Studies on the Colour Variation in Larvae of *Ephestia kühniella* ZELLER (Lepidoptera : Phycitidae)
II. Effect of Environmental Factors on the Larval Pigmentation

Osamu IMURA

*Entomology Laboratory, National Food Research Institute,
Yatabe, Tsukuba, Ibaraki 305, Japan*

(Received July 27, 1981)

Effects of six environmental factors, i.e. medium, lighting conditions, crowding, starvation, relative humidity, and temperature, on the larval pigmentation of *Ephestia kühniella* ZELLER were studied using two pure lines which differed in colour. Except for the lighting conditions, the other five environmental factors affected larval pigmentation. Degree of pigmentation in larval integument increased when larvae were reared on wheatfeed and rice bran and decreased when they were reared on corn flour. High population density, more than 3 days' starvation, and low relative humidity increased larval pigmentation. Especially temperature had the most marked effect, namely the higher the temperature, the darker the larval colour became. Interactions between genotype and environmental factors, such as medium, crowding, relative humidity, and temperature, were found.

INTRODUCTION

The Wild-type stock of the Mediterranean flour moth, *Ephestia kühniella* ZELLER, which is maintained in our laboratory, shows continuous colour variation ranging from white to deep pink in late instar larvae. In pink larvae red pigment is observed in epidermal cells, and before pupation some amount of pigment disappears from these cells. This red pigment was identified as a kind of ommochrome pigments, i.e. xanthommatin, and the degree of larval pigmentation was found to depend on the amount of pigment present in the larval epidermis (IMURA and SHIBUYA, 1979). IMURA (1980) demonstrated that this larval colour variation was a highly heritable quantitative character in which two pairs of alleles were considered to be involved. Although 87% of phenotypic variance could be explained by an additive genetic factor, the remainder was considered to be attributable to environmental factors.

In the present study, effects of six environmental factors (food medium, lighting conditions, crowding, starvation, relative humidity and temperature) on the larval pigmentation were investigated using two pure lines (white larval colour strain and red larval colour one).

MATERIALS AND METHODS

Experimental animals. Two genetically pure strains of *Ephestia kühniella* ZELLER, a strain with white larval colour (designated as PL-1) and a strain with red larval colour (designated as PL-10) were used. These two strains were selected by single-pair mating for ten generations respectively from a white larval strain and a red larval strain which were separated from the wild-type stock (IMURA, 1980). These two pure strains were maintained on wheatfeed in a room controlled at $25 \pm 0.5^\circ\text{C}$ and $67 \pm 5\%$ r.h. with a light regime of 16 hr light and 8 hr dark.

Experimental conditions. Experimental conditions are summarized in Table 1. Clear plastic containers (15 cm in diam., 8.5 cm deep) with a lid which had a hole covered with filter paper for ventilation were generally used to rear experimental animals. In the crowding experiment similar but much smaller containers (6.0 cm

Table 1. Experimental conditions

Environmental factors	Medium	Population density ^a	Temperature, Relative humidity and Light regime ^b
1. Medium	100 g of 1) Wheat flour 2) Wheat flour with 5% Brewer's yeast 3) Wheatfeed 4) Rice bran 5) Corn flour	100	$25 \pm 0.5^\circ\text{C}$, $67 \pm 5\%$ r.h., 16L8D
2. Lighting condition	100 g of wheatfeed with 5% Brewer's yeast	100	$25 \pm 0.5^\circ\text{C}$, $67 \pm 5\%$ r.h., 1) CD 2) 8L16D 3) 12L12D 4) 16L8D 5) CL
3. Crowding	20 g of wheatfeed with 5% Brewer's yeast	1) 10 2) 50 3) 100 4) 200 5) 400	$25 \pm 0.5^\circ\text{C}$, $67 \pm 5\%$ r.h., 16L8D
4. Starvation	100 g of wheatfeed with 5% Brewer's yeast	100	$25 \pm 0.5^\circ\text{C}$, $67 \pm 5\%$ r.h., 16L8D
5. Relative humidity	100 g of wheatfeed with 5% Brewer's yeast	100	$25 \pm 0.5^\circ\text{C}$, 16L8D, 1) 32.5% r.h. 2) 50.0% r.h. 3) 67.0% r.h. 4) 75.5% r.h. 5) 80.0% r.h.
6. Temperature	100 g of wheatfeed with 5% Brewer's yeast	100	$67 \pm 5\%$ r.h., 16L8D 1) $12.5 \pm 0.5^\circ\text{C}$ 2) $15.0 \pm 0.5^\circ\text{C}$ 3) $17.5 \pm 0.5^\circ\text{C}$ 4) $20.0 \pm 0.5^\circ\text{C}$ 5) $22.5 \pm 0.5^\circ\text{C}$ 6) $25.0 \pm 0.5^\circ\text{C}$ 7) $27.5 \pm 0.5^\circ\text{C}$ 8) $30.0 \pm 0.5^\circ\text{C}$

^a Number of newly hatched larvae placed.

^b CD: continuous dark, 8L16D: 8 hr light and 16 hr dark, 12L12D: 12 hr light and 12 hr dark, 16L8D: 16 hr light and 8 hr dark, CL: continuous light.

in diam., 3 cm deep) were used.

In the experiment on lighting conditions, we used lightproof corrugated cardboard boxes ($37 \times 31 \times 27$ cm) over which a 6W fluorescent tube was mounted. These boxes were placed in a dark room at $25 \pm 0.5^\circ\text{C}$ and $67 \pm 5\%$ r.h. The light period was controlled by an electric timer and light intensity on the medium was about 160 lux.

In the starvation experiment, the early 5th instar larvae were removed from the medium and confined singly in small glass vials (1 cm in diam., 3 cm deep) without medium. The degree of larval pigmentation was determined after 1, 3 and 5 days' starvation.

In the humidity test, saturated solutions of the following compounds were used to control humidity in 5 l glass desiccators; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ for 32.5% r.h., $\text{NH}_4\text{NO}_3 + \text{NaNO}_3$ for 50.0% r.h., $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ for 67.0% r.h., NaCl for 75.5% r.h. and KBr for 80.0% r.h. (WINSTON and BATES, 1960).

In all experiments, media were treated with a microwave oven for 5 min. to avoid biotic contamination and conditioned at each temperature and humidity for about a fortnight before use.

Measurement of the degree of pigmentation. The last (5th) instar larvae which were reared under different environmental conditions were collected, and stored at -25°C until used. These frozen larvae were decapitated and viscerated, and 50 mg (fresh weight) of the integument was homogenized in cold methanol. After centrifugation at 12,000 r.p.m. for 10 min., the precipitate was washed twice with cold methanol and ethyl ether, respectively by repeated suspension and centrifugation. Then ethyl ether was evaporated under reduced pressure. The pigment was extracted from the residue with 2 ml of acidic methanol (2 ml conc. HCl in 100 ml) for one hour. After centrifugation, absorbance at 450 nm of the extracts was measured in the presence of 1% NaNO_2 with a spectrophotometer. This absorbance at 450 nm was defined as the degree of pigmentation (IMURA and SHIBUYA, 1979). Usually 6 samples were measured for each experimental condition.

RESULTS AND DISCUSSION

Fig. 1-A shows the effect of 5 media on the larval pigmentation of two strains. Pigmentation of larvae reared on corn flour was light and that of larvae reared on rice bran and wheatfeed was heavy, although increase of pigmentation was not significant in PL-1 larvae on wheatfeed. The addition of Brewer's yeast to wheat flour had no significant effect on pigmentation. These differences of pigmentation in PL-1, however, could hardly be distinguished with the naked eye.

It is well known that ommochrome pigments are synthesized from tryptophan in insects (See LINZEN, 1974). The degree of larval pigmentation was correlated fairly well with the reported tryptophan contents in the media (KASARDA et al., 1964; Science and Technology Agency, 1966; TAMURA et al., 1972; JURIANO, 1972) on which larvae were reared. KAYSER (1979) also reported that larvae of *Pieris brassicae* reared on an artificial diet supplemented with tryptophan accumulated significantly more xanthommatin than those reared on a standard artificial diet.

Lighting conditions influence pigment formation in some insects. In an earlier study, KÜHN (1939) reported that illumination increased pigmentation in the eyes of *dec dec* genotype *Ptychopoda seriata*. In *Carausius morosus*, darkness increased a bile

Fig. 1. Effect of (A) medium and (B) lighting conditions on the degree of larval pigmentation in two pure lines of *Ephestia kuehniella* (PL-1 and PL-10). Open circles indicate mean and vertical lines 95% confidence limits of absorbance at 450 nm.

Fig. 2. Effect of (A) crowding and (B) starvation on the degree of larval pigmentation in two pure lines of *Ephestia kuehniella* (PL-1 and PL-10). Open circles indicate mean and vertical lines 95% confidence limits of absorbance at 450 nm.

pigment and decreased carotenoid pigments in the epidermis (WILLIG, 1969), whereas ommochrome production in the epidermis was higher in larvae reared in darkness than in those reared under 15L-9D and natural photoperiod (DUSTMANN, 1964). But as can be seen from Fig. 1-B, any light regime examined did not have marked effect on the larval pigmentation in *Ephestia kühniella*.

In many insect species, the body colour becomes darker when the insects are reared under crowded condition (WIGGLESWORTH, 1972). In *Ephestia kühniella*, as the population density became higher, the degree of pigmentation increased in PL-10 and the pigmentation at the density of 400 larvae was significantly darker in both strains (Fig. 2-A). However, GOODWIN (1950) reported that solitary larvae had approximately the same amount of ommochrome pigment as that of gregarious ones in *Locusta migratoria migratorioides*.

Starvation period of more than 3 days increased significantly larval pigmentation (Fig. 2-B). In *Carausius morosus* the production of ommochromes also increased when larvae were starved (BERTHOLD, personal communication).

At a relative humidity ranging between 50% and 80% larvae did not show any difference in pigmentation, however at 32.5% r.h. larval pigmentation was noticeably increased (Fig. 3-A). According to BERTHOLD, ommochromes in *Carausius morosus* increased with high or low relative humidity (personal communication). In our experiment, however, humidity level higher than 80% was not tested due to mold development in the media.

Fig. 3. Effect of (A) relative humidity and (B) temperature on the degree of larval pigmentation in two pure lines of *Ephestia kühniella* (PL-1 and PL-10). Open circles indicate mean and vertical lines 95% confidence limits of absorbance at 450 nm.

As seen in Fig. 3-B, temperature had a marked effect on larval pigmentation. As the temperature rose, the degree of larval pigmentation increased. The effect of temperature on the production of ommochromes varies with the insect species. At high temperatures ommochrome production increases in the epidermis of *Carausius morosus* (DUSTMANN, 1964; BERTHOLD, 1973), whereas it increases at low temperature in *Ceruna vinula* (BÜCKMANN, 1963) and *Schistocerca gregaria* (GOODWIN, 1950; BELLAMY, 1958). CASPARI (1949) mentioned that pigmentation of the eyes and testes whose pigments were identified as ommochrome pigments (BECKER, 1939, 1942; BUTENANDT et al., 1958, 1960; KÜHN and EGELHAAF, 1959) increased at low temperature in *aa* and *a^ka^k* genotypes of *Ephesia kühniella*. At any rate, the effect of temperature on pigmentation requires further investigations.

Two pathways of kynurenine degradation have been identified in insects, namely ommochrome and kynurenic acid formation (STRATAKIS, 1979). Under unfavorable conditions, such as starvation, high population density, low relative humidity and high temperature, excretion of kynurenic acid may be delayed and lowered and consequently ommochrome synthesis may increase to remove toxic tryptophan *via* kynurenine (BERTHOLD, personal communication).

To examine the presence of genotype (G) $\times$ environment (E) interaction, direct measurements of larval pigmentation in PL-1 and PL-10 were plotted against temperatures (Fig. 4-A). Although logarithm transformation was performed to remove scale effects, the slope of the regression line in PL-10 was still steeper than that in PL-1 (Fig. 4-B), suggesting the presence of G $\times$ E interaction (FINLAY and WILKINSON, 1963). Analysis of variance table (Table 2-A) shows that a statistically significant interaction exists between the two lines and the temperature as environmental factor. This result indicates that the pigmentation in PL-10 larvae is more sensitive to the temperature difference than that in PL-1 larvae. Table 2-B also shows significant interactions between genotype and environmental factors; medium, crowding, and humidity in which homogeneity of variances was confirmed.

These results show that many environmental factors may interact with genotypes in the larval colour variation, and additive genetic variance and heritability estimates which were reported by IMURA (1980) might be somewhat overestimated.

Fig. 4. Relationship between temperature and the degree of larval pigmentation in two pure lines of *Ephesia kühniella*. A: The direct measurement of pigmentation (P) is plotted against the temperatures. B: The scale in the degree of pigmentation is transformed into $\log_{10}(P \times 10^4)$. b indicates regression coefficient.

Table 2. Analysis of variance tables

A) Temperature ^a			
Source of Variation	DF	SS	MS
Genotype	1	19.7007	19.7007**
Environment	6	0.5009	0.0835**
Interaction	6	0.0724	0.0121**
Regression	1	0.0458	0.0458*
Deviation from regression	5	0.0266	0.0053
Error	70	0.1776	0.0025
B) Medium, Crowding, and Humidity ^a			
	DF	SS	MS
Interaction			
(Medium)	4	0.1682	0.0421**
(Crowding)	4	0.0222	0.0055**
(Humidity)	4	0.1516	0.0379**

^a Environmental factors.** $P < 0.01$ * $P < 0.05$

In conclusion, environmental factors, such as medium, starvation, crowding, relative humidity and temperature, had a distinct effect on larval pigmentation in two pure lines. Among them, temperature had a conspicuous effect on it suggesting that temperature may be an important factor which causes the environmental deviation. The presence of $G \times E$ interaction indicates a clear difference in sensitivity of pigmentation to various environments between strains.

ACKNOWLEDGEMENTS

I wish to thank Drs. Y. YAMADA, National Institute of Animal Industry, and H. IKEMOTO, Tokyo Metropolitan Isotope Research Center, for their invaluable comments and discussion. Thanks are also due to Mr. T. ISHIMA for his advice on statistical analysis.

REFERENCES

- BECKER, E. (1939) Über die Natur des Augenpigments von *Ephestia kühniella* und seinen Vergleich mit den Augenpigmenten anderer Insekten. *Biol. Zbl.* **59** : 597–627.
- BECKER, E. (1942) Über Eigenschaften, Verbreitung und die genetisch-entwicklungsphysiologische Bedeutung der Pigmente der Ommatin- und Ommingruppe (Ommochrome) bei den Arthropoden. *Z. f. Vererbgs.* **80** : 157–204.
- BELLAMY, D. (1958) The structure and metabolic properties of tissue preparations from *Schistocerca gregaria* (The desert locust). *Biochem. J.* **70** : 580–589.
- BERTHOLD, G. (1973) Der Einfluß der Corpora allata auf die Pigmentierung von *Carausius morosus* Br.. *Wilhelm Roux' Archiv* **173** : 246–262.
- BÜCKMANN, D. (1963) Der Einfluß der Temperature auf das Epidermispigment der Raupen von *Cerura vinula* L.. *Z. Natrf.* **18b** : 255–264.
- BUTENANDT, A., E. BIEKERT, und B. LINZEN (1958) Über Ommochrome. XIV Zur Verbreitung der Ommine im Tierreich. *Hoppe-Seyler's Z. physiol. Chem.* **313** : 251–258.
- BUTENANDT, A., E. BIEKERT, H. KÜBLER, und B. LINZEN (1960) Über Ommochrome. XX Zur Verbreitung

- der Ommatine im Tierreich. Neue Methoden zu ihrer Identifizierung und quantitativen Bestimmung. *Hoppe-Seyler's Z. physiol. Chem.* **319** : 238–256.
- CASPARI, E. (1949) Physiological action of eye color mutants in the moths *Ephestia kühniella* and *Ptychopoda seriata*. *Q. Rev. Biol.* **24** : 185–199.
- DUSTMANN, J. H. (1964) Die Redoxpigmente von *Carausius morosus* und ihre Bedeutung für den morphologischen Farbwechsel. *Z. fergl. Physiol.* **49** : 28–57 (1964).
- FINLAY, K. W. and G. N. WILKINSON (1963) The analysis of adaptation in a plant-breeding programme. *Aust. J. Agric. Res.* **14** : 742–754.
- GOODWIN, T. W. (1950) Biochemistry of locusts. 4. Insectorubin metabolism in the desert locust (*Schistocerca gregaria* FORSK.) and the African migratory locust (*Locusta migratoria migratorioides* R. and F.). *Biochem. J.* **47** : 554–562.
- IMURA, O. (1980) Studies on the colour variation in larvae of *Ephestia kühniella* ZELLER (Lepidoptera, Phycitidae). 1. On the inheritance of colour variation. *Kontyû, Tokyo* **48** : 248–258.
- IMURA, O. and N. SHIBUYA (1979) Red pigment in the larval integument of the Mediterranean flour moth *Ephestia kühniella* Z. (Lepidoptera : Phycitidae). *Appl. Ent. Zool.* **14** : 221–223.
- JULIANO, B. O. (1972) The rice caryopsis and its composition. In Rice: chemistry and technology (D. F. HOUSTON ed.), Minnesota : American Association of Cereal Chemists, pp. 16–74.
- KASARDA, D. D., C. C. NIMMO, and G. O. KOHLER (1964) Proteins and the amino acid composition of wheat fractions. In Wheat: chemistry and technology. (Y. POMERANZ ed.), Minnesota: American Association of Cereal Chemists, pp. 227–299.
- KAYSER, H. (1979) Ommochrome formation and kynurenine excretion in *Pieris brassicae*: Relation to tryptophan supply on an artificial diet. *J. Insect Physiol.* **25** : 641–646.
- KÜHN, A. (1939) Eine durch Temperatur und durch Licht modifizierbare Augenfarbenmutante von *Ptychopoda seriata*. *Naturwissenschaften* **27** : 787–788.
- KÜHN, A. und A. EGELHAAF (1959) Über die Mutation *br* (braunäugig) bei *Ephestia kühniella*. *Z. f. Vererbgs.* **90** : 244–250.
- LINZEN, B. (1974) The tryptophan—ommochrome pathway in insects. *Adv. Insect Physiol.* **10** : 118–246.
- Science and Technology Agency (1966) The amino acid composition of foods in Japan 71p. (In Japanese).
- STRATAKIS, E. (1979) Ommochrome synthesis and kynurenic acid excretion in relation to metamorphosis and allatectomy in the stick insect, *Carausius morosus* Br.. *J. Insect Physiol.* **25** : 925–929.
- TAMURA, S., K. KENMOCHI, T. SUZUKI, K. SUGIMURA, S. HORII, and H. MORIMOTO (1972) Amino acids contents of 32 raw feed materials in Japan. *Rept. Food Res. Inst.* **27** : 35–40 (In Japanese with English summary).
- WIGGLESWORTH, V. B. (1972) The principles of insect physiology. 7th ed. London: Chapman and Hall, 827p.
- WILLIG, A. (1969) Die Carotinoide und der Gallenfarbstoff der Stabheuschrecke, *Carausius morosus* und ihre Beteiligung an der Entstehung der Farbmodifikationen. *J. Insect Physiol.* **15** : 1907–1927.
- WINSTON, P. W. and D. H. BATES (1960) Saturated solutions for the control of humidity in biological research. *Ecology* **41** : 232–237.