

Intraspecific variation in wing colour is related to larval energy reserves in monarch butterflies (*Danaus plexippus*)

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Abstract. The physiological basis for pigment synthesis in lepidopteran wing scales is well-studied, although less is known about the reasons why individuals of the same species vary in pigmentation. Monarch butterflies (*Danaus plexippus* L.) show subtle variations in the shade of orange on their wings and this is known to predict flight ability and mating success. The present study tests the possibility that the shade of orange is associated with the amount of residual energy reserves carried over from the larval stage. Using monarchs reared in captivity under identical conditions ($n = 207$), the residuals of a regression of wing size and mass at eclosion, which indicate larval energy reserves, are obtained. This measure is positively related to adult longevity without feeding, indicating the importance of this reserve to the monarchs, as well as the value of the measure for this investigation. The shade of orange (i.e. hue) is determined on scanned wings using image analysis. Importantly, orange hue is predicted significantly by residual mass at eclosion (individuals with more mass are redder). The linkage between these traits may explain previous findings whereby redder monarchs fly further and mate more because both behaviours would be enhanced with greater energy stores. The findings of the present study add to a growing body of work showing how intraspecific variation in pigmentation has biological significance to monarchs, and possibly other butterflies. Although much remains to be investigated regarding the physiological underpinnings of this variation, the results of the present study indicate that future efforts should be rewarding.

Key words. Condition, *Danaus plexippus*, larval reserves, monarch butterflies, wing colour.

Introduction

Integument colour is important for the biology of insects for a variety of reasons, including for intraspecific signalling of status (Wiernasz, 1995; Burghardt *et al.*, 2000), for signalling chemical defence (Bezzler *et al.*, 2007), and for thermal absorption and flight activity (Roland, 1982; Ellers & Boggs, 2004; Davis *et al.*, 2005; Hegna *et al.*, 2013). The physiological mechanisms behind the development of insect pigmentation represent a body of research that has spanned over a century (Mayer, 1896; Ford, 1944; Nijhout, 1991; Nijhout *et al.*, 2003; Casas & Simpson, 2010). Much of this work focuses on butterflies, which are well-suited to experimental manipulation and have brightly-coloured wing patterns. The colour patterns

of butterfly wings arise from a mosaic of individually-coloured scales on the wings. Pigment formation on the scales occurs during the final stages of metamorphosis, when scale-forming cells take up pigment precursors from the haemolymph and synthesize the pigment to deposit onto the scales (Nijhout, 1980; Koch, 1991). Because all of the raw materials for synthesizing pigment (and other adult tissues) are obtained during the larval stage, the quality of adult wing pigmentation depends largely on the ability of larvae to procure and store such materials. This remains an active area of research (Knüttel & Fiedler, 2001; Kemp, 2008; Lindstedt *et al.*, 2009; Johnson *et al.*, 2014).

Monarch butterflies (*Danaus plexippus* L.) (Fig. 1) have characteristic orange and black wings, and, although it is difficult to see with the naked eye, the shade of orange varies subtly among individuals, going from pale orange (more yellow) to darker, almost reddish tones. This variation appears to have considerable biological significance; monarchs that migrate in the autumn have redder shades of orange than summer breeding

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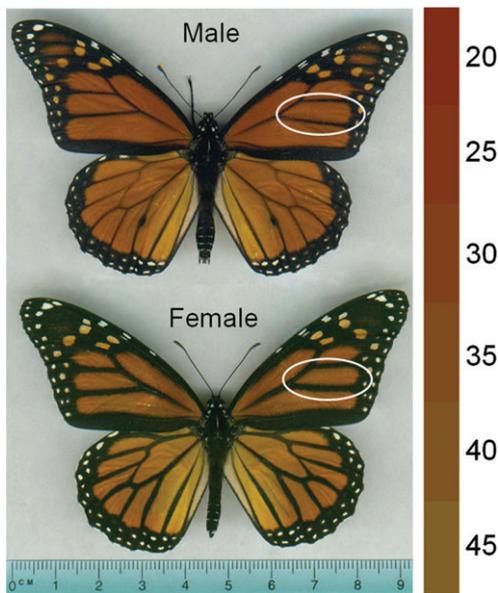


Fig. 1. Male and female monarch butterflies (*Danaus plexippus*). White circles indicate the wing cells in which orange colour (average pixel hue score of the cell) was measured using image analysis software (see Materials and methods). The orange bar on right shows the range of orange hue scores that are typical of monarchs (using image analysis).

monarchs (Davis, 2009) and the shade of orange predicts flight endurance and migration success (Davis *et al.*, 2012; Hanley *et al.*, 2013). Furthermore, males with redder orange wings also have higher mating success (Davis *et al.*, 2007). Although these studies demonstrate the importance of even minor variations in wing colour in *D. plexippus*, there is still no mechanistic explanation for what makes a redder monarch more successful at these behaviours. Importantly, recent work shows that orange colour is not associated with body structures important for flight, such as flight muscle size, wing shape or wing loading (A. K. Davis & M. Holden, unpublished observations). Therefore, the reason for the higher flight ability and mating success of redder monarchs appears not to be because of any outward, physical differences in these individuals. One strong possibility, then, is that wing colour relates to something internal or physiological. In the present study, the possibility is tested that the intraspecific variation in orange wing colour of monarchs is associated with the amount of energy reserves that is carried over from the larval stage.

In all holometabolous insects, adults emerge from their pupal stage with residual energy stores carried over from their larval stage in the form of the fat body (Arrese & Soulages, 2010). These stores are considered to sustain the adults until they can feed (Rivero *et al.*, 2001; Arrese & Soulages, 2010) and they also contribute to somatic maintenance of adults (Oberhauser, 1997). Furthermore, larval reserves are important for reproductive activities; in males, larval reserves help to form spermatophores (Oberhauser, 1989) and females use larval reserves for egg development (Oberhauser, 1997; Rivero *et al.*, 2001). Collectively then, the residual fat stores leftover

from the larval stage have a number of important functions for adult insects.

The possibility that adult wing pigmentation may be linked with fat deposits in *D. plexippus* is indicated by the redder shades of orange seen in the migratory generation (Davis, 2009). This generation, produced at the end of the summer and early autumn, migrates to central Mexico to spend the winter. To accomplish this, these adult monarchs must have considerable fat deposits to fuel their long-distance flights (Brown & Chippendale, 1974; Brower *et al.*, 2006; Satterfield *et al.*, 2013). Although much of this fat is likely deposited during the adult stage (i.e. from converting nectar to lipids), there must also be sizeable deposits that are carried over from the larval stage to help sustain the adults prior to starting the journey. Although there is insufficient research available on the early stage of the migration to be certain of the importance of larval fat reserves, larvae reared experimentally under cool temperatures (such as they would experience in early autumn) spend longer as larvae and become adults with larger fat stores (Wensler, 1977).

Materials and methods

Rearing and handling of *D. plexippus*

Monarchs (*D. plexippus*) from this project stemmed from an experiment conducted in 2005 examining effects of parasitism with *Ophryocystis elektroscirrha* (de Roode *et al.*, 2007), although, for the purposes of the present study, only the nonparasitized individuals from that experiment were considered ($n = 207$). Monarch larvae were the grand-progeny of a cross between wild-caught migrating butterflies obtained from Virginia, U.S.A. (September 2004) and wintering monarchs in Central Mexico (February 2005), thus representing monarchs from the eastern U.S. population. All larvae were reared individually in 1-L plastic containers and fed greenhouse-grown swamp milkweed (*Asclepias incarnata*) *ad libitum* until pupation. Rearing was carried out in a temperature controlled room (24 °C) with natural lighting from large windows. The hatching dates of all larvae from eggs were known and the dates of pupation and eclosion were recorded, so that the development time of all individuals could be obtained. When adults had eclosed (and when their wings had finished expanding and hardening), their sex was recorded and they were weighed with an electronic scale. Next, they were placed in a glassine envelope and stored in an incubator at 13 °C and then were checked daily to record the date of death, so that adult longevity (without feeding) could be obtained. This is a measure that is used routinely as a proxy for adult fitness in studies involving the *O. elektroscirrha* neogregarine parasite (de Roode *et al.*, 2008a,b; de Roode & Altizer, 2010). Note that longevity was not recorded for three individuals.

Wing measurements

At the termination of the above experiment, the forewings were removed from the monarchs and scanned with an

HP Scanjet flatbed scanner (Hewlett-Packard, Palo Alto, California) to produce a digital image for measurement (Davis *et al.*, 2012; Sander *et al.*, 2013). The scanner settings were constant for all individuals and no colour-correction was used. Then, using image analysis software (FOVEAPRO; <http://www.reindeergraphics.com>), the surface area of each forewing was measured (mm^2) and averaged for each individual. Next, the central orange cell of the forewing was selected (Fig. 1), and the average pixel hue score of the entire selection was obtained. Digital images are made up of millions of pixels and each pixel is associated with three colour scores (hue, saturation and brightness) that make up the overall appearance of the pixel. Prior work with monarchs showed how the hue score imparts the greatest significance on these (Davis, 2009; Davis *et al.*, 2012; Sander *et al.*, 2013), although saturation has also been used to score orange 'brightness' (Davis *et al.*, 2007). With computer software, hue is measured in degrees ($0\text{--}360^\circ$), with each degree representing a different colour shade. For *D. plexippus*, the orange wing colour (hue) typically varies from 20 to 45° , with lower scores indicating more reddish orange colours and higher scores indicating paler, more yellowed colours (Fig. 1). Hue was measured on both forewings and averaged for each individual.

This method of measuring wing colour differs from other studies where spectrophotometric techniques are employed to measure the amount of reflected light at a wide range of wavelengths, including the ultraviolet range (Morehouse *et al.*, 2007; Kemp *et al.*, 2008; Pegrarn *et al.*, 2013). However, hue scores produced from digital scans of monarch wings have been shown to be correlated with spectrophotometer readings between 580 and 780 nm (the wavelengths of yellow, orange and red colours; Davis *et al.*, 2012). Similar comparisons of hue scores from digital scans and spectrophotometer readings of monarch wings found consistent results (Hanley *et al.*, 2013). It is not known whether monarchs are capable of perceiving wavelengths in the ultraviolet range (Blackiston *et al.*, 2011), although they can clearly discriminate between colours in the visible spectrum (red, orange, yellow, green, blue and purple) and they have an innate preference for orange, yellow and red colours (Blackiston *et al.*, 2011).

Statistical analysis

The hue scores, wing sizes and adult longevity of monarchs were normally-distributed, based on visual inspection of their distributions. To estimate energy reserves for each monarch, the residuals were extracted from a regression of adult mass versus forewing size. This approach has been used before to estimate 'fatness' of monarchs after eclosion (Oberhauser, 1997). Both variables were positively correlated ($r = 0.78$, $P < 0.0001$) (Fig. 2). These residuals represented how much mass each adult butterfly had just after eclosion, and taking into account overall size (indexed by wing area). To determine how this energy reserve affects adult longevity, a general linear model was used with longevity as the response and sex and residual mass as predictors. Then, to determine whether larval energy reserves are a predictor of wing colour, orange hue score was used as

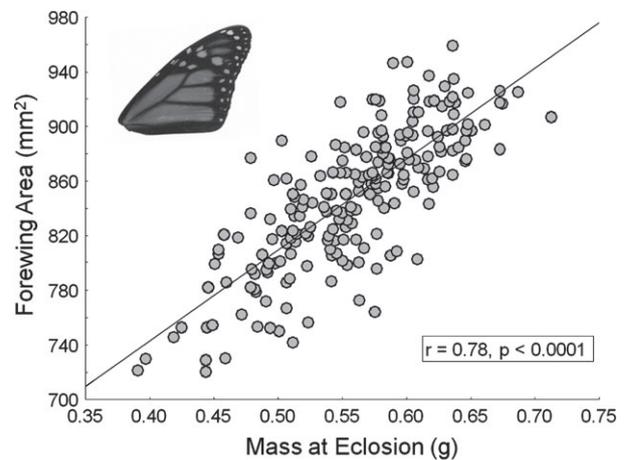


Fig. 2. Relationship between wet mass at eclosion and forewing surface area of monarch butterflies *Danaus plexippus* in the present study ($n = 207$). The residuals of this relationship were used to index larval energy reserves.

the response variable in a generalized linear model with sex and residual weight as predictors. Two-way interaction terms were included in both models but removed if not significant. All analyses were conducted using the STATISTICA, version 6.1 (<http://www.statsoft.com>).

Results

In the present study, adult monarchs lived between 12 and 25 days without feeding, and the overall mean \pm SD was 17.2 ± 2.6 days. There was no significant difference between males and females in terms of adult longevity ($F_{1,201} = 2.93$, $P = 0.089$), although there was a trend for males to live approximately 1 day longer than females. Residual body mass significantly predicted adult longevity ($F_{1,201} = 55.56$,

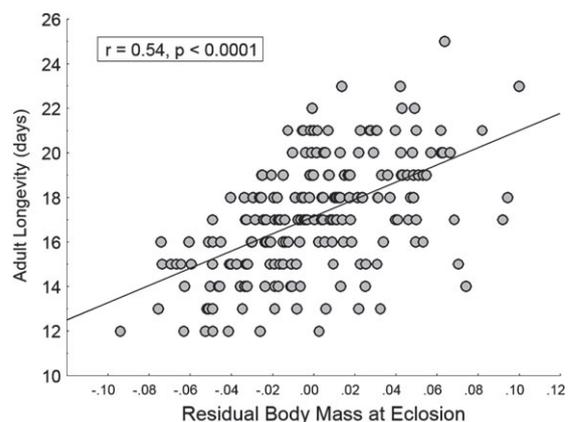


Fig. 3. Relationship between residual mass at eclosion (an index of larval energy reserves) and longevity of adult monarchs (without feeding). Residual mass was obtained from a regression of mass and forewing area (Fig. 2).

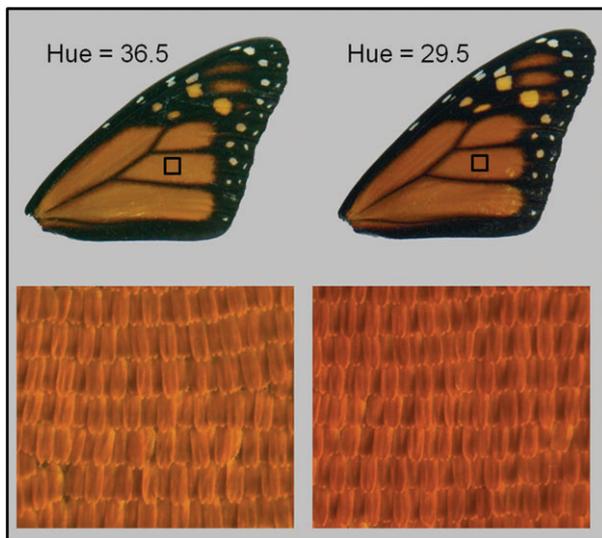


Fig. 4. Scanned images of male *Danaus plexippus* forewings that span the upper and lower range of hue scores (for males) in the present study. Note the difference in the shade of orange in the two wings. Lower boxes show magnified images of scales from the central wing cell of each forewing. All monarchs in the present study were reared under identical conditions and with matching diets.

$P < 0.0001$) and the two measures were positively correlated ($r = 0.54$, $P < 0.0001$) (Fig. 3). The interaction of sex and residual mass was not retained in the final model.

Orange hue scores of monarchs in the present study ranged from 32.1° to 40.2° in females and from 29.5° to 36.5° in males. This variation can be visualized in Fig. 4, which shows wings of two males that span the male hue scores. In the model examining orange hue, there was no support for the inclusion of the interaction term (sex \times residual mass; $F_{1,203} = 1.72$, $P = 0.1915$). The model with main effects only showed a significant effect of sex ($F_{1,204} = 138.4$, $P < 0.0001$), which was expected because males have lower hue scores in this species (Davis, 2009; Johnson *et al.*, 2014). Importantly, residual mass was a significant predictor of orange hue ($F_{1,204} = 6.80$, $P = 0.0010$). This relationship was such that monarchs with greater residual mass at eclosion tended to have lower hue scores (i.e. redder shades of orange) (Fig. 5). In other words, monarchs with greater energy reserves carried over from the larval stage also tended to have the reddest wing colour.

Discussion

The primary result of the present study, showing that individual variation in wing pigmentation is associated with the size of larval energy reserves in monarch butterflies *D. plexippus*, represents an area of research (within entomology) in its early stages. Although there is a solid foundation of knowledge about basic pigment synthesis in lepidopteran scales (Nijhout, 1980, 1991, 2010; Koch & Kaufmann, 1995), there is limited information about what factors influence intraspecific variation in pigment, or why two butterflies of the same species would

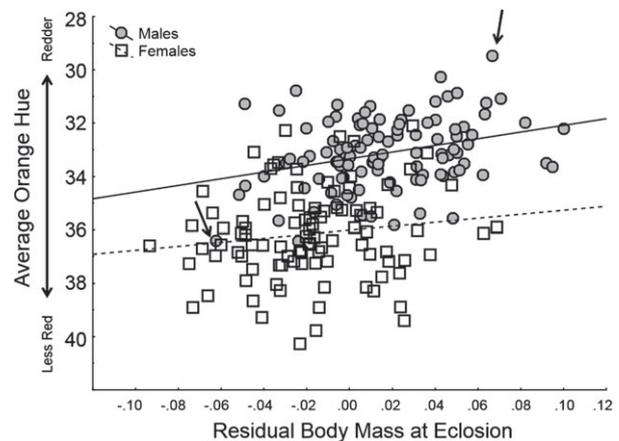


Fig. 5. Relationship between residual mass at eclosion (a proxy for larval energy reserves) and wing colour of monarch butterflies, *Danaus plexippus* (orange hue score). Values on the y-axis are reversed so that redder shades are at the top. Separate trend lines indicated for males and females. Arrows designate males that are shown in Fig. 4.

show differences in the shade of pigmentation on the same part of the wing. As a result of extensive research spanning well over a century (Mayer, 1896; Ford, 1944; Nijhout, 1980; Nijhout & Koch, 1991; Nijhout *et al.*, 2003), it is known that pigment is synthesized within scales during the final stages of metamorphosis, when scale-forming cells take up pigment precursors from the haemolymph (Koch, 1991). Furthermore, in Nymphalid butterflies (of which monarchs are a member), the orange–red pigments are identified as ommochromes, which are derived from tryptophan (Koch, 1991; Nijhout & Koch, 1991).

Despite the long history of research into pigment formation in butterflies, it is less clear why the shade of pigment varies between individuals, such as the two male monarchs shown in Fig. 4, especially when reared under identical conditions. One logical explanation is that the concentration of ommochrome pigment varies among individuals (redder individuals have more pigment). This same explanation does indeed hold true for other butterflies; pigment concentration (pterins) within scales is known to affect individual wing colour in pierid butterflies (Morehouse *et al.*, 2007). Given that all pigment is formed *de novo* from material acquired by larvae, it then is reasonable to assume that individual larvae differ in food intake (and by extension, quantity of pigment precursors) even given unlimited food (all larvae in this experiment were fed *ad libitum*). However, this idea may not hold true for *D. plexippus*. Although diet restrictions in the larval stage can influence adult wing colour in other Lepidoptera (Pegram *et al.*, 2013), larval experimental food restriction in monarchs appears to have little effect on wing colour (Johnson *et al.*, 2014). Further research is needed to clarify why certain individual *D. plexippus* appear better able to synthesize and deposit ommochrome pigments.

Regardless of the physiological mechanism behind the association between fatness and colour, this pattern does reinforce the linkage between individual condition and wing colour

in monarch butterflies, which is suggested in many prior studies (Davis *et al.*, 2007, 2012; Hanley *et al.*, 2013; A. K. Davis & M. Holden, unpublished observations). Moreover, it also sheds light into the mechanism behind these prior findings. For example, variation in orange colour is shown to predict both flight endurance and migration success in monarchs (Davis *et al.*, 2012; Hanley *et al.*, 2013). Data from the present study suggest that redder monarchs may accomplish this by having greater energy reserves for enabling long-distance flight. Moreover, this same mechanism could explain the redder colour of the migratory generation of monarchs (Davis, 2009); presumably, these individuals would also have greater larval reserves in preparation for the long-distance flight.

The results of the present study also help to explain other work showing how male monarchs with redder colour have higher mating success than paler individuals (Davis *et al.*, 2007). The mating system of monarchs is unusual among the Lepidoptera, such that males capture females in flight, wrestle them to the ground and attempt to copulate (Van Hook, 1993; Oberhauser & Frey, 1997). It is unclear whether females have any choice in this 'coercive mating' system (Solensky & Oberhauser, 2004), although they do vary in their resistance during the initial struggle (Frey *et al.*, 1998; Frey, 1999). Regardless of which sex is choosier, males with redder shades of orange appear to be more successful at mating (Davis *et al.*, 2007). In theory, these males could produce larger (or perhaps more) spermatophores, which are formed in part from larval resources (Oberhauser, 1989). Another (and simpler) interpretation is that the larger fat stores of redder males may give them an energetic advantage during the initial mating struggle, and/or when males carry the females as they remain coupled, which can take up to 14 h (Svard & Wiklund, 1988).

It should be noted that, although this line of research is relatively new, such questions regarding the relationship between larval resources and adult wing colour, as well as other relationships, should eventually become clearer now that technologies are available for assessing individual colouration in butterflies and other animals, such as image analysis (Davis *et al.*, 2004; Davis & Grayson, 2007; Davis & Castleberry, 2010) and spectrophotometric methods (Hill & Farmer, 2005; Quesada & Senar, 2006; Hanley *et al.*, 2013). Ornithologists are embracing these developing techniques rapidly to ask questions similar to that of the present study: what determines variation in plumage brightness within species (Hill, 2002; Saks *et al.*, 2003; Quesada & Senar, 2007; Maney *et al.*, 2008). The results of such efforts generally show how feather brightness is a signal of health or condition, especially in males.

In summary, the present study aims to determine the association between wing colour and larval energy reserves in butterflies using a cohort of healthy monarch butterflies reared under identical conditions. Residual mass at eclosion (as a proxy for larval reserves) is correlated positively with adult longevity when adults are not given food. Moreover, larval reserves are associated with the shade of orange pigmentation in adult wings. Monarchs with redder shades of orange tend to have greater larval energy reserves. This association helps to explain multiple prior studies showing linkages between pigmentation, flight and

mating success in monarch butterflies, and emphasizes the close connection between larval performance and adult fitness in butterflies.

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