

1 **Insect egg-killing: a new front on the evolutionary arms-race between Brassicaceae plants and**

2 **Pierid butterflies**

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12

13 **Abstract**

14 Evolutionary arms-races between plants and herbivores have been proposed to generate key
15 innovations that can drive diversification of the interacting species. Recent studies reveal that plant
16 traits that target herbivore insect eggs are widespread throughout the plant kingdom. Within the
17 Brassicaceae family, some plants express a hypersensitive response (HR)-like necrosis underneath
18 the eggs of specialist cabbage white butterflies (Pieridae) that leads to eggs desiccating or dropping
19 of the leaf. Here, we studied the evolutionary basis of this trait, its egg-killing effect on and
20 elicitation by specialist butterflies, by screening 31 Brassicaceae species and nine Pieridae species.

21 We show that induction of HR-like necrosis by pierid egg deposition is clade-specific in the
22 economically important Brassicaceae tribe (Brassica crops and close-relatives) and in the first-
23 branching genus *Aethionema*. The necrosis is elicited only by pierid butterflies that feed on
24 Brassicaceae plants; four *Pieris* and *Anthocharis cardamines* butterflies, of which the larvae are
25 specialists on Brassicaceae, elicited a HR-like necrosis. Eggs of pierid butterflies that feed on

26 Rhamnaceae (*Gonepteryx rhamni*) or Fabaceae (*Colias* spp.) however, did not elicit such a leaf
27 necrosis. Finally, eggs of *Agelais io*, a species of the sister group Nymphalidae, did not elicit any
28 visible response. Counter-adaptations to HR-like necrosis might have evolved by insect deposition
29 of eggs in clusters or on inflorescences. Our findings suggest that the plants' egg-killing trait is a
30 new front on the evolutionary arms-race between Brassicaceae and pierid butterflies beyond the
31 well-studied chemical defence traits against caterpillars.

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33 Key words: induced plant defences, counter adaptation, coevolution, plant-insect interaction, egg
34 deposition, hypersensitive response

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39 **Introduction**

40 The biodiversity on earth is shaped by numerous factors including inter-organismal interactions that
41 can result in coevolution of adaptive traits. For example, the coevolutionary interactions between
42 plants and insects as described by Ehrlich and Raven¹ has driven the diversification of plant
43 defensive metabolites^{2,3}. In turn, specialist herbivores have evolved detoxification mechanisms,
44 which allow them to feed on their host plants despite these toxic metabolites^{4,5}, e.g. monarch
45 butterflies can feed on cardenolide-containing milkweeds^{6,7}, and Pieridae and *Plutella* caterpillars
46 on glucosinolate-containing Brassicaceae⁸⁻¹⁰.

47

48 The role of plant defences against herbivore eggs has been understudied and underappreciated,
49 especially in a coevolutionary perspective between herbivores and plants. The majority of studies
50 on plant-insect interactions have focused on the feeding life stages of herbivorous insects. Yet,
51 plants can already perceive and respond physiologically to the presence of herbivore eggs before
52 they hatch¹¹. The evolution of plant defences against insect eggs is an important first line of
53 defence. In almost half of the ~400.000 known herbivorous insects, especially in case of
54 lepidopteran and sawfly species, eggs may be the first life stage to come into contact with the
55 targeted host plant. Every insect egg being detected and killed, is one less herbivorous larva or adult
56 insect feeding on the plant in the near future.

57

58 Different types of plant defences against insect eggs have been reported in more than thirty plant
59 species including gymnosperms and angiosperms (both monocots and eudicots)¹². In response to
60 insect egg deposition, plants can produce ovicidal substances¹³, form neoplasms^{14,15} or express a
61 hypersensitive response (HR)-like necrosis beneath the eggs¹⁵⁻¹⁹. Specifically, HR-like necrosis as
62 an egg-killing defence leading to eggs desiccating and/or falling off the leaf. It has so far been

63 observed in plants of the Pinaceae²⁰, Poaceae²¹, Fabaceae²², Solanaceae^{15,16} and Brassicaceae^{17-19,23}
64 families. However, the phylogenetic occurrence of the egg-killing trait across these plant families
65 and the phylogenetic co-occurrence in the reciprocal insect pest-clade has yet to be investigated in a
66 similar manner to recent studies of plants and their insect herbivores such as the Brassicaceae plants
67 and Pieridae caterpillars.

68

69 Sequence-based phylogenetic analysis²⁴⁻²⁶ has established that the Brassicaceae family is split into a
70 core clade containing 3680 species, sub-divided into three major lineages, and a smaller sister clade
71 containing only the genus *Aethionema* (61 species^{27,28}). The model plant *Arabidopsis thaliana* is a
72 representative of Lineage I and the *Brassica* crop plants are representatives of Lineage II. Lineage
73 III is a smaller group mostly restricted to Asia and lacking a model or crop species. Cleomaceae is
74 the sister family of the Brassicaceae²⁹. Within the Brassicaceae, defences against feeding herbivores
75 and the genetic basis of this defence have intensively been studied³⁰⁻³³. Aliphatic glucosinolates
76 evolved as defensive compounds near or at the origin of the Brassicales clade and became more
77 diverse and complex with plant species radiation. While these compounds play an important role in
78 defending the plants against herbivory, many feeding insects have specialized and evolved effective
79 glucosinolate detoxification and/or excretion mechanisms^{8,34-36}.

80

81 The Pieridae (whites and sulphurs), containing some 17000 species today, use two major host plants
82 belonging to the Fabales (Fabaceae) and Brassicales (Brassicaceae, Resedaceae, Capparaceae and
83 Cleomaceae); species in some clades also shifted to Rosales (Rhamnaceae, Rosaceae) or
84 Santalales^{9,37}. Recent phylogenetic reconstruction of the Pieridae indicate that the ancestral host
85 appears to be Fabaceae with multiple independent shifts to other orders. While the Dismorphiinae
86 and nearly all Coliadinae are Fabales feeders, the sister to the Coliadinae, Pierinae, primary feed on
87 Brassicales³⁸. The latter thus represent a single origin of glucosinolates feeding⁹. Shortly after the

88 initial evolution of the order Brassicales, some ancestral Pierinae were able to evolve nitrile-
89 specifier proteins (NSPs) that detoxify glucosinolates. This enabled a host shift from their prior
90 Fabaceae hosts to the Brassicales roughly 80 million years ago^{9,37}. Similarly, the evolution of
91 glucosinolate sulfatase in *Plutella xylostella* allowed the caterpillar of these moths to feed on
92 Brassicaceae⁸. It has been shown that speciation-rate shifts, as well as genome-duplication events
93 with gene birth-death dynamics, occurred in both Brassicales and Pieridae, usually following a key
94 defence (glucosinolates) or counter-defence (NSPs and sulfatase) invention in one of the
95 coevolutionary partners³⁷. To pinpoint the evolution of transitions and innovations, it is necessary to
96 have investigate the trait(s) of interest in a proper phylogenetic context. Defence responses targeting
97 eggs might add a new layer of traits evolved in response to herbivore specialization. Egg-killing
98 responses could then be understood as a first-line-of-defence on top of the later acting glucosinolate
99 defence system.

100

101 Eggs of the specialist herbivore *Pieris brassicae* induce HR-like necrosis in the crop plants *Brassica*
102 *rapa*, *B. napus* and *Raphanus sativus*^{12,39}. However, egg-induced responses have mainly been
103 studied in the black mustard *Brassica nigra* and the model plant *A. thaliana*. On *A. thaliana* egg
104 deposition induces a localized cell death response and higher expression of defence genes
105 resembling HR against pathogens, but a visible necrosis is not expressed and egg-killing never been
106 shown^{40,41}. Egg-killing due to a strong necrosis has been shown for the black mustard *B. nigra*.
107 Within *B. nigra*, HR-like necrosis shows high intraspecific variation. Several *B. nigra* accessions
108 were tested with regard to their ability to express HR-like necrosis in response to egg depositions,
109 with some accessions being more likely to express this trait than others^{17,18,23}.

110

111 The current study explores whether egg-killing necrosis evolved as a specific response to pierid egg
112 deposition in a subset of Brassicaceae. So far, no large-scale screening has been done within the

113 family to determine how common the egg-killing necrosis is expressed within the family.
114 Furthermore, no effort has ever been made to map the phylogenetic history of any egg defence trait
115 for any plant family. Doing so would be a first necessary step to show an adaptive response to egg
116 deposition. For this study we first established that egg wash generated from eggs of *P. brassicae*
117 butterflies and egg deposition on plants yielded a similar plant response on *B. nigra* plants. We then
118 used a representative collection of species in the Brassicaceae (mainly lineage I and II) and three
119 species in the Cleomaceae to investigate the phylogenetic occurrence of egg-killing necrosis across
120 the family. Furthermore, we explored the reciprocal phylogenetic co-occurrence in the Pieridae
121 clade and related species. We compared elicitation of HR-like response by egg deposition and egg
122 wash of three other *Pieris* butterflies (Pierinae) as well as by three relatives, *Anthocharis*
123 *cardamines* (Pierinae) feeding on *Cardamine* plants of Lineage I, *Colias* spp. (Coliadinae) feeding
124 on Fabaceae and *Gonopteryx rhamni* (Coliadinae) feeding on *Rhamnus* plants belonging to
125 Rhamnaceae. As an outgroup, we used the butterfly *Aglaia io* (Lepidoptera: Nymphalidae) that
126 feeds on *Urtica* plants (Urticaceae). We addressed the following questions: (i) Is HR-like necrosis
127 induced in a clade-specific manner within the Brassicaceae? (ii) Is the observed necrosis lowering
128 egg survival under greenhouse and field conditions? (iii) Is elicitation of HR-like necrosis by eggs
129 specific to a particular clade of butterfly species (e.g. genus, subfamily or family) and/or specific to
130 species that co-evolved with the Brassicaceae?

131

132 **Material and Methods**

133 *Plants and insects*

134 For our study, we obtained seeds of twenty-eight Brassicaceae and three Cleomaceae species from
135 various sources. The selected plants represent the major lineages in each family. For each plant
136 species, between one and eleven accessions were obtained (Table S1). Per accession, between three
137 and seventeen plants were phenotyped across members of the two families. Two accessions of *B.*

138 *nigra* (SF48, SF19) were used to assess elicitation of the HR-like necrosis by different butterfly
139 species. Finally, egg-killing was tested for four responsive plant species with the same number of
140 genotypes per species. In preliminary trials, plant species with unknown developmental times were
141 grown to assess their flowering time after sowing. Then, plants were sown in a scheme to ensure
142 similar life stages, i.e. vegetative growth, and sizes if possible. Therefore, plants were between three
143 and six weeks old when being treated with butterfly eggs or egg wash.

144 For phenotyping the Brassicaceae we used the wash of *Pieris brassicae* eggs. To assess induction of
145 HR-like necrosis on *B. nigra* plants, we used egg deposition from two populations of *P. brassicae*,
146 *P. napi* L. and *P. rapae* L. and one population of *P. mannii* Mayer (Table S2). Furthermore, we
147 tested egg wash from three populations of *A. cardamines* L., and one population of *G. rhamni* L.
148 and *A. io* L. (Lepidoptera: Nymphalidae) (Table S2). Finally, survival was measured for eggs of *P.*
149 *brassicae*, *P. napi* and *P. rapae*.

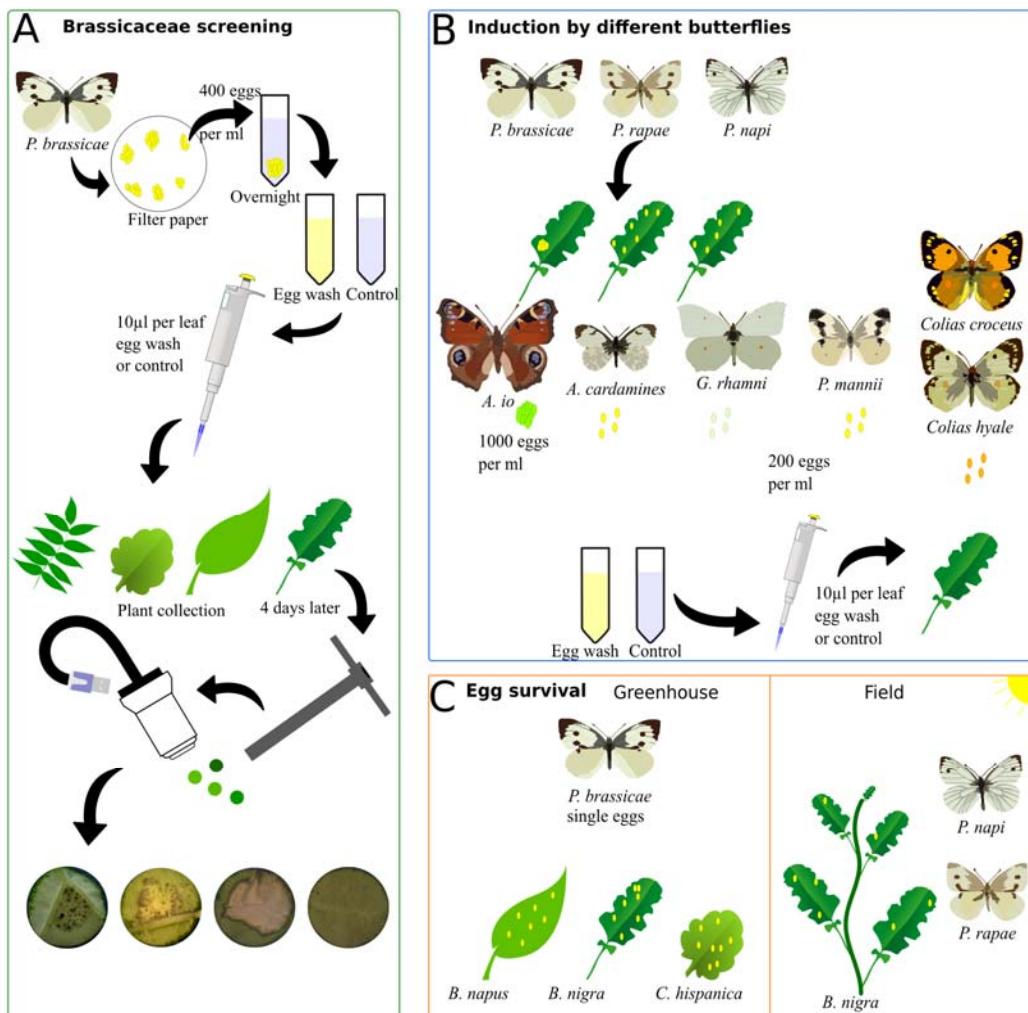
150 *Pieris brassicae*, *P. napi* and *P. rapae* were reared on *Brassica oleracea* var. *gemmaifera* cv. Cyrus
151 in a greenhouse compartment ($21 \pm 4^\circ\text{C}$, 60–80% RH, LD 16: 8). *Pieris mannii* was reared in the
152 same greenhouse, but instead on flowering *Iberis* spp. plants. One population of *A. cardamines* was
153 obtained from a butterfly farm Farma Motyli Zielona Dolina (Babidół, Poland) as hibernating
154 pupae. Hibernation was broken by storing the pupae at 4°C in a cold storage room for five months
155 and another month outdoors. After hibernation, the butterflies were kept in a greenhouse
156 compartment ($18 \pm 2^\circ\text{C}$, 50–60% RH, LD 16: 8) with flowering *Cardamine hirsuta* and *Sisymbrium*
157 *irio* plants to obtain eggs. *Aglais io* butterflies were kept in cages outside (May to June 2018) with
158 cuttings of *Urtica* sp. plants on which they oviposited. Eggs and/or adults of *A. cardamines*, *Colias*
159 spp. and *G. rhamni* were also collected outdoors (for locations see table S2); adults were released
160 again when sufficient egg depositions were obtained. *Pieris brassicae* and *A. io* both lay egg
161 clutches, *P. napi* sometimes lays eggs in small groups, while *A. cardamines*, *G. rhamni*, *P. mannii*
162 and *P. rapae* lay single eggs.

163

164 *Egg wash preparation*

165 Wash from *P. brassicae* eggs was made by fostering females to oviposit on filter paper by pinning
166 the paper to the underside of leaves of *B. oleracea* (Fig. 1a). Within 24 hours after oviposition, the
167 filter paper with the eggs was cut and placed into a 15 ml Falcon tube with purified water
168 (purification system from Millipore Company) at a concentration of 400 eggs per ml. The eggs were
169 left overnight at room temperature. The next morning the supernatant was pipetted off and stored at
170 -20 °C. Before using the egg wash, Tween20 was added at a 0.005 % concentration. The addition of
171 Tween20 was necessary to lower the surface tension of the water droplets, therefore improving the
172 distribution of the egg wash on the waxy leaf surface of some plant species.

173



174 **Figure 1:** Scheme for plant treatments and phenotyping of HR-like necrosis. A) Production and use
175 of wash from *P. brassicae* egg clusters for a screening of 31 plant species, each of which consisted
176 of 1 to 10 plant accessions. B) Use of eggs or egg wash from different butterfly species to determine
177 which species elicits a necrosis in *B. nigra* accessions. C) Use of singly laid *P. brassicae* eggs to
178 determine the egg-killing effect of HR-like necrosis on *B. napus*, *B. nigra* and *C. hispanica*
179 accessions. From the field *P. napi* and *P. rapae* eggs were collected from *B. nigra* and hatching
180 (survival) observed.

181

182 Wash from *A. io*, *G. rhamni* and *A. cardamines* eggs was made by removing eggs from leaves of
183 *Urtica* sp. (*A. io*) or *Rhamnus* sp. (*G. rhamni*) and floral inflorescences of *C. hirsuta* or *S. irio* (*A.*

184 *cardamines*). These eggs were immersed in pure water (*A. io*) or 20 mM 2-(*N*-morpholino) ethane-
185 sulfonic acid (MES) buffer (*A. cardamines*) and left overnight. We chose a concentration of 1000
186 eggs per ml for *A. io*, as egg size is lower than of Pierini eggs (compare database on egg size from
187 more than 10.000 insect species: <https://shchurch.github.io/dataviz/index.html>). As controls, clean
188 *Urtica* sp. leaves for *A. io*, a mixture of *C. hirsuta* and *S. irio* inflorescence stems for *A. cardamines*,
189 clean leaves of *Rhamnus frangula* L. for *G. rhamni*, and inflorescence stems of *Iberis* spp. For *P.*
190 *mannii* were washed in the same manner. Eggs and leaves were kept in the solution overnight, after
191 which the supernatant without eggs was pipetted off and stored at -20 °C. As these egg washes were
192 tested on *B. nigra* plants, no Tween20 was added to the washes.

193

194 *Phenotyping of HR-like necrosis of Brassicales plants*

195 Experiments were carried out in a greenhouse compartment to standardize plant-growth conditions
196 (22-27°C, Rh: 50-90%, L:D: 16:8). For the screening of twenty-eight Brassicaceae and three
197 Cleomaceae plant species, 5 µl of *P. brassicae* egg wash was pipetted on a fully mature leaf (the
198 third or fourth leaf from the top) of each plant. Another fully matured leaf (the third or fourth from
199 the top) received pure water with Tween20 as a control. After four days, leaf disks were harvested
200 of the area where egg wash had been applied using a cork borer (1 cm) and put in a rectangular Petri
201 dish with wet blue filter paper. Pictures were taken using a Dino-Lite digital microscope (AnMo
202 Electronics Corporation). These pictures were visually scored for expression of HR-like necrosis
203 (Fig. 1a).

204

205 *Testing for elicitation of HR-like necrosis by diverse Pieridae species*

206 Female butterflies of *P. brassicae* (2 populations), *P. napi* and *P. rapae* (2 populations) were
207 allowed to lay between five to ten eggs on two different *B. nigra* accessions (SF19 and SF48)
208 (Supplementary Table 1). Accession SF19 is known as a low responder with respect to egg HR-like

209 necrosis and SF48 as a strong responder¹⁸. *Anthocharis cardamines*, *Colias* sp. and *G. rhamni* egg
210 wash was pipetted on both *B. nigra* accessions (Supplementary Table 1). The nymphalid Peacock
211 butterfly *A. io* was used as an outgroup. Eggs laid on *Urtica* leaves were collected and an egg wash
212 made as well as a control wash made from *Urtica* leaves and pipetted on plants of the same *B. nigra*
213 accessions. Between 17 and 40 plant replicates per *B. nigra* accession were used for each butterfly
214 population (Fig. 1b). After four days, HR-like necrosis was scored using a slightly adapted scoring
215 system previously described by Griese et al.¹⁸. For this scoring system a number between 0 (no
216 response) and 4 (very strong response on both sites of the leaf) is assigned to the observed necrosis.
217

218 *Pieris brassicae* egg survival on HR-like expressing plants

219 Experiments were done in greenhouse conditions ($21 \pm 5^\circ\text{C}$, Rh: 45 - 70%, L16 : D8). HR-like
220 necrosis has been shown to have weaker effects on egg-survival under greenhouse conditions than
221 under natural conditions^{17,18}. *Pieris brassicae* females were manipulated to lay five to fifteen
222 separated eggs (not touching each other) on all lines of *B. napa*, *B. nigra* and *C. hispanica* used in
223 the screening of Brassicaceae species. Previous studies revealed that *P. brassicae* egg survival was
224 only affected when eggs were laid singly, not touching each other¹⁸. The oviposition of separated
225 eggs was accomplished by observing the females and taking them off the leaf after they laid one
226 egg. After this, the females were put on a different spot of the same leaf. The eggs were left on the
227 plant and four days after oviposition HR-like necrosis was scored as present or absent. After five
228 days, survival of eggs was noted by counting the number of hatched caterpillars (Fig. 1c).
229

230 *Pieris brassicae* egg survival assessed by field survey

231 A survey was conducted to record survival of *Pieris* eggs on individual *B. nigra* plants in a natural
232 population (compare Fatouros, et al.¹⁷). The survey was conducted at an established *B. nigra* patch
233 along the River Rhine in Wageningen (Steenfabriek), The Netherlands (coordinates: 51.96°N,

234 5.68°E) in one season and butterfly generation (August—September 2017). The total area
235 monitored was approximately 100 m² consisting of ~1000 plants. Plants were monitored for eggs at
236 the edges of a patch or on isolated growing plants So that not all ~1000 plants were monitored.
237 Eggs were collected on leaves and checked for the presence of a HR-like necrotic zone on the leaf.
238 After collection, eggs were kept in a climate chamber (25 ± 1°C, 50–70 % RH, L16 : D8) until
239 caterpillars emerged. All hatched and dead eggs were recorded (Fig. 1c).

240

241 *Phylogenetic analysis of Brassicales and Pieridae species*

242 We used a consensus tree to place our tested Brassicales species according to the species (or genera)
243 reported by two recent studies^{25,26}. Both studies analyse representatives of the three distinct lineages
244 of the core Brassicaceae clade and the first-branching *Aethionema* and the outgroup Cleomaceae.
245 We used the established three-lineage classification when planning and conducting our experiments.
246 As some species and genera were not present in either study, we established their relationships with
247 other included species by calculating our own phylogenetic tree using DNA sequences of two
248 chloroplast markers (*rbcL* and *matK*) and one nuclear genome marker (*ITS2*). The sequences were
249 obtained from the BOLD system website (ID numbers see Supplementary Table 3)⁴². The
250 phylogenetic tree was inferred under maximum likelihood using RaxML v 8.2.4 (GTR+GAMMA,
251 random seed and 1000 bootstrap pseudo-replicates) on the CIPRES science gateway^{43,44}. The three
252 Cleomaceae species were used as outgroups for the phylogenetic tree.
253 The phylogenetic tree of the butterfly species was created using the mitochondrial *COI* gene and the
254 nuclear *EF1α* (Supplementary Table 4). The phylogenetic tree was inferred using maximum
255 likelihood through the IQ TREE website⁴⁵⁻⁴⁷. The models selected here for each of the partitions
256 were GTR+F+I+G4:part1, TIM2e+G4:part2, random seed and 1000 ultrafast bootstrap pseudo-
257 replicates. We verified that each clade of butterflies in the tree contained more species than were
258 used in our test to improve separation. *Plutella xylostella* L. was used as an outgroup. The

259 phylogeny showed support for splits within the Pieridae family and the genera were well supported.
260 The phylogeny is very similar to a more extensive study with more species that used two more
261 markers, *wingless* and *28S*⁴⁸.
262 A Bayesian approach was also performed for phylogenetic inference of the butterflies using the
263 program MrBayes version 3.2⁴⁹ on the same dataset using as priors the parameters from the models
264 selected by IQ TREE and using the same partition of the data. Four simultaneous chains (one cold,
265 three heated) were run for ten million generations, and trees were sampled every 1,000 generations.
266 To check the convergence and stability of the parameter estimates and to determine the burn-in
267 value, Tracer v1.5⁵⁰ was used to explore the log files. Initial trees generated in the burn-in phase
268 (i.e., before establishing stable estimates of parameters) were discarded (burn-in value= 2500, 25 %
269 of the trees). The remaining trees were used to estimate tree topology, branch lengths, and
270 substitution parameters. The phylogenetic relationships inferred from this bayesian approach were
271 congruent with the ML tree obtained from the analysis above.

272

273 *Statistical analysis*

274 To test for statistical significance, R version 3.3.2 “Sincere Pumpkin Patch”⁵¹ was used. For the
275 screening of plant accessions, χ^2 -tests were used to determine which plant species/genotypes
276 significantly expressed HR-like necrosis after egg wash treatment compared to the control
277 treatment. The contingency tables for the χ^2 -tests consisted of the number of egg wash-treated
278 leaves expressing HR-like necrosis, the number of egg wash-treated leaves not expressing HR-like
279 necrosis, the number of control wash-treated leaves expressing HR-like necrosis and the number of
280 control wash-treated leaves not expressing HR-like necrosis. With this set-up, all plant accessions
281 from each plant species were tested independently.

282 Egg survival was analysed using binomial generalized linear models (GLMs) in which first all
283 variables (plant species, flowering state, HR expression and all interactions between the factors)

284 were used and then based on Akaike information criterions (AICs) removed to simplify the model
285 (plant species, HR expression and interaction). After this, EMMEANS test or Mann-Whitney-U
286 tests were performed as post-hoc tests. Differences in induction of HR-like necrosis by different
287 butterflies were tested using binomial GLMs and, to test differences in strength, GLMs with
288 Poisson distribution Dunn tests with Bonferroni-Holm correction were used as post-hoc tests.

289

290 **Results**

291 *Establishing egg wash as an alternative treatment for natural egg deposition*

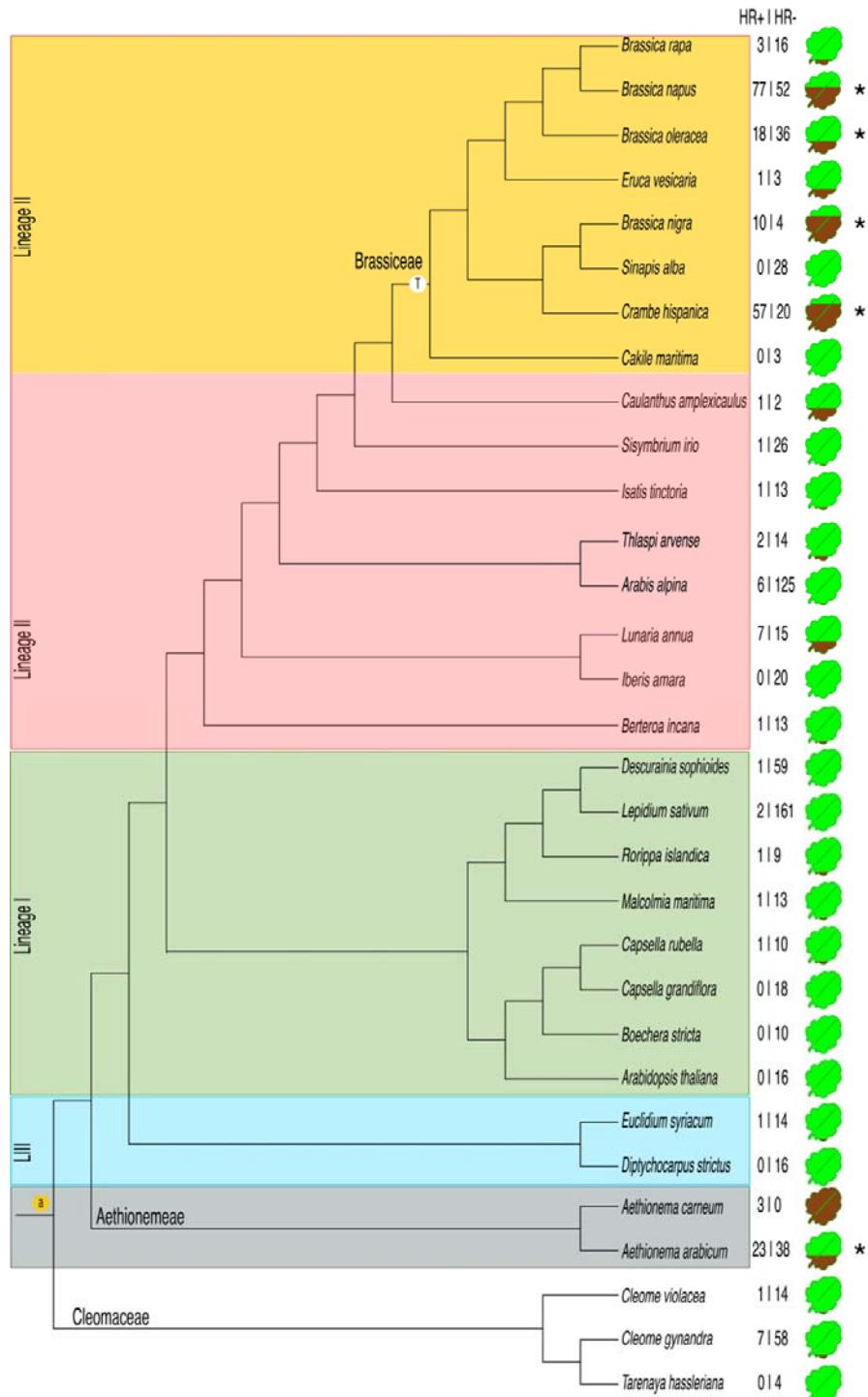
292 Not all tested butterfly species naturally deposit eggs on (all) brassicaceous species. In order to be
293 able to test eggs of those species and screen a large number of brassicaceous species efficiently, we
294 developed a standard method to wash eggs and treat plants with egg wash. We first compared the
295 effect of eggs and egg wash on *B. nigra*, and scored symptoms induced by oviposition or egg wash,
296 scoring a number between 0 (no response) and 4 (very strong response). The accession SF48
297 responded with a score between 1-4 in all plants (Supplementary Figure 1). There was no statistical
298 difference between class of symptoms induced by eggs or egg wash (GLM: $\chi^2 = 1.43$, df = 1, $P =$
299 0.232), and so we concluded that we could use egg wash to test the effect on all species.

300

301 *Origin of HR-like necrosis in the core Brassicaceae, Aethionema and Cleomaceae*

302 Of all thirty-one species tested, five species responded significantly with HR-like necrosis to *P.*
303 *brassicae* egg wash. This included species of the genus *Aethionema* and of the tribe Brassiceae (Fig.
304 2). In the tribe Brassiceae, egg wash treatment significantly enhanced expression of HR-like
305 necrosis in specific accessions of four species: *B. napus* (25-86%), *B. nigra* (63-83%), *B. oleracea*
306 (20-40%) and *C. hispanica* (0-86%) (Supplementary Table 5). There was no significant enhanced
307 HR-like necrosis after egg wash treatment for all other tested plant species tested compared to
308 control leaves. Necrosis was expressed in single plants of some accessions in lineage I and III (0

309 and 29%) (Fig. 2, Supplementary Table 5). HR-like necrosis of *Aethionema arabicum* varied among
310 the tested accessions between 0 and 60 % (Supplementary Table 5). In some cases, e.g. for
311 *Aethionema carneum*, plants responded with HR-like necrosis to egg wash, however, due to the low
312 number of replicates (*A. carneum*: three plants) difference between control and egg wash treatment
313 was not significant (Supplementary Table 5). For *Lunaria annua*, up to 40% expressed HR-like
314 necrosis, but for this plant species only few replicates were tested, making it impossible to test for
315 significant differences (Supplementary Table 5).



316

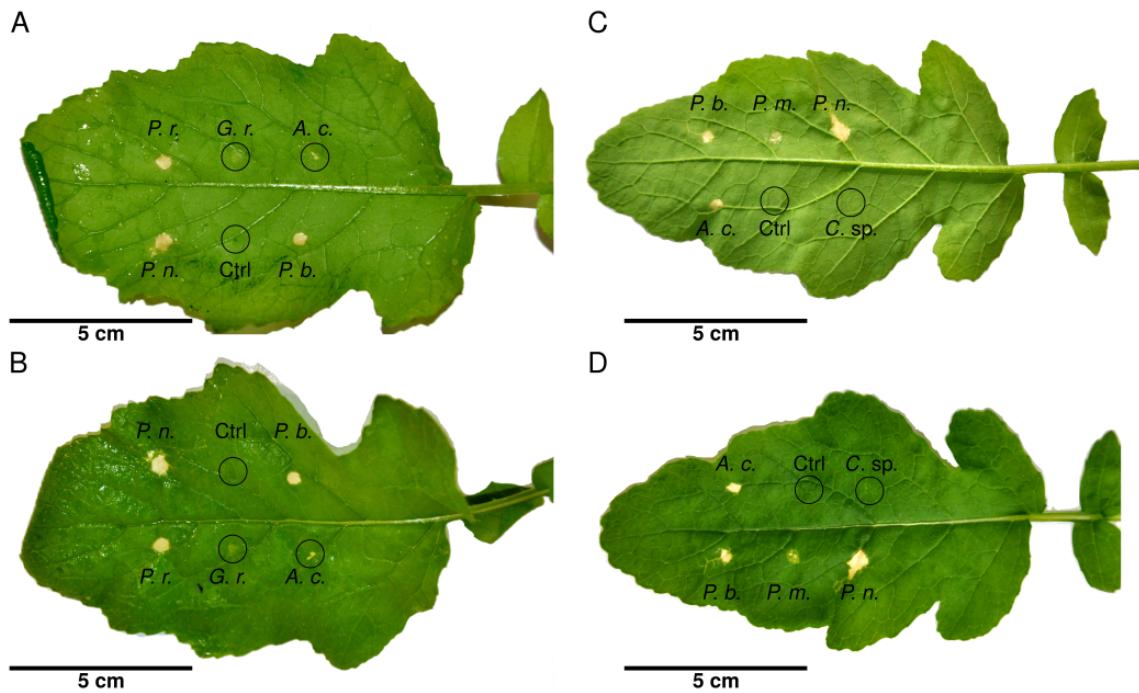
317 **Figure 2:** Phylogenetic tree of all plant species treated with *P. brassicae* egg wash and the
318 resulting- fraction of necrosis after 4 days. Consensus phylogeny based on literature and our own
319 analysis of 3 marker genes: *rbcL* and *matK* and one nuclear genome marker: ITS2 used. The brown

320 part of the leaf shape represents the percentage of tested plants per plant species responding to egg
321 wash with necrosis. Asterisks indicate that at least one plant accession within the species showed
322 significantly more HR-like necrosis on leaves treated with egg wash than on control treated leaves
323 (χ^2 -tests, $P < 0.05$). Phylogenetic clades are coloured differently in the tree. The whole genome
324 duplication WGD (a) and genome triplication (T) the Brassiceae tribe specific events are marked in
325 the tree.

326

327 *Elicitation of HR-like necrosis by different butterfly species correlated with phylogenetic signal*
328 Egg deposition by all *Pieris* spp. and egg wash of *A. cardamines* elicited a HR-like necrosis on both
329 tested *B. nigra* accessions; the low responding SF19 and as the strong responding SF48. Egg wash
330 of *G. rhamni* and *Colias* spp. did not elicit a HR-like necrosis. Notably, egg wash of both species
331 induced the formation of chlorotic tissue (Fig. 3). Egg wash from *A. io* neither elicited a chlorosis
332 nor HR-like necrosis on either *B. nigra* accession (Table 1). When several populations were
333 available for butterfly species, all populations elicited HR-like necrosis in similar frequency (GLM:
334 $\chi^2 = 1.36$, $df = 3$, $P = 0.71$) and severity (GLM: $\chi^2 = 2.60$, $df = 3$, $P = 0.46$).

335



336

337 **Figure 3:** Leaves from *B. nigra* treated with egg wash of different butterfly species and controls
338 inducing or not a HR-like necrosis. *Pieris brassicae* (*P. b.*), *P. mannii*, (*P. m.*), *P. napi* (*P. n.*), and
339 *P. rapae* (*P. r.*) and *Anthocharis cardamines* (*A. c.*) induce a strong HR-like necrosis. Egg wash of
340 *G. rhamni* (*G. r.*) and *Colias* sp. (*C. sp.*) induces a very faint response resembling a chlorosis and
341 does not fit into the established scoring system (faintness indicates 1, but showing up on both sides
342 of the leaf indicates 2). The control (buffer without eggs) does not elicit a HR-like necrosis. All egg
343 washes had the same concentration (200 eggs per ml) and amount applied onto the leaf (5 μ l). Two
344 leaves were needed as not all egg washes were available at the same time. A) and C) Abaxial side of
345 the leaf where the egg washes were applied onto. B) and D) Adaxial side of the leaf showing how
346 strong the HR-like response is on the side which was not treated with egg wash.

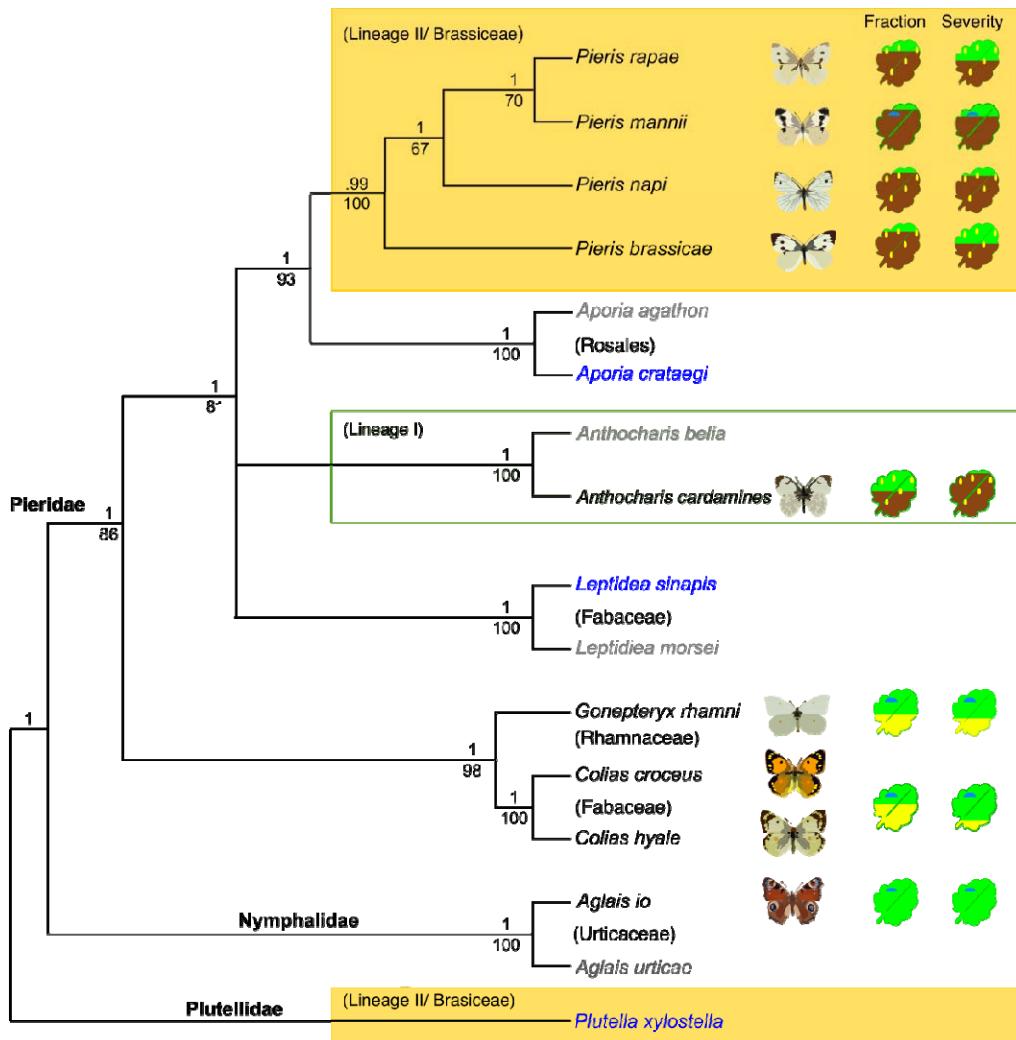
347 **Table 1:** HR- like necrosis (score ranging from 0 to 4) expressed by *B. nigra* plants elicited by
348 different butterfly species. HR- plants did not express HR-like necrosis, while HR+ plants did.
349 Different letters indicate significant differences (different when $P < 0.025$) between butterfly
350 species, Dunn-test, Bonferroni Holm corrected.

351

Butterfly species	HR score (SE)	Plants HR+	Plants HR-	HR fraction (SE)
<i>Anthocharis cardamines</i>	1.63 (0.10) a	61	5	0.92 (0.03) a
<i>Aglais io</i>	0 (0) b	0	40	0 (0) b
<i>Colias spp.</i>	0.67 (0.10) ab	4	5	0.56 (0.18) a
<i>Gonepteryx rhamni</i>	1.11 (0.33) a	8	10	0.44 (0.12) c
<i>Pieris brassicae</i>	1.69 (0.13) a	53	12	0.82 (0.05) a
<i>Pieris mannii</i>	2.14 (0.40) ac	6	1	0.86 (0.14) ac
<i>Pieris napi</i>	2.46 (0.16) c	33	4	0.89 (0.05) a
<i>Pieris rapae</i>	1.64 (0.15) a	42	14	0.75 (0.06) ac

352

353 Eggs of all brassicaceous specialists, *Pieris brassicae*, *P. napi*, *P. rapae* and *A. cardamines* induced
354 an equally high fraction of HR-like necrosis in *B. nigra* (Supplementary Tables 1 and 6). *Pieris napi*
355 elicited a significantly stronger HR-like necrosis (2.46 ± 0.16) compared to all other butterfly
356 species (Supplementary Tables 1 and 7). The fraction and severity of chlorotic tissue formation
357 elicited by *Colias* spp. and *G. rhamni* was generally lower than HR-like necrosis by the eggs of
358 *Pieris* spp and *A. cardamines* (0.44 ± 0.12 ; 1.11 ± 0.33 respectively) (Table 1 and Supplementary
359 Tables 6-7). When we plotted the fraction of HR-like necrosis and its severity per butterfly species
360 on our phylogeny, the likelihood and severity of HR-like necrosis is stronger in butterfly species
361 that are the more closely related to *Pieris* sp. (Fig. 4). Thus, all tested Pieridae elicited an egg
362 response while the nymphalid butterfly *A. io* of the sister group never did.



363

364 **Figure 4:** Phylogeny of a subset of Pieridae and elicitation of HR-like necrosis on *B. nigra* leaves
365 by pierid egg wash or eggs. The phylogeny is based on the maximum likelihood and Bayesian
366 posterior probability analysis of the nuclear marker EF1 α and mitochondrial marker COI subunit 1.
367 As outgroups, the nymphalid *Aglais io* and the plutellid moth *Plutella xylostella* were chosen. The
368 pictograms of leaves on the right of the cladogram represent the fraction of HR-like necrosis
369 elicitation (left) and severity of HR-like necrosis expressed (right). The average fraction (between 0
370 and 1) and severity (between 0 and 4) elicited by either eggs or egg wash is represented by the
371 brown part of the leaf, while the yellowing in the leaves represents a different type of response
372 (chlorosis). The phylogenetic tree consists of species used in the experiments (black), species that

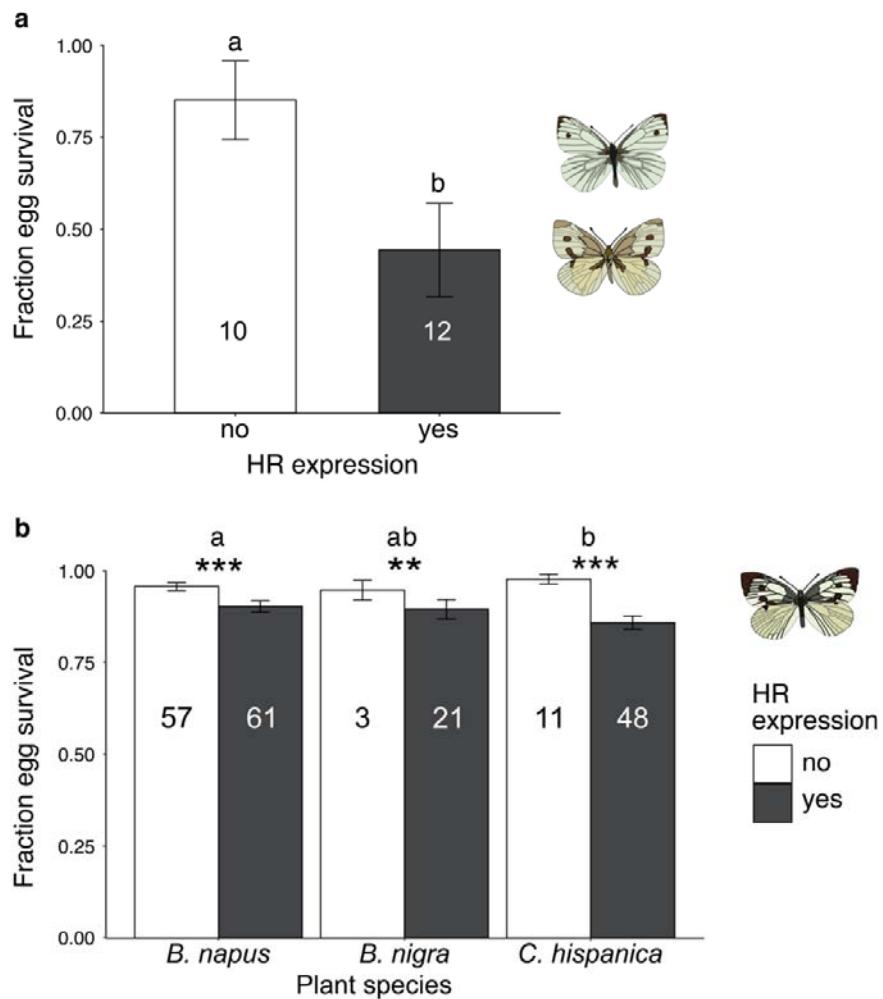
373 would answer open questions when tested (blue) and species added to more fully represent the
374 phylogenetic tree (grey). Coloured boxes indicate the Brassicaceae lineage which the butterflies use
375 as main host plants. Lepidopteran families are written on their nodes where they separate from the
376 rest of the clades. Bootstrap values for the nodes are given below nodes, Bayesian values are given
377 above.

378

379 *Effect of HR-like necrosis on Pieris egg survival on different Brassicaceae plants*

380 First, we also monitored egg survival of the abundant (in the Netherlands) *Pieris* species (both *P.*
381 *napi* and *P. rapae*) under natural field conditions. Egg survival was 40 % lower when eggs induced
382 HR-like necrosis compared to survival of eggs that did not induce a leaf necrosis (GLM: $\chi^2 = 11.02$,
383 $df = 1$, $P < 0.001$, Fig. 5a). As not all eggs on a given plant elicited a necrosis, the fraction of eggs
384 eliciting HR-like necrosis was tested as well.

385 Second, we tested egg survival on three highly responding plant species from the first screening
386 under greenhouse conditions. HR-like necrosis significantly lowered the survival of singly laid *P.*
387 *brassicae* eggs on all three plant species (GLM: $\chi^2 = 38.41$, $df = 1$, $P < 0.001$, fig. 5b). Plant species
388 alone significantly affected egg survival (GLM: $\chi^2 = 6.38$, $df = 2$, $P = 0.04$), while the interaction
389 did not (GLM: $\chi^2 = 3.25$, $df = 2$, $P = 0.20$). On *C. hispanica* plants egg survival was significantly
390 lower than on *B. napus* plants (pairwise MWU: $P = 0.006$, Fig. 5b).



391

392 Figure 5: Survival rates of singly laid *P. brassicae*, *P. rapae* or *P. napi* eggs and effect of
393 expression of HR-like necrosis on different plant species. A) Survey of *P. napi* and *P. rapae* eggs
394 on *B. nigra* plants located near the river Rhine in Wageningen. One to 13 eggs were sampled per
395 plant, total number of collected eggs n = 96. Fraction of survival depending on the expression of
396 HR-like necrosis by the plant. If the plant expressed HR-like necrosis under at least one egg it was
397 counted as HR-expressing 'yes'. Different letters indicate significant differences (GLM: $P < 0.001$).
398 Numbers in bars indicate the number of plants surveyed within each category. B) Effect of HR-like
399 necrosis on survival rates (mean \pm SE) of singly laid *P. brassicae* eggs on *B. napus*, *B. nigra* and *C.*
400 *hispanica*. Asterisks indicate differences in egg survival between plants expressing HR-like necrosis
401 and non-HR within a plant species. Different letters indicate significant differences in egg survival

402 between plant species, without taking HR-like necrosis into account. ns: not significant, **: $P <$
403 0.01, ***: $P < 0.001$. (GLM).

404

405 **Discussion**

406 Pierid butterflies and their brassicaceous host plants are a fascinating model system of co-
407 evolutionary interactions; research so far has explored its evolutionary and genetic basis by
408 focusing on the diversifying selection on plant chemical defences, i.e. glucosinolates, and insect
409 NSP detoxification genes^{9,37,52}. Here, we attempt for the first time to map the phylogenetic history
410 of an egg-induced plant defence trait and its reciprocal co-occurrence in the herbivore clade. We
411 show that pierid egg-induced HR-like necrosis evolved in two clades within the Brassicales. Half of
412 the tested plant species from the Brassiceae tribe in lineage II express strong HR-like necrosis to
413 egg wash. Moreover, all tested *Aethionema* species, the sister clade to the core Brassicaceae,
414 expressed leaf necrosis. Of the *Brassica* and *Crambe* plants (tribe Brassiceae) that were tested, the
415 HR-like necrosis lowered egg survival both under natural and greenhouse conditions. Furthermore,
416 we showed for the first time that only egg wash of *Pieris* butterflies and *A. cardamines*, specialist
417 feeders on the Brassicaceae, elicit a strong HR-like necrosis on *B. nigra*. While *Colias* spp. and *G.*
418 *rhamni* elicited a chlorotic response similar to that of *Solanum dulcamara* to *Spodoptera* eggs⁵³.
419 Our results demonstrate that the egg-induced HR-like necrosis evolved as a new trait at least twice
420 in the Brassicales, but also show that plants specifically evolved this trait to lower egg survival of
421 those pierid species that evolved effective glucosinolate detoxification mechanisms.

422

423 Four out of eight tested Brassiceae species, as well as two tested *Aethionema* species showed
424 consistent HR-like necrosis to *Pieris* egg wash in at least one of the genotypes tested. In other plant
425 species, occasionally a single plant showed a light HR-like necrosis. Likely, those plants are false
426 positives, as some plants expressed a light necrosis to control (buffer) wash as well. Alternatively, it

427 could be a general perception response of insect eggs as described for *A. thaliana*⁵⁴. In the latter
428 species it was shown that a lectin receptor kinase, LecRK-I.8, might be involved in early perception
429 of eggs from two widely divergent species, *P. brassicae* and *Spodoptera littoralis*. The ancient
430 genome triplication event in the Brassiceae tribe might have facilitated the evolution of the HR-like
431 necrosis to eggs in this group by increasing the number of resistance genes underlying the trait.
432 Work is underway to identify the genes, which will contribute to a better understanding on the
433 evolution of HR-like necrosis. It is unlikely that the triplication event is the only factor involved in
434 the evolution of HR-like, because *Aethionema* plants respond to *Pieris* eggs with necrosis as well.
435 *Aethionema* species tested here are annuals that occur in dry habitats during a very short time of the
436 year⁵⁵. Interestingly, most tested Brassiceae plants and *Aethionema* are host plants for different
437 *Pieris* butterflies. Both *P. rapae* and *P. napi* eggs are abundant in nature on *B. nigra* and its close
438 relatives like *Sinapis arvensis*^{17,19,55,56}. *Pieris ergane* is described to feed on several *Aethionema*
439 species in their south eastern European habitat⁵⁷.

440

441 Not all tested plant species within the Brassiceae tribe within Lineage II expressed HR-like
442 necrosis. This could be because we only selected non-responsive genotypes of these plant species or
443 genus. For example, *Sinapis alba*, did not show HR-like necrosis. However, previous work on the
444 close relative *S. arvensis* showed that eggs of *P. rapae* and *P. brassicae* strongly induced HR-like
445 necrosis³⁹. This means that in some genera there is trait variation between species.
446 Alternatively, some plant species might have lost the ability to express HR-like necrosis. Those
447 plants could be less frequently used as host plants for pierid butterflies e.g. because of a
448 phenological mismatch between the plant species and its potential specialist herbivores, as e.g. in
449 the case of *A. thaliana*⁵⁸. In central Europe, *A. thaliana* is usually not attacked by pierid butterflies,
450 as it is rather small and usually completes its life-cycle before caterpillars could develop on the
451 plant⁵⁸. Notably, *A. cardamines* was observed to deposit eggs on *A. thaliana* in North Sweden

452 where both life cycles briefly overlap⁵⁹. Yet, *Pieris* eggs have not been reported to induce a leaf
453 necrosis lowering *Pieris* egg survival on different genotypes of *A. thaliana* including some Swedish
454 accessions^{39,40,60}, neither did we observe a visible necrosis on the tested genotype (Col-0) in our
455 experiments when using *P. brassicae* egg wash.

456

457 Strong induction of HR-like necrosis seems to be highly specific to *Pieris* butterfly species
458 belonging to the Pierinae clade and feeding on hosts belonging to the Brassiceae clade.
459 Interestingly, another Pierinae species, *A. cardamines*, induced HR-like but feeds on hosts
460 belonging to lineage I of the Brassicaceae (e.g. *Cardamine* sp.⁹). In the latter lineage we did not find
461 species responding with HR-like necrosis. When collecting *A. cardamines* eggs from the
462 inflorescence of *Cardamine* spp. we did not observe any HR-like necrosis (N.E. Fatouros, personal
463 observation). Wash from eggs of species from the non-brassicaceous Coliadinae subfamily, *Colias*
464 spp. and *G. rhamni* and the nymphalid *A. io* did not elicit HR-like necrosis. This suggests that the
465 elicitor for HR-like necrosis is specific for Pierinae butterflies that evolved with Brassicaceae plant
466 species rather than a general molecule present in butterfly eggs. Testing more pierid species from
467 different clades and host plant families is needed to confirm this hypothesis. So far, we also do not
468 know if slight differences of HR-like necrosis elicitation between different *Pieris* species is caused
469 by quantitative differences of the elicitor(s), or by changes in the chemical composition of the
470 elicitor(s). Currently, we are analysing the chemical composition of the egg wash from the different
471 butterfly species to identify the compounds inducing HR-like necrosis.

472

473 Previous work has shown that the NSP glucosinolate detoxification gene was a key innovation in
474 the ancestral Pierinae enabling them to shift host plant from Fabaceae to Brassicaceae^{9,37}. A recent
475 study revealed another intriguing counter-adaptation to NSP genes: the speciose genus *Erysimum*
476 has recently gained a novel type of chemical defences, the toxic cardenolides. So far, no known

477 specific adaptations to cardenolides have evolved in insect herbivores, including the Pieridae⁶¹. On
478 the other hand, pierid butterflies may already have found ways to counter-adapt to the egg-killing
479 HR-like necrosis. Clustered eggs of *P. brassicae* were shown to negate the egg-killing effect of the
480 HR-like necrosis¹⁸. While other advantages of egg clustering have been proposed before⁶², it clearly
481 is helpful in dealing with HR-like necrosis. Although the direct mechanisms of how clustering can
482 protect against egg-killing HR-like necrosis are unknown, it has been shown that desiccation can be
483 slowed down by clustering eggs^{18,63}. This might be mitigated by the reduced egg surface area
484 exposed to the environment, compared with single eggs. Other pierid butterflies like *A.*
485 *cardamines*⁶⁴, *P. mannii* and *P. napi* have been observed to deposit their eggs near or on
486 inflorescence stems of their host plants (N.E. Fatouros, personal observation).

487

488 In conclusion, our findings demonstrate that various Brassicaceae plants can mount defences
489 against insect eggs and that these might be under similar selective pressures as plant defences
490 against feeding insects. A coevolutionary arms-race between *Pieris* butterfly eggs and plant species
491 within the Brassicaceae clade as well as species within the sister clade *Aethionema* is likely to have
492 occurred. These plants make use of necrotic lesions to lower egg survival and might just have
493 evolved a new mechanism, possibly hijacked from disease resistances, to combat specialist
494 herbivores adapted to their host plants' toxins. Being a very early, premeditated defence, the
495 mechanism of HR-like necrosis is currently studied as a novel defensive trait to improve resistance
496 of *Brassica* crops against *Pieris* pests.

497

498

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510

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