How symbiosis and ecological context influence the variable expression of transgenerational wing induction upon fungal infection of aphids

Wen-Hao Tan[1], Miguel L. Reyes[1], Kim L. Hoang[1], Tarik Acevedo[1,#], Fredrick Leon[1], Joshua D. Barbosa[1], Nicole M. Gerardo[1*].

[1]Department of Biology, Emory University, Atlanta, Georgia, United States of America.

[#]Department of Ecosystem Science and Management, Penn State University, State College, Pennsylvania, United States of America.

*Corresponding author

Email: ngerard@emory.edu (NMG)

Abstract

Aphids, like most animals, mount a diverse set of defenses against pathogens. For aphids, two of the best studied defenses are symbiont-conferred protection and transgenerational wing induction. Aphids can harbor bacterial symbionts that provide protection against pathogens, parasitoids and predators, as well as against other environmental stressors. In response to signals of danger, aphids also protect not themselves but their offspring by producing more winged than unwinged offspring as a way to ensure that their progeny may be able to escape deteriorating conditions. Such transgenerational wing induction has been studied most commonly as a response to overcrowding of host plants and presence of predators, but recent evidence suggests that pea aphids (*Acyrthosiphon pisum*) may also begin to produce a greater proportion of winged offspring when infected with fungal pathogens. Here, we explore this phenomenon further by asking how protective symbionts, pathogen dosage and environmental conditions influence this response. Overall, while we find some evidence that protective symbionts can modulate transgenerational wing induction in response to fungal pathogens, we observe that transgenerational wing induction in response to fungal infection is highly variable. That variability cannot be explained entirely by symbiont association, by pathogen load or by environmental stress, leaving the possibility that a complex interplay of genotypic and environmental factors may together influence this trait.

Introduction

Animals have evolved several forms of defense against pathogens. Although the most studied defenses are mediated by cellular and humoral immune mechanisms, some defenses are mediated by behavioral mechanisms or symbiotic, microbial partners [1]. These alternative forms of defense may act in isolation or may influence one another.

Pea aphids (*Acyrthosiphon pisum*) utilize an array of defense strategies, and thus are a suitable system for studying how alternative forms of defense interact with one another. One defense, referred to here as transgenerational wing induction, arises from pea aphids' reproductive biology. Under summer conditions, pea aphids asexually produce clonal copies of themselves, and, though genetically identical, these clonal offspring can be either unwinged (apterous) or winged (alate). For aphids, one commonly mounted defense against environmental stress, predators, and parasites is increased production of winged (relative to unwinged) offspring that can hopefully escape to better conditions [2–4]. Such transgenerational wing induction is similar to other transgenerational defenses, a phenomenon seen in many insect systems, in which defenses against pathogens are mounted not to protect oneself but to protect one's offspring [5]. For example, immune-challenged bumblebees (*Bombus terrestris*) produce offspring with higher levels of antibacterial activity [6], and when infected with a protozoan parasite, monarch butterflies preferentially lay their eggs on plants that increase their offsprings' resistance to the parasite [7]. Though aphid

51 transgenerational wing induction is best known as a response to host plant overcrowding and
52 presence of predators [3], recent work suggests that aphids utilize transgenerational wing
53 induction in response to pathogen infection as well. Specifically, Hatano et al. [8]
54 demonstrated that pea aphids can increase production of winged offspring in response to
55 infection with the natural aphid pathogen, *Pandora neoaphidis.*

56 The finding that transgenerational wing induction is a potential aphid defense against
57 fungal pathogens suggests that this form of defense could interact with another common
58 aphid defense against fungal pathogens, namely association with protective bacterial
59 symbionts. All pea aphids harbor an obligate endosymbiont, *Buchnera aphidicola,* that is
60 essential for their survival and reproduction. In addition, individuals can harbor one to a few
61 different facultative symbionts that can increase their hosts' fitness [9–11]. Specifically,
62 *Regiella insecticola*, a facultative endosymbiont, confers resistance against fungal pathogens
63 [12], including *Pandora neoaphidis* [10], an important natural enemy in wild populations
64 [13]. Interestingly, *R. insecticola* has been shown to impact transgenerational wing induction
65 in response to crowding, suggesting the potential for the symbiosis to influence
66 transgenerational wing induction in response to pathogens as well [14].

67 Our primary goal was to leverage the aphid - *Regiella* symbiont - *Pandora* pathogen
68 system to explore how protective symbionts influence transgenerational defense. In our
69 preliminary investigations, however, transgenerational wing induction in response to fungal

infection was not consistently observed. To attempt to explain this variability, we also conducted a series of experiments to explore whether *R. insecticola* genotypes vary in their influence on transgenerational wing induction upon fungal infection, and whether the degree of pathogen exposure or environmental quality influences transgenerational wing induction upon fungal infection.

Materials and methods

Aphid lines

We used five lines of pea aphids (*Acyrthosiphon pisum*) previously established in the laboratory that have the same aphid genetic background but that harbor different genotypes of the secondary, facultative symbiont, *Regiella insecticola*. The five lines were established by experimentally infecting a single clonal aphid lineage, LSR1 [15], which did not have *Regiella*, with five genetically distinct *Regiella* (Ri, 313, 5.15, CO21, and U1), collected in previous studies [16–18], using established protocols [19,20]. This created lines LSR1-Ri, LSR1-313, LSR1-5.15, LSR1-CO21, and LSR1-Ui, which we abbreviate here as LRi, L313, L515, LCO21, and LUi. In addition to these five lines, we also maintained a line without *Regiella* (LSR1-01, abbreviated as L01). Upon establishment, all aphid lines were reared asexually on fava beans (*Vicia faba*) in a temperature-controlled growth chamber at 20 °C under a 16/8h L/D cycle, which maintains them as asexual clones. Presence of symbionts was

89 confirmed via PCR prior to conducting experiments [18,21].

90

91 *Pandora* fungal pathogen infections

92 *Pandora neoaphidis* ARSEF 2588 was obtained from the Agricultural Research Service
93 Collection of Entomopathogenic Fungal Cultures, USA. We maintained *Pandora* in the
94 laboratory by *in vivo* culturing, storing dead, infected aphids at 4 °C following methods
95 described in Parker et al. [17]. We performed the fungal infection experiments using an
96 established protocol [22] that mimics the natural route of pathogen transmission. Infected
97 aphid cadavers, the fungal source, were placed on 1.5% tap water agar at 18 °C for 14-16
98 hours, providing sufficient time for the fungus to sporulate prior to aphid infections.
99 Recently-molted (10-day old) adult aphids were experimentally infected by placing them in
100 the bottom of an infection chamber (a PVC tube, 28 mm diameter and 40 mm height) on top
101 of which we placed an agar plate with sporulating cadavers, allowing the experimental aphids
102 to receive a fungal spore shower. Agar plates were rotated among infection chambers to
103 homogenize the infection dosage, and a grid slide was used to estimate the infection dosage
104 (number of spores / mm^2). The infection period was 3-hr unless otherwise specified. Control
105 aphids were handled similarly but were instead placed under agar plates without infected
106 cadavers. After infection, we transferred aphids to two-week-old fava plants to monitor
107 survival and offspring production. During the first four days post-infection, the plants were

covered with solid plastic cups in order to keep the environment moist, as *Pandora* requires high humidity to infect aphids [23]. Afterward, the plants were covered by plastic cups with mesh tops.

Overview of survival and wing induction measurements

We used survivorship to quantify the differences in *Pandora* resistance between aphid lines and measured induction of winged offspring production as a transgenerational defense trait. For survival assays, we inspected infected and uninfected aphids daily to record survival. Dead aphids were checked for visible signs of sporulation. We monitored survival for 9-10 days, as infection-caused mortality and sporulation usually occur between 4 -10 days after exposure in this system [22]. For transgenerational wing induction, we collected offspring produced in the four days post fungal infection by transferring each adult aphid to a new plant every other day. We recorded the number of offspring produced each day. The proportion of offspring that were winged was recorded after each cohort reached adulthood.

Experiment 1: Influence of *Regiella* presence on transgenerational wing induction upon *Pandora* infection

We used two aphid lines, LRi (harboring *Regiella*) and L01 (without *Regiella*). We exposed 34 aphids of each line to *Pandora*, and monitored 34 control, uninfected aphids per

line as well. For each treatment group, 10 aphids went to individual plants to monitor offspring production, and 24 aphids were monitored (8 aphids on each of three plants) for survival. We monitored survival of the exposed (F0) aphids and assessed the proportion of their offspring (F1) that were winged using methods detailed above. After the F1 aphids became adults, we typically randomly selected six unwinged, F1 offspring of each F0 individual and transferred them to individual plants to monitor the winged status of the F2 generation. Twenty-two F0 individuals produced fewer than six unwinged offspring however, and we thus used fewer offspring from these individuals (F1 per F0: range = 1-6, median = 5).

Experiment 2: Influence of alternative *Regiella* lines on transgenerational wing induction upon *Pandora* infection

Given that we did not observe wing induction in either *Regiella*-present or *Regiella*-absent lines in Experiment 1 in response to fungal infection, and that *Regiella*-mediated resistance against *Pandora* is dependent on genotype-by-genotype specificity [17], we hypothesized that *Regiella*'s influence on transgenerational wing induction could also be genotype-specific. In this experiment, we tested for the effect of symbiont genotype on resistance and wing induction upon fungal infection. We used all six lines of aphids described above (five lines harboring different genotypes of *Regiella* and one

146 without *Regiella*). We performed the experiment twice. We first conducted the experiment
147 using previously established lines, and then repeated this experiment with re-established lines
148 to ensure that the host genetic background was identical across all lines (while aphids
149 clonally reproduce, mutations can occur and become fixed in lab lines). We conducted
150 infections and monitored survival of F0 individuals, and measured the proportion of their F1
151 offspring that were winged following methods described above, with the exception that in the
152 first experiment we did a 3-day rather than 4-day collection of F1 offspring due to logistical
153 constraints. Although the same protocol and infection period was used, the fungal dosages
154 were very different between the two replicates. The fungal infection dosage was 12.3
155 spores/mm^2 in Replicate A and 105.6 spores/mm^2 in Replicate B. For Replicate A, sample
156 sizes ranged from 3 – 10 (median = 6.5) per treatment group; for Replicate B, sample sizes
157 ranged from 11 – 12 (median = 12) per treatment group. For Replicate B, aphid line L313
158 was removed from analyses due to low survival of the control group (all died within 10 days).
159

160 Experiment 3: Influence of pathogen dose on transgenerational
161 wing induction upon *Pandora* infection

162 Given that the two replicates of Experiment 2 showed different results in terms of
163 transgenerational offspring production in response to *Pandora* infection, we attempted to
164 examine the potential factors influencing this response. A previous study demonstrated that

higher infection dosage leads to higher pathogen burden and higher mortality in this system [22]. Due to the fact that the infection dosage was markedly different in the two replicates of Experiment 2, we hypothesized that induction of winged offspring production might be dependent on pathogen load. To test this, we exposed two aphid lines (LRi and L01) to three infection dosages: high (144.1 spores/mm^2), medium (12.5 spore s/mm^2), and low (1.6 spores/mm^2) by altering the infection period to manipulate infection dosage. That is, a higher dosage group was infected for a longer time. In order to control for a confounding effect that staying longer in a chamber could increase stress, we added an uninfected control group for each infection period. Thus, this experiment was a fully factorial design of three infection periods (which correlates with dosage), two infection treatments (infected and uninfected), and two aphid lines. Sample sizes ranged from 10 – 12 (median = 11) per treatment group. After fungal infection, we followed the methods described above to monitor survival and offspring production.

Experiment 4: Influence of environmental condition on transgenerational wing induction upon *Pandora* infection

Our results in Experiment 3 did not detect increased production of winged offspring under high pathogen load, suggesting that pathogen dosage alone is not the main factor triggering the expression of this response. Through comparing conditions in the above

experiments, we hypothesized that other factors, such as host plant quality and aphid health, could influence the production of winged offspring. To manipulate condition, in Experiment 4, we used starvation and drought as abiotic stresses, both of which have been shown to negatively affect pea aphids [24–26]. For the starvation treatment, prior to fungal infections, we starved aphids for 12 hours when they were four days old (young nymph) and again when they were 10 days old (newly-molted adults). To do so, we moved aphids that were reared on the same plant to another pot with moist soil but no plants. Aphids were transferred back to their original plant after the starvation treatment. For drought treatments, we transplanted fava plants to dry soil the same day we transferred aphids onto them. Those two treatments caused the aphids to look pale, which is an indication of poor condition [27]. This experiment was thus a fully factorial design of three environmental conditions (starvation, drought, and control), two infection treatments (infected, uninfected) and two lines (LRi and L01). We used seven aphids for each treatment group. We followed the same infection protocol as above, using an infection period of eight hours to reach a medium dosage (21.0 spores/mm^2). After fungal infection, we assayed survival and offspring production as above.

Statistical analyses

Across all experiments, we measured survivorship and the proportion offspring that were winged and tested for the effects of several factors. Survival analyses were performed

using Cox Proportional Hazardous models using the R package survival 2.41-3 [28]. The proportions of offspring that were winged were analyzed using General Linear Models (GLMs) with either binomial or quasi-binomial error structures, depending on the dispersion parameter. All analyses were performed using R version 3.4.1 [29].

Results

Experiment 1: Influence of *Regiella* presence on transgenerational wing induction upon *Pandora* infection

Fungal infection significantly reduced aphid survival, and aphid lines differed in their survival; however, there was no significant interaction between infection status and aphid line (Fig 1A; infection: $\chi^2 = 8.198$, df = 1, $P = 0.004$; line: $\chi^2 = 14.938$, df = 1, $P < 0.001$; interactions: $\chi^2 = 0.270$, df = 1, $P = 0.604$). *Pandora* fungal infection of F0 generation aphids did not induce production of winged offspring relative to control, uninfected aphids (Table 1). Specifically, while some F1 offspring were winged, and symbiont status significantly affected this trait, it was not influenced by fungal infection of their mothers (Table 1; Fig 1B). The F2 generation consisted of few winged: two out of 2854 F2 offspring were winged. Specifically, out of a total of 195 F1 aphids, only two of them produced a winged offspring.

Table 1. Results of analysis of deviance of GLMs for the proportion of offspring that

were winged for the four experiments.

	Deviance	df	*P*-value
Experiment 1: F1			
Infection	0.110	1	0.873
Line	18.940	1	**0.037***
Infection * Line	5.502	1	0.260
Experiment 2: Replicate A			
Infection	0.170	1	0.886
Line	62.747	5	0.064
Infection * Line	102.073	5	**0.005****
Experiment 2: Replicate B			
Infection	296.120	1	**< 0.001*****
Line	24.065	5	**< 0.001*****
Infection * Line	12.276	5	**0.021***
Experiment 3			
Infection	0.054	1	0.901
Dosage	36.349	2	**0.005****
Line	0.372	1	0.743
Infection * Dosage	29.490	2	**0.014***
Infection * Line	1.427	1	0.522
Dosage * Line	5.988	2	0.422
Infection * Dosage * Line	27.801	2	**0.018***
Experiment 4			
Infection	0.039	1	0.910
Stress	285.361	2	**< 0.001*****
Line	0.714	1	0.629
Infection * Stress	34.158	2	**0.004****
Infection * Line	5.298	1	0.188
Stress * Line	2.991	2	0.614
Infection * Stress * Line	0	2	1.000

"Infection" refers to fungal infection (uninfected control, infected). "Line" refers to aphid line (L01 and LRi in Experiment 1, 3, 4; all six lines in Experiment 2 Replicate A; 5 of 6 lines (L313 excluded) in Experiment 2 Replicate B). "Dosage" refers to the degree of pathogen exposure, and "Stress" refers to rearing environment (no-stress control, drought, starvation).

In Experiment 1, F1 are offspring of experimentally infected F0 mothers. While we also collected data on the F2 offspring of F1 aphids, the results for F2 were not analyzed because very few F2 winged offspring were produced: out of a total of 195 F1 aphids used, only two of them produced a winged offspring (2 out of 2854 offspring in total).

Fig 1. Experiment 1. Fungal infection reduced survival but had no effect on the proportion of offspring that were winged across two aphid lines. F1 are offspring of experimentally infected or control F0 mothers. (A) F0 aphid survival upon *Pandora* infection. Solid lines indicate aphid line LRi (with *Regiella*) and dotted lines indicate line L01 (without *Regiella*); (B) the proportion of F1 offspring that were winged. An average of 10.3 offspring were produced per F0 aphid over four days. Sample sizes range from 9 - 10 (median = 10) F0 monitored for winged offspring production per treatment group. Points are proportion of winged offspring for each F0 individual; bars represent mean ±1 SEM.

Experiment 2: Influence of alternative *Regiella* lines on transgenerational wing induction upon *Pandora* infection

Replicate A

Fungal infection significantly reduced aphid survival (Fig 2A; $\chi^2 = 64.907$, df = 1, $P < 0.001$), and symbiont genotype had a significant effect on host survival upon fungal infection

246 (χ^2 = 29.908, df = 5, P < 0.001). Resistance against *Pandora* differed between the six
247 aphid-lines: LRi, LUi, and L313 had significantly higher survival than L01, while L515 and
248 LCO21 did not (Fig 2B, Table 2). Neither fungal infection nor aphid line had significant main
249 effects on the proportion of offspring that were winged; however, the interaction term had a
250 significant effect, suggesting a role for symbionts in altering induction of winged offspring
251 production (Fig 2C, Table 1).

252

253 **Table 2. Differences in survival between aphid lines in Experiment 2.**

	Z	***P*-value**
Experiment 2: Replicate A		
L313 – L01	-2.408	**0.016***
L515 – L01	-0.389	0.700
LCO21 – L01	-1.555	0.120
LRi – L01	3.862	**< 0.001*****
LUi – L01	-3.496	**< 0.001*****
Experiment 2: Replicate B		
L515 – L01	-0.942	0.346
LCO21 – L01	0.540	0.589
LRi – L01	-3.441	**< 0.001*****
LUi – L01	-3.462	**< 0.001*****

254 Each aphid line with *Regiella* was compared to L01, which did not harbor *Regiella*. In
255 Replicate B, aphid line L313 was removed from the analysis due to low survival of
256 uninfected controls (all died within 10 days).

257

258 **Fig 2. Experiment 2. Lines with different symbionts varied in terms of survival upon**

fungal infection but did not consistently vary in terms of winged offspring production. Replicate A shown in (A) – (C), and Replicate B shown in (D) – (F). **(A) and (D):** survival upon *Pandora* infection relative to control, uninfected aphids - aphids lines combined. In both replicates, fungal infection reduced aphid survival. **(B) and (E):** survival upon *Pandora* infection – aphid lines plotted separately for pathogen infected groups only. In both replicates, *Regiella* genotype impacts aphid survival upon infection. Note that in (B), the survival curve for L01 and L515 are shown as the same color because the curves completely overlap. **(C) and (F):** the proportion of offspring that were winged upon fungal infection. In Replicate A, an average of 22.1 offspring per experimental aphid were produced across three days; in Replicate B, an average of 25.2 offspring were produced across four days. In Replicate A, fungal infection did not consistently induce an increase in winged offspring production; however, in Replicate B, fungal infection consistently induced an increase in winged offspring production. Although the two replicates showed strikingly different patterns, there was a significant interaction between infection and aphid line found in both replicates, suggesting that symbiont genotypes alter responses to fungal infections in different ways. Error bars in (C) and (F) represent mean ±1 SEM. For Replicate A, sample sizes range from 3 – 10 (median = 6.5) experimental aphids monitored for survival and proportion of winged offspring produced per treatment group. For Replicate B, sample sizes range from 11 – 12 (median = 12) experimental aphids monitored for survival and proportion of winged offspring

278 produced per treatment group.

279

280 **Replicate B**

281 Consistent with Replicate A, fungal infection significantly reduced aphid survival (Fig
282 2D; $\chi^2 = 115.301$, df = 1, $P < 0.001$), and symbiont genotype had a significant effect on
283 survival upon fungal infection ($\chi^2 = 26.099$, df = 4, $P < 0.001$). Resistance against *Pandora*
284 differed between aphid lines: LRi and LUi had significantly higher survival than L01, while
285 L515 and LCO21 did not (Fig 2E, Table 2). Though patterns of survival were similar to
286 Replicate A, transgenerational wing induction was strikingly different. The proportion of
287 offspring that were winged was significantly influenced by fungal infection, aphid line, and
288 their interaction (Fig 2F, Table 1).

289

290 # Experiment 3: Influence of pathogen dose on transgenerational
291 # wing induction upon *Pandora* infection

292 Fungal infection, infection dosage, and aphid line all had significant effects on host
293 survival upon fungal infection (Fig 3; infection: $\chi^2 = 44.542$, df = 1, $P < 0.001$; dosage: $\chi^2 =$
294 13.378, df = 1, $P < 0.001$; line: $\chi^2 = 12.264$, df = 1, $P < 0.001$); higher dosages caused higher
295 mortality in both lines. Fungal dosage had a significant effect on the proportion of offspring
296 that were winged, as did the interaction between dosage and infection and the three-way

297 interaction between infection, dosage, and aphid line (Fig 4, Table 1). However, the
298 proportion of offspring that were winged was generally low: across all fungally infected
299 aphid treatments only 46 of 1666 offspring (2.76%) were winged.
300
301 **Fig 3. Experiment 3. Higher fungal dosage led to lower survival upon infection across**
302 **two aphid lines.** Each infection dosage has a corresponding control group in order to control
303 for the effect of infection period (*i.e.*, the time aphids stayed in infection chambers). Low
304 dose represents 1.6 spores/mm^2, medium dose represents 12.5 spores/mm^2, and high dose
305 represents 144.1 spores/mm^2. (A) aphid lines combined; (B) aphid lines plotted separately for
306 infected groups only. Solid lines indicate aphid line LRi (with *Regiella*), and dotted lines
307 indicate line L01 (without *Regiella*). Sample sizes range from 10 – 12 (median = 11) per
308 treatment group.
309
310 **Fig 4. Experiment 3. The effects of infection, fungal dosage, and aphid lines on the**
311 **proportion of offspring that were winged.** Each infection dosage has a corresponding
312 control group in order to control for the effect of infection period (*i.e.,* the time aphids stayed
313 in infection chambers). Low dose represents 1.6 spores/mm^2, medium dose represents 12.5
314 spores/mm^2, and high dose represents 144.1 spores/mm^2. (A) aphid line L01 (B) aphid line
315 LRi. In this experiment, the proportion of offspring that were winged was generally low:

316 across all fungally infected aphid treatments only 46 of 1666 offspring (2.76%) were winged
317 (Note that the y-axis scale is 0 to 0.6). An average of 24.4 offspring were produced per
318 experimental aphid across four days. Sample sizes range from 10 – 12 (median = 11)
319 experimental aphids monitored for proportion of winged offspring produced per treatment
320 group. Points represent proportion of offspring that were winged for a particular individual;
321 bars represent mean ±1 SEM.
322

323 Experiment 4: Influence of environmental condition on
324 transgenerational wing induction upon *Pandora* infection

325 A high background death rate was observed across treatments in this experiment.
326 Neither the main effects of infection, environmental condition, nor aphid line had significant
327 effects on host survival upon infection (Fig 5; infection: $\chi^2 = 0.001$, df = 1, $P = 0.982$; stress:
328 $\chi^2 = 0.437$, df = 1, $P = 0.804$; line: $\chi^2 = 0.695$, df = 1, $P = 0.404$). Environmental condition,
329 however, had a significant effect on the proportion of offspring that were winged, as did the
330 interaction between stress and infection (Fig 6, Table 1). The most striking influence on
331 winged offspring was starvation of mothers, which stimulated transgenerational winged
332 offspring production in both the presence and absence of fungal infection.
333

334 **Fig 5. Experiment 4. Stress, infection, and aphid line had no effect on aphid survival**

335 **upon fungal infection.** Solid lines indicate aphid line LRi (with *Regiella*), and dotted lines
336 indicate aphid line L01 (without *Regiella*). (A) no-stress (control) treatment; (B) drought
337 treatment; (C) starvation treatment; (D) survival of infected aphids only, all treatments.
338 Sample sizes equal to seven individuals per treatment group.
339
340 **Fig 6. Experiment 4. Starvation induced an increase in the production of offspring that**
341 **were winged but fungal infection did not.** (A) aphid line L01, and (B) aphid line LRi. An
342 average of 21.2 offspring were produced per experimental aphid across four days. Sample
343 sizes equal to seven experimental aphids per treatment group. Points represent proportion of
344 offspring that were winged for a particular individual; bars represent mean ±1 SEM.
345

346 Discussion

347 The utilization and efficacy of defenses is often dependent on genotype-by-genotype
348 interactions and on environmental context [30,31]. In this study, we tested for the influence of
349 multi-generational effects, symbiont genotype, pathogen dosage, and two abiotic stressors on
350 the expression of an induced defense trait – production of winged offspring in response to
351 fungal infection. Though transgenerational wing induction in response to fungal infection was
352 reported in a previous study [8], we did not consistently observe wing induction. Our results
353 suggest that wing induction of pea aphids upon *Pandora* infection may be strongly dependent

354 on particular factors that were not captured in our experimental design. Given that the aphid
355 and *Pandora* genotypes we used were different from the ones used in Hatano et al. [8], one
356 possibility is that the response may vary between host genotypes, may vary based on
357 pathogen genotypes, or may be influenced by genotype-by-genotype interactions. However,
358 in our study, replicate experiments (*e.g.,* Experiment 2 Replicates A and B) exhibited
359 considerable differences, suggesting that environmental variation not captured here also
360 influences expression of this defense.

361 In Experiment 1, we tested whether exposure to pathogens leads to production of more
362 winged daughters and/or more winged granddaughters. Our consideration of the potential
363 influence on granddaughter physiology stems from the interesting reproductive biology of
364 aphids. In days with relatively high levels of light, pea aphids reproduce via parthenogenesis,
365 producing offspring as first instar nymphs (viviparous). Prior to birth, their developing
366 embryos already have embryos developing within them, a condition known as telescoping
367 generations [32]. As a result, it creates an opportunity for aphids to receive maternal and even
368 grandmaternal signals related to environmental conditions and pathogen exposure [33].
369 Because of previous findings that aphids produced a greater proportion of winged offspring
370 in response to fungal infection [8], we asked whether this pathogen exposure could have
371 longer lasting effects across multiple generations. As we saw no influence of pathogen
372 exposure on winged offspring production in either generation, this is not a consistently

373 observed phenomenon. However, future work should test this interesting hypothesis in
374 relation to other pea aphid defense traits.

375 Given that we saw little evidence of transgenerational wing induction in response to
376 fungal infection in Experiment 1, we carried out a set of experiments to explore how genetic
377 and non-genetic (environmental) variation might influence this trait. In Experiment 2, we
378 asked whether symbiont genotypes varied in their effects on transgenerational wing induction
379 in response to fungal infection. This built on previous research showing that *R. insecticola*
380 genotypes vary in the level of protection that they confer against *P. neoaphidis* [17]. In the
381 first replicate of the experiment, we saw no significant main effect of either fungal infection
382 or symbiont genotype on the production of winged offspring, but the interaction between
383 infection and symbiont genotype was significant, suggesting that lines with alternative
384 symbionts varied in their response to infection. In contrast, in the second replicate, all aphid
385 lines, regardless of symbiont genotype, produced more winged offspring when fungally
386 infected, though the strength of the response varied across lines. Despite the inconsistent
387 main effects of fungal infection and symbiont genotype, the fact that both experimental
388 replicates demonstrated significant symbiont genotype by fungal infection interactions
389 suggests that *Regiella* may play a role in regulating the response and that this regulation is
390 likely symbiont genotype-specific.

391 Given the differences observed between the outcomes of the two replicates of

392 Experiment 2, we asked what environmental conditions could have differed between the two
393 replicates as a way to begin to understand the variable expression of transgenerational wing
394 induction in response to fungal infection. We first noted that fungal pathogen virulence was
395 higher in Replicate B than in Replicate A (Fig 2 (A) and (D)), consistent with the fact that
396 fungal dosage in Replicate B (105.6 spores/mm^2) was much higher than that in Replicate A
397 (12.25 spores/mm^2). We thus hypothesized that pathogen virulence and/or dosage could
398 influence the expression of the defense trait. Results of Experiment 3 showed that higher
399 fungal dosages led to lower survival, which is consistent with previous studies [22]. However,
400 higher dosage did not result in higher proportions of winged offspring. Indeed, we instead
401 observed fewer winged offspring produced when aphids were exposed to a higher pathogen
402 dose. A recent study suggested that wing polyphenism in pea aphids is controlled by the
403 ecdysone pathway – downregulation of the ecdysone pathway leads to increased winged
404 offspring and vice versa [34]. Ecdysone is also identified as a positive regulator of innate
405 immune mechanisms in other systems [35]. Therefore, it is possible that enhanced immune
406 responses in the face of stronger pathogen challenge could lead to suppression of wing
407 induction; however, future studies are required to disentangle the physiological mechanisms
408 underlying this response.

409 Host defenses are often dependent on environmental-context [36]. For example, in
410 honey bees, birds and humans decreasing nutrient availability decreases immunocompetence

411 [37–39]. We hypothesized that the variation that we observed between the two replicates of
412 Experiment 2 could be a result of environment variation that influenced host condition.
413 Specifically, control, uninfected aphids had lower survival in Experiment 2 Replicate B
414 compared to Replicate A, suggesting that their overall condition may have been worse. In
415 Experiment 4, we attempted to modulate host condition by rearing aphids under control,
416 drought and starvation conditions. While starvation triggered a strong induction of winged
417 offspring production, neither starvation nor drought enhanced responses to fungal infection.
418 We should note, however, that fungal infection did not significantly impact aphid survival in
419 this experiment, though we did observe sporulating aphids suggesting that the aphids were
420 indeed infected. Thus, it is possible environmental stress could enhance transgenerational
421 winged offspring production in response to fungal pathogen under different infection
422 conditions.
423

424 # Conclusions

425 In this study, through a series of experiments that tested the influence of multiple factors
426 on transgenerational wing induction in response to *Pandora* infection, we did not consistently
427 observe the increased production of winged offspring by infected individuals. Our results
428 confirmed that *Regiella* genotypes differ in the strength of protection that they confer to
429 aphids, and showed that wing induction, though not consistently expressed, may be

dependent on symbiont genotype to some extent. Our study further suggested that *Pandora*-induced winged offspring production may be strongly dependent on other environmental or non-genetic factors not captured in our experiments, and may have strong specificity across host, symbiont, and pathogen genotypes.

Acknowledgments

We thank Dr. Ben Parker for assistance with aphid symbiont infections, fungal infection protocols, and thoughtful feedback. We thank Manasa Peddineni for assistance with experiments. We thank Gerardo lab members for providing helpful comments on the manuscript and Tiger Li for comments on statistical analyses.

Author contributions

WHT designed and carried out experiments, and wrote the manuscript. MR, KH designed and carried out experiments, and edited the manuscript. NMG designed experiments and edited the manuscript. TA, FL, and JB assisted with experiments and provided comments on the manuscript.

References

1. Parker BJ, Barribeau SM, Laughton AM, de Roode JC, Gerardo NM.

449 Non-immunological defense in an evolutionary framework. Trends Ecol Evol. 2011;26:
450 242–248. doi:10.1016/j.tree.2011.02.005
451 2. Lees AD. The production of the apterous and alate forms in the aphid *Megoura viciae*
452 Buckton, with special reference to the rôle of crowding. J Insect Physiol. 1967;13:
453 289–318. doi:10.1016/0022-1910(67)90155-2
454 3. Müller CB, Williams IS, Hardie J. The role of nutrition, crowding and interspecific
455 interactions in the development of winged aphids. Ecol Entomol. 2001;26: 330–340.
456 doi:10.1046/j.1365-2311.2001.00321.x
457 4. Kunert G, Otto S, Röse USR, Gershenzon J, Weisser WW. Alarm pheromone mediates
458 production of winged dispersal morphs in aphids. Ecol Lett. 2005;8: 596–603.
459 doi:10.1111/j.1461-0248.2005.00754.x
460 5. Grindstaff JL, Brodie ED, Ketterson ED. Immune function across generations:
461 Integrating mechanism and evolutionary process in maternal antibody transmission.
462 Proc R Soc B Biol Sci. 2003;270: 2309–2319. doi:10.1098/rspb.2003.2485
463 6. Sadd BM, Kleinlogel Y, Schmid-Hempel R, Schmid-Hempel P. Trans-generational
464 immune priming in a social insect. Biol Lett. 2005;1: 386–388.
465 doi:10.1098/rsbl.2005.0369
466 7. Lefèvre T, Oliver L, Hunter MD, De Roode JC. Evidence for trans-generational
467 medication in nature. Ecol Lett. 2010;13: 1485–1493.

doi:10.1111/j.1461-0248.2010.01537.x

8. Hatano E, Baverstock J, Kunert G, Pell JK, Weisser WW. Entomopathogenic fungi stimulate transgenerational wing induction in pea aphids, *Acyrthosiphon pisum* (Hemiptera: Aphididae). Ecol Entomol. 2012;37: 75–82. doi:10.1111/j.1365-2311.2011.01336.x

9. Oliver KM, Degnan PH, Burke GR, Moran NA. Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. Annu Rev Entomol. 2010;55: 247–266. doi:10.1146/annurev-ento-112408-085305

10. Scarborough CL, Julia F, H.C.J G. Aphid protected from pathogen by endosymbiont. Science. 2005;310: 2005. doi:10.1126/science.1120180

11. Oliver KM, Moran NA, Hunter MS. Variation in resistance to parasitism in aphids is due to symbionts not host genotype. Proc Natl Acad Sci. 2005;102: 12795–12800. doi:10.1073/pnas.0506131102

12. Parker BJ, Spragg CJ, Altincicek B, Gerardo NM. Symbiont-mediated protection against fungal pathogens in pea aphids: A role for pathogen specificity. Appl Environ Microbiol. 2013;79: 2455–2458. doi:10.1128/AEM.03193-12

13. Van Veen FJF, Müller CB, Pell JK, Godfray HCJ. Food web structure of three guilds of natural enemies: Predators, parasitoids and pathogens of aphids. J Anim Ecol. 2008;77: 191–200. doi:10.1111/j.1365-2656.2007.01325.x

14. Leonardo TE, Mondor EB. Symbiont modifies host life-history traits that affect gene flow. Proc R Soc B Biol Sci. 2006;273: 1079–1084. doi:10.1098/rspb.2005.3408

15. Caillaud MC, Boutin M, Braendle C, Simon JC. A sex-linked locus controls wing polymorphism in males of the pea aphid, *Acyrthosiphon pisum* (Harris). Heredity. 2002;89: 346–352. doi:10.1038/sj.hdy.6800146

16. Łukasik P, Dawid MA, Ferrari J, Godfray HCJ. The diversity and fitness effects of infection with facultative endosymbionts in the grain aphid, *Sitobion avenae*. Oecologia. 2013;173: 985–996. doi:10.1007/s00442-013-2660-5

17. Parker BJ, Hrček J, McLean AHC, Godfray HCJ. Genotype specificity among hosts, pathogens, and beneficial microbes influences the strength of symbiont-mediated protection. Evolution. 2017;71: 1222–1231. doi:10.1111/evo.13216

18. Vorburger C, Gehrer L, Rodriguez P. A strain of the bacterial symbiont *Regiella insecticola* protects aphids against parasitoids. Biol Lett. 2010;6: 109–111. doi:10.1098/rsbl.2009.0642

19. Fukatsu T, Tsuchida T, Nikoh N. Spiroplasma symbiont of the pea aphid, *Acyrthosiphon pisum* (Insecta: Homoptera). Appl Environ Microbiol. 2001;67: 1284–1291. doi:10.1128/AEM.67.3.1284

20. Tsuchida T, Koga R, Sakurai M, Fukatsu T. Facultative bacterial endosymbionts of three aphid species, *Aphis craccivora*, *Megoura crassicauda* and *Acyrthosiphon pisum*,

506 sympatrically found on the same host plants. Appl Entomol Zool. 2006;41: 129–137.
507 doi:10.1303/aez.2006.129
508 21. Henry LM, Peccoud J, Simon JC, Hadfield JD, Maiden MJC, Ferrari J, et al.
509 Horizontally transmitted symbionts and host colonization of ecological niches. Curr
510 Biol. 2013;23: 1713–1717. doi:10.1016/j.cub.2013.07.029
511 22. Parker BJ, Garcia JR, Gerardo NM. Genetic variation in resistance and fecundity
512 tolerance in a natural host-pathogen interaction. Evolution. 2014;68: 2421–2429.
513 doi:10.1111/evo.12418
514 23. Papierok B, Hajek AE. Fungi: Entomophthorales. Manual of techniques in insect
515 pathology. L. A. Lace. London: Academic Press; 1997. pp. 187–212.
516 24. McVean RIK, Dixon A. The effect of plant drought-stress on populations of the pea
517 aphid *Acyrthosiphon pisum*. Ecol Entomol. 2001;26: 440–443.
518 doi:10.1046/j.1365-2311.2001.00341.x
519 25. Nelson EH. Predator avoidance behavior in the pea aphid: Costs, frequency, and
520 population consequences. Oecologia. 2007;151: 22–32.
521 doi:10.1007/s00442-006-0573-2
522 26. Tariq M, Wright DJ, Rossiter JT, Staley JT. Aphids in a changing world: Testing the
523 plant stress, plant vigour and pulsed stress hypotheses. Agric For Entomol. 2012;14:
524 177–185. doi:10.1111/j.1461-9563.2011.00557.x

525 27. Tabadkani SM, Ahsaei SM, Hosseininaveh V, Nozari J. Food stress prompts dispersal
526 behavior in apterous pea aphids: Do activated aphids incur energy loss? Physiol Behav.
527 2013;110–111: 221–225. doi:10.1016/j.physbeh.2012.12.004
528 28. Therneau TM. A package for survival analysis in S. 2015. Available:
529 https://cran.r-project.org/package=survival
530 29. R Core Team. R: A language and environment for statistical computing. R Foundation
531 for Statistical Computing. Vienna, Austria; 2017.
532 30. Echaubard P, Leduc J, Pauli B, Chinchar VG, Robert J, Lesbarrères D. Environmental
533 dependency of amphibian-ranavirus genotypic interactions: Evolutionary perspectives
534 on infectious diseases. Evol Appl. 2014;7: 723–733. doi:10.1111/eva.12169
535 31. Tétard-Jones C, Kertesz MA, Gallois P, Preziosi RF. Genotype-by-genotype
536 interactions modified by a third species in a plant-insect system. Am Nat. 2007;170:
537 492–499. doi:10.1086/520115
538 32. Brisson JA, Stern DL. The pea aphid, *Acyrthosiphon pisum*: An emerging genomic
539 model system for ecological, developmental and evolutionary studies. BioEssays.
540 2006;28: 747–755. doi:10.1002/bies.20436
541 33. Ogawa K, Miura T. Aphid polyphenisms: Trans-generational developmental regulation
542 through viviparity. Front Physiol. 2014;5 JAN: 1–11. doi:10.3389/fphys.2014.00001
543 34. Vellichirammal NN, Gupta P, Hall TA, Brisson JA. Ecdysone signaling underlies the

pea aphid transgenerational wing polyphenism. Proc Natl Acad Sci. 2017;114: 1419–1423. doi:10.1073/pnas.1617640114

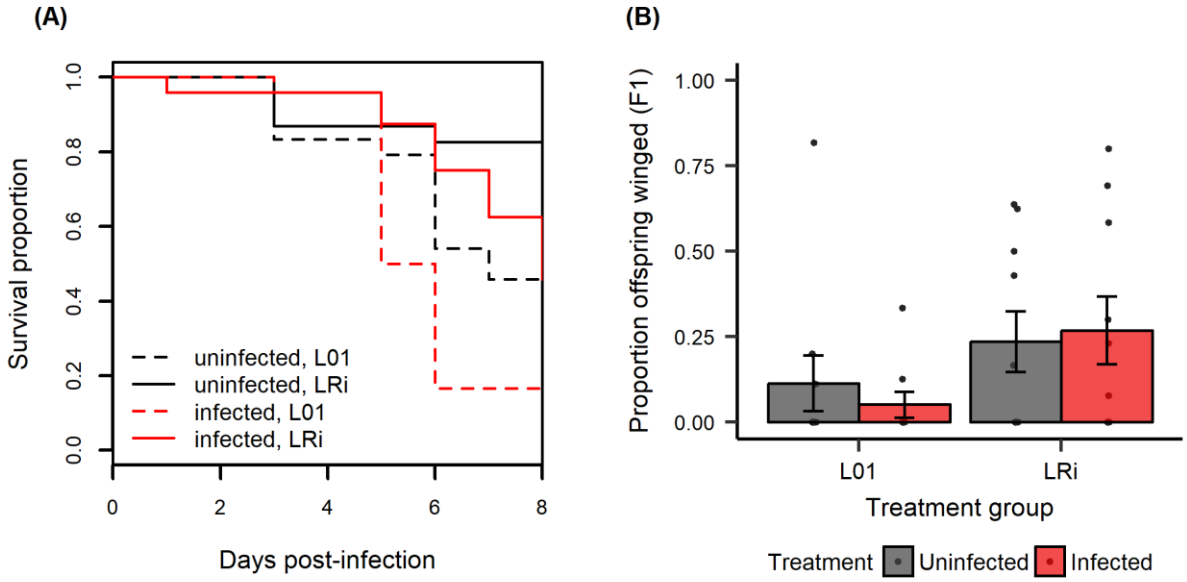
35. Ahmed A, Martin D, Manetti AGO, Han S-J, Lee W-J, Mathiopoulos KD, et al. Genomic structure and ecdysone regulation of the prophenoloxidase 1 gene in the malaria vector *Anopheles gambiae*. Proc Natl Acad Sci. 1999;96: 14795–14800. doi:10.1073/pnas.96.26.14795

36. Sandland GJ, Minchella DJ. Costs of immune defense: An enigma wrapped in an environmental cloak? Trends Parasitol. 2003;19: 571–574. doi:10.1016/j.pt.2003.10.006

37. Lochmiller RL, Vestey MR, Boren JC. Relationship between protein nutritional status and immunocompetence in Northern Bobwhite Chicks. AUK. 1993;110: 503–510. doi:10.2307/4088414

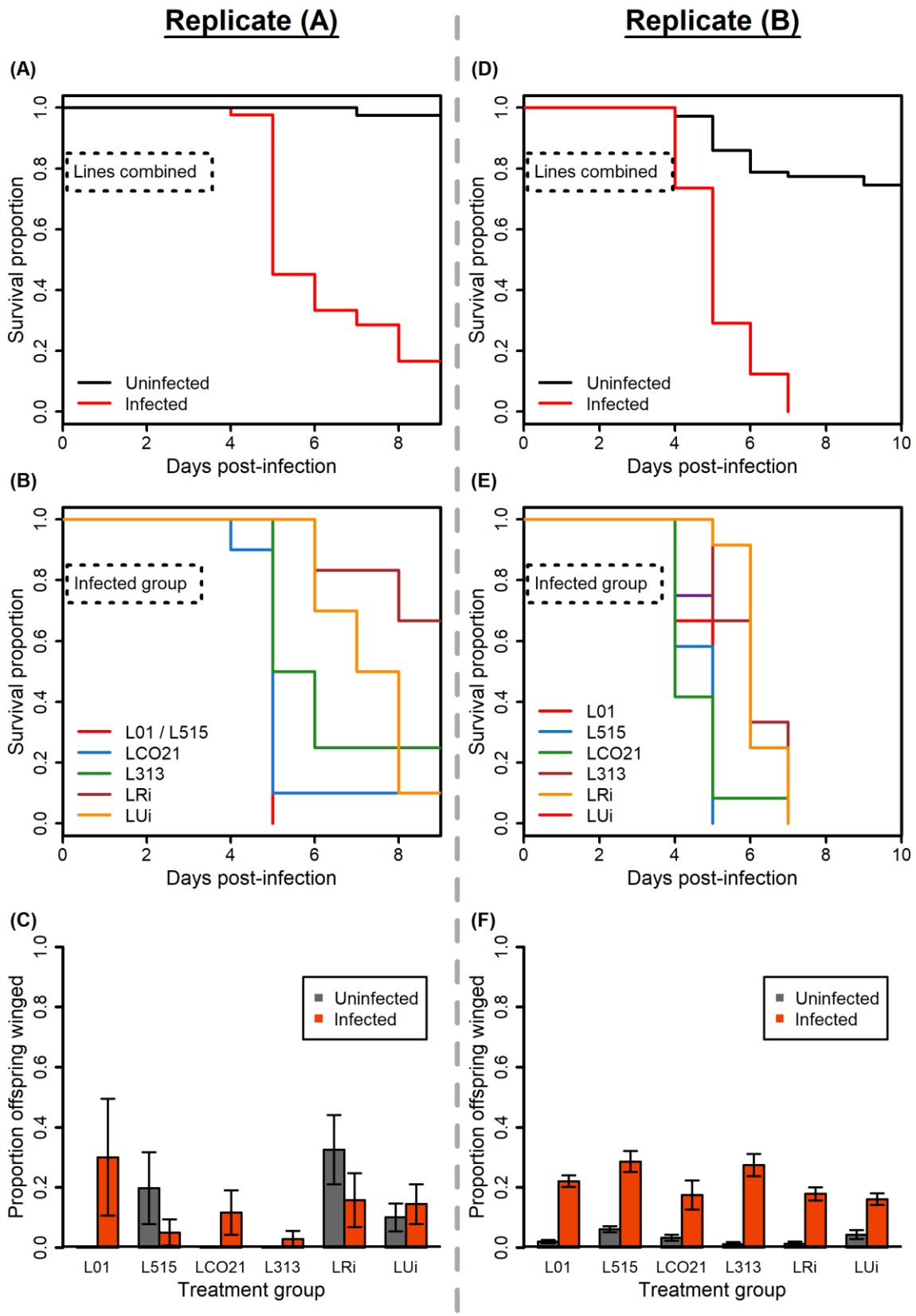
38. Alaux C, Ducloz F, Crauser D, Conte Y Le. Diet effects on honeybee immunocompetence. Biol Lett. 2010;45. doi:10.1098/rsbl.2009.0986

39. Shankar AH, Genton B, Semba RD, Baisor M, Paino J, Tamja S, et al. Effect of vitamin A supplementation on morbidity due to *Plasmodium falciparum* in young children in Papua New Guinea: A randomised trial. Lancet. 1999;354: 203–209. doi:10.1016/S0140-6736(98)08293-2



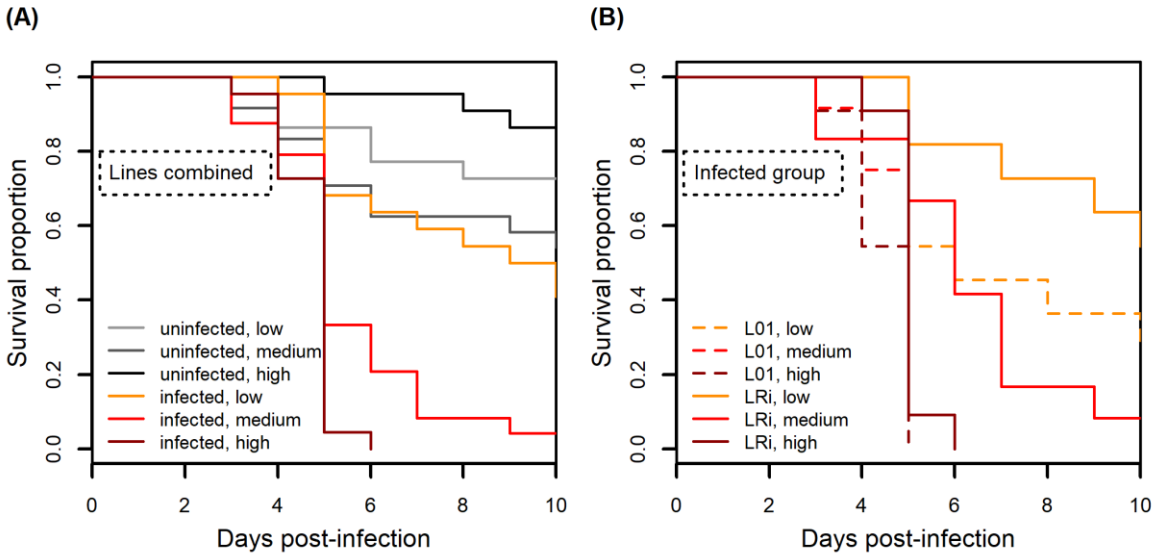
562

563 **Fig 1.**



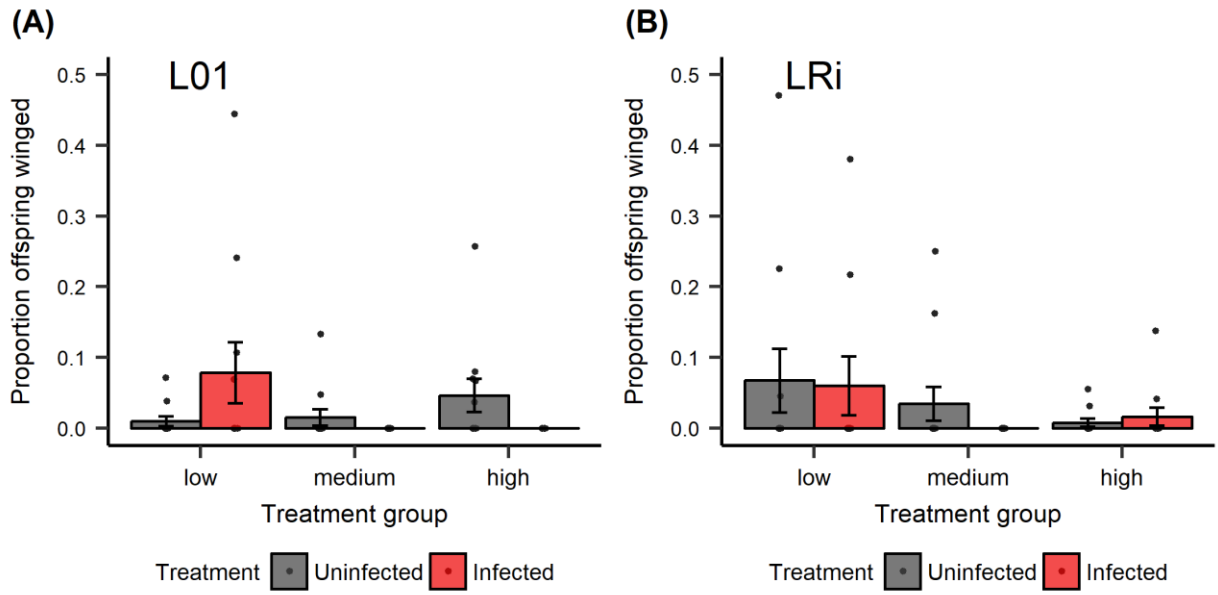
564

565 **Fig 2.**



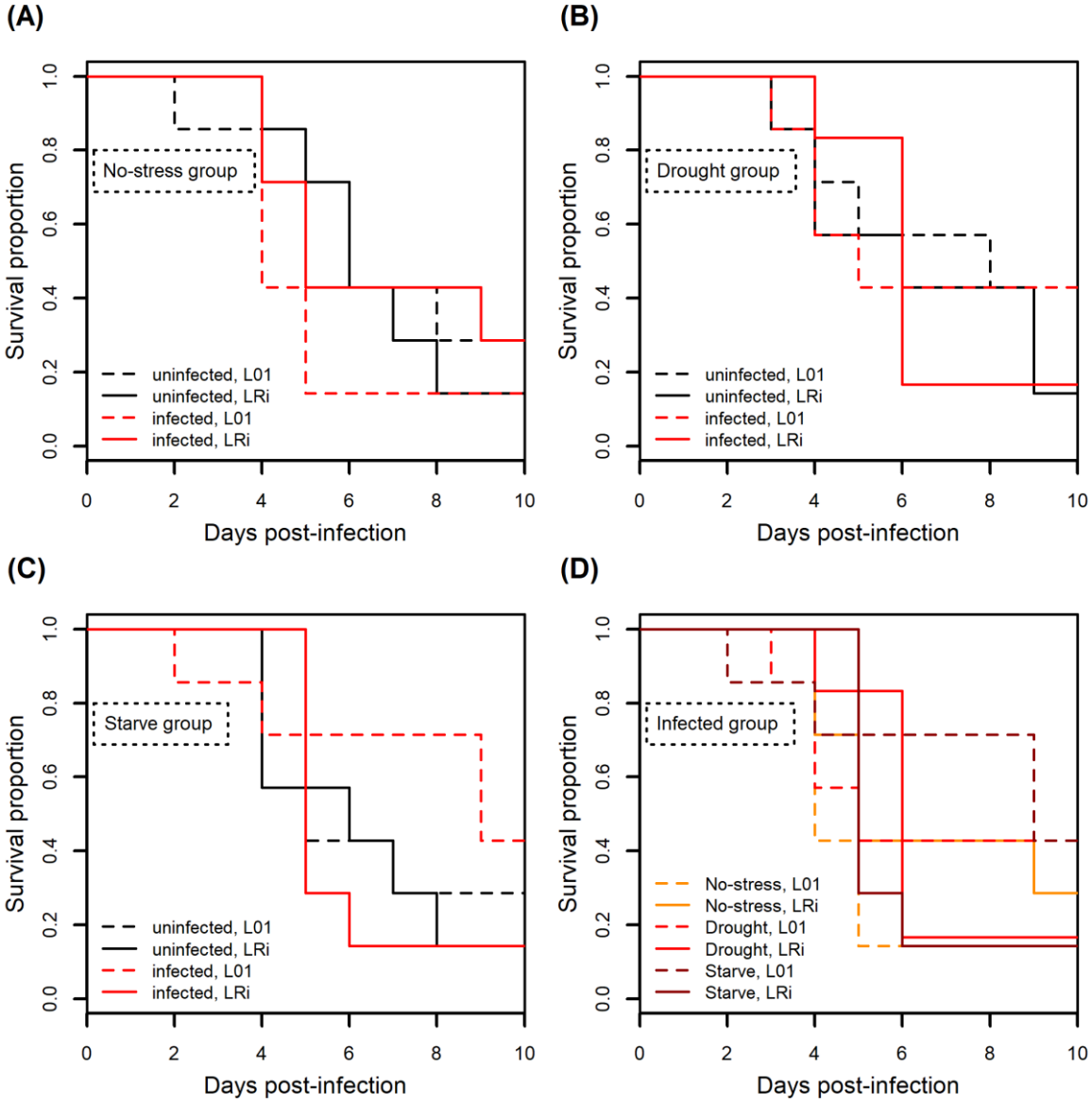
566

567 **Fig 3.**



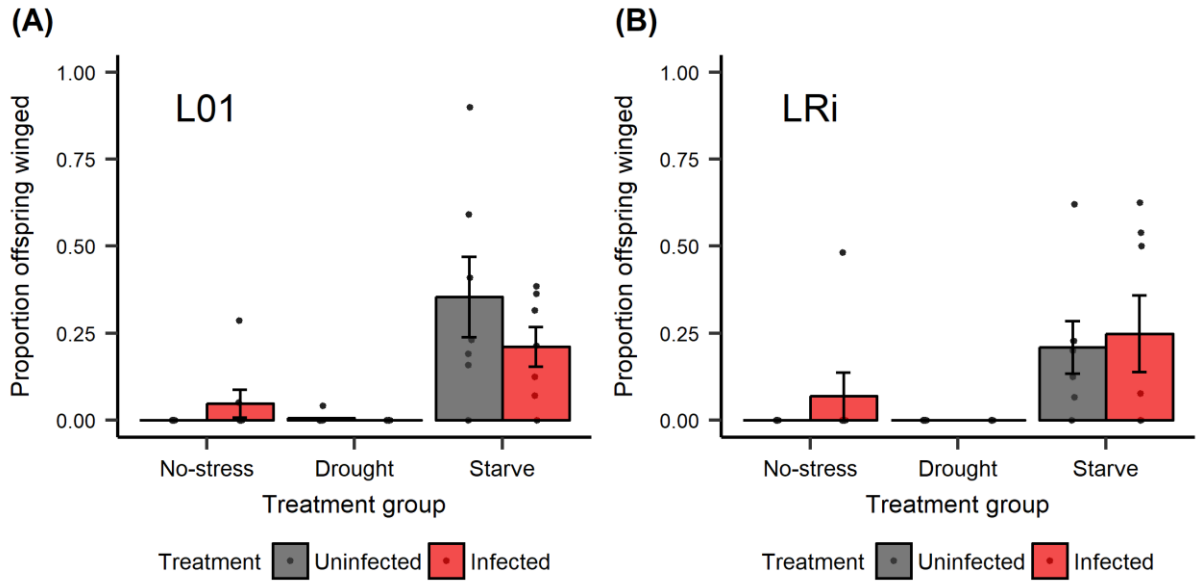
568

569 **Fig 4.**



570

571 **Fig 5.**



572

573 **Fig 6.**