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GENETICS OF A "ZEBRA" PIGMENT MUTATION IN THE LARVAE OF *DANAUS PLEXIPPUS*, L. (NYMPHALIDAE: DANAINAE)

KAREN S. OBERHAUSER[1]

Department of Ecology, Evolution and Behavior, University of Minnesota, 1987 Upper Buford Circle, Saint Paul, Minnesota 55108, USA

DE CANSLER

Willow Creek Middle School, Rochester, Minnesota 55904, USA

AND

ANN FEITL

Sunrise Park Middle School, White Bear Lake, Minnesota 55110, USA

ABSTRACT. We report a mutation in monarch butterflies that results in a lack of yellow pigment in larvae, pupae and eggs. Genetic studies reveal that this phenotype is caused by a recessive autosomal allele with normal Mendelian segregation. We did not detect an effect on butterfly size or asymmetry, but mutant individuals of both sexes appear to suffer lower reproductive success than normal individuals. We hypothesize that the mutants are unable to sequester carotenoids from their larval host plants.

Additional key words: carotenoids, pigmentation.

Lepidopteran coloration plays important roles in camouflage, warning, mimicry, and mate recognition, and knowledge of the genetic and biochemical mechanisms of pigmentation will lead to greater understanding of evolution in this group. Here we report a mutation in the monarch butterfly, *Danaus plexippus* L. (Nymphalidae), that affects egg, larval, and pupal pigmentation. This mutation shows simple Mendelian inheritance, and we hypothesize that it is caused by a recessive autosomal allele that prevents carotenoid sequestration in larvae.

MATERIALS AND METHODS

In May 1994, we received 30 monarch pupae from Tennessee to use as parents of butterflies for several experiments. These were all the offspring of three wild females, but the parentage of individuals was not known. All of the Tennessee pupae had normal appearance, as did the adults that emerged from them. Adults were kept in glassine envelopes for four days after emergence, then put into a large outdoor screen cage,

[1] To whom correspondence should be addressed

where eleven pairs mated. Mated females were kept in individual outdoor cages with cuttings of *Asclepias syriaca* L. (Asclepiadaceae), and between 75 and 150 eggs from each were reared to adulthood. Larvae from each female were kept in separate screen cages in a screened room where they experienced ambient temperatures and photoperiod. They were fed fresh cuttings of *A. syriaca* daily. One day after experimental adults emerged, we weighed them to the nearest 0.01 mg on a Mettler analytical balance, and measured the length of both forewings to the nearest 0.01 mm with a vernier caliper.

RESULTS

Zebra phenotype. One of the eleven F_1 sibling broods from the Tennessee butterflies contained larvae that lacked the yellow stripes characteristic of normal monarch pigmentation. Because of their alternate white and black stripes, we called this unusual phenotype "zebra." Other larvae in the brood were normally colored. Zebra pupae were blue-green, and their metallic spots silver. Siblings with a normal phenotype produced typical green pupae with gold spots. We detected no differences between zebra and normal adults, but zebra females laid white eggs, while typical monarch eggs are cream-colored.

We compared the size (mass and right forewing length), and forewing asymmetry (the absolute value of the difference between the right and left forewing lengths) of zebra individuals with other experimental butterflies. Comparisons were made both between sibships (that contained and did not contain zebra individuals) and within zebra sibships. These two comparisons were made because there are sometimes family effects on size (Oberhauser 1989, and unpubl. data) that could confound effects of the zebra genotype. We analyzed males and females separately, since females were slightly smaller than males. The results of two-tailed t-tests of these comparisons are summarized in Table 1. There were no mass or asymmetry differences between any of the groups, but the family that contained zebras had significantly shorter forewings than the rest of our butterflies. This was true of both males and females. Within this family, however, forewing lengths of zebra individuals were not significantly different from their normal siblings.

We have not carried out detailed studies of life history characteristics of zebra individuals, but our observations suggest that there are fitness consequences of this mutation. Zebra males rarely mate when put into cages with females, and are more difficult to hand-pair than normal captive males. Zebra females lay fewer eggs than normal females and a higher proportion of their eggs fail to hatch.

Transmission of the zebra trait. The proportions of normal and zebra individuals in the initial zebra brood and the likelihood that their

TABLE 1. Size and asymmetry comparisons of zebra to normal larvae of *Danaus plexippus*. A, males. B, females. All measurements compared using 2-tailed two sample t-tests. Measurements given are group means followed by one SD (in parentheses).

	N	mass (mg)	right forewing length (mm)	abs.value forewing asymmetry (mm)
A: males				
zebra family	36	525.76 (48.10)	50.88 (1.47)	0.28 (0.21)
all others	341	518.00 (55.24)	51.98 (1.89)	0.32 (0.35)
p		0.418	0.003	0.514
zebra individuals	12	531.20 (36.20)	51.15 (0.91)	0.25 (0.21)
normal siblings	24	523.03 (53.58)	50.68 (1.78)	0.31 (0.21)
p		0.618	0.407	0.476
B: females				
zebra family	28	480.00 (52.54)	50.20 (1.89)	0.36 (0.29)
all others	404	479.71 (58.46)	51.39 (2.17)	0.32 (0.36)
p		0.958	0.008	0.560
zebra individuals	10	480.49 (59.50)	50.22 (2.30)	0.32 (0.24)
normal siblings	18	479.13 (39.90)	50.16 (1.14)	0.39 (0.31)
p		0.947	0.939	0.503

parents were siblings suggested that the trait was caused by a recessive allele. To test this, we set up matings among zebra adults, their siblings with normal pigmentation, their offspring, and non-siblings from our experimental stock. All matings that involved zebra males were hand-paired, as were about half of the other matings (we hand-pair butterflies often when we want specific individuals to mate, and have no evidence that this affects mating success). We reared offspring from all crosses as described above, with the exception of those in Table 2f. These crosses were done in our laboratory in the fall and winter, and the larvae reared on *A. currasavica* (Asclepiadaceae) or *Cynachum laeva* (Asclepiadaceae) grown in a greenhouse.

Table 2 summarizes the results of these crosses, and gives the expected proportion of offspring with the zebra phenotype from each type of cross. Expected values were calculated as follows: We called the recessive zebra allele *z*, and the dominant, wild-type allele Z. Thus the genotype of a zebra individual is hypothesized to be *zz*, and a normal individual ZZ or *Zz*. A *Zz* x *Zz* cross should result in a brood that contains 1/4 ZZ, 1/2 *Zz* and 1/4 *zz* individuals. Two-thirds of zebra x zebra sibling matings from a brood with this genotype ratio should be *zz* x *Zz*, and thus produce 1/2 zebra individuals, while one-third should be *zz* x ZZ and produce only individuals with a normal phenotype. Crosses between

TABLE 2. Phenotypic ratios of the zebra trait in laboratory crosses in *Danaus plexippus*. Zeb=zebra individual, Sib=normal sibling of zebras, NS=individual that is a non-sibling of zebra family, F_2=offspring of Zeb x NS cross; letters followed by the same superscript represent the same individual (all other individuals are unique). Confidence intervals when proportion of zebras = 0 or 1 calculated according to Blythe and Still (1978).

	♀ x ♂ phenotypes	# zebra larvae	# normal larvae	observed proportion zebras	95% CI for proportion zebras	expected proportion zebras
a	initial cross	45	105	0.30	0.23 – 0.37	0.25
b	Zeb x Zeb	45	0	1.0	0.902 – 1.0	1.0
c	Zeb x Sib[1]	0	10	0	0 – 0.38	2/3 broods 0.5, 1/3 0
	Zeb x Sib[1]	0	9	0	0 – 0.43	
	Sib x Zeb[2]	0	72	0	0 – 0.063	
	Sib x Zeb[2]	0	46	0	0 – 0.096	
d	Sib x Sib	15	36	0.29	0.17 – 0.41	4/9 broods 0.25, 5/9 0
	Sib x Sib	14	24	0.37	0.22 – 0.52	
e	NS x Zeb[3]	0	97	0	0 – 0.048	most broods 0
	NS x Zeb[3]	0	74	0	0 – 0.062	
f	F_2 x F_2	41	133	0.24	0.21 – 0.27	0.25
	F_2 x F_2	26	77	0.25	0.17 – 0.33	

two zebra siblings should only result in any zebra offspring if both parents are *Zz*; this should be true 4/9 of the time. Depending on the contribution of the three wild females to our initial stock, from 1/2 (if the female containing the zebra gene [or mated to a male containing the gene] produced all thirty individuals) to 1/30 (if this female only produced a single individual) of our stock are expected to have been zebra heterozygotes. If all three females contributed equally to the stock, we would expect 1/6 of this stock to be zebra heterozygotes. Thus it is impossible to calculate precisely the expected outcomes of matings that include non-siblings from our initial stock.

The initial zebra brood, which we assumed resulted from a *Zz* x *Zz* mating, contained 32% zebra larvae (Table 2a). The matings summarized in Table 2b-2e were either between siblings from this brood or one individual from this brood and a non-sibling. All offspring from a mating between two zebra parents (*zz* x *zz*) were themselves zebras (Table 2b). All of the offspring from crosses between zebras and their normal siblings (*zz* x Z_) were normal (Table 2c). Two crosses between normal siblings from the initial mating (Z_ x Z_) resulted in broods that contained both larval types (Table 2d). Crosses between zebras and non-siblings resulted in normal larvae (Table 2e).

We used F_2 offspring from the crosses between zebras and unrelated individuals represented in Table 2e for the matings in Table 2f. While all of these individuals had a normal phenotype, they should have been

heterozygotes. As predicted, presumed *Zz* x *Zz* matings resulted in broods that contained both types of larvae (Table 2f).

The 95% confidence intervals for the percentages of zebra larvae in crosses between presumed heterozygotes (Table 2a, 2d and 2f) included 25%, as predicted by our hypothesis. The bias toward higher than expected percentages of zebras in crosses represented in Table 2a and 2d could be explained by our summer rearing technique. We keep individual broods together, but in some cases larvae escape from their cages or wander away while we change their plants. Larvae of unknown origin are discarded so that we do not mix broods. Wandering zebra larvae were not discarded, because their origin was usually clear. The F_2 crosses shown in Table 2f were done in the fall, when we keep larvae on potted plants, thus reducing the likelihood of escape; in both of these crosses the proportion of zebra larvae was close to the prediction.

The fact that all of the progeny from crosses in Table 2c (between three normal individuals and their zebra siblings) were normal suggests that the three normal siblings chosen for these matings were all *ZZ*. The probability of three *ZZ* individuals being drawn for these matings is 0.037 (0.333^3), given our genetic hypothesis. Even though two of these crosses resulted in few viable offspring, *zz* x *Zz* crosses should result in broods that are half zebras, and neither of the 95% confidence intervals for these small broods included 50%.

Both of our crosses between normal siblings produced mixed broods (Table 2d), suggesting that both crosses were *Zz* x *Zz*. The probability of this occurring is 0.197 (0.667^4).

Sex ratios in all broods did not differ significantly from 50% (data not shown), suggesting that there is no sex linkage of this trait.

DISCUSSION

Our results support the hypothesis that the zebra trait in monarchs is caused by a recessive autosomal allele. One successful cross between two zebra individuals resulted in the only brood that consisted solely of zebra individuals (Table 2b), crosses between zebra and non-siblings resulted in broods that all appeared normal (Table 2e), and crosses between their presumed heterozygote progeny resulted in broods that had one quarter zebra offspring (Table 2f). There was no sex bias in the transmission of the trait.

We should note that crosses represented in Table 2c between zebra individuals and three of their siblings with normal phenotypes are inconsistent with the hypothesis; two thirds of the normal siblings are expected to be heterozygous carriers, yet we saw no zebra individuals in any of these broods. The only explanations that fit the rest of our results are that, against statistical odds, we chose three homozygous normal in-

dividuals for these matings, or the small broods from two matings misrepresent their possible phenotypes.

We have not studied the biochemical mechanisms that are responsible for the lack of yellow pigment in our zebra larvae and pupae, and their eggs. However, based on studies of larval pigmentation in other Lepidoptera, we hypothesize that these larvae do not sequester the carotenoids they obtain from their host plant. Carotenoids (carotenes and xanthophylls) are often responsible for yellow coloration in insects, including Lepidoptera (e.g., Chapman 1982, Kayser 1985). When the yellow carotenoids are combined with blue bile pigments, they result in the green colors that are common in many lepidopteran larvae and pupae (Feltwell 1978, Rothschild 1978, Kayser 1985). Although we have found no reported cases of mutant larvae lacking yellow coloration, other workers have implicated autosomal recessive genes in the blue-green coloration of larvae that are normally green (*Colias philodice*, Gerould 1921, Hoffman & Watt 1974; *Hyalophora cecropia*, Waldbauer & Sternburg 1972; and *Papilio memnon*, Clarke & Sheppard 1973). Phenocopies of these mutants can be produced by feeding larvae artificial diets lacking carotenoids (Clarke 1971, Valadon et al. 1975, Feltwell 1978, Rothschild 1978). In addition, the larvae of other Danainae raised on carotenoid-free diets produced pupae in which the gold sheen was silver (Rothschild et al. 1978), and *Pieris brassicae* larvae reared on diets that contained almost no carotenoids laid white, but fertile eggs (Rothschild 1978).

Hoffman and Watt (1974) demonstrated that the hemolymph of normal *Colias philodice* larvae contains a yellow pigment, presumably the carotenoid lutein, bound to a protein with a molecular weight of about a million daltons. This carotenoid/protein complex was absent in the hemolymph of blue-green mutants. Bergman and Chippendale (1992) demonstrated that carotenoids in larvae of the southwestern corn borer, *Diatraea grandiosella*, are transported in the hemolymph by lipophorin. It is likely that a mutation in the gene coding for this or a similar protein is responsible for the lack of yellow pigment in our zebra line and other mutant lepidopteran lines described above.

In addition to their role in coloration, carotenoids have two well-documented functions that could be implicated in the lower fitness that we observed in zebra monarchs. These include protecting cells from photo-oxidative damage caused by the absorption of visible and near UV light (Krinsky 1979), and serving as precursors for the formation of visual pigments that are needed for normal vision (Kayser 1985). Rothschild (1978) noted that adult *Pieris brassicae* reared on carotenoid-deficient diets were rather inactive, and suggested that this was due to defective vision. This could explain the low mating ability of zebra males in cages,

but is unlikely to be responsible for our lack of success in hand-pairing them. It is also possible that they had suffered photo-oxidative damage.

It is interesting that there are no clear effects on adult size associated with the trait. We suspect that the shorter winglength in the family that produced the zebras is unrelated to the trait, since it is not more pronounced in zebra individuals themselves, but it is possible that heterozygous individuals also are affected in an unknown manner.

The degree of fluctuating asymmetry (FA), or small, random deviations from bilateral symmetry in morphological traits, has attracted a great deal of recent attention as an indicator of overall fitness in animals (e.g., Parsons 1992). Many traits can be measured to indicate the symmetry of an organism, but the choice of wing length is common to many studies of FA in insects (e.g., Thornhill 1992, Harvey & Walsh 1993, Ueno 1994). FA is a measure of developmental stability, and can result from both environmental and genomic stresses (Parsons 1992). In some insects, homozygosity of rare major genes results in increased FA, presumably because this causes major genetic perturbations (Reeve 1960, Clarke & McKenzie 1987). The lack of greater FA in the forewing lengths of zebra monarchs indicates that some genes that decrease fitness may have no effect on the developmental processes that affect wing symmetry.

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LITERATURE CITED

BERGMAN, D. K. & G. M. CHIPPENDALE. 1992. Carotenoid transport by the larval lipophorin of the southwestern corn borer, *Diatraea grandiosella*. Entomol. Exp. Appl. 62:81–85.

BLYTHE, C. R. & H. A. STILL. 1983. Binomial confidence intervals. J. Am. Stat. Assoc. 78:108–116.

CHAPMAN, R. F. 1982. The insects: structure and function. Harvard University Press, Cambridge, Massachusetts. 991 pp.

CLARKE, C. A. 1971. Pigmentation of *Hyalophora cecropia* larvae fed artificial diets containing carotenoid additives. J. Insect Physiol. 17:1593–1598.

CLARKE, C. A. & P. M. SHEPPARD. 1973. The genetics of four new forms of the mimetic butterfly *Papilio memnon* L. Proc. Roy. Soc. Lond. B. 184:1–14.

CLARKE, G. M. & J. A. MCKENZIE. 1987. Developmental stability of insecticide resistant phenotypes in the blowfly; a result of canalizing selection. Nature 325:345–346.

FELTWELL, J. 1978. The distribution of carotenoids in insects, pp 277–307. *In* Harborne, J. B. (ed.), Biochemical aspects of plant and animal coevolution. Academic Press, London.

GEROULD, J. H. 1921. Blue-green caterpillars: the origin and ecology of a mutation in hemolymph color in *Colias (Eurymus) philodice*. J. Exp. Zool. 34:385–415.

HARVEY, I. F. & K. J. WALSH. 1993. Fluctuating asymmetry and lifetime mating success are correlated in males of the damselfly *Coenagrion puella* (Odonata: Coenagrionidae). Ecol. Entomol. 18:198–202.

HOFFMAN, R. J. & W. B. WATT. 1974. Naturally occurring variation in larval color of *Colias* butterflies: isolation from two Colorado populations. Evolution 28:326–328.

KAYSER, H. 1985. Pigments, Vol. 10, pp. 367–415. *In* Kerbut, G. A. & L. I. Gilbert (eds.), Comprehensive insect physiology, biochemistry and pharmacology. Pergamon Press.

KRINSKY, N. I. 1979. Carotenoid protection against oxidation. Pure Appl. Chem. 51: 649–660.

OBERHAUSER, K. S. 1989. Material investment in mating by male monarch butterflies (*Danaus plexippus*). Unpubl. Ph.D. Thesis, Univ. Minnesota.

PARSONS, P. A. 1992. Fluctuating asymmetry: a biological monitor of environmental and genomic stress. Heredity 68:361–364.

REEVE, E. C. R. 1960. Some genetic tests on asymmetry of sternopleural chaeta number in *Drosophila*. Genet. Res. 1:151–172.

ROTHSCHILD, M. 1978. Carotenoids in the evolution of signals: experiments with insects (1974-1976), pp. 259–276. *In* Harborne, J. B. (ed.), Biochemical aspects of plant and animal coevolution. Academic Press, London.

ROTHSCHILD, M., B. O. C. GARDINER & R. MUMMERY. 1978. The role of carotenoids in the "golden glance" of danaid pupae. J. Zool. Lond. 186:351–358.

THORNHILL, R. 1992. Fluctuating asymmetry and the mating system of the Japanese scorpionfly, *Panorpa japonica*. Anim. Behav. 44:867–879.

UENO, H. 1994. Fluctuating asymmetry in relation to two fitness components, adult longevity and male mating success in a ladybird beetle, *Harmonia axyridis* (Coleoptera: Coccinellidae). Ecol. Entomol. 19:87–88.

VALADON, L. R. G., R. S. MUMMERY & M. ROTHSCHILD. 1975. Carotenoids in blue mutant and normal green pupae of *Hyalophora cecropia*. J. Entomol. (A) 50:169–173.

WALDBAUER, G. P. & J. G. STERNBURG. 1972. A mutant blue *Cecropia* larva. Entomol. Exp. Appl. 15:250–251.

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