The Inheritance of Cuticular Coloration in the Tobacco Hornworm (Lepidoptera: Sphingidae)[1]

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ABSTRACT Laboratory colonies of white and black color-strains of the tobacco hornworm, *Manduca sexta* (L.) were used to study the inheritance of cuticular coloration where color is under the control of genes located on separate chromosomes. The white and black color traits are inherited as autosomal recessive and sex-linked recessive traits, respectively. Because the genes for these traits are located on separate chromosomes, the tobacco hornworm system may serve as a model for investigating the interactions between genes. Coloration of progeny of crosses between individuals from the white and black laboratory colonies, F_1 self crosses, F_1 reciprocal crosses and F_1 backcrosses was examined to determine the inheritance of these two traits and potential epistasis between them. Genetic analysis showed that the two color traits are inherited independently and result in a brown phenotype in the double homozygous, recessive condition.

KEY WORDS Insecta, tobacco hornworm, *Manduca sexta*, cuticle color inheritance.

Cuticular pigmentation is important in the camouflage and survival of many insects in the wild. The tobacco hornworm, *Manduca sexta* (L.), is a large, green insect that feeds upon several host plants and like many insects relies heavily upon camouflage to escape predators and parasites (Kawooya et al. 1985). Because tobacco hornworms feed upon several important agricultural crops in the southeastern US, they are important pests. They have the potential to be one of the most devastating tobacco pests (Davidson and Lyon 1979, Jones 1990, Manley 1991). As a research animal, the hornworm is a very practical insect to study due to its large size, short life cycle, specifically timed life stages, and the ease with which it may be reared in the laboratory (Bell and Joachim 1976, Yamamoto 1969).

In 1972, black larvae were found in a laboratory colony of wild-type (green) tobacco hornworms (Safranek and Riddiford 1975). This black phenotype was studied and the coloration was determined to be the result of a recessive, sex-linked gene. The production of insufficient amounts of juvenile hormone in the last two instars has been shown to be the cause of this black pigmentation (Safranek and Riddiford 1975). Recently, several white larvae of the tobacco hornworm were found in the wild-type (green) laboratory colony maintained by North Carolina State University. A colony of the white-mutant was established and the white cuticular coloration was found to be inherited as an autosomal recessive gene (Lampert et al. 1990). The white coloration is thought to be the result of reduced production of insecticyanin (Dahlman 1969).

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The objective of this study was to investigate the interaction of the genes responsible for the white and black coloration in the larval stages of tobacco hornworms. Specifically, this paper presents results of experiments to determine the interaction of these two recessive color traits, operating on separate chromosomes, and the resulting phenotype when both genes are present in the double homozygous-recessive condition.

Materials and Methods

Parental individuals were selected randomly from the laboratory colonies of white and black tobacco hornworms that are maintained by the Department of Entomology, North Carolina State University, on artificial diet (Yamamoto 1969) at a 15:9 (L:D) photoperiod, 60% RH, and an average temperature of 26.7 ± 1°C. Reciprocal crosses between groups (4 males:3 females) of adults from the existing white and black colonies were made using previously published techniques (Lampert et al. 1990). The eggs from the different matings were collected daily and maintained separately in labelled Petri dishes. After hatching, larvae were reared on artificial diet (Yamamoto 1969) at a photoperiod of 16:8 (L:D), 60% RH, and 23.2 ± 1°C. It has been reported (Lampert et al. 1990) that the white larvae enter diapause under a longer photophase as compared with wild-type larvae, therefore, the slightly longer photophase was used in the rearing room to reduce the likehood of diapause in the white larvae or their progeny. The phenotypic ratios were determined and recorded when larvae reached either fourth or fifth instar because the black color-form does not exhibit melanization until these stages. Sex of the offspring from the various crosses was determined by examination of the pupae. When the F_1 adults emerged, they were either reciprocally backcrossed with adults from the parental colonies or the F_1 individuals were allowed to interbreed. The eggs from these matings were collected and reared as described above to determine the phenotypes produced. The resulting phenotypes were recorded and compared to the expected phenotypic frequencies using χ^2 analysis (Sokal and Rohlf 1969).

Expected phenotypes. Based on the knowledge that the white coloration is inherited as an autosomal recessive trait and the black coloration is inherited as a sex-linked recessive trait, Punnett squares (Strickberger 1985) were constructed. From these Punnett squares, expected genotypes and resulting phenotypes of the offspring from various crosses of the black and white individuals were determined. Tables 1 and 2 present the expected larval phenotypes for the following crosses: white male and black female, black male and white female, F_1 self crosses from these parental crosses, and F_1 reciprocal and backcrosses to the respective parental colonies. In Table 1, the expected phenotypes following an initial cross between white males and black females are presented, while the expected phenotypes of the reciprocal initial cross, black males and white females, are presented in Table 2.

Data Analysis. Using χ^2 analysis, the expected and observed numbers of individuals of each phenotype were compared to determine goodness of fit and agreement with the hypothesis of independent inheritance. In certain crosses, larvae were observed to have unstable production of cuticular pigment (over a

period of 2 days, some fifth instar larvae were observed changing between black and green cuticle color), therefore, positive differentiation between these phenotypes was not always possible. In all cases, there were more green phenotypes and fewer black phenotypes than expected. The black strain of the tobacco hornworm does not develop the black cuticular pigmentation until the fourth instar (Safranek and Riddiford 1975). Because of these observed changes in cuticular pigment in the fifth instar, it is possible that some black phenotypes where incorrectly categorized as green phenotypes. The white and brown phenotypes were positively identified because their cuticle pigmentation is expressed during the second instar and remains for the entire larval stage. For this reason, the green and black phenotypes in some crosses were pooled into an 'other' category. The phenotypes then were regrouped into white, brown, and other, and the χ^2 was calculated for the regrouped phenotypic data.

Results and Discussion

Most crosses of white and black strains of the tobacco hornworms produced the expected phenotypic ratios. Crossing white males (genotype wwBB) with black females (genotype WWb0) (Table 1; cross 1) resulted in 100% green F_1 phenotypes (genotypes WwBb and WwB0). The reciprocal of that cross, black males (genotype WWbb) with white females (genotype wwB0) (Table 2; cross 1), produced the expected phenotypic ratio of 1 green male (genotype WwBb): 1 black female (genotype Wwb0) ($\chi^2 = 0.02$, df = 1, $P > 0.90$).

Crosses between the green F_1 individuals from the white male and black female (Table 1; cross 2) have an expected larval phenotypic ratio of 9 green: 3 black: 3 white: 1 unknown. The unknown phenotype represents the genotype that is homozygous recessive for both color traits, and this phenotype was revealed to be brown. The observed phenotypes were significantly different than expected ($\chi^2 = 19.16$, df = 3, $P < 0.005$), however, positive differentiation between the black and green larvae was not always possible, and individual larvae from this cross were observed to change colors between black and green as they grew. The reason for this unstable cuticle coloration is unknown and similar changes are not known to occur in the parental colonies. Phenotypes from this cross were regrouped as either white, brown, or other. The use of this grouping led to agreement between the expected and observed phenotypes ($\chi^2 = 2.40$, df = 2, $0.25 < P < 0.50$). The results of the cross between green F_1 males and black F_1 females (Table 2; cross 2) also showed significant departure from the expected phenotypic ratio ($\chi^2 = 20.55$, df = 3, $P < 0.005$). Once again, categorizing the larvae as white, brown, or other resulted in the expected phenotypic ratios ($\chi^2 = 5.39$, df = 2, $0.10 < P < 0.05$).

Backcrosses to both parental types were used to verify the dominance of the green color trait. When F_1 females were backcrossed with males from the white parental colony (Table 1; cross 3 and Table 2; cross 3), a phenotypic ratio of 1 green: 1 white larva was expected and observed ($\chi^2 = 0.17$, df = 1, $0.50 < P < 0.75$, Table 1, cross 3; and $\chi^2 = 0.87$, df = 1, $0.50 < P < 0.75$, Table 2, cross 3). Backcrossing the F_1 males with females from the white parental colony produced the expected ratio of 3 green: 3 white: 1 black: 1 unknown (Table 1; cross 4) ($\chi^2 = 0.97$, df = 3, $0.75 < P < 0.90$). The other backcross of F_1 males to

Table 1. Expected and observed phenotypes resulting from crossing white male with black female tobacco hornworms, including F_1 crosses and reciprocal and backcrosses to parental colonies.

Cross	Male	Female	Expected Phenotypes	Observed Phenotypes	Chi Square*
1	White wwBB	Black WWb0	All green	161 green	—
2	F_1 WwBb	F_1 WwB0	9 green: 3 black: 3 white: 1 unknown	180 green: 27 black: 43 white: 12 brown	19.162 s (2.398 ns)†
3	White wwBB	F_1 WwB0	1 green: 1 white	13 green: 11 white	0.167 ns
4	F_1 WwBb	White wwB0	3 green: 3 white: 1 black: 1 unknown	15 green: 19 white: 4 black: 6 brown	0.974 ns
5	Black WWbb	F_1 WwB0	1 green male: 1 black female	78 green: 54 black	4.396 s
6	F_1 WwBb	Black WWb0	1 green: 1 black	103 green: 88 black	1.178 ns

* ns = not significant, s = significant at P = 0.05.
† Chi square values in parenthesis were obtained by regrouping phenotypes as white, brown, or other.

females from the white parental colony (Table 2; cross 4) was not in agreement with the expected ratio ($\chi^2 = 11.584$, df = 2, $0.005 < P < 0.01$). As before, phenotypes were regrouped and expected phenotypic ratios were obtained ($\chi^2 = 2.36$, df = 2, $0.50 < P < 0.75$).

Crosses between males from the black parental colony and the F_1 females (Table 1; cross 5) did not produce the expected phenotypic ratios of 1 green: 1 black ($\chi^2 = 4.40$, df = 1, $P < 0.05$). This deviation was presumed to be due to the difficulty in distinguishing between the black and green phenotypes, which could not be regrouped in this instance. This cross should have produced 1 green male: 1 black female. The sex of 10 randomly selected pupae of each color was determined and; as expected, all the black larval phenotypes were female and all green larval phenotypes were male. If the deviation in the expected phenotypic ratios was due exclusively to the inability to correctly distinguish between the two phenotypes, then some of the opposite sex should have been discovered when the sexes of the pupae were determined. It is possible that there was unequal sperm competition among these genotypes, but that was not investigated in the present study.

Mating F_1 male with females from the black parental colony produced the expected phenotypic ratio, 1 green: 1 black (Table 1; cross 6, $\chi^2 = 1.18$, df = 1, $0.25 < P < 0.50$ and Table 2; cross 5, $\chi^2 = 0.47$, df = 1, $0.25 < P < 0.50$). Finally,

Table 2. Expected and observed phenotypes resulting from crossing black male with white female tobacco hornworms, including F_1 crosses and reciprocal and backcrosses to parental colonies.

Cross	Male	Female	Expected Phenotypes	Observed Phenotypes	Chi Square*
1	Black WWbb	White wwB0	1 green male: 1 black female	32 green males: 33 black females	0.016 ns
2	Green F_1 WwBb	Black F_1 Wwb0	3 green: 3 black: 1 white: 1 unknown	66 green: 28 black: 10 white: 23 brown	20.549 s (5.388 ns)†
3	White wwBB	Black F_1 Wwb0	1 green: 1 white	288 green: 266 white	0.874 ns
4	Green F_1 WwBb	White wwB0	3 green: 3 white: 1 black: 1 unknown	73 green: 58 white: 9 black: 13 brown	11.584 s (2.364 ns)†
5	Green F_1 WwBb	Black WWb0	1 green: 1 black	15 green 19 black	4.471 ns
6	Green F_1 WwBb	Green WWB0	3 green: 1 black	27 green: 15 black	0.411 ns

* ns = not significant, s = significant at P = 0.05.
† Chi square values in parenthesis were obtained by regrouping phenotypes as white, brown, or other.

backcrossing the green F_1 males with females from the wild-type green colony (Table 2, cross 6) resulted in a phenotypic ratio of 3 green: 1 black, as predicted ($\chi^2 = 0.411$, df = 1, $0.50 < P < 0.75$).

The interaction of the two color traits was determined: the black and white color traits are inherited independently and when simultaneously expressed, result in a brown phenotype. Results from the F_1 crosses, reciprocal crosses, and backcrosses to the black males or white females were in agreement with the initial set of crosses and verified that an individual which is homozygous recessive for either genotype combination (wwbb and wwb0) has a brown cuticle coloration. Several of the crosses did not produce the expected phenotypic ratios and in all cases more green larvae and fewer black larvae than expected were observed. This deviation from the expected phenotypic ratios may be explained partially by the fact that it was not always possible to distinguish between green and black larvae, as some black larvae were later observed to develop green cuticle color for unknown reasons. No pattern to this color change was observed, i.e., stage of larvae, season of year, outside temperature, etc. It is possible that these individuals may have been heterozygous for the green and/or black traits and some interactions with or inhibitions due to the rearing conditions may have taken place. However, these questions were not addressed

in the current study. The brown individuals, that are in the double homozygous recessive condition, may serve as a biological model for studying the simultaneous expression of two independently inherited traits.

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