Maternal melanization defines wing spot area of male *Drosophila nepalensis*: supporting evidence through genetic crosses

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Received 3 December 2021; revised 5 August 2022; accepted 10 August 2022

Abstract. Males of *Drosophila nepalensis* show dimorphism in wing melanization, but how do they evolve and coordinate during evolution is unknown. Heterogeneity in the environment helps individuals to adapt accordingly either through genetic polymorphism or through phenotypic plasticity. In this study, we tried to untangle the genetic architecture underlying differences in wing melanization in males because in nature the frequency of spotted and spotless males is different. We investigated the genetic basis of the inheritance of the sexually dimorphic characteristic of wing spot area (WSA) in *D. nepalensis* males with the aid of genetic crosses. We found spot formation on wings in male is directly correlated to female body melanization. In addition to this, we studied the phenotypic plasticity in the degree of wing spot melanization in males with respect to changes in temperature. We observed that increased WSA is negatively correlated with higher temperature. We found dark and light females only at 21°C. Dark females always produced males with spotted wing, whereas lighter females always produced males without wing spots. Finally, we found wing spot is highly correlated in reciprocal progeny due to linkage or pleiotropy which could help in evolution.

Keywords. wing melanization; genetic cross; genetic polymorphism; phenotypic plasticity; *Drosophila nepalensis*.

Introduction

Ecology is primarily concerned with understanding the adaptation, as organisms (which do not move fast in changing climate envelopes) rely on adaptation to escape extinction (Bell and Gonzalez 2011; Hoffmann and Sgro 2011). One of the main mechanisms of adaptation is phenotypic plasticity based on gene expression (Pigliucci et al. 2006). The study of mechanisms that allow organisms to cope with changing environments is being prioritized when anthropogenic changes are considered responsible for species extinctions.

*Drosophila* species is abundant in Indian subtropics and it possesses melanization variability (Parkash et al. 2008a, b). Drosophilids pigmentation has been measured by various methods, including measuring the width of the abdominal stripe, measuring the intensity of the thoracic trident, and analysing each fly’s melanin content at specific points (Robertson et al. 1977; Majerus 1998; Pool and Aquadro 2007; Wittkopp et al. 2011). There are traits like sexual dimorphism, abdominal melanization and wing melanization which have independently developed through evolutionary succession (Kopp et al. 2003). The genesis of wing melanization have long been the subject of speculation and has significantly contributed to adaptation of various species of butterflies via mate choice, providing defense against predators through mimicry and thermoregulation (Watt 1968; Roland 1982; Kingsolver 1987; Wiernasz 1989; Ellers and Boggs 2002, 2003). A genetic study has investigated the differences between the sibling species *Drosophila elegans* and *D. gunungcola* in their wing pigmentation and mating displays (Massey et al. 2020) and another study has examined that *D. biarmipes* can retain sexual dimorphism of wing spots when the environment (wet versus dry) changes (Parkash et al. 2013). However, the inheritance of wing spot dimorphism in other *Drosophila* species (*D. nepalensis*) is little understood for evaluating the impact of climate selection.

The *Drosophila* species shows variations in melanization (either in the body or in the wing) and hence in association with abiotic factors, there is existence of different phenotypes (dark, light body pigmentation or spotted and spotless wings) in nature. It is observed that the appearance of the spot on wing of *D. nepalensis* is restricted in a particular area and this wing spot area (WSA) is limited to males only. The genetic basis of
WSA is not yet clear. In the previous 22 years, 308 studies were reported in the Web of Science database (https://www.webofscience.com/wos/wosce/summary/a46ce381-0d17-4ca4-a9b9-829d80cda765-44d90cea/relevance/1) using the keyword ‘wing spot area’. The majority of studies involved pests, wing size, sex, evolution, development, host, frequency, age, yeast, courtship, variation, and habitat of the USA in D. suzukii or butterflies only. Nevertheless, gaps in the literature exist for different Drosophila species, especially D. nepalensis, with regard to studies of wing melanization, wing spots, sex, structure, evolution, plasticity, inheritance of wing melanization, as well as habitat in diverse islands.

Genetic inheritance determines an individual’s characteristics, which means that the quality transmission occurs generation by generation from parents to progeny. In this study, we used different body colour females (Dark and light) for genetic crosses to study parental inheritance and genetic basis of WSA in males of D. nepalensis, and simultaneously checked any change in WSA parameters, especially in F1 and F2 progenies. We examined the effect of growth temperature on WSA to check the developmental plasticity. Natural sexual dimorphism exists in D nepalensis for melanization (wing spots in males and abdominal spots in females). Therefore, D. nepalensis is an appropriate model for examining (i) maternal/paternal inheritance of WSA (ii) the plasticity of WSA at different temperatures (iii) frequency variation between spotted and spotless males through genetic crosses. Lastly, we compared our findings about WSAs from genetic crosses with field data for possible mechanisms, since natural selection could take place with constant climate change in D. nepalensis.

Material and methods

Collection and culturing

In prewinter (Oct–Nov) and winter (Dec–Jan), we collected ~100–150 individuals of D. nepalensis in highlands (Kasauli and Shimla) of the Western Himalayas using bait traps and net sweeping from fruit markets and godowns. Wild caught flies were maintained at 21°C in all the crosses. After the egg laying in eight vials having 60–80 eggs were maintained at three growth temperatures (17, 21 and 25°C) in all the crosses. We measured the plastic effects on melanization (abdominal pigmentation in females and wing melanization in males) due to the developmental growth temperatures (17, 21 and 25°C) in all the crosses. After the egg laying in eight vials from 30 pairs of each crosses for 8 h at 21°C, two replicate vials having 60–80 eggs were maintained at three growth

Identification of colour dimorphism in both sexes

In D. nepalensis, females show variation in abdominal melanization while males show variation in wing melanization. Females correspond phenotypically to dark and light if 6th and 7th abdominal segments show either completely melanization or no melanization. Alternatively, males are classified as spotted or spotless phenotypically if their wings are with apical dark patch or without apical dark patch respectively.

Dark and light isofemale lines

Abdominal melanization variation is visible in females of D. nepalensis while males of this species are totally black. Vice-versa, wing melanization (spotted and spotless wings) is visible only in males and not in females. We observed that collections done in the winter months, showed totally darker flies, whereas a few lighter flies were obtained along with darker flies from collections done in prewinter months. From the 50 established IF lines at 21°C, we checked true breeding IF lines from the successive progeny and up to 11 generations homozygosity of each IF line was checked for dark and light phenotypes. We checked the genetic basis of melanization through F1 and F2 by crossing dark and light individuals of homozygous IF lines (the lines which were showing 1:2:1 ratio) and found that in F1 the light allele was dominant at 25°C while dark allele was dominant at 17°C. Hence, we labelled all the phenotypes as DD (dark), DL (intermediate/heterozygote) and LL (light).

Mendelian crosses to determine genetic basis

To assess the genetic basis of divergence in melanization for abdominal pigmentation and for wing melanization, we set up the genetic crosses with individuals of true breeding dark IF lines and light IF lines. We collected virgin males (♂) and virgin females (♀) from homozygous true-breeding dark (DD) and light (LL) IF lines of D. nepalensis. Further, we made following crosses to determine the genetic basis of WSA: (i) cross I = DD ♀ × S ♂; (ii) cross II = LL ♀ × SL ♂; (iii) reciprocal cross I = DD ♀ × SL ♂; and (iv) reciprocal cross II = LL ♀ × S ♂. Single male and single female of each phenotype (DD and LL) were allowed for single mating and in the F1 progeny, 100 female flies were scored for abdominal melanization to obtain F2 progeny. Fifty males and 50 females from F1 were selected randomly and 10 pairs were used in five replicates.

Assessment of phenotypic plasticity

We measured the plastic effects on melanization (abdominal pigmentation in females and wing melanization in males) due to the developmental growth temperatures (17, 21 and 25°C) in all the crosses. After the egg laying in eight vials from 30 pairs of each crosses for 8 h at 21°C, two replicate vials having 60–80 eggs were maintained at three growth
temperatures (17, 21 and 25°C). Six-day old fly of the progeny from each vial were analysed for melanization (abdominal in females and wings in males).

**Assessment of abdominal melanization in females**

The following two methods were opted for analysing the body melanization in females: (i) visual scoring using stereo-zoom microscope where body melanization of the female abdomen was scored from dorsal view and values from 0 (no melanization) to 10 (complete melanization) were given for each abdominal segments. (ii) The abdomen of each fly without its viscera was mounted on a slide and analysed using Biowizard software.

**Assessment of wing parameters in males**

After emergence, adult flies were transferred to fresh food, aged 6–7 days. Ten males were randomly taken; their wings were removed and mounted on a microscope slide. The two linear wing measurements made were (figure 1): (i) P, from proximal tip of III vein to anterior cross vein; (ii) D, from anterior cross vein to distal tip of vein III. The length of P and D were measured as wing length (WL) under Olympus stereozoom microscope SZ-11, Japan (https://www.olympus.com) fitted with a micrometer at magnification 3×. As index of wing width, we used the distance between distal ends of the second and fifth longitudinal veins.

The wing area (in mm²) was estimated as WA = WL × WW. Further, WSA (mm²) per fly was calculated through image analysis when male wing was subjected to Biowizard image software (https://www.dewinterindia.com). The validity of visual method is based on highly significant correlation (r = 0.99) with data from image analysis.

Finally, we scored wing melanization(%)

\[
\text{Wing spot area} \times 100
\]

\[
\frac{\text{Wing area}}{\text{Wing spot area}} \times 100.
\]

**Statistical analysis**

We used the \(\chi^2\) test for all crosses to compare expected phenotypes with observed phenotypes. Mean values were used for the representation of data. In F1, to check the dominance effect for melanization (abdominal melanization in females and wing melanization in males) total melanization value of parents was subtracted from F1 values. Statistica 5.0 (Statsoft Inc., Tulsa, Oklahoma, USA) was used for analysis.

**Results**

Data on per cent abundance of wild-caught spotted and spotless males of *D. nepalensis* in prewinter and winter seasons are shown in table 1. Spotted males are always abundant in both seasons but in winters they occurs more frequently. The winters are moderately colder and drier (Tave 17°C, 42.5% relative humidity) whereas the prewinters are

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**Figure 1.** Schematic representation showing measurement of WL and WW.
warmer with higher relative humidity ($T_{ave} 25°C$, 62.8% relative humidity). Thus, significant reductions in $T_{ave}$ and relative humidity may act as selection agents, affecting abundance of spotted and spotless males.

**Genetic analysis of wing melanization in males**

Total number of male individuals in progeny of crosses (I and II) and their reciprocal crosses along with their replicates are shown in table 3. We observed two types of males in progeny of both the crosses based on size of WSA. Inheritance of male wing melanization of *D. nepalensis* in true breeding lines is shown in figure 2 and table 3. Homozygous darker females crossed with spotted males (cross I) always produces spotted wing in males and the homozygous lighter females crossed with spotless males (cross II) always produces spotless wings in males. The trait inheritance in these crosses (I and II) is stable from parent to offspring due to continuous self-breeding in successive generations. Hence, these crosses (I and II) shows stable expression of characters through several generations (figure 2; table 3).

To test the role of parental sex on wing spot area inheritance patterns, reciprocal crosses were performed. Darker females when crossed with spotted males (reciprocal cross I) the $F_1$ progeny is always with spotted wings and their $F_2$ progeny also showed spotless males (figure 3; table 2). Maternal effect in WSA is visible from these reciprocal crosses. The mother’s genotype affects the phenotype of the offspring and hence maternal effect is an important source of phenotypic diversity. Thus, the wing dimorphism (spotted and spotless) in *D. nepalensis* is due to the maternal inheritance.

**Phenotypic plasticity for melanization**

To assess whether melanization differ in plasticity owing to growth temperatures in the range of 17–25°C, data on per cent melanization (abdominal melanization in both females and males while wing melanization only in spotted males) across three growth temperatures are shown in figure 4. For males, there was a complete lack of phenotypic plasticity for abdominal melanization because they remained darker on all growth temperatures (figure 4a). In contrast, there was significant plasticity for the abdominal segments (6th–7th) in $F_1$ female flies. For DL phenotypes, female plasticity level was quite low in the anterior abdominal segments (2nd–5th). However, plasticity of the last two abdominal segments (6th and 7th) was significantly high (figure 4a; % melanization was 80% at 17°C, 60% at 21°C and only 7% at 25°C).

In spotted male flies, the plasticity of wing melanization was significantly higher (figure 4b). Spotted males showed pattern of plasticity (figure 4b) in % wing melanization, i.e., much higher % wing melanization at 17°C ($\sim 38\%$) compared with % wing melanization at 25°C (only 12%). Thus, the significant variations in the melanization (abdominal in females and wing in males) were due to plasticity (figure 4).

**Variation in wing size parameters of males**

The body colour of *D. nepalensis* males shows no plastic effect, but the wing spots show plasticity. The wing size variations (wing length, wing width, wing area and WSA) across different temperatures are shown in table 2. An

### Table 1. Frequency of total males (spotted and spotless) in field in varying seasons (prewinter and winter).

| Seasons    | Prewinter | Winter |
|------------|-----------|--------|
|            | Spotted male | Spotless male | Spotted male | Spotless male |
| Number of individuals | 158 | 52 | 352 | 21 |
| Frequency  | 75.23% | 24.76% | 94.36% | 5.63% |

![Figure 2](image-url)  
*Figure 2.* Genetic crosses in *D. nepalensis* true breeding lines. Homozygous darker females crossed with spotted males (cross I) and the homozygous lighter females crossed with spotless males (cross II). The trait inheritance is stable from parent to offspring due to continuous self-breeding in successive generations.
increase in wing length and wing width was quite low across temperatures. The WSA of spotted males has increased five times at 17°C as compared to 25°C (table 2; \( P < 0.001 \)). Consequently, there was no relationship between the plasticity of abdominal melanization and the plasticity of wing melanization in males.

**Discussion**

Our study aimed to determine the genetic basis and phenotypic plastic effects of wing spot of *D. nepalensis*. By examining the wing spot we were able to determine whether climatic variables may have mediated natural selection. Present work on inheritance of WSA is interesting in several respects (i) quite high plasticity for WSA in *D. nepalensis*. (ii) Increase in number of males with WSA at lower temperature and increase in number of males without WSA at higher temperature. Our result confirms that number of *D. nepalensis* male, eclosing in nature differs significantly in accordance to wing melanization (table 1). Thus, we can say that the thermal variation is playing a significant role in natural selection, as presence of intense WSA in males at lower temperature increases the mating propensity. Our analysis of the genetics of male wing spot inheritance is one of the first attempts to genetically dissect a difference within a species.

The \( F_2 \) male and female progeny of reciprocal genetic crosses fit a two-locus model with a maternal effect on body melanization because we found differences in the Mendelian ratio of the reciprocal crosses I and II (table 3). Further, the cross direction has a more pronounced effect on \( F_1 \) males. Based on these observations, either (i) one of the pigmentation loci is X-linked or (ii) males experience a greater

\[ \text{Wing area} = \frac{\text{Wing spot area}}{\text{Wing area}} \times 100 \]

\[ \text{Wing melanization} (\%) = \frac{\text{Wing spot area}}{\text{Wing area}} \times 100 \]

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maternal effect. An analysis of our data shows that F2 males are showing the large effect of cross direction that was seen in F1 males. The results of our study support the hypothesis that males are more affected by maternal factors. We found that F1 phenotypes of reciprocal crosses showed significantly different levels of thermal plastic effects (abdominal melanization in females and wing melanization in males; figure 4).

The effects of maternal genotypes on evolutionary outcomes are more variable than those of simple Mendalian inheritance (Wolf 2000). According to Schluter and Gustafsson (1993), maternal effects were responsible for more than 25% of the variation in clutch size in collared flycatchers. Life-history traits including total fitness for red deer have been reported to be affected by maternal effects (Kruuk et al. 2000). Melanism was not affected by rearing temperature in pygmy grasshoppers, but rather by genes or epigenetic maternal effects (Forsman 2011). D. melanogaster crosses between European and African strains displayed maternal effects on thoracic trident pigmentation in F1 flies (Capy et al. 1988). There is a maternal effect on body melanization in genetic crosses between laboratory selected dark and light strains of D. melanogaster (Parkash et al. 2012). The present study finds that cross direction has a much greater impact on F1 males. A large effect of cross direction is also retained in F2 males. It is therefore possible that our observations are the result of an epigenetic maternal effect. It is, however, necessary to conduct further research to elucidate such a possibility.

Brisson and co-workers found body melanism correlation with habitat in D. polymorpha of Brazil and gave evidence for the presence of dark and light morph in open and forested environments, respectively (Brisson et al. 2005). However, in the present studies, habitat selection of wing spot males was not investigated but our results show that male wing spot is correlated with female body colour, i.e., if females are darker in colour; males are always with spotted wings and if females are light in colour then males are with spotless wings (figure 3).

Our result (table 1) explains the variation in frequency of total males (spotted and spotless) in field in varying seasons

![Figure 4](image_url). Effect of thermal rearing on % melanization, i.e., abdominal melanization in both females and males (a), while wing melanization in spotted males only (b) across different temperatures (17, 21 and 25°C).

| Crosses | Replicates | Total males $(n)$ | Spotted males | Spotted % | Spotless males | Spotless % | Mendelian ratio | $\chi^2$ test |
|---------|------------|-------------------|---------------|----------|----------------|-----------|----------------|--------------|
| (I) DD ♀ × S ♂ | F1 | 175 | 175 | 100 | 0 | 0 | – | – |
| (II) LL ♀ × SL ♂ | F1 | 139 | 0 | 0 | 139 | 100 | – | – |
| Reciprocal crosses | | | | | | | | |
| (I) DD ♀ × SL ♂ | F1 | 115 | 115 | 100 | | | | |
| F2 | 1 | 105 | 79 | 75.24 | 26 | 24.76 | 3.04 : 1 | ns |
| 2 | 110 | 83 | 75.45 | 27 | 24.55 | 3.07 : 1 | ns |
| 3 | 113 | 85 | 75.22 | 28 | 24.78 | 3.03 : 1 | ns |
| 4 | 106 | 79 | 74.53 | 27 | 25.47 | 2.92 : 1 | ns |
| 5 | 95 | 71 | 74.74 | 24 | 25.26 | 2.96 : 1 | ns |
| (II) LL ♀ × S ♂ | F1 | 100 | 0 | 0 | 100 | 100 | – | – |
| F2 | 1 | 106 | 106 | 100 | – | – | – | – |
| 2 | 113 | 113 | 100 | – | – | – | – |
| 3 | 116 | 116 | 100 | – | – | – | – |
| 4 | 95 | 95 | 100 | – | – | – | – |
| 5 | 115 | 115 | 100 | – | – | – | – |

DD ♀, homozygous dark female; LL ♀, homozygous light female; S ♂, spotted male; SL ♂, spotless male; ns, nonsignificant.
(prewinter and winter). Winter season select spotted flies because wing spot formation on wings in male is directly correlated to female body melanization and females in winter (lower temperature) are always dark. A phenotypic manifestation of wing melanization in \textit{D. nepalensis} has two interesting features: (i) WSA in nature as well as in laboratory effects (Brisson et al. 2005); and (ii) for populations of two-spot ladybird, lack of geographical variation in morphs (nonmelanic being the dominant allele) (Brakefield 1985; Majerus 1994). The occurrence of such genetic strategies across insect taxa should have adaptive significance. The dominance of spotted male over spotless male might help \textit{D. nepalensis} to adjust environmental conditions.

For \textit{D. nepalensis} occurrence of wing colour polymorphism and its inheritance has not been reported previously. It is worth consideration to check fitness of an individual when phenotype of organism is in accordance with behaviour. The common trait of insect taxa is melanism and it has mixed evolutionary effects. Ecological significance of melanism in cold-adapted temperate insect taxa including cosmopolitan and temperate endemic drosophilids favours thermal-melanism hypothesis (Sabath et al. 1973; Majerus 1998). \textit{D. nepalensis} have adapted to climatic stresses by modifying the frequencies of wing spot males through assortative mating. It may be concluded that seasonal changes can help to maintain frequency of spotted and spotless males in \textit{D. nepalensis}.

The datasets used and/or analysed during the current study are included in this article and its supplementary information files available from the corresponding author on reasonable request.

Acknowledgements

Authors are grateful to Chandigarh University. SR and DS both are grateful to the Department of Science and Technology (DST), Ministry of Science and Technology, New Delhi for the financial support through the SP/YO/775/2018G and SP/YO/2019/1165(G) project respectively.

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Corresponding editor: DURGADAS P. KASBEKAR