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Impacts of Body Colour, Symbionts and Genomic Regions on the Pea Aphid Wing Plasticity Variation

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ABSTRACT

Adaptive phenotypic plasticity describes the phenomenon in which a single genotype can produce a variety of phenotypes that match their environments. Like any trait, plasticity is a phenotype that can exhibit variation, but despite the ecological importance of plasticity variation, little is known about its genetic basis. Here we use the pea aphid to investigate the genetic basis of wing plasticity variation. Previous reports have suggested an ecological association between body coloration and wing plasticity strength in the pea aphid, so we tested the hypothesis that the body colour determination locus (*tor*) associated with wing plasticity variation. We discover that there is no relationship between body colour and wing plasticity in natural populations or in a genetic mapping population. We also localise the *tor* locus to the third autosome, whereas it was previously thought to be on the first autosome, a finding that will be important for future studies of the locus. We find that the presence of the bacterial symbiont *Regiella* is associated with higher levels of wing plasticity. Genome-wide association analysis of wing plasticity variation did not reveal an impact of the *tor* locus, consistent with independence of body colour and wing plasticity. This analysis implicated one possible candidate gene—a Hox gene, *abdominal-A*—underlying wing plasticity variation, although SNPs do not reach the level of genome-wide significance and therefore will require further study. Our study highlights that plasticity variation is complex, impacted by a bacterial symbiont and genetic variation, but not influenced by body colour.

1 | Introduction

Many organisms have evolved the ability to respond to changing environmental conditions by altering development to produce adaptive phenotypes. Such developmental plasticity can be highly advantageous, allowing individuals within populations to adjust to changing environmental circumstances on short, non-evolutionary time scales (Bradshaw 1965; West-Eberhard 2003). Considerable studies have addressed the factors that promote the evolution of plasticity versus genetic adaptation (Moran 1992; Gavrilets and Scheiner 1993; Sultan and Spencer 2002) as well as the potential costs and limits of plasticity (e.g., DeWitt, Sih, and Wilson 1998; Murren et al. 2015). These studies have greatly advanced our understanding of the ecological and evolutionary role of plasticity, especially as a potentially critical phenomenon for buying time in the face of rapidly changing environments (Chevin, Lande, and Mace 2010; Fox et al. 2019; Vinton et al. 2022) or for enabling adaptive processes (Pfennig et al. 2010; Moczek et al. 2011).

Like other traits, plastic traits exhibit genetic variation (e.g., Roff 1996; Lively 1999; West-Eberhard 2003; Ehrenreich and

Pfennig 2016). The sensitivity to environmental cues differs among genotypes, such that some genotypes are highly responsive to the environment and react by producing environment-specific phenotypes, while others are not. Underlying this differential phenotypic response must be allelic variation at environmentally responsive loci, creating gene-by-environment interactions. And, like all phenotypic variation, the underlying genetic variation is subject to natural selection and is critical for adapting a plastic response to its fitness optimum. Despite the importance to studies of evolution and interest from theoretical perspectives (e.g., Hazel, Smock, and Johnson 1990; Leimar and McNamara 2015), how variation in plasticity is generated at the developmental genetic level largely remains an open question. For example, what kinds of genes/genetic pathways and mechanisms are the targets of selection with respect to plasticity? This lack of knowledge represents a significant roadblock to understanding how plastic traits evolve (Schlichting and Smith 2002; Sommer 2020; Ledon-Rettig and Ragsdale 2021).

Here we use the pea aphid (*Acyrthosiphon pisum*) wing plasticity to investigate genetic variation for plasticity strength. Wing plasticity occurs naturally in pea aphids during the spring and summer months. During this time, females reproduce parthenogenetically, producing clonal daughters for multiple generations. Females are viviparous, giving live birth to genetically identical daughters via a modified meiosis that bypasses recombination (Blackman 1987). These females are flightless, lacking wings. When a host plant becomes overcrowded, mothers produce winged daughters which disperse to other host plants (Sutherland 1969). Because the winged and wingless morphs are genetically identical and induced by an environmental cue, this is called wing plasticity.

Previous work has established that there is considerable natural variation for the pea aphid wing plasticity, with some lines producing many winged offspring in response to environmental cues and others very few (e.g., Lamb and MacKay 1979; Grantham et al. 2016; Parker et al. 2021). Some of that work initially focused on the possible influence of body colour on wing plasticity (Lowe and Taylor 1964; Sutherland 1969; Weisser and Braendle 2001). Pea aphids are red or green, controlled by a single locus, the *tor* locus, with red dominant to green (Moran and Jarvik 2010). The red colour results from the action of a carotene desaturase gene, laterally transferred from a fungal origin, producing the red colour (Moran and Jarvik 2010). The colour polymorphism is thought to balance predation and parasitism: red aphids on green plants are easier for visual predators like ladybird beetles to see (Losey et al. 1997), while parasitoid wasps prefer to attack green aphids (Libbrecht, Gwynn, and Fellowes 2007). Multiple studies have found that red pea aphid lines produce more winged offspring in response to high densities (Lowe and Taylor 1964; Sutherland 1969; Weisser and Braendle 2001). High densities, in turn, are associated with increased predation by ladybird beetles (Schellhorn and Andow 2005; Honěk, Martinková, and Štrobach 2018). In this way, wing plasticity and colour are linked at the ecological level, although it is not known if they are linked at the genetic level.

Aphid wing plasticity occurs via a multi-step process from environmental sensing to offspring morph determination (reviewed in Deem et al. 2024) and variation could theoretically accrue anywhere in that process. A pea aphid mother must first sense that her environment is overcrowded. This is reported to be a tactile stimulation response (Sutherland 1969; Müller, Williams, and Hardie 2001), so it likely involves gene products responsible for transmitting mechanosensory information. After environment sensing, the mother must signal to her developing embryos to be winged or wingless, which is mediated by mechanisms including the biogenic amine dopamine (Liu and Brisson 2023) and the ecdysone signalling pathway (Vellichirammal et al. 2017), and the embryos must respond to that signal. Variation could impact any part or multiple parts of this series of events: the mechanosensory sensitivity, the strength of the maternal signal, and/or the sensitivity of the embryonic response.

One potential clue to the mechanistic basis of pea aphid wing plasticity variation can be found in the work of Parker et al. (2021), who examined the maternal transcriptional response to environmental crowding in a high wing plasticity pea aphid line compared to a low plasticity pea aphid line. We found a strong response, in terms of many differentially expressed genes, in the former and a lack of a detectable response in the latter, with zero differentially expressed genes. This result indicated that the low plasticity line was likely unable to sense the high density of its environment and/or the cue could not be acted upon, and therefore was not mounting a plasticity response. It is likely, therefore, that genes responsible for environmental sensing, for example, genes with mechanosensory neuronal functions, may harbour the genetic variation for plasticity in this system.

Here, we explored factors that may impact wing plasticity variation. To do this, we used plasticity variation among 178 unique pea aphid lines [phenotype assayed in Parker and Brisson (2019)] along with the genotyping data on these lines found in Chung et al. (2020). We performed genome-wide association studies (GWAS) to independently map red/green colour variation and wing plasticity variation to investigate their potential genetic linkage, within the wider context of studying the evolution of plasticity using the pea aphid system.

2 | Materials and Methods

2.1 | Pea Aphids

Pea aphids were collected from two alfalfa fields separated by ~800 m in Ithaca, NY. Unique genotypes were selected for further analysis (Chung et al. 2020). We determined the body colour of a line by visual inspection. Colour data is available in Table S1.

2.2 | Wing Plasticity Phenotyping

Two replicates of a standardised crowding assay (Grantham et al. 2016; Vellichirammal, Madayiputhiya, and Brisson 2016) were carried out as detailed and reported in Parker and Brisson (2019). Briefly, we reared aphids on fava bean plants (*Vicia faba*) at low densities (< 7 individuals per plant) for

three generations. We then placed adult aphids that had begun reproducing in 3.5 cm Petri dishes for 24 h at a density of 12 aphids per dish. Aphids were then placed on a fava bean plant for a further 24 h and allowed to reproduce, and the percentage of winged offspring produced during this period was recorded. The average offspring winged percentage from the two replicates was used for analyses. Note that most pea aphid lines produce very few offspring under low-density conditions on plants (e.g., see data in Grantham et al. 2016), so using the per cent winged offspring produced under crowded conditions is a proxy for the difference in winged offspring percentages produced under these two conditions. Wing plasticity data is available in Table S1.

2.3 | Symbiont Detection

To assess their potential involvement in wing plasticity variation, we used data on bacterial symbiont presence/absence as assessed in Chung et al. (2020). Briefly, 12-μl reactions included 40 ng DNA, 10 μM primers and Master Mix Quick-Load Taq (New England BioLabs), with the 'Touchdown' PCR cycle: 94°C for 2 min; 11 cycles of 94°C for 20 s, 56°C (declining by 1°C in each cycle) for 50 s and 72°C for 30 s; 25 cycles of 94°C for 2 min, 45°C for 50 s, and 72°C for 2 min; and a final extension of 72°C for 5 min. Amplification products were separated by electrophoresis in a 2% agarose gel and visualised with SYBR Safe DNA Gel Stain (Invitrogen). Primer sequences are provided in Table S1 of (Chung et al. 2020). Symbiont presence or absence is available in Table S1.

2.4 | Restriction Fragment Length Polymorphism (RFLP) Analysis

To test if body colour associated with two different autosomal regions, we used RFLP analysis. We designed primers that amplified two different SNPs resulting in restriction enzyme cut site differences. The primers for the amplicon on autosome 1 are AAGATACACGAGACGACAATGG and AGTGTC CAAATCGATCACCTCC, which cuts with MspI (New England BioLabs), and for the amplicon on autosome 3 are CACTCTACAACAGTTCTCGTCG and ATCGGATTTGGAA ACTGTGTGG, which cuts with HpyCH4V (New England BioLabs). DNA from individual backcross progeny was amplified with both primer sets separately with a standard PCR reaction with a 55°C anneal, then cut for 1 h with the relevant enzyme, and run on a 3% gel for visualisation.

2.5 | Genotype by Sequencing (GBS) Analysis

gDNA was extracted from 10 aphids for each line. GBS was conducted according to the procedure given by (Elshire et al. 2011). Briefly, the purified DNA was digested with the methylation-sensitive restriction enzyme *Ape*KI (New England BioLabs) at 75°C for 2 h, and the fragments were ligated with barcode adaptors, and then amplified, purified and sequenced on an Illumina HiSeq platform as 100-bp single-end reads.

Raw GBS reads were retrieved from PRJNA497124 (Chung et al. 2020) and were demultiplexed and trimmed of barcodes using Sabre (github.com/najoshi/sabre). Reads were aligned to the pea aphid reference genome (v3.0, Li et al. 2019) with default settings using bowtie2 (v2.4.5, Langmead and Salzberg 2012). SAM files were sorted using samtools v1.15.1 and reads were piled using bcftools mpileup (Li 2011). Calls were made using bcftools call with the '-m' flag for a multiallelic calling model. Called SNPs were filtered using vcftools for a minimum Phred score of 20. Genotypes were sorted using the TASSEL5 'SortGenotypeFilePlugIn' (v5.2.80, Glaubitz et al. 2014).

2.6 | Linkage Disequilibrium (LD) Analysis

We used PopLDdecay (Zhang et al. 2018) to calculate LD decay across the pea aphid genome. LD data are available in Table S2. LD pruning was conducted using PLINK 1.9 (Chang et al. 2015) using a window size of 50, step size of 1, and an r^2 threshold of 0.75 (Gusareva and Van Steen 2014; Joiret et al. 2019). LD pruning yielded 22,217 SNPs.

2.7 | Genome-Wide Association Study

Genome-wide association analyses were carried out in TASSEL5 (v5.2.80) and filtered for site minimum count of 90% of samples and site minor allele frequency of 0.05. After filtering, 31,991 sites remained for downstream analyses.

Compressed mixed linear models (MLM) with the optimal grouping setting were used for all GWA analyses in TASSEL5. Population structure was estimated in TASSEL5 using principal component analysis (PCA) on genetic data with the first five principal components included as fixed effect covariates in the MLM. A kinship matrix was determined in TASSEL5, using the centred IBS setting, and included as a covariate in the MLM to account for cryptic relationships among SNPs. Genetic marker data was included as a fixed effect. Genetic and residual variance for each trait and marker were estimated using the P3D method in TASSEL5.

For the body colour GWA, body colour was assigned as the sole phenotype data ($n = 182$). For the wing plasticity GWA, average per cent wingedness was assigned as the primary trait data ($n = 182$), with *Regiella* status as a cofactor ($n = 178$). In each analysis, population structure and kinship matrices were included as covariates as listed above.

MLMs run in TASSEL5 can be described in Henderson's matrix notation as $Y = X\beta + Zv + \varepsilon$; where Y is a vector of phenotypic observations, X and Z are known design matrices, β is a vector with fixed effects (marker and population structure), v is a vector with random additive genetic effects, and ε is a vector of random residual effect (Henderson 1975; Bradbury et al. 2007). Manhattan plots and q-q plots were created in R (v2023.06.0 + 421) using the 'qqman' package (Turner 2018). Genome-wide significance threshold for SNPs was determined using Bonferroni correction ($-\log_{10}(0.05/31{,}991)$).

3 | Results

3.1 | Association Between Body Colour and Wing Plasticity

To investigate any potential linkage between body colour and wing plasticity, we examined if these phenotypes were associated. As noted in the Introduction, red lines have a hypothesized stronger response to wing-inducing cues (Lowe and Taylor 1964; Sutherland 1969; Weisser and Braendle 2001). However, using our 182 phenotyped lines from a natural population, we found no significant difference in the wing plasticity response strength between red and green lines (Figure 1A; Mann–Whitney U test, $p = 0.09$). We then asked this same question using individuals from a laboratory cross between a green and red line (Driscoll, Liu, McDonough, Schmidt, and Brisson (in press)). In this cross, the red parent exhibited a strong plasticity response, while the green parent did not. Among the backcross progeny, we observed no wing plasticity differences between red and green lines (Figure 1B; Mann–Whitney U test, $p = 0.86$). We conclude that there is no linkage between body colour and plasticity response, either as displayed through linkage disequilibrium in natural populations (Figure 1A) or in the large linkage blocks generated in our laboratory cross (Figure 1B).

3.2 | Linkage Disequilibrium in the Pea Aphid

Before we performed genome-wide association analyses, we first updated the genotype-by-sequencing data previously used by Chung et al. (2020) by mapping raw reads to a more recent

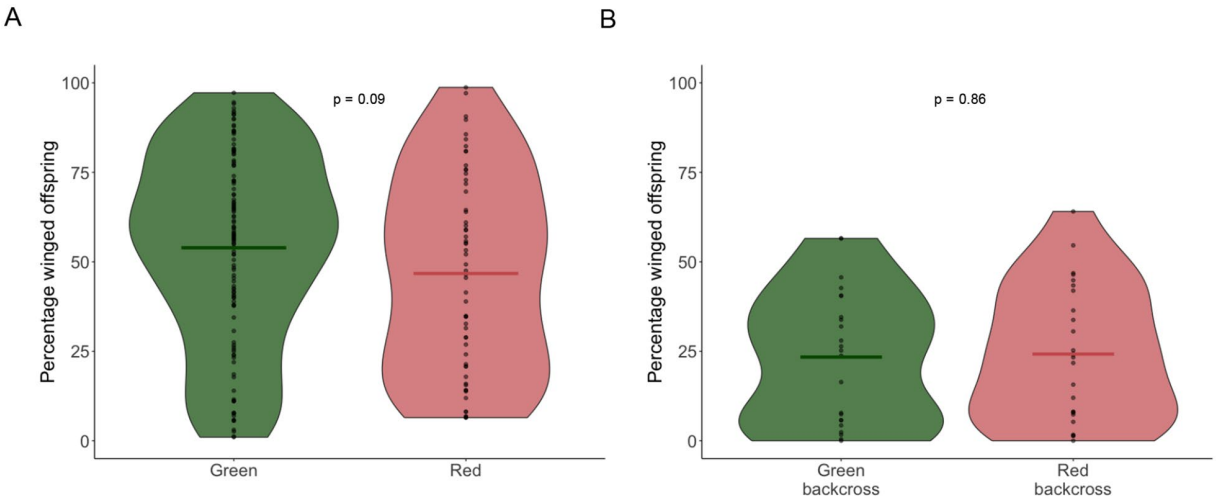

FIGURE 1 | Colour does not impact the wing plasticity response. (A) Colour phenotype of 182 unique lines from natural populations, with no difference in mean plasticity response as measured by the percentage of winged offspring following maternal high-density treatment. (B) Backcross progeny in a lab-generated cross between a red, high plasticity line and a green, low plasticity line shows no linkage between colour and plasticity variation ($n = 49$). p-Values for Mann–Whitney U tests are shown for each panel. [Colour figure can be viewed at wileyonlinelibrary.com]

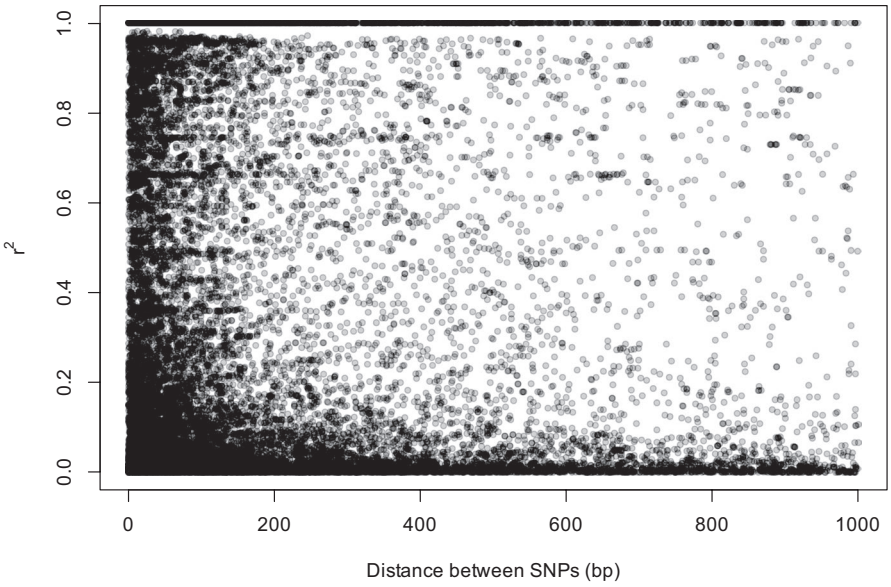

FIGURE 2 | Linkage disequilibrium levels in the pea aphid fall quickly within 1000 bp. Shown is the correlation (r^2) between every two SNPs and their corresponding distance in base pairs (bp) for all SNPs within a 1 kb window as calculated by PopLDdecay (Zhang et al. 2018).

version of the pea aphid genome [v3.0 (Li et al. 2019)]. Our analysis identified 31,991 single-nucleotide polymorphisms (SNPs), which averaged to one SNP every 16.2 kb across the ~517 Mb genome. To understand the extent of genomic variation we captured with this SNP density, we calculated linkage disequilibrium among the lines using the pairwise correlation (r^2) between any two SNPs. We found that r^2 falls quickly within 1 kb (Figure 2), and each SNP in our dataset is not, on average, in strong linkage disequilibrium with neighbouring SNPs.

3.3 | Genome-Wide Association Analysis of Body Colour

We performed GWAS of red/green body colour to find the location of the *tor* locus insertion which causes the red colour (Moran and Jarvik 2010). This locus is not assembled into the chromosome-level contigs for the pea aphid genome v3.0 but rather is on an unassembled 59 kb contig (ID: NW_021771109.1). Mandrioli et al. (2016) previously suggested an autosome 1 placement for the *tor* locus based on cytogenetic analysis. Our GWAS using 182 pea aphid lines identified a single highly significant peak of associated SNPs on autosome 3 that spanned about 0.5 Mb (Figure 3A, Table S3). We observed no SNPs on autosome 1 that were significantly associated with body colour differences. We also did not observe a significantly associated SNP on the scaffold containing the *tor* locus (NW_021771109.1), although closer examination revealed that the only sequencing reads that matched the scaffold came solely from red aphids. The quantile-quantile plot corresponding to the observed versus expected *p*-values for these data indicated low Type I errors (Figure 3B).

To ask if the *tor* locus was located on autosome 1 or 3, we used the laboratory cross mentioned above that we generated between a green and a red line (Driscoll, Liu, McDonough, Schmidt, and Brisson (in press)). From the F1 progeny of that cross, we produced backcross progeny to the green line. We then designed and assayed restriction fragment length polymorphism (RFLP) markers near the previously reported *tor* location on autosome 1 (Mandrioli et al. 2016; RFLP primers target a SNP at position 109,101,444) and our discovered peak on autosome 3 (RFLP primers target a SNP at position 26,668,175). The RFLP marker on autosome 1 showed no association with red/green colour ($n = 48$, Fisher's exact test, $p = 0.53$; Table S4) in backcross progeny while the marker on autosome 3 showed complete association ($n = 47$, Fisher's exact test, $p < 0.001$; Table S4). We conclude that the *tor* locus is within the peak at autosome 3, and not on autosome 1 as previously thought.

3.4 | Association Between Bacterial Symbionts and Wing Plasticity

We next turned our attention to wing plasticity variation as a phenotype. Data on the wing plasticity for our 182 focal lines was previously generated by Parker and Brisson (2019). As shown in that paper, pea aphids from this single population display a full range of phenotypic variation from near 0%–100% of winged offspring produced in response to a maternal crowding treatment. Before performing a GWAS on wing plasticity, we first considered a factor thought to impact wing plasticity in natural populations: the presence of bacterial symbionts. Pea aphids can harbour a variety of facultative bacterial symbionts that convey fitness costs and benefits (Renoz 2024), including influencing the wing plasticity response (Leonardo and Mondor 2006).

Chung et al. (2020) previously assayed the focal pea aphid lines for the presence of seven bacterial symbionts, detecting five of them (*Regiella*, *Hamiltonella*, *Spiroplasma*, X-type and *Ricketsia*) in at least two lines. *Rickettsiella* was one of the symbionts that was not detected. This is important because *Rickettsiella* is known to change aphid body colour from red to green (Tsuchida et al. 2010). Because it was not detected in these lines, it is not a confounding factor in our analyses of body colour differences. Of the five detected symbionts, lines carrying *Regiella* showed a significantly higher (Mann–Whitney U test; $p = 0.02$) wing plasticity compared to those without *Regiella* (Figure 4), as reported in Reyes et al. (2019). All other symbionts showed no effect on the wing plasticity.

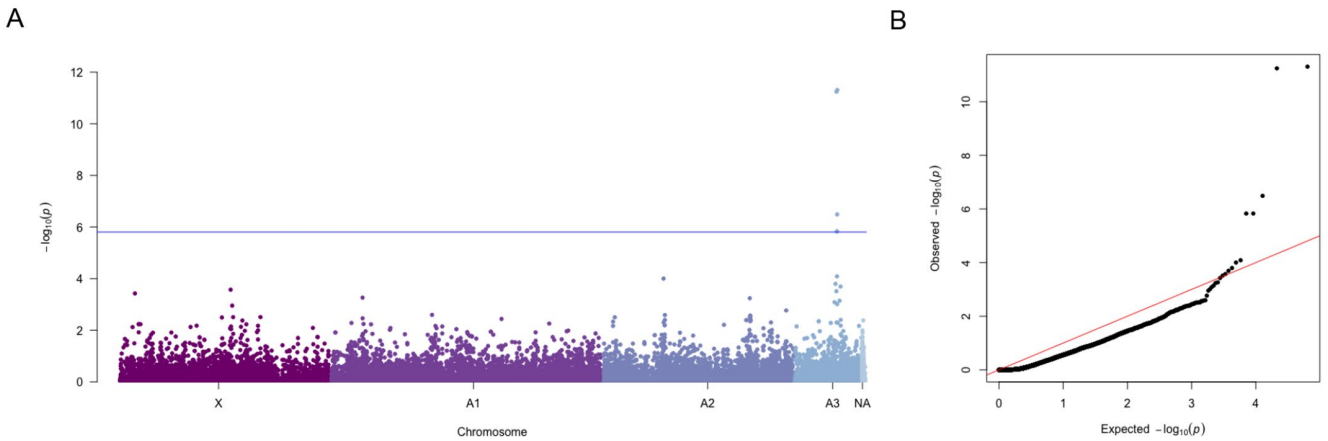

FIGURE 3 | A single genomic region contains SNPs that have highly significant associations with red/green colour differences. (A) GWAS identifies a single, highly significant peak on the third autosome, A3. The blue horizontal line indicates the genome-wide significance cut-off. (B) Quantile-quantile plot of observed to expected *p*-values for SNP/phenotype associations shows a general underestimation of observed p-values. [Colour figure can be viewed at wileyonlinelibrary.com]

3.5 | Genome-Wide Association Analysis of Wing Plasticity Strength

We performed a GWAS between SNPs and the wing plasticity phenotype, while controlling for the presence or absence of *Regiella*. We were able to use information from 178 genotypes for this analysis, as *Regiella* data was missing from four of the lines with plasticity phenotype data. Unlike the body colour GWAS, we did not observe a single, highly significant SNP peak. Indeed, we discovered no SNPs that rose above the genome-wide level of significance (Figure 5A), and an accompanying quantile-quantile plot showed linear correspondence between observed and expected p-values, indicating a roughly normal distribution of *p*-values and no strong outliers. (Figure 5B). Notably, we observed no SNPs at the body colour locus emerging from this analysis.

Two SNPs, however, neared the level of significance: scaffold NC_042494.1, positions 144,389,817 and 144,389,827 on autosome 1 (Table S5). They are in complete linkage, so their p-values are identical (1.7×10^{-5}). They fall within an intergenic region that is upstream of *abdominal-A* (*abd-A*) and downstream of *Abdominal-B* in the pea aphid's single Hox complex. They are, therefore, labelled 'abd-A' in Figure 5A. Again, these SNPs do not rise above the level of significance, but we observed that they are always the most significant SNPs from our GWAS analyses, regardless of how we vary filters for minor allele frequency or representation at sites, if we use linkage disequilibrium pruning, or if we vary the GWAS model to include colour (see data in Table S6).

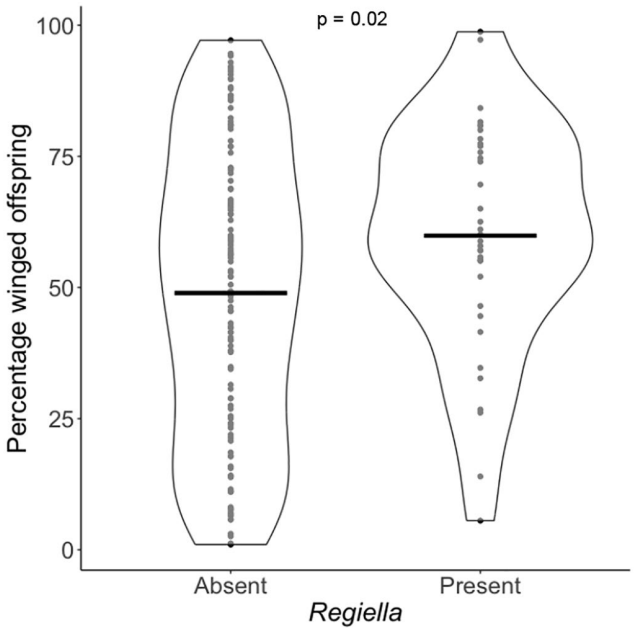

FIGURE 4 | The presence of the symbiont *Regiella* impacts the wing plasticity. Across all natural lines, those with *Regiella* have significantly higher offspring wing percentages when crowded ($n = 178$). *p*-Value for a Mann–Whitney U test is shown.

4 | Discussion

The wing plasticity is tightly tied to the ecology of aphids, given that it results in morphs that trade-off fecundity for dispersal as in most wing dimorphic insects (Zera and Denno 1997). By producing more wingless morphs, a clone can maximise reproduction but will quickly overwhelm a host plant, necessitating the production of dispersing winged morphs. But if too many winged morphs are produced, overall clonal fecundity will decrease. The propensity to produce winged or wingless morphs, that is, the plasticity strength, is therefore closely tied to clonal fitness (Weisser and Stadler 1994). Our study investigated factors relevant to the strength of the wing plasticity response and revealed a number of important insights.

In contrast to previous studies which worked with a relatively small number of pea aphid lines (Lowe and Taylor 1964; Sutherland 1969; Weisser and Braendle 2001), we did not find any evidence for a link between body colour and the wing plasticity response in our comparably large number of lines. Our GWAS does, however, provide a location for the pea aphid *tor* locus that

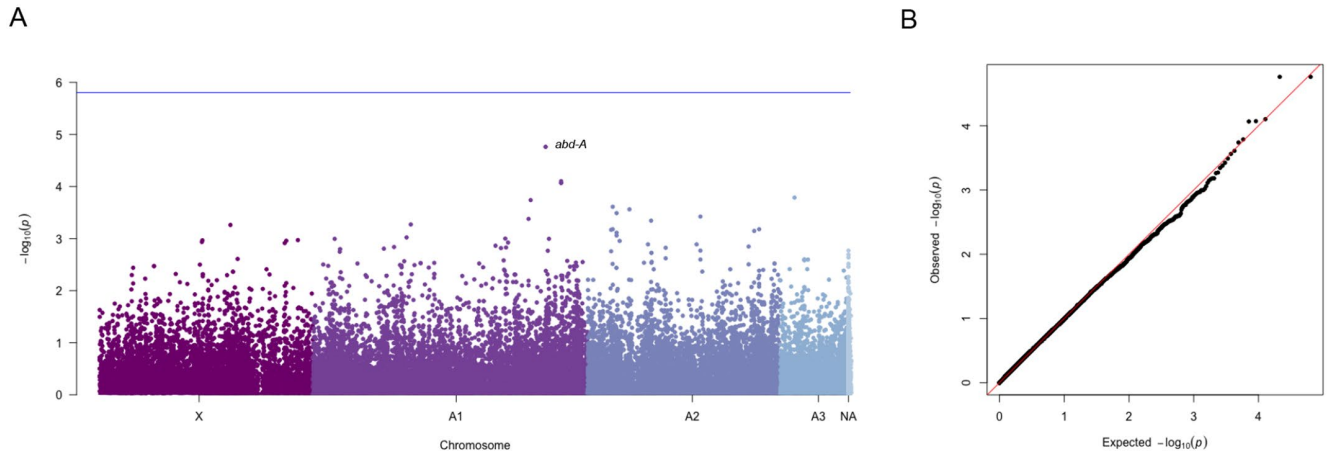

FIGURE 5 | Results of GWAS for the wing plasticity phenotype, controlling for presence/absence of *Regiella*. (A) Individual SNPs and their associated significance value, with the genome-wide significance threshold shown as a horizontal line. The strongest associated SNPs across analyses, in an intergenic region upstream of *abdominal-A* (*abd-A*), are labelled (two SNPs 10 bp apart with identical significance values). (B) Quantile–quantile plot of observed to expected p-values for SNP/phenotype associations showed a general concordance. [Colour figure can be viewed at wileyonlinelibrary.com]

may be useful for future studies investigating aphid body colour genetics and its co-variation with other ecologically relevant phenotypes. To determine if other pea aphid genome assemblies were able to integrate the *tor* region, we searched two other long-read pea aphid genome assemblies [the winged and wingless male-specific assemblies of (Li et al. 2020); another assembly (Mathers et al. 2020) sequenced a green aphid and therefore that genome does not have the *tor* insertion]. In both cases, we were able to find the *tor* locus, but the assembled regions were relatively short (< 50 kb) and did not agree with one another. It is likely, therefore, that the *tor* locus is flanked by highly repetitive regions that make assembly into the larger genome challenging.

We did observe that pea aphid lines carrying the bacterial symbiont *Regiella* had a stronger wing induction response compared to those without (Figure 4). *Regiella* protects aphids from aphid-specific fungal pathogens (Scarborough, Ferrari, and Godfray 2005; Parker et al. 2013). The impact of *Regiella* on wing plasticity result is in line with another study showing that lines cured of *Regiella* produced fewer winged offspring (Reyes et al. 2019) but contradicts earlier studies showing the opposite effect (Leonardo and Mondor 2006; Liu, Lei, and Chen 2019). These contrasting results may be due to different *Regiella* genotypes, which can have different effects on the wing plasticity (Reyes et al. 2019). We did not assay for the *Regiella* genotypes in our pea aphid population, so whether or not the *Regiella* genotype differentially affected the wing plasticity remains unknown. Also unknown is the mechanism by which *Regiella* impacts the wing plasticity phenotype.

We implicated a potential candidate gene for wing plasticity variation, with the strong caveat that it approached but did not reach the level of genome-wide significance: a Hox gene, *abd-A*. The relevant SNPs at *abd-A* are upstream of the gene's transcription start site. In *Drosophila melanogaster*, *abd-A* is within the Bithorax complex, which contains the genes *Ultrabithorax (Ubx)*, *abd-A*, and *Abd-B*; these genes control thoracic and abdominal segmental identity (Duncan 1987; Peifer, Karch, and Bender 1987; Martin et al. 1995). The region upstream of *abd-A* in *Drosophila* houses an extensive amount of sequence containing cis-regulatory elements that control the expression of both *abd-A* and *Ubx* (Bender et al. 1983; Duncan 1987), so it is possible that the SNP we have identified is part of a regulatory unit that impacts expression of *abd-A*, *Ubx*, or even both. *Ubx* has an important role in wing development, including repression of the *wingless* gene in haltere formation (Weatherbee et al. 1998), and the pea aphid's *wingless* gene homologue is necessary for wing formation (Zhou et al. 2023). We hypothesize that this genomic region might contribute to the embryonic response to the mother's signal to be winged or wingless, setting up that embryo to either develop or not develop wings as an adult. Nucleotide variation within an enhancer or silencer region could mediate this response by activating or inhibiting *Ubx* expression in relevant cells. This finding warrants further investigation with functional studies.

The lack of finding a single, highly significant locus for wing plasticity variation indicates that the variation that we have measured is likely caused by several loci of relatively small effect and/or epistatic interaction among loci. Our results are consistent with the continuous range of plasticity variation that was observed in this pea aphid population (Parker and Brisson 2019), and with small effect variants that commonly contribute to variation in complex phenotypes (Rockman 2012). This result is also consistent with results from the pea aphid mapping study mentioned above, which examined wing plasticity via a cross between a high and low plasticity line (Driscoll, Liu, McDonough, Schmidt, and Brisson (in press)) and found that variation was likely due to multiple loci.

Parker and Brisson (2019) previously found that two laterally transferred viral genes were expressed at higher levels in response to crowding, specifically in lines with strong versus weak plastic responses (subsets of the lines used here). We did not find any SNPs at these two loci (Apns1: ACYPI085607 autosome 2, position 116.3 Mb and ACYPI36509: unplaced scaffold NW_021769898, position 7.5 kb) that were associated with plasticity variation. Upon close examination of SNPs in these two regions, we noted that the closest marker to each was 20 kb and 8 kb away, respectively. If these genes are associated with plasticity variation in this pea aphid population, we were unlikely to detect them given the levels of linkage disequilibrium at these distances (see Figure 2).

Overall, here we have discovered that both non-genomic (a bacterial symbiont) and genomic sources likely impact the ecologically important trait of wing plasticity variation. Our study joins others showing that plasticity variation is complex (Mackay and Lyman 2005; Lafuente, Duneau, and Beldade 2018; Ørsted et al. 2018). In the future, whole or nearly whole genome sequencing will be necessary to provide the density of SNPs required, given the very low levels of linkage disequilibrium across the genome (Figure 2). Also, a much larger sample size is crucial for detecting genes of smaller effect (Wray et al. 2013), as well as epistatic interactions (Gauderman 2002).

Author Contributions

L.E.G., R.M.H.D., B.J.P., and J.A.B. conceived of the study; L.E.G., R.M.H.D., and B.J.P. performed the experiments; L.E.G., R.M.H.D., B.J.P., and J.A.B. analysed the data; L.E.G., R.M.H.D., and J.A.B. wrote the paper, and B.J.P. contributed to revisions.

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Data Availability Statement

Raw sequence reads and associated metadata are deposited in the SRA (BioProject PRJNA497124). Other data is available in the Supporting Information.

Benefit-Sharing Statement

The benefits of this research stem from the sharing of our data and findings through public databases, as described above.

Conflicts of Interest

The authors declare no conflicts of interest.

References

Bender, W., M. Akam, F. Karch, et al. 1983. "Molecular Genetics of the Bithorax Complex in *Drosophila melanogaster*." *Science* 221: 23–29.

Blackman, R. L. 1987. "Reproduction, Cytogenetics and Development." In *Aphids: Their Biology, Natural Enemies & Control*, edited by A. K. Minks and P. Harrewijn, 163–195. Amsterdam: Elsevier.

Bradbury, P. J., Z. Zhang, D. E. Kroon, T. M. Casstevens, Y. Ramdoss, and E. S. Buckler. 2007. "TASSEL: Software for Association Mapping of Complex Traits in Diverse Samples." *Bioinformatics* 23: 2633–2635.

Bradshaw, A. D. 1965. "Evolutionary Significance of Phenotypic Plasticity in Plants." *Advances in Genetics* 13: 115–155.

Chang, C. C., C. C. Chow, L. C. Tellier, S. Vattikuti, S. M. Purcell, and J. J. Lee. 2015. "Second-Generation PLINK: Rising to the Challenge of Larger and Richer Datasets." *GigaScience* 4: 7.

Chevin, L.-M., R. Lande, and G. M. Mace. 2010. "Adaptation, Plasticity, and Extinction in a Changing Environment: Towards a Predictive Theory." *PLoS Biology* 8: e1000357.

Chung, S. H., B. J. Parker, F. Blow, J. A. Brisson, and A. E. Douglas. 2020. "Host and Symbiont Genetic Determinants of Nutritional Phenotype in a Natural Population of the Pea Aphid." *Molecular Ecology* 29: 848–858.

Deem, K. D., L. Gregory, X. Liu, O. Saleh Ziabari, and J. A. Brisson. 2024. "Evolution and Molecular Mechanisms of Wing Plasticity in Aphids." *Current Opinion in Insect Science* 61: 101142.

DeWitt, T. J., A. Sih, and D. S. Wilson. 1998. "Costs and Limits of Phenotypic Plasticity." *Trends in Ecology & Evolution* 13: 77–81.

Driscoll, R. M. H., X. Liu, J. McDonough, J. Schmidt, and J. A. Brisson. (in press). "Pea Aphid Wing Plasticity Variation has a Multigenic Basis."

Duncan, I. M. 1987. "The Bithorax Complex." *Annual Review of Genetics* 21: 285–319.

Ehrenreich, I. M., and D. W. Pfennig. 2016. "Genetic Assimilation: A Review of Its Potential Proximate Causes and Evolutionary Consequences." *Annals of Botany* 117: 769–779.

Elshire, R. J., J. C. Glaubitz, Q. Sun, et al. 2011. "A Robust, Simple Genotyping-By-Sequencing (GBS) Approach for High Diversity Species." *PLoS One* 6: e19379.

Fox, R. J., J. M. Donelson, C. Schunter, T. Ravasi, and J. D. Gaitán-Espitia. 2019. "Beyond Buying Time: The Role of Plasticity in Phenotypic Adaptation to Rapid Environmental Change." *Philosophical Transactions of the Royal Society, B: Biological Sciences* 374: 20180174.

Gauderman, W. J. 2002. "Sample Size Requirements for Association Studies of Gene–Gene Interaction." *American Journal of Epidemiology* 155: 478–484.

Gavrilets, S., and S. M. Scheiner. 1993. "The Genetics of Phenotypic Plasticity. VI. Theoretical Predictions for Directional Selection." *Journal of Evolutionary Biology* 6: 49–68.

Glaubitz, J. C., T. M. Casstevens, F. Lu, et al. 2014. "TASSEL-GBS: A High Capacity Genotyping by Sequencing Analysis Pipeline." *PLoS One* 9: e90346.

Grantham, M. E., C. J. Antonio, B. R. O'Neil, Y. Z. Zhan, and J. A. Brisson. 2016. "A Case for a Joint Strategy of Diversified bet Hedging and Plasticity in the pea Aphid Wing Polyphenism." *Biology Letters* 12: 20160654.

Gusareva, E. S., and K. Van Steen. 2014. "Practical Aspects of Genome-Wide Association Interaction Analysis." *Human Genetics* 133: 1343–1358.

Hazel, W. N., R. Smock, and M. D. Johnson. 1990. "A Polygenic Model for the Evolution and Maintenance of Conditional Strategies." *Proceedings of the Royal Society B* 242: 181–187.

Henderson, C. R. 1975. "Best Linear Unbiased Estimation and Prediction Under a Selection Model." *Biometrics* 31: 423–447.

Honěk, A., Z. Martinková, and J. Štrobach. 2018. "Effect of Aphid Abundance and Urbanization on the Abundance of *Harmonia axyridis* (Coleoptera: Coccinellidae)." *European Journal of Entomology* 115: 703–707.

Joiret, M., J. M. Mahachie John, E. S. Gusareva, and K. Van Steen. 2019. "Confounding of Linkage Disequilibrium Patterns in Large Scale DNA Based Gene–Gene Interaction Studies." *Biodata Mining* 12: 11.

Lafuente, E., D. Duneau, and P. Beldade. 2018. "Genetic Basis of Thermal Plasticity Variation in *Drosophila melanogaster* Body Size." *PLoS Genetics* 14: e1007686.

Lamb, R. J., and P. A. MacKay. 1979. "Variability in Migratory Tendency Within and Among Natural Populations of the Pea Aphid, *Acyrthosiphon pisum*." *Oecologia* 39: 289–299.

Langmead, B., and S. L. Salzberg. 2012. "Fast Gapped-Read Alignment With Bowtie 2." *Nature Methods* 9: 357–359.

Ledon-Rettig, C. C., and E. J. Ragsdale. 2021. "Physiological Mechanisms and the Evolution of Plasticity *in* D." In *Phenotyipc Plasticity & Evolution: Causes, Consequences, Controversies*, edited by W. Pfennig. Boca Raton: Taylor & Francis.

Leimar, O., and J. M. McNamara. 2015. "The Evolution of Transgenerational Integration of Information in Heterogeneous Environments." *American Naturalist* 185: E55–E69.

Leonardo, T. E., and E. B. Mondor. 2006. "Symbiont Modifies Host Life-History Traits That Affect Gene Flow." *Proceedings of the Royal Society B: Biological Sciences* 273: 1079–1084.

Li, B., R. D. Bickel, B. J. Parker, et al. 2020. "A Large Genomic Insertion Containing a Duplicated Follistatin Gene Is Linked to the Pea Aphid Male Wing Dimorphism." *eLife* 9: e50608.

Li, H. 2011. "A Statistical Framework for SNP Calling, Mutation Discovery, Association Mapping and Population Genetical Parameter Estimation From Sequencing Data." *Bioinformatics* 27: 2987–2993.

Li, Y., H. Park, T. E. Smith, and N. A. Moran. 2019. "Gene Family Evolution in the Pea Aphid Based on Chromosome-Level Genome Assembly." *Molecular Biology and Evolution* 36: 2143–2156.

Libbrecht, R., D. M. Gwynn, and M. D. E. Fellowes. 2007. "Aphidius Ervi Preferentially Attacks the Green Morph of the Pea Aphid, *Acyrthosiphon pisum*." *Journal of Insect Behavior* 20: 25–32.

Liu, X., and J. A. Brisson. 2023. "Dopamine Mediates the Pea Aphid Wing Plasticity." *Biology Letters* 19: 20230024.

Liu, X. D., H. X. Lei, and F. F. Chen. 2019. "Infection Pattern and Negative Effects of a Facultative Endosymbiont on Its Insect Host Are Environment-Dependent." *Scientific Reports* 9: 4013.

Lively, C. M. 1999. "Developmental Strategies in Spatially Variable Environments: Barnacle Shell Dimorphism and Strategic Models of Selection." In *The Ecology and Evolution of Inducible Defences*, edited by R. Tollrian and C. D. Harvell. Princton, NJ: Princeton University.

Losey, J. E., J. Harmon, F. Ballantyne, and C. Brown. 1997. "A Polymorphism Maintained by Opposite Patterns of Parasitism and Predation." *Nature* 388: 269–272.

Lowe, H. J. B., and L. R. Taylor. 1964. "Population Parameters, Wing Production and Behaviour in Red and Green *Acyrthosiphon pisum* (Harris) (Homoptera: Aphididae)." *Entomologia Experimentalis et Applicata* 7: 287–295.

Mackay, T. F., and R. F. Lyman. 2005. "Drosophila Bristles and the Nature of Quantitative Genetic Variation." *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 360: 1513–1527.

Mandrioli, M., V. Rivi, A. Nardelli, and G. C. Manicardi. 2016. "Genomic and Cytogenetic Localization of the Carotenoid Genes in the Aphid Genome." *Cytogenetic and Genome Research* 149: 207–217.

Martin, C. H., C. A. Mayeda, C. A. Davis, et al. 1995. "Complete Sequence of the Bithorax Complex of Drosophila." *Proceedings of the National Academy of Sciences of the United States of America* 92: 8398–8402.

Mathers, T. C., R. H. M. Wouters, S. T. Mugford, D. Swarbreck, C. Van Oosterhout, and S. A. Hogenhout. 2020. "Chromosome-Scale Genome Assemblies of Aphids Reveal Extensively Rearranged Autosomes and Long-Term Conservation of the X Chromosome." *Molecular Biology and Evolution* 38, no. 3: 856–875.

Moczek, A. P., S. Sultan, S. Foster, et al. 2011. "The Role of Developmental Plasticity in Evolutionary Innovation." *Proceedings of the Royal Society B: Biological Sciences* 278: 2705–2713.

Moran, N. A. 1992. "The Evolutionary Maintenance of Alternative Phenotypes." *American Naturalist* 139: 971–989.

Moran, N. A., and T. Jarvik. 2010. "Lateral Transfer of Genes From Fungi Underlies Carotenoid Production in Aphids." *Science* 328: 624–627.

Müller, C. B., I. S. Williams, and J. Hardie. 2001. "The Role of Nutrition, Crowding, and Interspecfic Interactions in the Development of Winged Aphids." *Ecological Entomology* 26: 330–340.

Murren, C. J., J. R. Auld, H. Callahan, et al. 2015. "Constraints on the Evolution of Phenotypic Plasticity: Limits and Costs of Phenotype and Plasticity." *Heredity* 115: 293–301.

Ørsted, M., P. D. Rohde, A. A. Hoffmann, P. Sørensen, and T. N. Kristensen. 2018. "Environmental Variation Partitioned Into Separate Heritable Components." *Evolution* 72: 136–152.

Parker, B. J., and J. A. Brisson. 2019. "A Laterally Transferred Viral Gene Modifies Aphid Wing Plasticity." *Current Biology* 29: 2098–2103.

Parker, B. J., R. M. H. Driscoll, M. E. Grantham, J. Hrcek, and J. A. Brisson. 2021. "Wing Plasticity and Associated Gene Expression Varies Across the Pea Aphid Biotype Complex." *Evolution* 75: 1143–1149.

Parker, B. J., C. J. Spragg, B. Altincicek, and N. M. Gerardo. 2013. "Symbiont-Mediated Protection Against Fungal Pathogens in Pea Aphids: A Role for Pathogen Specificity?" *Applied and Environmental Microbiology* 79: 2455–2458.

Peifer, M., F. Karch, and W. Bender. 1987. "The Bithorax Complex: Control of Segmental Identity." *Genes & Development* 1: 891–898.

Pfennig, D. W., M. A. Wund, E. C. Snell-Rood, T. Cruickshank, C. D. Schlichting, and A. P. Moczek. 2010. "Phenotypic Plasticity's Impacts on Diversification and Speciation." *Trends in Ecology & Evolution* 25: 459–467.

Renoz, F. 2024. "The Nutritional Dimension of Facultative Bacterial Symbiosis in Aphids: Current Status and Methodological Considerations for Future Research." *Current Research in Insect Science* 5: 100070.

Reyes, M. L., A. M. Laughton, B. J. Parker, et al. 2019. "The Influence of Symbiotic Bacteria on Reproductive Strategies and Wing Polyphenism in Pea Aphids Responding to Stress." *Journal of Animal Ecology* 88: 601–611.

Rockman, M. V. 2012. "The QTN Program and the Alleles That Matter for Evolution: All That's Gold Does Not Glitter." *Evolution* 66: 1–17.

Roff, D. A. 1996. "The Evolution of Threshold Traits in Animals." *Quarterly Review of Biology* 71: 3–35.

Scarborough, C. L., J. Ferrari, and H. C. Godfray. 2005. "Aphid Protected From Pathogen by Endosymbiont." *Science* 310: 1781.

Schellhorn, N. A., and D. A. Andow. 2005. "Response of Coccinellids to Their Aphid Prey at Different Spatial Scales." *Population Ecology* 47: 71–76.

Schlichting, C. D., and H. Smith. 2002. "Phenotypic Plasticity: Linking Molecular Mechanisms With Evolutionary Outcomes." *Evolutionary Ecology* 16: 189–211.

Sommer, R. J. 2020. "Phenotypic Plasticity: From Theory and Genetics to Current and Future Challenges." *Genetics* 215: 1–13.

Sultan, S. E., and H. G. Spencer. 2002. "Metapopulation Structure Favors Plasticity Over Local Adaptation." *American Naturalist* 160: 271–283.

Sutherland, O. R. W. 1969. "The Role of Crowding in the Production of Winged Forms by Two Strains of the Pea Aphid, *Acyrthosiphon pisum*." *Journal of Insect Physiology* 15: 1385–1410.

Tsuchida, T., R. Koga, M. Horikawa, et al. 2010. "Symbiotic Bacterium Modifies Aphid Body Color." *Science* 330: 1102–1104.

Turner, S. D. 2018. "Qqman: An R Package for Visualizing GWAS Results Using Q-Q and Manhattan Plots." *Journal of Open Source Software* 3: 731.

Vellichirammal, N. N., P. Gupta, T. A. Hall, and J. A. Brisson. 2017. "Ecdysone Signaling Underlies the Pea Aphid Transgenerational Wing Polyphenism." *Proceedings of the National Academy of Sciences* 114: 1419–1423.

Vellichirammal, N. N., N. Madayiputhiya, and J. A. Brisson. 2016. "The Genome-Wide Transcriptional Response Underlying the Pea Aphid Wing Polyphenism." *Molecular Ecology* 25: 4146–4160.

Vinton, A. C., S. J. L. Gascoigne, I. Sepil, and R. Salguero-Gómez. 2022. "Plasticity's Role in Adaptive Evolution Depends on Environmental Change Components." *Trends in Ecology & Evolution* 37: 1067–1078.

Weatherbee, S. D., G. Halder, J. Kim, A. Hudson, and S. Carroll. 1998. "Ultrabithorax Regulates Genes at Several Levels of the Wing-Patterning Hierarchy to Shape the Development of the Drosophila Haltere." *Genes & Development* 12: 1474–1482.

Weisser, W. W., and C. Braendle. 2001. "Body Colour and Genetic Variation in Winged Morph Production in the Pea Aphid." *Entomologia Experimentalis et Applicata* 99: 217–223.

Weisser, W. W., and B. Stadler. 1994. "Phenotypic Plasticity and Fitness in Aphids." *European Journal of Entomology* 91: 71–78.

West-Eberhard, M. J. 2003. *Developmental Plasticity and Evolution*. New York: Oxford University Press.

Wray, N. R., J. Yang, B. J. Hayes, A. L. Price, M. E. Goddard, and P. M. Visscher. 2013. "Pitfalls of Predicting Complex Traits From SNPs." *Nature Reviews. Genetics* 14: 507–515.

Zera, A. J., and R. F. Denno. 1997. "Physiology and Ecology of Dispersal Polymorphism in Insects." *Annual Review of Entomology* 42: 207–230.

Zhang, C., S.-S. Dong, J.-Y. Xu, W.-M. He, and T.-L. Yang. 2018. "PopLDdecay: A Fast and Effective Tool for Linkage Disequilibrium Decay Analysis Based on Variant Call Format Files." *Bioinformatics* 35: 1786–1788.

Zhou, P., X. Zong, S. Yan, J. Zhang, D. Wang, and J. Shen. 2023. "The Wnt Pathway Regulates Wing Morph Determination in *Acyrthosiphon pisum*." *Insect Biochemistry and Molecular Biology* 161: 104003.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.