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Pea aphid winged and wingless males exhibit reproductive, gene expression, and lipid metabolism differences

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ABSTRACT

Alternative, intraspecific phenotypes offer an opportunity to identify the mechanistic basis of differences associated with distinctive life history strategies. Wing dimorphic insects, in which both flight-capable and flight-incapable individuals occur in the same population, are particularly well-studied in terms of why and how the morphs trade off flight for reproduction. Yet despite a wealth of studies examining the differences between female morphs, little is known about male differences, which could arise from different causes than those acting on females. Here we examined reproductive, gene expression, and biochemical differences between pea aphid (*Acyrthosiphon pisum*) winged and wingless males. We find that winged males are competitively superior in one-on-one mating circumstances, but wingless males reach reproductive maturity faster and have larger testes. We suggest that males tradeoff increased local matings with concurrent possible inbreeding for outbreeding and increased ability to find mates. At the mechanistic level, differential gene expression between the morphs revealed a possible role for activin and insulin signaling in morph differences; it also highlighted genes not previously identified as being functionally important in wing polymorphism, such as genes likely involved in sperm production. Further, we find that winged males have higher lipid levels, consistent with their use as flight fuel, but we find no consistent patterns of different levels of activity among five enzymes associated with lipid biosynthesis. Overall, our analyses provide evidence that winged versus wingless males exhibit differences at the reproductive, gene expression, and biochemical levels, expanding the field's understanding of the functional aspects of morph differences.

Introduction

Insect wing dimorphisms have long served as models for studying the trade-offs associated with alternative life history strategies (Zera and Denno 1997). These dimorphisms have evolved multiple times across a variety of insect orders (Johnson 1969; Harrison 1980; Roff 1986; Zera and Denno 1997), demonstrating the repeated success of this strategy. In wing dimorphisms, individuals typically trade-off reproductive for dispersal abilities, associated with a flight deficient or incapable morph and a flight capable morph, respectively.

Wing dimorphic systems are useful experimentally because individuals with alternative phenotypes occur in the same population. Consequently, phenotypic differences can be compared without complicating factors like population or species divergence. In the case of species with asexuality as part of their life cycle, these differences can even be studied within the same genotype. Because of this, wing dimorphism studies have been fruitful for discovering the morphological, developmental, biochemical, molecular, and life history differences that underlie alternative morphs (Zera and Denno 1997; Zhang et al. 2019). Identifying these differences is critical for understanding how these dimorphisms have evolved and for future studies examining what factors may or may not constrain further phenotypic divergence of the morphs.

Most mechanistic wing dimorphism studies have focused on females, where strong tradeoffs between the morphs are observed because females invest so heavily in reproduction. Wing dimorphisms in males are far less well studied (reviewed in Langellotto et al. 2000), although Fawcett et al. (2018) found that short-winged males of the soapberry bug were less fecund than long-winged males. The reproductive cost to males may not be as great as it is in females, since males often have a more limited energetic investment in offspring (Holtmeier and Zera 1993; Zera and Denno 1997; Guerra 2011). On the other hand, investment in searching for mates may be higher in males relative to fe-

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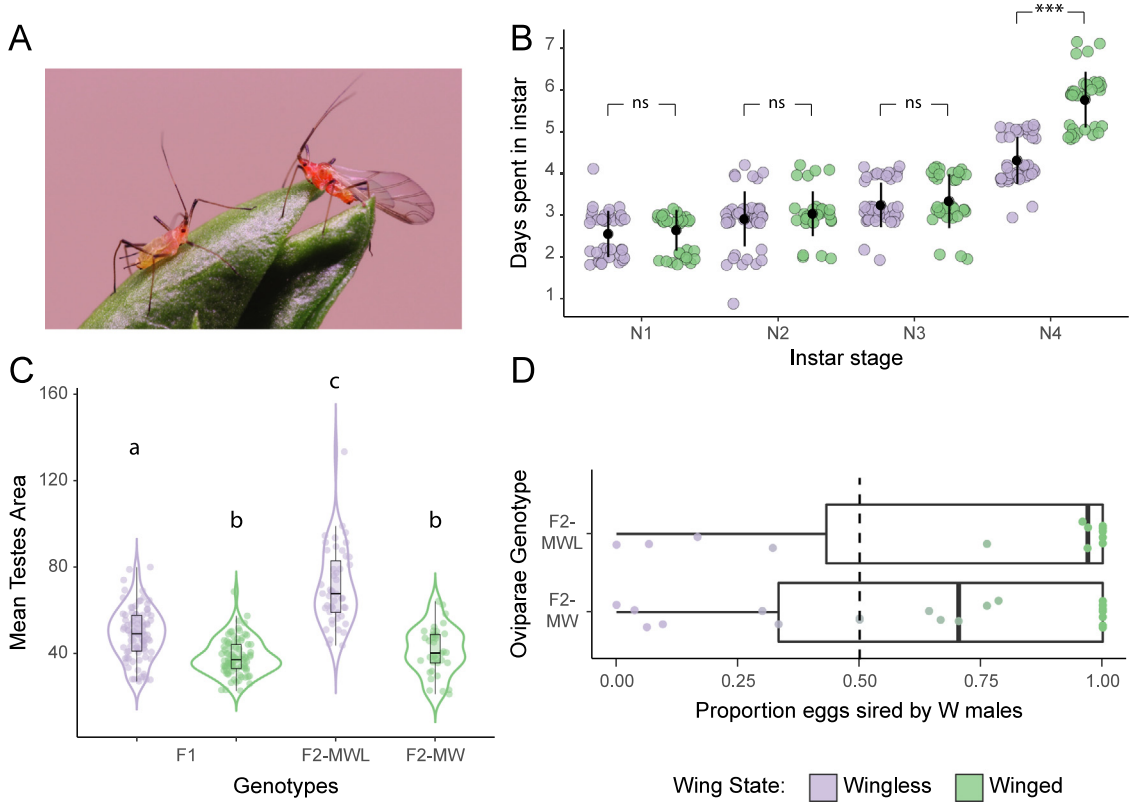

Fig. 1. Differences between winged and wingless males in factors important for reproductive success. (A) Wingless (left) and winged (right) males are morphologically distinct. For (B)-(C) data from wingless males are shown in purple and winged in green. Points shown are individual biological replicate data points. (B) Days spent in each nymphal instar (N1, N2, N3, N4) by wingless and winged males of the F1 line. Differences between each instar was determined by the Wilcoxon rank sum test (asterisks denote P<0.0001; ns=not significant). We applied a horizontal and vertical jitter to show dispersion, but days and instar stage are discrete variables. (C) Mean testes area for F1 (n=94 for wingless, n=86 for winged), F2-MWL (n=49) and F2-MW (n=36) winged and wingless males. Testes area is measured in mm^2. Different letters denote significant differences using Tukey's post-hoc test. (D) Shows the proportion of fertilized eggs fathered by winged (W) males when winged and wingless F1 males compete for oviparous females from the F2-MWL genotype (top, n=13 females) or F2-MW genotype (bottom, n=18 females). For each data point, the proportion was determined from 10-36 eggs per oviparous female with 750 eggs genotyped in total. The vertical, dotted line shows the expected proportion of winged male fathers if winged and wingless males had equal mating success. The mean proportion is shown as a thick black vertical line. For each genotype, the Wilcoxon signed rank test was significantly different ($p < 0.05$) from the expected equal proportion. The points are shown as a purple-green gradient corresponding to a majority of offspring as wingless (left, purple) or winged (right, green).

males, and the energy devoted to searching may differ between morphs (Dixon, 1985).

Here we investigate winged versus wingless morph differences as the basis for possible evolutionary tradeoffs in wing dimorphic males of the pea aphid (*Acyrthosiphon pisum)*. In these males, the winged morph is fully flight capable, while the wingless morph lacks wings (Fig. 1A) and most wing musculature (Ogawa et al. 2012). Both winged and wingless males are found within most pea aphid populations (Frantz et al. 2009; Li et al. 2020). Aphid wing dimorphisms are generally well-known because of the intensively studied wing plasticity in aphid females (environmentally determined winged and wingless morphs; Dixon and Howard 1986; Braendle et al. 2006; Brisson 2010), but pea aphid male wing morphs are not induced by environmental signals. Rather, their morphology is genetically controlled by a single locus on the X chromosome (Caillaud et al. 2002; Braendle et al. 2005), wherein wingless males have a 120kb insertion containing a duplicated follistatin gene compared to the winged males (Li et al. 2020). Male aphid wing dimorphisms have likely evolved repeatedly across the aphid phylogeny, although only approximately 4% of extant species are male wing dimorphic (Saleh Ziabari et al. 2022) and it's unknown if these dimorphisms are under genetic or environmental control. While the segregating allele frequencies of the pea aphid dimorphism (shown in Li et al. 2020) suggest maintenance under balancing selection, the possibility remains that the polymorphism is non-adaptive.

We use an integrative approach to study the alternative morphs of the pea aphid male wing dimorphism. We first focus on three characteristics that likely contribute to differences in reproductive success and thus maintenance of the morphs in natural populations: time to adulthood, testes size, and competitive mating ability. We then turn to gene expression and biochemical studies to better understand the mechanistic basis of male morph differences. Our results contribute substantial information about how the tradeoffs in a wing dimorphic species are achieved at multiple levels of biological organization.

Materials and Methods

Aphid lines

The life cycle of the pea aphid alternates between asexual reproduction in the spring/summer and sexual reproduction in the fall, followed by overwintering eggs. A single locus on the X chromosome (males are XO and females are XX) determines whether pea aphid males are winged or wingless (Li et al. 2020). The bulk of the experiments here were performed using males from line "F1", a line derived from a cross of two wild isolates originally used to map the genetic basis of winged versus wingless male differences in this species (Braendle et al. 2005, Li et al. 2020). Females of this F1 line are heterozygous for the male wing dimorphism locus and therefore produce both winged and wing-

less males. Two other lines used – F2-MWL and F2-MW – are F2 offspring lines derived from the F1 line crossed to itself. F2-MWL females are homozygous for the wingless allele and produce only wingless males (where "MWL" = males wingless), while F2-MW females are homozygous for the winged allele and produce only winged males (where "MW" = males winged). Finally, the RNA-Seq experiment (details below) used an additional two lines that are heterozygous for the male dimorphism locus (BK11, Ithaca18). We maintained asexual lines of each in cages containing *Vicia faba* seedlings at 18°C on a 16:8 (light:dark) cycle.

Male induction

To induce asexual females to produce sexual offspring (males and sexual females), six asexual female nymphs were transferred to a cage and placed in an incubator set to 15°C on a 13:11 light:dark cycle to simulate autumn. Aphids were transferred to new plants every generation (~10 days), dividing groups of aphids into multiple cages to reduce overcrowding and to increase the population. Around 21 days after transfer, adult asexual females begin producing sexual offspring. Male offspring can be detected as fourth instar nymphs by their elongated abdomens relative to females. Winged versus wingless males can be detected at this stage due the prominent wing pads of the winged males. Once asexual females start producing males, they continue producing males until death. For the assays described below, we used these male-producing females to generate male individuals.

Developmental time

Asexual females producing sexual offspring of the F1 line were placed on *Vicia faba* plants. Each day, newly emerged nymphs were removed to 40mm plates containing a single *Vicia faba* leaf with its stem placed in agar. Nymphs were monitored daily for molting. Some nymphs grew up to be females; data from these individuals were discarded, while male data were retained. We compared days until molt in winged (n=33 individuals) and wingless (n=42) males for each instar using the Wilcoxon rank sum test using wilcox.test in the R stats package.

Testes size measurements

The F1, F2-MWL, and F2-FMW lines were used for this experiment. Nymphs of male-producing asexual females from each line were reared to adulthood on plates containing a single *Vicia faba* leaf in agar. The day after a male nymph became an adult, it was placed in 70% ethanol, and the testes were dissected and mounted in a drop of 50% balsam/50% citrus oil mixture.

ImageJ (Schneider et al. 2012) was used to analyze testes. The calibration was set using a picture of a 1mm calibration slide taken with a 10x objective, so the measured pixel values of the images could be transformed into μm^2. We used the polygon selection tool to mark the outlines of each of the six follicles of the two testes of each male, and the follicle size was measured as the area inside the polygon. Statistical comparisons of testes area were made using an ANOVA and a multiple comparisons Tukey's post-hoc test.

Competition assay

Each competition was set up by placing one F2-MWL or F2-MW oviparous (sexual, egg-laying) female, two wingless F1 males, and two winged F1 males on a leaf plate. The oviparous females were isolated as nymphs to ensure that they were virgins before the competition. The males were selected at random and all were at least two-day-old adults. The plates were checked daily for the presence of eggs. After around four days, the females started laying eggs. The fertilized eggs melanized one to two days after they are laid. Each day, the melanized eggs were removed and stored. To ensure that the female was equally exposed to the two types of males, when a male of one morph died, one male of the other morph was also removed from the plate.

DNA from each egg was extracted in a Tris-EDTA salt buffer with a proteinase-K digestion. The genotype of each egg (750 eggs total), and thus its parentage, was determined using primers that detect polymorphism for winged versus wingless males. The primer set (5' TGGTACATATCAGCTATCAGCACA 3' and 5' ACACAAGTTATTTCAGTTGCTTAGG 3') amplifies a region containing a heterozygous restriction enzyme (Taqα) recognition sequence on the X chromosome that is in linkage with the indel that determines winged versus wingless males. Males thus display one of two possible bands (wingless males have the "low" bands because they have the cut allele and winged males have the "high" band because they have the uncut allele). As the oviparae (egg-laying females) of the two different lines used (F2-MWL or F2-MW) are each homozygous, parentage could be determined by the presence of the alternative band. For example, F2-MW oviparae are homozygous for the "low" bands. If an offspring is fathered by a wingless male, then the egg will display the "low" bands. If an offspring is fathered by a winged male, then the egg will display both the "low" and "high" bands.

Sampling for gene expression analysis

For this study, we re-analyzed data from Purandare et al. (2014). Whole body samples of winged and wingless males were collected and RNA sequenced as previously reported (Purandare et al. 2014). Briefly, we collected age-matched, whole bodies of 30 adult aphids of each male morph (winged or wingless) from three unique lines (total of six samples, 30 adult aphids in each sample, each for library preparation) by flash freezing them in liquid nitrogen. Individuals were collected on the second day after their molt into adulthood. We used three different lines (including the "F1" line) as biological replicates for the study rather than three replicates of the same genotype to identify genes that were systematically expressed in a particular morph, not just a particular morph of a particular genotype. The expectation of this design was that it would result in a smaller list of differentially expressed genes than three biological replicates of the same genotype, but that the discovered genes would be representative of differences between any winged or wingless male, regardless of genotype. RNA-Seq data can be found in NCBI's GEO archive under accession number GSE56830.

RNA-Seq data mapping and analysis

We mapped sequencing reads to the v3.0 version of the pea aphid reference genome using STAR 2.7 (Dobin et al. 2012). Reads were mapped under default STAR parameters. The number of mapped reads per library ranged from 59 to 70 million (average 65 million reads). We used the Trim Galore! wrapper for cutadapt (Martin, 2011) to filter low quality reads (-q 20). Reads were summarized over genomic features using the featureCounts program from the subread package (Liao et al. 2013). The DESeq2 R package (Love et al. 2014) was used to identify differentially expressed genes among winged and wingless male samples between three biological replicates (six libraries total). Genes with less than 10 total reads across all 6 samples were removed. We disabled the default independent filtering criteria in the 'results' function (independentFiltering=FALSE) to include lowly expressed, but differentially expressed genes. We did this so that we could capture the most complete list of possible differences between the morphs for hypothesis generation. Differential expression p-values were based on the Wald statistic, and FDR-adjusted through the DESeq2 package. Enrichment analyses using Fisher's Exact Tests were performed by Blast2Go (Conesa et al. 2005). GO terms with p-values $\leq$0.0.01 (uncorrected) were considered enriched and Table 1 reports GO terms after the "Reduce to Most Specific" action was implemented. Table S2 reports the unreduced output.

Table 1
Enriched gene ontology (GO) terms associated with differentially expressed genes.

GO ID	GO Name	GO Category	P-Value*	# in Test Set	# in Reference Set
GO:0007018	microtubule-based movement	Biological Process	7.16E-10	14	62
GO:0005874	microtubule	Cellular Component	1.71E-10	16	79
GO:0000276	mitochondrial proton-transporting ATP synthase complex, coupling factor F(o)	Cellular Component	0.002	3	9
GO:0005868	cytoplasmic dynein complex	Cellular Component	0.002	3	10
GO:0005858	axonemal dynein complex	Cellular Component	0.009	2	5
GO:0003777	microtubule motor activity	Molecular Function	1.35E-07	10	42
GO:0005200	structural constituent of cytoskeleton	Molecular Function	1.40E-04	5	19
GO:0003713	transcription coactivator activity	Molecular Function	5.21E-04	4	14
GO:0005524	ATP binding	Molecular Function	0.002	32	822
GO:0031177	phosphopantetheine binding	Molecular Function	0.004	2	3
GO:0004675	transmembrane receptor protein serine/threonine kinase activity	Molecular Function	0.007	2	4
GO:0045505	dynein intermediate chain binding	Molecular Function	0.009	2	5

* As determined by Fisher's exact test; P-value uncorrected

Lipid and enzyme assays

F1 males were identified as fourth instars by their elongated abdomens relative to females and transferred onto individual fava seedlings and inspected daily for emergence into adulthood. Males were flash frozen on either the first or fourth day of adulthood.

Total, whole-aphid lipid was estimated using the standard vanillin assay (see Zera et al. 1994 for details). Briefly, 3-12 aphids were weighed and homogenized in 500 uL 2:1 chloroform/methanol. The homogenate was washed with $\frac{1}{4}$ volume of 0.88 % KCl solution, the homogenate was vortexed for a few seconds, and centrifuged at 15,000 x g for 5 minutes. 50 uL of the lower chloroform/methanol layer was transferred to a glass test tube, the chloroform/methanol was evaporated, and 30 uL of H2SO4 was added. The tube was heated at 90°C for 10 min, cooled to room temperature, vanillin reagent was added, and, after 10 min, absorbance was read on a spectrophotometer (three replicates per each aphid sample). The vanillin assay involves the conversion of double bonds or free hydroxyl groups in extracted lipids to stable intermediates by sulfuric acid at high temperature (McMahon et al, 2013). These intermediates then produce a pink chromophore when vanillin reagent (vanillin and phosphoric acid) is added, the concentration of which is quantified spectrophotometrically. Lipid levels in aphid samples were estimated via linear regression (r^2 values always > 0.95) by comparison of absorbances relative to those of six known standards (0, 20, 40 60, 80 and 100 ug triolein) assayed in the same time and manner.

Specific activities of $NADP^+$- dependent isocitrate dehydrogenase (IDH), $NADP^+$-dependent malate dehydrogenase (ME, malic enzyme), ATP-citrate lyase (ACL), and glucose-6-phosphate dehydrogenase (G-6-PDH) were quantified using standard spectrophotometric assays described in (Zera and Zhao 2003). The α-glycerophosphate dehydrogenase (α-GPDH) assay cocktail contained 100 mM MOPS [3-(N-Morpholino)-Propanesulfonic Acid] buffer, pH 7.0, 2 mM dihydoxyacetone phosphate, 150 uM NADH, and 2 mM K^+-cyanide. IDH, ME and G-6-PDH activities were quantified spectrophotometrically by measuring the increase in absorbance at 339 nm versus time, due to the production of NADPH from NADP+ by these enzymes. a-GPDH activity was measured at the same wavelength by quantifying the decrease in absorbance of NADH, which is directly oxidized by this enzyme. ACL activity was quantified indirectly by measuring the rate at which oxaloacetate, one of the products of the ACL reaction, reacts with NADH and malate dehydrogenase contained in the assay solution. This results in the decrease in absorbance at 339 nm which was quantified spectrophotometrically. The decrease in absorbance due to the oxidation of NADH is directly proportional to the production of oxaloacetate by ACL. NADH solutions were made just prior to assays. Protein concentrations were measured using the Bradford assay using bovine serum albumin as the standard.

For enzyme assays, three to eight aphids were homogenized in 200 uL of 100 mM Na+-phosphate buffer, pH 7.4, containing 5 mM EDTA and 0.1% 2-mercaptoethanol using a hand-held pestle followed by sonication for 5 sec, and centrifugation at 15,000 x g for 2 minutes. Depending upon the enzyme to be assayed the supernatant was further diluted in homogenization buffer or was used undiluted. Five-10 uL of enzyme homogenate was added to wells of a microtiter plate containing 190-195 uL assay cocktail pre-heated to 28° C. The microtiter plate was inserted into the spectrophotometer and allowed to thermally equilibrate to 28° C for 2 minutes. Change in absorbance was then followed for 4-8 minutes. Degree of dilution of enzyme homogenate and volume of enzyme added to the assay cocktail were chosen such that change in absorbance versus time was linear (i.*e.*, measured rates were initial rates). Preliminary studies were also undertaken to insure that measured activities of each enzyme were within the range in which activity was linearly proportional to enzyme concentration.

Results

Morph differences in measures related to reproductive success

We investigated three factors suspected to be relevant for reproductive success. First, we measured the development time to adulthood, reasoning that a faster time to reproduction would result in increased reproductive success. Second, we measured the relative size of the testes in both morphs, with the rationale that bigger testes make more sperm. And finally, we evaluated success in mating via competitive mating trials between winged and wingless males.

For development time, we found that winged males took significantly longer to develop to adulthood compared to wingless males, and this difference was attributable to the time spent in the penultimate, fourth-instar nymphal stage (Fig. 1B, Wilcoxon rank sum test (W=90, p-value=1.4e-11) with an average of 5.8 days for winged compared to 4.3 days for wingless males. In that stage, much of wing pad development occurs. Since wingless males develop faster, they likely begin reproduction earlier than winged males.

We then measured mean testes area using winged and wingless adult males from line F1, the same line used in the developmental time analysis. We also used two additional lines that are progeny lines of an F1xF1 mating. One of those lines, F2-MWL is homozygous for the wingless allele at the wing dimorphism locus and thus only produces wingless males. The other, F2-MW, is homozygous for the opposite allele and only produces winged males. When comparing winged and wingless males of the F1 line to each other, or the wingless males to the winged males of the F2s (lines F2-MWL and F2-MW, respectively), wingless males had larger testes than winged males (Fig. 1C).

For male mating success, we used competitions between two winged and two wingless males placed with one female in a small plate (55mm) containing a *Vicia faba* leaf. Winged and wingless F1 males competed to achieve matings with F2-MW females, or in a separate competition, with F2-MWL females. We directly measured which male fathered each resulting egg by genotyping each egg using a restriction fragment length polymorphism marker (see Methods). Regardless of the genotype of the mother, winged males fathered more eggs than wingless males (Fig. 1D).

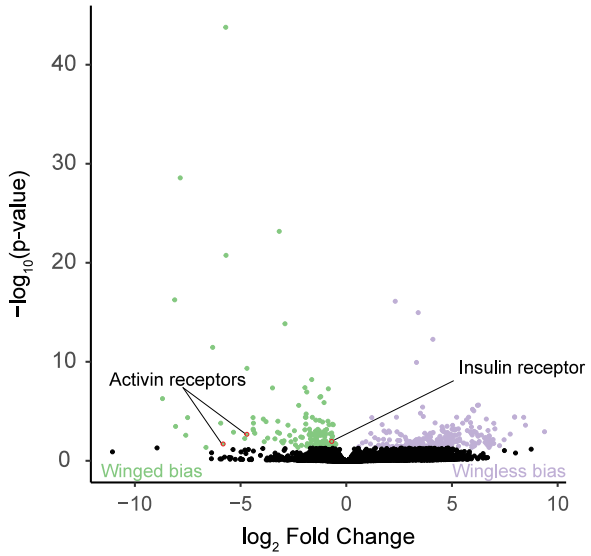

Fig. 2. Gene expression level differences between male morphs. Volcano plot showing genes with significantly higher (FDR-adjusted p-value < 0.05) expression in winged males (left, green) or significantly higher expression in wingless males (right, purple). Red points show specific genes from the discussion (activin and insulin receptors).

Qualitatively, we note that winged males were more active moving around the enclosure than the wingless males.

Gene expression differences between morphs

From the winged and wingless adult male RNA-Seq data, we found 14,355 expressed genes for comparative analysis. We discovered 357 genes significantly differentially expressed between the male wing morphs (Wald test, FDR-adjusted p-value < 0.05; Table S1). Of these, 139 were at higher levels in winged males, and 218 were at higher levels in wingless males (Fig. 2A).

We identified the genes that likely code for each of the enzymes examined below (IDH: XP_029347226; ME: XP_001943996; ACL: XP_008179483; G-6-PDH: XP_001951527 & XP_001951527; α-GPDH: XP_001945715). Of these, only α-GPDH was significantly differentially expressed (FDR-adjusted pvalue=0.001), with higher expression in the winged males. Several gene ontology terms were enriched among the 357 differentially expressed genes (p-value < 0.01; Table S2 for full list, Table 1 for condensed list).

Lipid and related enzyme differences between morphs

We next turned to the biochemical level, focusing on lipids because they are an important component of flight (Beenakkers et al. 1985; see Discussion). We first measured whole body lipid levels of adult winged and wingless males (again using line F1) at day one and day four after the final molt, finding higher lipid levels in winged males on both of these days (Fig. 3A).

We then assayed activities of five important enzymes involved in lipid biosynthesis. Malic enzyme (ME), isocitrate dehydrogenase (IDH), and glucose-6-phosphate dehydrogenase (G-6-PDH) are dehydrogenases that produce the NADPH required for lipid biosynthesis (Zera 2005). Glyceraldehyde-3-phosphate dehydrogenase (α-GPDH) produces the glycerophosphate backbone of triglycerides, in addition to playing an important role in carbohydrate catabolism in insect flight muscle, another key aspect of flight energetics. For all five enzymes, we again assayed day one and day four of adulthood. We found α-GPDH was significantly higher in winged males (Wilcoxon ranked sum test, see Table S3 for p-values and W statistics), while ACL, G-6-PDH, ME, and IDH exhibited no significant differences between morphs (Fig. 3B-3F).

Discussion

Here we examined reproductive differences and possible underlying mechanisms in alternative male morphs as a basis for understanding their possible evolutionary tradeoffs.

For reproductive output, wingless males appear to have an advantage because they reach adulthood faster and have larger testes. In particular, wingless males reached adulthood a day and a half faster than winged males, as previously observed in another pea aphid line (Ogawa et al. 2012). Given this faster development time, wingless males could potentially find and mate with females before winged males reach reproductive maturity. This could lead to a large competitive advantage for wingless males at the population level. And larger testes suggest the production of more sperm (Pitnick 1996, and references within). Testes size is an indirect measure of total sperm ejaculate volume, and testes size positively correlates with sperm competition across a variety of species (Parker 1970).

Despite these wingless male advantages, winged males sire more offspring. We directly measured mating success by genotyping fertilized eggs. A previous study indirectly measured pea aphid male morph mating success (Sack and Stern 2007) and found mixed results: when single winged or wingless males competed to mate with females, winged males achieved more matings, but when 10 of each morph competed there was no clear advantage. This study was an excellent first foray into this topic, but low sample sizes (only 10 observed matings for the first experiment and 10 competitions for the second) and indirect measures of success (observations of video recordings for the first, transfer of powdered dyes for the second) limited the certainty of interpretations. Our experiment had the advantage of a larger number of observations over two different pea aphid lines using genotyping as a direct measure of success.

Mate finding and outbreeding may be driving male morph differences. All sexual females are wingless, and therefore they cannot disperse to find males. If males do not disperse and thus mate locally, they may be mating with their asexually-produced siblings. This is genetically equivalent to self-fertilization. So if the faster developing wingless males attain more local matings, this may come with the cost of increasing inbreeding depression. In contrast, winged males that migrate and outcompete wingless males in a new population enjoy the benefits of outcrossing at the cost of migration itself. While the reproductive pressures driving male morph differences align with the expectations of male winged states under sex ratio theory (see discussion in Saleh Ziabari et al., 2022), we cannot rule out male dispersal evolving as a stochastic process.

Our gene expression analysis provides insight into the building blocks underlying the alternative male morphologies and lifestyles. The genes that were significantly differentially expressed between the morphs (Fig. 2, Table 1, Table S1-S2) were enriched for the molecular function of microtubule motor activity. These were largely genes with higher expression in wingless males. A closer look at more specific gene ontology terms revealed cellular components like the “cilium” and the “axonemal dynein complex”, among other related terms. One of the few cells in insects with a cilium is the sperm (Zur Lage et al. 2019) and sperm proteomes have overrepresentation of genes with many these same gene ontology categories (Dorus et al. 2006; Fabian and Brill 2012; Whittington et al. 2015). Thus, the general descriptors of the genes with higher expression in wingless males indicate that wingless males are likely producing more sperm than winged males, a result that ties directly to our observation that wingless males have larger testes volumes.

We can also tie our gene expression results to what is known about the genetic basis of male morph differences. Wingless males have a duplicated follistatin gene that winged males lack (Li et al. 2020). In *Drosophila melanogaster*, the Follistatin protein binds to activin to inhibit

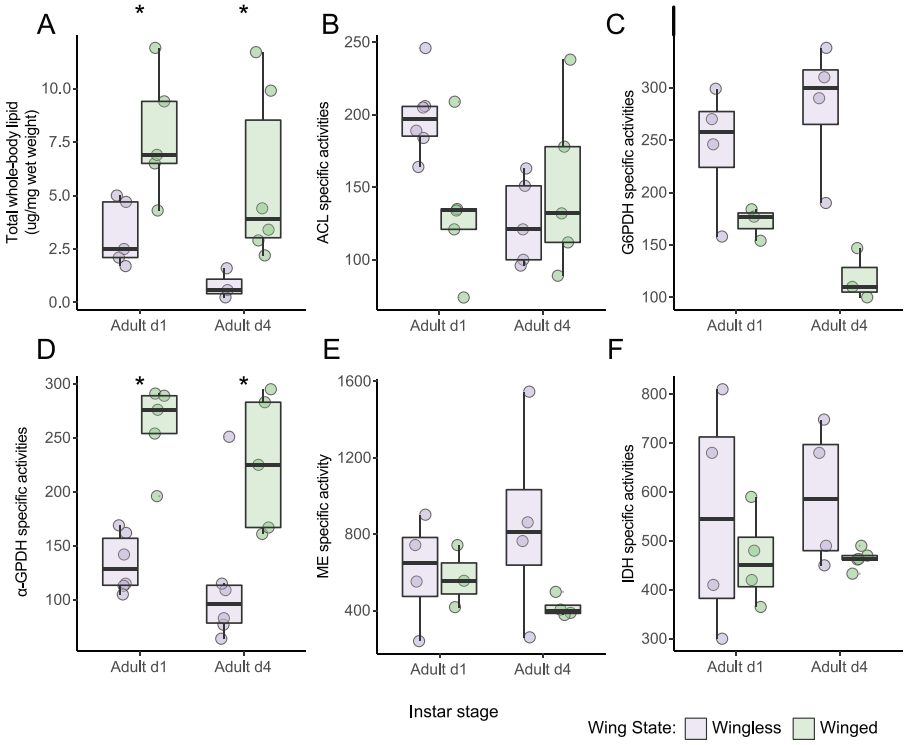

Fig. 3. Winged and wingless-specific measures of whole body lipid levels and lipid-related enzyme activities. All data are from wingless (purple) and winged (green) males from the F1 line from day one (d1) and day four (d4) of adulthood. Points shown are individual biological replicate data points. (A) Shows whole-body lipid amounts and (B)-(F) shows activity levels of different lipid-related enzymes. (A) n=5 for day one males, n=6 for day four males. (B) n=6 for both days. (C) n=4 for both days. (D) n=6 for both days. (E) n=4 for both days. (F) n=4 for day one males and n=5 for day four males. All comparisons were made with a Wilcoxon rank sum tests, where asterisks denote significance with $p<0.05$ (p-values and W statistics can be found in Table S3).

TGFβ signaling, a pathway that plays a critical role in the patterning and growth of numerous tissue types (Bickel et al. 2008; Pentek et al. 2009). This duplicated follistatin gene stays expressed throughout much of the development of the wingless morph and therefore presumably inhibits the activin signaling pathway. In comparison, the winged males do not have this gene and therefore their TGFβ signaling pathway is likely not inhibited. In our expression data, we observed two activin receptor genes (XP_029347930.1 and XP_029345750.1) with significantly higher expression in winged compared to wingless males. These two genes appear on the enriched GO list with the molecular function term "transmembrane receptor protein serine/threonine kinase activity". Thus, the activity of these activin receptors may be a transcriptional indicator of this winged/wingless genetic difference that persists into adulthood.

Another notable gene with higher expression in winged males is the insulin receptor gene (XP_008179974.1). In planthoppers, differential expression of two different insulin receptors modulate the production of long versus short-winged morphs in response to environmental conditions (Xu et al. 2015), and Fawcett et al. (2018) found a role for insulin signaling in the regulation of the soapberry wing polyphenism. A search of the pea aphid genome revealed only this single insulin receptor, and its differential expression here indicates that it may help regulate winged versus wingless differences in pea aphid males, too. And in *Drosophila melanogaster*, activin and insulin signaling interact in several contexts (e.g., Gibbens et al. 2011; Bai et al. 2013; Luo et al. 2020), suggesting a possible link between the activin receptors mentioned above and insulin signaling.

At the biochemical level, studies of wing polymorphic crickets (*Gryllus firmus*; Zera and Zhao 2003; Zera 2005) have identified dramatic differences between winged and wingless female morphs in whole organism lipid level and in the activities of enzymes that produce this compound. Prior to this study, however, no detailed comparable biochemical studies have been undertaken in male morphs. The higher total lipid in adult winged versus wingless males at day one and day four of adulthood (Fig. 3A) is consistent with the winged morph requiring a higher level of lipid for flight. The significantly higher α-GPDH activity in the winged morph is also consistent with up-regulation of biochemical aspects of metabolism required for flight because it plays a key role in maintaining high glycolytic flux during flight. This, in turn, produces the elevated levels of carbohydrate-derived compounds (Krebs Cycle intermediates) that are required for the full utilization of lipid in insects that burn primarily lipid during sustained flight (e.g., locusts; Beenakkers et al. 1985). Differential use of α-GPDH was reflected at the gene expression level as well, with levels significantly higher in winged males. This concordance between gene expression and protein differences is notable because it strengthens the argument that other examples of morph differences in gene expression, for which information at the protein level is lacking, are functionally important

Results obtained thus far do not identify the biochemical processes that are responsible for the elevated lipid level in the winged morph. Activities of most key lipid biosynthetic enzymes did not differ between the morphs (Fig. 3). α-GPDH is a dual-functioning enzyme which can play an important role in lipid biosynthesis because it produces the glycerophosphate backbone of triglycerides. However, the reduced level of total lipid and the absence of elevated levels of other lipid biosynthetic enzymes suggests that the elevated activity of α-GPDH is primarily involved in flight metabolism, as discussed above, rather than lipid biosynthesis. More direct information on the role of α-GPDH in flight energetics versus lipid biosynthesis can be obtained by subsequent studies of organ-specific gene expression and enzyme activity. If α-GPDH is primarily involved in flight energetics, as proposed here, it should be expressed primarily in the flight muscle and have high activity there. On the other hand, if this enzyme is primarily involved in lipid biosynthesis, it should be more highly expressed in the fat body, the primary site of lipid biosynthesis. Insect flight muscle typically has very low lipid biosynthetic capacity (Zera 2005). One possibility for the elevated lipid level in the winged morph, in the absence of elevated lipid biosynthesis in that morph, is reduced lipid oxidation in the winged morph. In *G. firmus*, the elevated lipid level in long-winged females is due to both reduced lipid oxidation as well increased lipid biosynthesis, compared to the flightless short-winged morph (Zera 2005). Alternatively, the elevated lipid level in the winged morph could result from elevated biosynthesis during an earlier stage of development or from increased fatty acid assimilation from the diet.

By simultaneously studying this wing dimorphism at multiple levels, here we were able to provide new insights into the differences between winged and wingless pea aphid males. Wingless males have faster development time, larger testes and likely higher sperm production, while winged males sire more offspring, have higher lipid levels, and likely higher activin and insulin signaling levels. Unlike the well-investigated wing dimorphic females that tradeoff reproduction for dispersal, wing dimorphic males may tradeoff increased local matings and potential inbreeding for outbreeding and increased ability to find mates.

Data Accessibility

RNA-Seq data can be found in NCBI's GEO archive under accession number GSE56830. Remaining data can be found on Dryad at doi:10.5061/dryad.pc866t1rf.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Omid Saleh Ziabari: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Qingyi Zhong:** Investigation. **Swapna R. Purandare:** Investigation. **Joel Reiter:** Investigation, Methodology. **Anthony J. Zera:** Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. **Jennifer A. Brisson:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cris.2022.100039.

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