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Intrinsic pre-zygotic reproductive isolation of distantly related pea aphid host races

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File S4. Details of statistical analysis and comparisons within cross types.

Statistical analysis

Statistical analysis was performed in RStudio, with R (R Core Team 2018, version 3.5.1).

Normality tests

We tested normality of residuals using Shapiro-Wilk test [1] by running **shapiro.test** in R. Results are summarized in the table below.

Variable	For all cross types	
	W	P-value
Number of copulations	0.88	22.3E-09
Total copulation time	0.93	2.7E-06
Female survival time after mating	0.92	8.1E-07
Total number of eggs laid	0.88	2.3E-09
Number of eggs with dark serosal cuticle	0.49	< 2.2E-16

Kruskal-Wallis tests

Kruskal-Wallis [2] tests were performed using **kruskal.test** in R. Significant Kruskal-Wallis tests were followed by Dunn's test [3], without correction for multiple testing as we performed small number of planned comparisons (n = 3: "close" vs "same", "close" vs "distant", "same" vs "distant").

Comparison of proportion of trials with observed copulations

The proportion of trials that lead to mating was not lower in "distant" compared to "same" or "close" comparisons $\chi^2 = 0.31, 2\ \text{d.f.}, p = 0.85$, which suggests absence of recognition mechanisms that prevent mating. Tests for equality of proportion was performed using **prop.test** in R, with confidence level 0.95. The alternative hypothesis for the test was that the proportion of copulations is different among the three types of crosses.

The proportions of trials with observed copulations were similar to ones from previous studies, which performed crosses within host races [4,5]. Overall, we performed 296 mating trials, and we did not observe copulation in 153 trials. Out of these, in 55 trials we did not observe physical contact between male and female – this happened mostly due to males staying still during the 90min observation period. In the rest of the trials with no copulations, the contact has been made due to random encounter rather than intentional movement of males towards females and normally did not include mounting attempts. In our preliminary mating trials, we have seen that increasing density of aphids (i.e. placing larger number of aphids into Petri dishes) and providing the inactive males with choice of females (including those which mated successfully previously) did not make them active. Based on these observations, one might speculate about absence of copulation being linked to low male performance. However, firm conclusions require further experiments.

Comparisons within cross types

In our experiment, we were interested to test if the distantly related host races would show more reproductive isolation than the closely related ones – as expected due to absence of hybrids in the field, and strong genetic differentiation of the *Lathyrus* host race. That is why we pooled the data based on the cross type. We also performed "same" crosses, which allow us to study variables related to behavior, survival and eggs without confounding effects of reproductive isolation.

We compared proportions of trials that resulted in mating within the "same" cross type. The proportions were not significantly different ($\chi^2 = 5.3, 2\ \mathrm{d.f.}, p = 0.07$). Thus, the pattern observed (isolation in distant crosses) does not appear to result from less mating willingness of *Lathyrus* specifically. Tests for equality of proportion was performed using **prop.test** in R, with confidence level 0.95. The alternative hypothesis for the test was that the proportion of copulations is different among the three host races.

We also compared the total number of eggs within the "same" crosses. Within the "same" crosses, the number of eggs did not vary significantly between *Vicia, Medicago* and *Lathyrus* females (Kruskal-Wallis Test, $H = 4.56, 2\ \mathrm{d.f.}, p = 0.1$). Thus, our result on decrease of the total number of eggs in "distant" crosses (figure 2d) cannot come from "unwillingness" of *Lathyrus* females to lay eggs on broad bean.

We analyzed the relationship between the mean copulation time and the number of eggs with dark serosal cuticle laid in the "same" group using Spearman correlation (**cor.test** in R). There was no correlation between these variables $r_s = -0.018, p = 0.92$). Thus, longer copulations do not seem to lead to higher number of eggs with dark serosal cuticle.

References:

1. Shapiro SS, Wilk MB. 1965 An Analysis of Variance Test for Normality (Complete Samples). *Biometrika* **52**, 591. (doi:10.2307/2333709)
2. Kruskal WH, Wallis WA. 1952 Use of Ranks in One-Criterion Variance Analysis. *J. Am. Stat. Assoc.* **47**, 583. (doi:10.2307/2280779)
3. Dunn OJ. 1961 Multiple Comparisons Among Means. *J. Am. Stat. Assoc.* **56**, 52. (doi:10.2307/2282330)
4. Sack C, Stern DL. 2007 Sex and Death in the Male Pea Aphid, *Acyrthosiphon pisum* : The Life-History Effects of a Wing Dimorphism. *J. Insect Sci.* **7**, 1–9. (doi:10.1673/031.007.4501)
5. McLean AHC, Ferrari J, Godfray HCJ. 2018 Do facultative symbionts affect fitness of pea aphids in the sexual generation? *Entomol. Exp. Appl.* **166**, 32–40. (doi:10.1111/eea.12641)

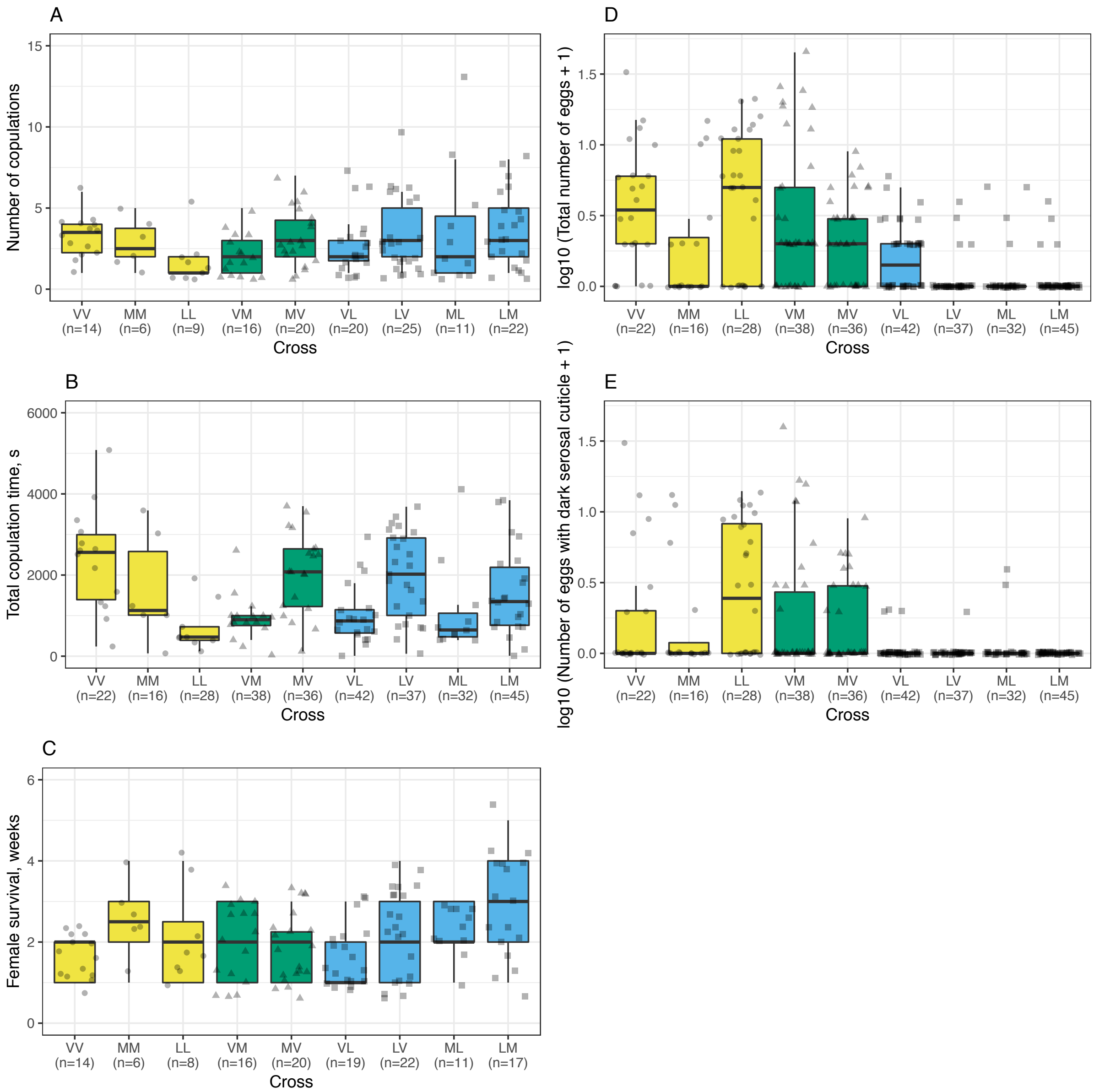

Figure S4.1. Graphical summary of the mating experiment for each cross.

Number of copulations (**A**); Total copulation time (**B**); The number of weeks females survived after the start of the mating experiment (**C**); Total number of eggs per female on Day 35 (**D**); Number of fertile eggs per female on Day 35 (**E**). The boxes denote interquartile range (IQR). Lower whiskers extend to lowest data points $\geq first\ quartile - 1.5 * IQR$. Upper whiskers extend to largest observation $\leq third\ quartile + 1.5 * IQR$. Markers represent individual data points ("same" – circles; "close" – triangles, "distant" – squares). Number in brackets show number of observations. In x-axis labels, the first letter stands for female and the second one for male host race.