while weight of seven-day-old *P. brassicae*
345 larvae and oviposition choices correlated significantly (Figure 6A), no such significant relationship
346 was found when analyzing this relationship for three-day-old larvae and egg load ($S = 30$, $\rho = 0.46$,
347 $P = 0.30$, Spearman correlation, Supplementary Figure S4A). As for seven-day-old caterpillars, the
348 weight of three-day-old *P. rapae* larvae feeding upon HR-expressing plant species gained
349 significantly more weight than larvae on non-HR-expressing plants ($\chi^2 = 8.12$, $df = 1$, $P = 0.004$,
350 LMM, Supplementary Figure S3C). Plant species and interaction between both factors did not have
351 any effect ($\chi^2 = 7.44$, $df = 6$, $P = 0.28$ and $\chi^2 = 0.35$, $df = 3$, $P = 0.95$, LMM, respectively).
352 Correlation analysis for three-day-old *P. rapae* caterpillars did not reveal a significant correlation
353 between oviposition choices and larval performances ($S = 62$, $\rho = -0.11$, $P = 0.84$, Spearman
354 correlation, Supplementary Figure 4B).

355

356 **Discussion**

357 The results of our study support the PPH when relating oviposition preferences and larval
358 performances and show that oviposition-induced plant responses partly explain the PPH.
359 Caterpillars of both species gained most biomass on those plant species that received most eggs.
360 However, oviposition choices of both *Pieris* species do not correlate with egg survival. In response
361 to singly laid *P. rapae* eggs, HR-like leaf necrosis was always induced in *B. nigra*, a plant species
362 on which the eggs of this pierid species showed lowest survival rates. Unexpectedly, the solitary
363 butterfly coped with this egg-induced plant defense by depositing most eggs on this plant species;
364 *B. nigra* was most preferred for oviposition. Larval biomass of *P. rapae* was higher on plants
365 expressing egg-induced HR-like necrosis compared to plants without necrosis. In contrast, the
366 gregarious *P. brassicae* showed no significant oviposition preference for any of the tested plant

367 species, and egg survival was hardly affected by HR-like necrosis. Weight of *P. brassicae*
368 caterpillars feeding on previously egg-deposited plants was lower than of those feeding on egg-free
369 plants. Such an egg-mediated plant effect on larval performance was not found in interactions with
370 *P. rapae*. Hence, our data do not confirm the PPH when considering the relationship between
371 oviposition preference and egg performances (egg survival). But we confirm the PPH for the
372 relationship between preference and performance when considering the plant's response to egg
373 deposition that may affect larval performances.

374

375 Our finding that *P. rapae* females laid most eggs on a plant species (*B. nigra*) on which survival of
376 eggs was lowest can hardly be considered an “oviposition mistake” (Larsson and Ekbom, 1995).
377 Because *B. nigra* shows phenotypic variation in the expression of HR-like necrosis it might be
378 difficult for the butterflies to discriminate between egg-resistant (HR+) and egg-susceptible (HR-)
379 genotypes. Oviposition choices are influenced by different cues over long and short distances
380 (Schoonhoven et al., 2005). Cues that signal intraspecific variation in suitability might be absent or
381 of low detectability (Larsson and Ekbom, 1995). Yet, our data show that weight of *P. rapae*
382 caterpillars was highest on *B. nigra* plants expressing HR where egg survival was reduced most.
383 However, then the question arises why do the butterflies not lay most eggs on those plants where
384 egg survival rates *and* larval performance are best? High egg survival rates on plants with a high
385 egg load might result in several problems for the many hatching larvae, i.e. fast food depletion, easy
386 detectability of caterpillars by parasitoids, increased cannibalism and the spread of pathogens
387 (Prokopy and Roitberg, 2001). Therefore, the females might adjust their oviposition rate to the egg
388 survival rate, which determines the extent of intraspecific competition, which caterpillars might
389 experience on these plants.

390

391 Similar preferences and performances were obtained in studies with the polyphagous *Anastrepha*
392 *ludens* fruit fly tested on six different host plants belonging to different families. The second most

393 preferred plant species for oviposition, *Casimiroa edulis* (white sapote), was also the host on which
394 larvae performed best. However, approximately half of all egg clutches laid on *C. edulis* were killed
395 by a wound tissue growth response that led to egg encapsulation (Aluja et al., 2004; Birke and
396 Aluja, 2018). We suggest that *P. rapae* butterflies can afford laying most eggs on the most nutritive
397 plants, because here intraspecific competition among even numerous caterpillars is expected to be
398 low due to the rich nutritional quality of the plant. While survival rates of *P. rapae* eggs on the most
399 preferred host *B. nigra* were lowest, survival rates of *P. brassicae* eggs on the plant species with
400 most egg depositions (*B. montana*) were similar to the plant species with fewer ovipositions. This
401 suggests that *P. brassicae* does not adjust its oviposition choices to egg survival rates. *Pieris*
402 *brassicae* might afford to be not choosy when selecting a host plant for oviposition because
403 gregariously laid eggs have certain advantages with regard to egg survival over singly laid eggs.
404 Gregariousness may for example contribute to protection from desiccation (Clark and Faeth, 1998;
405 Stamp, 1980; Griese et al. 2017).

406

407 A positive relationship was found between the plant's capability to respond to the singly laid *P.*
408 *rapae* eggs by HR-like leaf necrosis and the oviposition preference of *P. rapae*. No such
409 relationship was found between oviposition choices of *P. brassicae* and the occurrence of HR-like
410 leaf necrosis in the various plant species. It is possible that those *P. rapae* eggs surviving potentially
411 egg-killing plant responses harbored the fittest larvae that also face less competition, eventually
412 leading to heavier growing larvae. Another possibility could be that caterpillars perform best on
413 those plant species showing strong HR-like necrosis, because these plants provide high nutritional
414 quality. Based on a meta-analysis, Wetzal et al. (2016) suggested that host plant nutritional quality
415 might be more important for offspring performance than plant defenses against larvae; however, the
416 analysis did not consider studies on plant defenses against insect eggs. Lastly, because less damage
417 is inflicted to the leaf, less defenses might be induced, leading to heavier larvae.

418

419 Egg-mediated reinforcement of the plant's defense against the caterpillars was only shown in the
420 case of the gregarious *P. brassicae*, but not for the solitary *P. rapae*. When comparing weight of
421 caterpillars on egg-free and previously egg-deposited plants (all species), *P. brassicae* caterpillars
422 gained less weight on the latter. These results confirm previous results shown for *P. brassicae*
423 (Bonnet et al., 2017; Geiselhardt et al., 2013; Pashalidou et al., 2015a; Pashalidou et al., 2015b;
424 Pashalidou et al., 2015c; Pashalidou et al., 2013). Similarly, reinforced plant defense against insect
425 larvae mediated by prior egg deposition has been shown in several interactions between plants and
426 insects depositing eggs in clutches and feeding gregariously in early larval development, namely
427 *Diprion pini* sawflies on pine (Beyaert et al., 2012) ; *Spodoptera littoralis* caterpillars on wild
428 tobacco, (Bandoly et al., 2016; Bandoly et al., 2015) and *Xanthogaleruca luteola* leaf beetles on elm
429 (Austel et al., 2016). Singly laid eggs of *P. rapae* did not prime plant defense against caterpillars.
430 The reason why only plant responses to egg clutches of the gregarious *P. brassicae* negatively
431 affected the performance of subsequently feeding caterpillars and not the singly laid eggs of *P.*
432 *rapae* remains to be investigated.

433

434 Our study revealed that preferences and performances differ between the two butterfly species,
435 which might be due to differences in oviposition modes of the butterfly species. The egg-laying
436 mode might have affected egg survival when the plants expressed HR-like necrosis. When the
437 plants received the singly laid eggs of *P. rapae*, we found a positive correlation between oviposition
438 preference (fraction of eggs laid) and expression of HR-like necrosis (fraction of plants expressing
439 HR). In contrast, no correlation was detected between these parameters when the plants received
440 gregariously laid eggs of *P. brassicae*. In a previous study (Griese et al., 2017), we showed that
441 expression of HR-like by *B. nigra* in response to *P. brassicae* did not affect survival of *P. brassicae*
442 eggs when laid in clutches; however, when *P. brassicae* eggs were experimentally kept singly, they
443 clearly suffered from low humidity at the oviposition site, which is characteristic of necrotic leaf
444 tissue (Shapiro and DeVay, 1987). Hence, our current study supports the assumption that egg-

445 induced HR-like has negative effects especially on survival of singly laid eggs (as those of *P.*
446 *rapae*) rather than on clustered eggs (as those of *P. brassicae*). This is further supported by previous
447 studies which showed that single eggs of *P. rapae* as well as of *P. napi* suffer high mortality when
448 the host plant expresses HR-like necrosis (Fatouros et al., 2014; Shapiro and DeVay, 1987).

449

450 With respect to our initial questions, our study has shown that both *P. rapae* and *P. brassicae* laid
451 most eggs on those plant species which provide best larval performance, while these plant species
452 do not provide best egg survival rates. The plant species' capability to activate HR-like necrosis in
453 response to egg deposition affected the oviposition choice of *P. rapae* in so far, as this butterfly
454 species laid most eggs on a plant species where HR-expression frequently occurred, and egg
455 survival rates were low. This behavior might be considered a counter-adaptation because a high egg
456 load on a plant with a high egg-killing capability ensures survival of at least some offspring. Since
457 survival of eggs of *P. brassicae* was not affected by the plant's HR-like, this butterfly species can
458 afford laying many eggs on a plant species with high HR-expression frequency. Our data indicate
459 that the gregarious oviposition mode of *P. brassicae* allows this butterfly species to be less choosy
460 than *P. rapae* in selecting an oviposition site because the gregariously laid eggs are not affected by
461 HR.

462

463 Future studies need to further address the question whether the differences in the effects of plant
464 responses to these two pierid species are due to the different oviposition modes and larval feeding
465 behaviors (singly vs. gregariously) or whether other insect species-specific traits are important as
466 well. It remains to be investigated by studying interactions between more Brassicaceae and pierid
467 species whether a gregarious oviposition mode as shown by *P. brassicae* and/or a high oviposition
468 rate as shown by *P. rapae* laying single eggs on HR-expressing plant species may be considered as
469 adaptations to the egg-induced leaf necrosis and whether these oviposition modes have evolved as
470 countermeasures to this egg-inducible plant defense trait. Other countermeasures of pierid

471 butterflies against egg-induced plant defenses include, e.g. oviposition on inflorescence stems
472 instead of leaves, like observed in some other pierid species feeding on Brassicaceae (*P. napi* and
473 *Anthocharis cardamines*) (NE Fatouros, personal observation) and feeding preference upon flowers
474 if available (*A. cardamines* and *P. brassicae*) (Smallegange et al., 2007; Wiklund and Åhrberg,
475 1978). Because of the impact of insect oviposition on plant defense against hatching larvae shown
476 in our and many other studies, we recommend to consider the egg phase when testing the PPH in
477 herbivorous insects.

478

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487

488 **Author contribution statement**

489 NEF, AP, FGP and EPI designed the experiments. EPI, AP and FGP performed the experiments.
490 EG conducted statistical analysis and wrote the first draft of the paper. All authors interpreted
491 results, drafted and revised the manuscript.

492

493 **Supporting information**

494 Additional supporting information may be found online in the Supporting Information section at the
495 end of the article.

496 **Results.** Effect of plant species, egg infestation and HR on performance of three-day-old larvae

497 **Table S1.** Results of the post hoc test on oviposition preference of *P. brassicae* and *P. rapae*
498 differences between species.

499 **Table S2.** Results of post hoc test on egg survival of *P. brassicae* when compared between plant
500 species.

501 **Table S3.** Results of single GLMs for each plant species tested for the influence of HR expression
502 separated for *P. brassicae* on egg survival, used as a post hoc test.

503 **Figure S1.** Correlation between the number of eggs laid and plant fresh weight.

504 **Figure S2.** Correlation between egg laying preference and mea

505 **Figure S3.** Effect of brassicaceous plant species (A), egg-mediated plant effects (B), and HR-like
506 necrosis (C) on weight (mean \pm SE) of three-day-old *Pieris* caterpillars. n weight (\pm SE) of seven-
507 day-old caterpillars on non-egg infested (F) plant species.

508 **Figure S4.** Linear regression between the fraction of eggs laid and the mass of 3-day old
509 caterpillars (mean \pm SE) on egg-infested (EF) plant species.

510

511

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671

672

Table 1: Number of infested plants and percentage of these plants expressing HR-like necrosis for both butterfly species separated for all plant species.

Plant species	<i>P. brassicae</i>		<i>P. rapae</i>	
	No. infested	% HR yes	No. infested	% HR yes
<i>B. montana</i>	9	22.22	6	16.67
<i>B. nigra</i>	9	88.89	6	100.00
<i>B. oleracea</i>	8	0.00	6	0.00
<i>B. rapa</i>	8	0.00	7	57.14
<i>H. incana</i>	9	55.56	6	33.33
<i>R. sativus</i>	10	80.00	6	16.67
<i>S. arvensis</i>	9	22.22	6	33.33

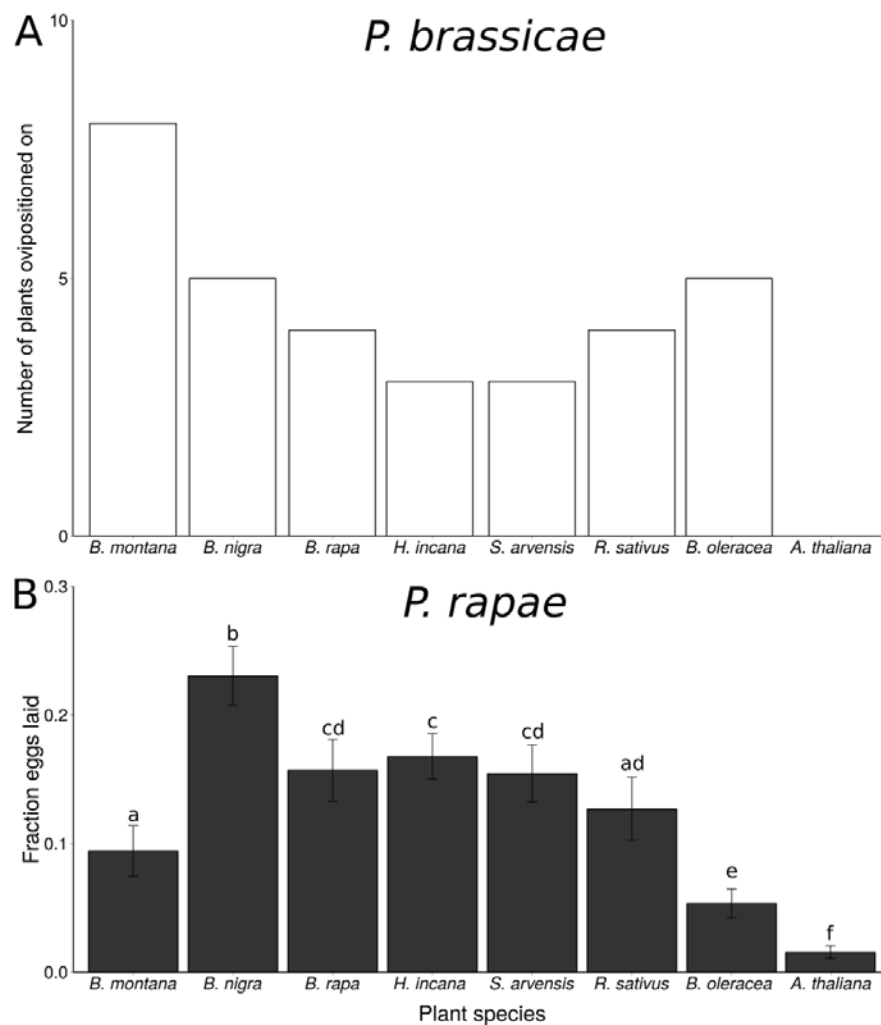
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675

676 **Figure 1:** Developmental stages of the studied *Pieris* species. Differences in appearance and life history of
677 the gregarious *P. brassicae* (top) and solitary *P. rapae* (bottom). From left to right: Eggs, neonates, L5
678 caterpillars, pupae, adults. *Pieris brassicae* larvae are feeding gregariously until the third larval stage. While
679 eggs and adults of both species look similar and mainly differ in size, caterpillars of the two species are
680 differently colored. Larvae of *P. brassicae* larvae are aposematically colored (an indication for
681 unpalatability), which allows them to feed in groups, whereas *P. rapae* larvae are cryptically colored, a trait
682 which is often linked with a solitary feeding behavior in butterflies (Sillén-Tullberg, 1988). Photo credits: E.
683 Griese.

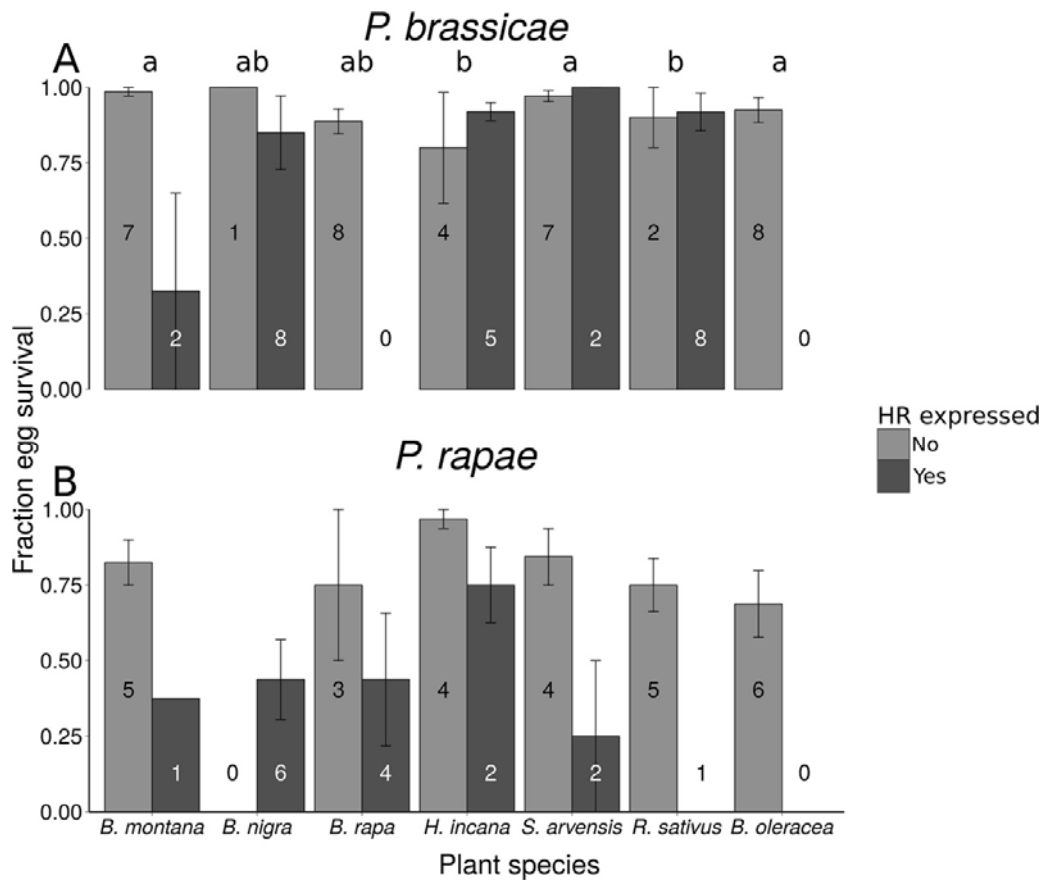
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685

686 **Figure 2:** Oviposition preference bioassay. Fraction of plants, which received an egg deposition by *Pieris*
687 *brassicae* (A) and fraction of eggs laid by *P. rapae* on different brassicaceous plant species (B). Mean
688 fraction \pm SE is given. (A) Female *P. brassicae* always laid at maximum of one egg clutch per plant;
689 therefore, here the number of egg-deposited plants is given, whereas in (B) the number of single eggs of *P.*
690 *rapae* were counted per plant species. In total, 18 plants per species were tested in random setups. Small
691 letters indicate significant differences between plant species with $P < 0.05$, GLM.

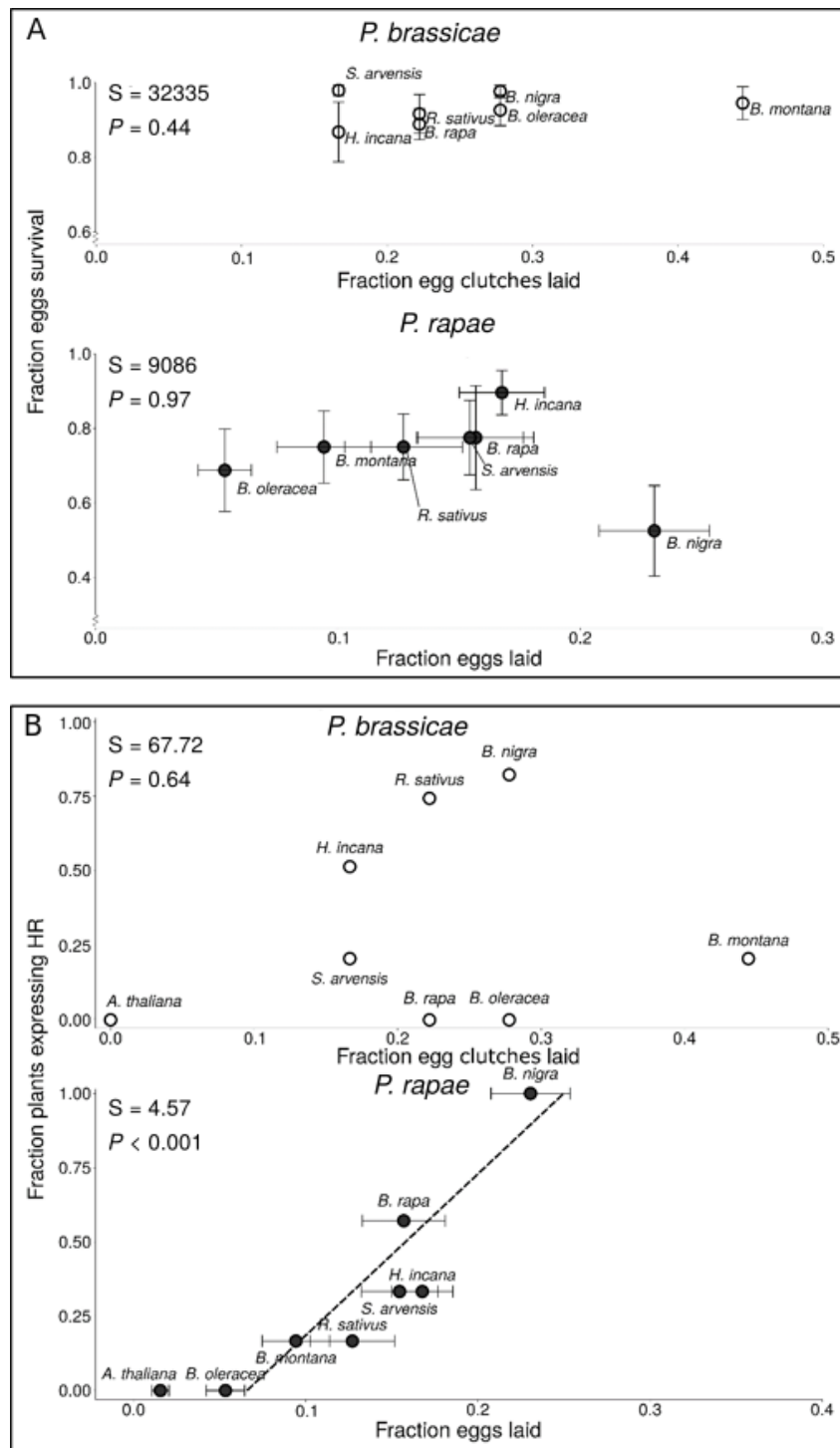
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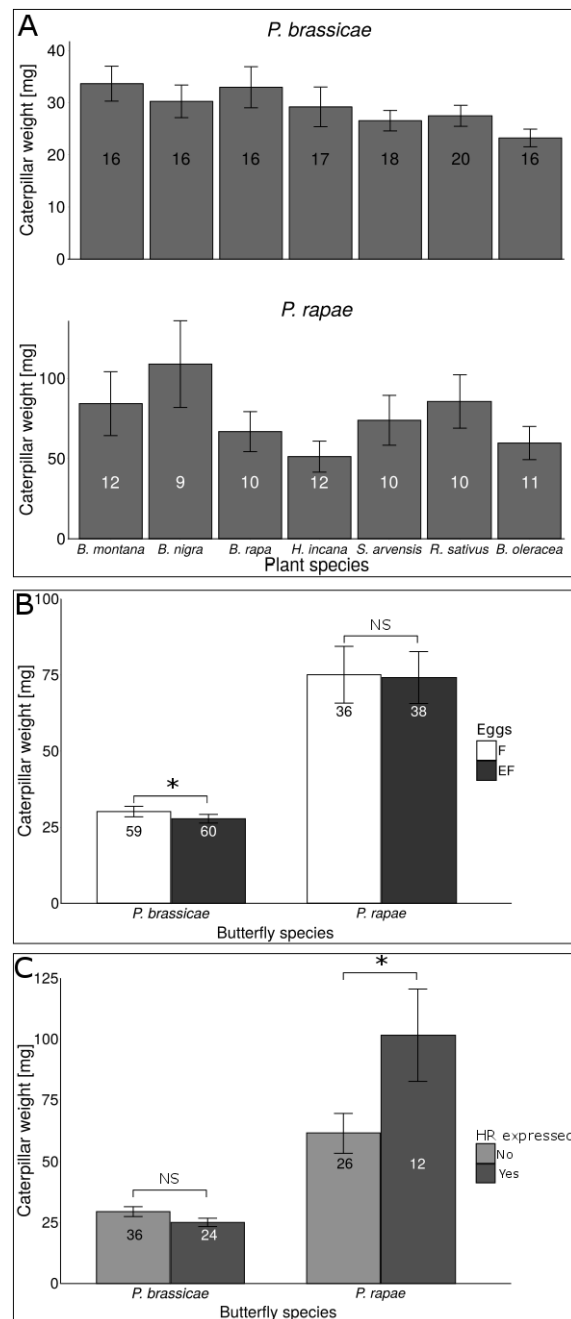
694 **Figure 3:** Effect of HR-like necrosis on survival of eggs of two *Pieris* species on different plant species
 695 (mean fraction \pm SE). Numbers given in the bars indicate the number of plants. Different letters indicate
 696 significant differences ($P < 0.05$, GLM) between plant species regardless of HR-like necrosis. (A) Fraction
 697 survival of *P. brassicae* eggs, with each egg clutch consisting of 20 eggs. (B) Fraction survival of *P. rapae*
 698 eggs, with eight eggs being laid per plant.

699



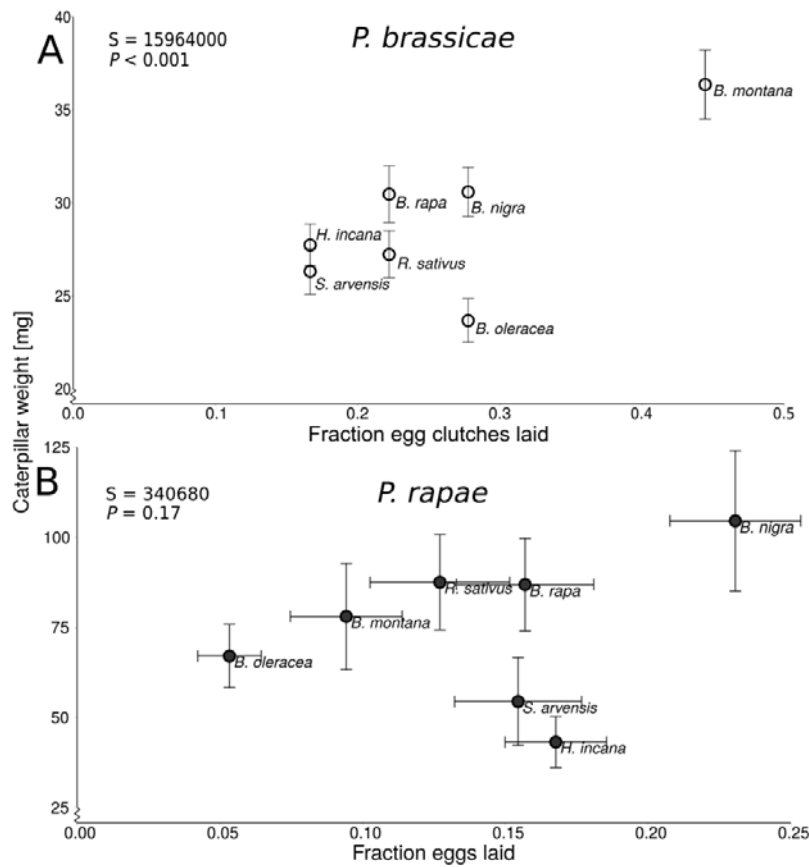
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701 **Figure 4:** Correlation between fraction of eggs laid on different brassicaceous plants by two *Pieris*
 702 butterflies and (A) fraction of egg survival (mean \pm SE) or (B) fraction of HR-like necrosis (mean \pm SE).
 703 Text boxes show correlation results. Fraction of HR+ plants has no error bars, and for *P. brassicae*, no error
 704 bars for the preferences are available. N = 8-10 plants for *P. brassicae*, N = 5-6 plants for *P. rapae*.



705

706 **Figure 5:** Effect of brassicaceous plant species (A), egg-mediated plant effects (B), and HR-like necrosis (C)
707 on weight (mean \pm SE) of seven-day-old *Pieris brassicae* or *P. rapae* caterpillars. In (A), weights of
708 caterpillars feeding upon egg-free and previously egg-deposited plants are pooled. In (B), weights of
709 caterpillars feeding upon egg-free and previously egg-deposited plants are shown separately. In (C), weights
710 of caterpillars feeding upon previously egg-deposited plants are shown separately for plants expressing HR-
711 like necrosis or not in response to egg deposition. The numbers in the bars represent the number of plants
712 within the group. The weight of caterpillars was averaged per plant. Asterisks indicate significant
713 differences. * $P < 0.05$ ns: not significant G.I.M.M



714

715 **Figure 6:** Correlation between oviposition preference and larval performance of seven-day-old *Pieris*
716 caterpillars on different previously egg-infested EF brassicaceous plant species. Caterpillar weight (\pm
717 SE) and fraction of eggs/ egg clusters laid is shown. (A) Fraction of *Pieris brassicae* egg clutches laid per 18
718 test plants were used as preference measurement. (B) Fraction of *Pieris rapae* eggs laid per plant species (\pm
719 SE) were used as preference data. Results of the Spearman correlation test are shown in text boxes. The y-
720 axes do not start at zero to show the graph in greater detail.

721