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# Hormonal control of GTP cyclohydrolase I gene expression and enzyme activity during color pattern development in wings of *Precis coenia*

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## Abstract

Color patterns of butterfly wings are composed of single color points represented by each scale. In the case of *Precis coenia*, at the end of pupal development, different types of pigments are synthesized sequentially in the differently colored scales beginning with white (pterins) followed by red (ommata) and then black (melanin). In order to explain how formation of these different colors is regulated, we examined the expression of an mRNA-encoding guanosine triphosphate-cyclohydrolase I (GTP-CH I; EC 3.5.4.16), the first key enzyme in the biosynthesis of pteridines, during pigment formation in the wings of *P. coenia*. The strongest positive signal was recognized around pigment formation one day before butterfly emergence. This *GTP-CH I* gene expression is paralleled by GTP-CH I enzyme activity measured in wing extracts. We also investigated the effect of 20-hydroxyecdysone on the expression of *GTP-CH I* mRNA and the enzyme activity during color formation. The results strongly suggest that the onset and duration of the expression of a *GTP-CH I* mRNA is triggered by a declining ecdysteroid hormone titer during late pupal development. © 2002 Elsevier Science Ltd. All rights reserved.

**Keywords:** Color formation; Ecdysteroids; GTP-cyclohydrolase I; *Precis coenia*; Pteridines; Melanin

## 1. Introduction

Color patterns of lepidopteran wings are composed of single color points represented by each scale. The single scale appears to be monochrome and in most instances contains only one type of pigment (Nijhout, 1985; Nijhout and Koch, 1991). Many different kinds of pigments may occur, most of them belonging to pteridines, ommochromes and melanins (Nijhout, 1985; Koch, 1992). These pigments not only cause the color of wing patterns but also cause the color of the insect body. In the scorpion fly, *Panorpa japonica*, different coloration

in seasonal dimorphism is due to sepiapterin and melanin (Nakagoshi et al. 1984, 1992). In the case of the larval silkworm, *Bombyx mori*, the body colors are also due to pteridines, ommochromes and melanin (Ohashi et al., 1983; Sawada et al. 1994, 1998). Colors of the larval body and central nervous system are caused directly by amounts and composition of pigments present in cytosolic granules as has been shown in the normal type, *quail* mutant and *lemon* mutant of the silkworm (Tsusue et al., 1990; Sawada et al. 1990, 2000).

Among the pteridine derivatives, tetrahydrobiopterin (BH<sub>4</sub>) plays a very important physiological role (Thöny et al., 2000). It serves as an electron donor for the hydroxylation of the aromatic amino acids phenylalanine, tyrosine, and tryptophan and thus is the essential cofactor for the synthesis of neurotransmitters (Kaufman, 1986). BH<sub>4</sub> is also an essential cofactor for nitric oxide synthase (Marletta, 1993; Stuehr, 1997). Thus, pteridine derivatives have an important role in both biological and medical aspects.

**Abbreviations:** BH<sub>4</sub>, tetrahydrobiopterin; DOPA, dihydroxyphenylalanine; GTP-CH I, guanosine triphosphate-cyclohydrolase I; 20E, 20-hydroxyecdysone.

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Guanosine triphosphate-cyclohydrolase I (GTP-CH I; EC 3.5.4.16) is the first enzyme in the biosynthesis of pteridines, including pteridine pigments and BH<sub>4</sub>. Therefore, GTP-CH I is an excellent marker for the investigation of color pattern formation or the biosynthesis of neurotransmitters. Takikawa and Nakagoshi (1994) have reported the significance of developmental changes of the enzyme activity of GTP-CH I, 6-pyruvoyl-tetrahydropterin synthase (EC 4.6.1.10; Takikawa et al., 1986) and sepiapterin reductase (EC 1.1.1.153; Iino et al., 1992) in the toad, *Bufo vulgaris*. In *Danio rerio*, changes in enzyme activity of GTP-CH I are related to amounts of pteridines during embryogenesis (Ziegler et al., 2000). These results demonstrate that measurement of GTP-CH I enzyme activity is a useful indicator for the development of neurons and pigment cells.

For insect color pattern formation the developing wings of *Precis coenia* and other butterfly species are well-established model systems (Nijhout, 1980b; Koch, 2000). In *P. coenia* locally different activity of dihydroxyphenylalanine (DOPA) decarboxylase leads to different amounts of gray or black melanin (Koch and Kaufmann, 1995) and differentiated onset of activity is due to the decreasing ecdysteroid hormone titer (Koch, 1995). On the other hand, the seasonal phenotypic change in the pigmentation of the ventral hind wing is due to ommochromes. The summer pigmentation is due to an accumulation of xanthommatin, whereas the winter pigmentation is due to a mixture of dihydro-xanthommatin and ommatin-D (Nijhout, 1997). This diphenic switch in pigment development is controlled by ecdysteroids during a critical period between 28 and 48 h after pupation (Rountree and Nijhout, 1995). In *Papilio glaucus* expression and activation of a key enzyme,  $\beta$ -alanyl-dopamine synthase, seems to shift production of melanin (black) to a different pigment papiliochrome (yellow) (Koch et al., 1998; Koch et al., 2000a,b).

In contrast, nothing is known on the regulation of pterin synthesis and we have chosen *P. coenia* for a first molecular approach. This was an important task, since different wing pigments are synthesized in an invariable time course starting with white (pterins), followed by red (ommatsins) and black and gray (melanins) (Nijhout, 1980a; Koch, 1992; Koch et al., 1998). In order to explain how formation of these wing colors is regulated, we examined the *GTP-CH I* gene expression and measured the enzyme activity during pigment synthesis in the forewing of *P. coenia*. Additionally, we investigated the effect of 20-hydroxyecdysone (20E) on expression of *GTP-CH I* mRNA and the enzyme activity in wings during late pupal development and color formation. This is the first report on the regulation of the *GTP-CH I* gene expression by steroid hormones.

## 2. Materials and methods

### 2.1. Animals and tissue samples

The North American Buckeye, *P. coenia*, was reared on semiartificial diet as described previously (Koch and Kaufmann, 1995). Larvae and pupae were kept under long-day conditions (16L:8D) at 25.5 °C with lights on at 07:00 and lights off at 23:00. Pupal development including the onset of wing coloration is gated by photoperiod (Nijhout, 1980a). Only those pupae which developed within 7 days were used for experiments. Forewings were dissected from pupae and the wing was washed quickly with Grace's medium (Sigma), then immediately stored at –80 °C until extraction of RNA or protein.

### 2.2. Treatment of pupae with 20-hydroxyecdysone

A dose of 10 µg of 20E (Sigma) dissolved in 10 µl Grace's medium (Sigma) was injected into pupae laterally in a posterior abdominal segment at day 4 evening after pupal molt. Control pupae were injected only with 10 µl of Grace's medium at the same stage. Pupae were placed in covered dishes and kept until use.

### 2.3. GTP-CH I enzyme assay

Two forewings were extracted by homogenization with a homogenizer (1 ml Dounce) in 50 mM Tris buffer (pH 8.0). The centrifuged homogenates (13 000g, 15 min) were passed through Micro Spin G25 columns. For determination of GTP-CH I activity, the reaction product H<sub>2</sub> neopterin triphosphate was oxidized to neopterin triphosphate by acidic iodine solution. After reaction of excess iodine by ascorbic acid and dephosphorylation with alkaline phosphatase (0.8 unit/200 µl), neopterin was determined by high performance liquid chromatography as described previously (Ziegler et al., 2000).

### 2.4. Isolation of RNA and Northern blot hybridization

Total RNA was prepared from the pupal wings by the TriPure Isolation Reagent (Boehringer) method described previously by Chomczynski and Sacchi (1987).

An aliquot (20 µg) of total RNA was denatured by incubation with 20 mM 3-(*N*-morpholino)propane-sulfonic acid (MOPS) (pH 7.0), 5mM sodium acetate, 0.1 mM EDTA, 6.5% formaldehyde and 50% formamide for 15 min at 68 °C (Sambrook et al., 1989). The denatured RNA was electrophoresed on a 1.0% agarose gel containing 20 mM MOPS (pH 7.0), 5 mM sodium acetate, 0.1 mM EDTA and 6.5% formaldehyde, and blotted onto nylon membrane. The membrane was hybridized with <sup>32</sup>P-labelled *P. coenia* *GTP-CH I* anti-

sense-riboprobe at 60 °C. The <sup>32</sup>P-labelled antisense-riboprobe was prepared for pCR-Script vector in the fragment of *P. coenia* *GTP-CH I* cDNA using RNA transcription kit (Stratagene). After hybridization, the membrane was washed at 60 °C and dried and exposed for autoradiography.

### 2.5. RT-PCR and DNA sequence analysis

Total RNA (10 µg) was reverse-transcribed by MMLV-reverse transcriptase (Gibco BRL) using oligo dT primer in 25 µl of reaction solution. PCR experiment was performed using a 5-µl aliquot of first-stranded cDNA solution as template. Degenerate consensus PCR-primers specific for GTP-CH I were designed on the basis of highly conserved regions of the open reading frames in the sequences of man, rat, mouse, *E. coli* and *B. subtilis* as described by Maier et al. (1995). The sequences of degenerate PCR-primers were: sense, 5'-AYGARGAYCAYGAYGARATGG-3'; antisense, 5'-ACCNCGCATNACCATRCAC-3'.

RT-PCR consisted of 30 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C. The PCR product was electrophoresed on 1.5% agarose gel and stained with ethidium bromide. A product was then cloned into the pCR-Script vector (Stratagene) and sequenced using the ALF express autoread sequencing kit (Pharmacia) and the Pharmacia ALF express DNA sequencer system.

### 2.6. Whole-mount in situ hybridization

For in situ hybridization to whole-mounted butterfly wings, *P. coenia* wings were dissected and then fixed immediately in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 4 h. After washing with PBS in 0.1% Tween, the wings were incubated in PBS containing 0.3% Triton-X 100 at 4 °C for 18 h. The wings were then treated with proteinase K (10 µg/ml) in 30 mM Tris buffer containing 10 mM EDTA, 50 mM NaCl and 10 mM MgCl<sub>2</sub>, pH 8.5 for 30 min. The wings were hybridized with <sup>35</sup>S-labelled *P. coenia* *GTP-CH I* riboprobe at 55°C for 18 h. After hybridization, wings were washed at 55 °C and treated with RNase A and RNase T1 in 10 mM Tris buffer containing 5 mM EDTA, 0.3 M NaCl, pH 7.5 for 30 min at 37 °C. After digestion of unbound probe, the wings were washed at 55 °C and subjected to autoradiography.

## 3. Results

### 3.1. Sequencing of partial *P. coenia* *GTP-CH I*

A partial cDNA of *P. coenia* *GTP-CH I* was obtained by the homology cloning technique. The predicted amino acid sequence of the cDNA showed a core region of

GTP-CH I and a high degree of identity when compared with known sequences for GTP-CH I (Fig. 1, boxed amino acids).

### 3.2. Developmental expression of *GTP-CH I* mRNA and enzyme activity

The time course of *GTP-CH I* mRNA expression during the late pupal development of *P. coenia* was investigated by probing a blot of total RNA from forewings with an antisense in vitro transcript of the partial *GTP-CH I* cDNA. The probe hybridized specifically to two RNA species of approximately 1.0 and 2.7 kb, respectively. The strongest positive signal was recognized around pigment formation one day before butterfly emergence at day 6 evening (Fig. 2).

We also examined GTP-CH I enzyme activity in whole forewings during the late pupal development. The activity increased dramatically during day 6, and the increase continued until just before adult emergence at day 7 morning (Fig. 3, closed circles).

### 3.3. Effect of 20-hydroxyecdysone on *GTP-CH I* gene expression and enzyme activity

To study the effect of 20E on the expression of *GTP-CH I* mRNA, 20E was injected into day 4 evening pupa and the level of the *GTP-CH I* mRNA was followed by Northern blot analysis. This 20E treatment delayed the time point of adult emergence for one day and shifted

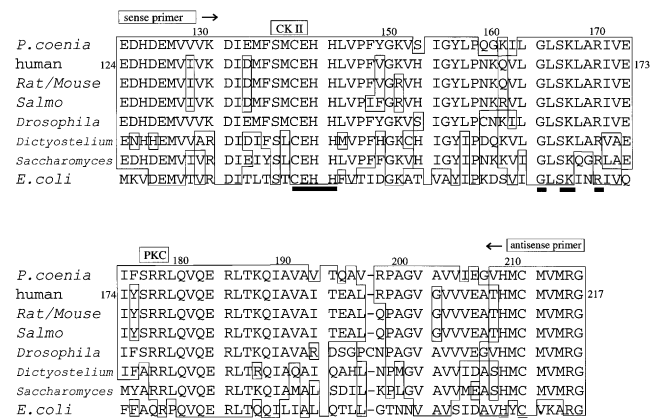


Fig. 1. Deduced partial amino acid sequence of the *P. coenia* *GTP-CH I* protein and alignment of amino acid sequences of the core region of *GTP-CH I*. Comparison of the aligned amino acid sequences of GTP-CH I from human (Togari et al., 1992), rat/mouse (Hatakeyama et al., 1991; Nomura et al., 1993), *Drosophila melanogaster* (McLean et al., 1993), *Salmo gairdneri*, *Saccharomyces cerevisiae* (Maier et al., 1995), *Dictyostelium discoideum* (Witter et al., 1996) and *E. coli* (Katzenmeier et al., 1991) sources. Identical amino acids are boxed. The numbering above the alignment is according to the human sequence (Gütlich et al., 1992). The amino acids contributing to the GTP-binding pocket are underlined. Possible phosphorylation sites for protein kinase C (PKC) and casein kinase II (CK II) and primers are indicated on top of the alignment.

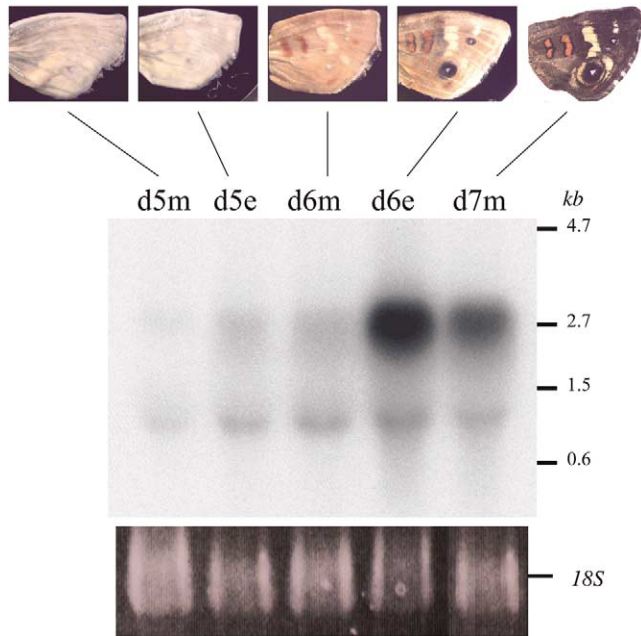


Fig. 2. Developmental change of *GTP-CH I* mRNA in wings. Total RNA was prepared from wings during the final stages of pupal development. Each 20  $\mu$ g of total RNA was electrophoresed and blotted onto nylon membrane. The blot was hybridized with  $^{32}$ P-labeled antisense *GTP-CH I* riboprobe. Developmental stages are indicated as follows: d5m, on day 5 at 8:00; d5e, on day 5 at 20:00 white pigment appeared; d6m, on day 6 at 8:00 red pigment synthesis started; d6e, on day 6 at 20:00 black eyespot appeared; d7m, on day 7 at 8:00 wing pigmentation appeared to be complete about 3 h before adult emergence. The pictures above indicate the wings of each developmental stage. The picture below the Northern blot shows the 18S RNA region stained with ethidium bromide.

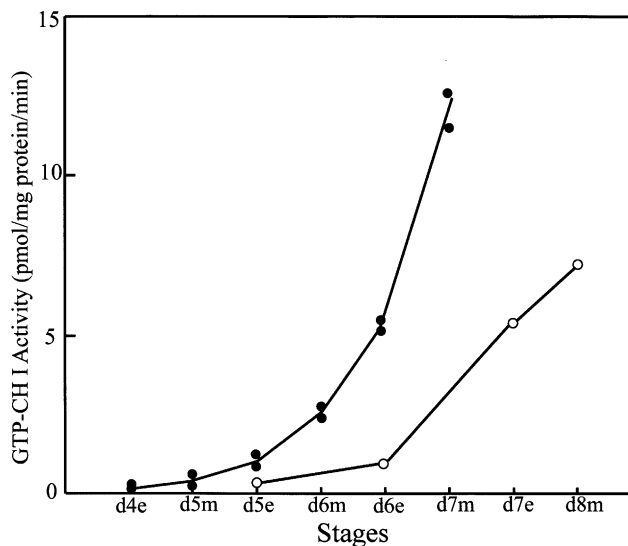


Fig. 3. Effect of 20-hydroxyecdysone on *GTP-CH I* activity in developmental stages of wings. The pupae on day 4 at 20:00: the pupae were injected with 20E (open circle) or Grace's medium as controls (closed circle) as described in Section 2. Developmental stages are indicated as in Fig. 2. Both control and injected with 20E pupae: d7m (closed circle) and d8m (open circle), which is about 3 h before adult emergence.

the *GTP-CH I* expression (2.7 kb signal) from day 6 to day 7 (Fig. 4, arrow). Consequently, the 20E treatment also delayed the *GTP-CH I* enzyme activity. The activity increased during day 7 slower than in controls, and the increase continued until delayed adult emergence at day 8 morning (Fig. 3, open circles).

### 3.4. Spatial expression of *GTP-CH I* mRNA by whole-mount *in situ* hybridization

We tried to investigate the spatial distribution of *GTP-CH I* mRNA in forewings at day 6 evening which was recognized as the stage of strongest expression by whole-mount *in situ* hybridization. However, we have failed in this experiment at the present time, probably because the wings, or more specifically the scales, at day 6 evening and day 7 morning are extremely hydrophobic. Accordingly, we tried to examine the spatial distribution of *GTP-CH I* mRNA at an earlier stage of day 5 evening (Fig. 5C) using the core region of the *GTP-CH I* antisense-riboprobe. High levels of the *GTP-CH I* mRNA were specifically detected as shown in Fig. 5A (arrows) corresponding to the position of the presumptive white center and the white eyespot ring and some general gray areas (compare to Fig. 5D). We found no positive signals in control wings using a sense-riboprobe (Fig. 5B).

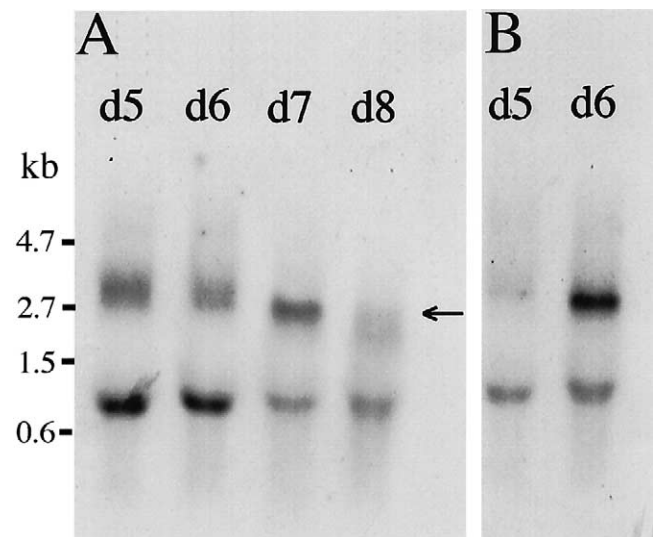


Fig. 4. Effect of 20-hydroxyecdysone on expression of *GTP-CH I* mRNA. The pupae on day 4 at 20:00: the pupae were injected with 20E (A) or Grace's medium as controls (B). Total RNA was prepared from wings during the final pupal development. Each 20  $\mu$ g of total RNA was electrophoresed and blotted onto nylon membrane. The blot was hybridized with  $^{32}$ P-labeled antisense *GTP-CH I* riboprobe. Developmental stages are indicated as follows: d5, on day 5 at 14:00; d6, on day 6 at 14:00; d7, on day 7 at 14:00; d8, on day 8 at 14:00.

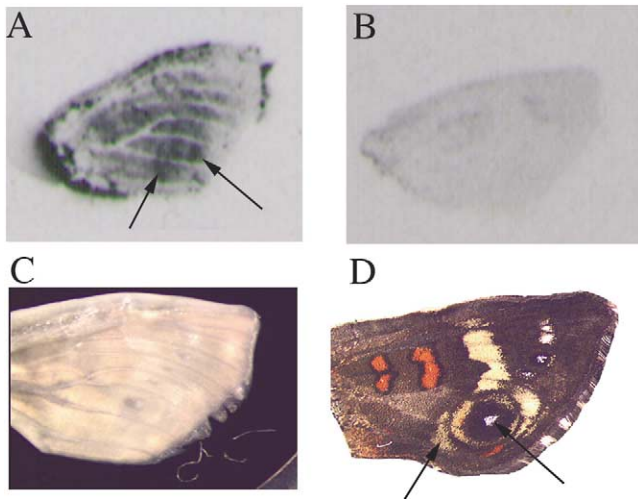


Fig. 5. Spatial expression of *GTP-CH I* mRNA by whole-mount in situ hybridization. Autoradiographs A and B result from the antisense-riboprobe and sense-riboprobe, respectively, of the *GTP-CH I* gene fragment on day 5 at 20:00 (d5e) of the pupal wing. The arrows in A show positive signals. Panel C shows the pigmentation of the day 5 wing just before fixation. Panel D shows the pigmentation on day 7 at 8:00 which is about 3 h before adult emergence. The arrows in panel D indicate the prospective position for the arrows in panel A.

#### 4. Discussion

In the present study, we obtained the partial cDNA of *GTP-CH I* from wings of *P. coenia*. The deduced amino acid sequence of the cDNA showed highly conserved regions of GTP-CH I which contains the GTP-binding pocket and possible phosphorylation sites by protein kinase C (S/T-x-R/K) (Woodgett et al., 1986) and casein kinase II (S/T-xx-D/E) (Pinna, 1990). The deduced amino acid sequence of the partial *P. coenia* GTP-CH I was found to be homologous to that of human (83%), rat/mouse (80%), *Salmo gairdneri* (78%), *Drosophila melanogaster* (88%), *Dictyostelium discoideum* (68%), *Saccharomyces cerevisiae* (68%), *E. coli* (48%) and other species of lepidoptera (97–99%, data not shown). Maier et al. (1995) suggested that this core region was necessary for correct enzyme activity and protein folding.

During color pattern formation of the wing, the dramatic changes in the expression level of *GTP-CH I* were observed during final pupal development of *P. coenia* when the pigment synthesis occurs. The strongest positive signal was recognized around one day before butterfly emergence. This *GTP-CH I* gene expression is paralleled by GTP-CH I enzyme activity measured in the wing extracts. During this last day of pupal development before adult emergence the black and gray (melanin) pigmentation appears (Koch and Kaufmann, 1995). Therefore, GTP-CH I activity in this period may be due to BH<sub>4</sub> synthesis, the cofactor for the hydroxylation of tyrosine. BH<sub>4</sub> is reported to be necessary for biosynthesis of neurotransmitters by tyrosine 3-monooxygenase (EC

1.14.16.2) in the nerve cells in mammals (Kaufman, 1986). However, in the case of the biosynthesis of melanin in the mammalian melanocytes, formation of DOPA from tyrosine is catalyzed by tyrosinase (EC 1.14.18.1), and the reaction does not require BH<sub>4</sub> as a cofactor (Riley, 1997; Hearing, 2000). In contrast, in the case of *Drosophila*, BH<sub>4</sub> is essential to the enzyme which catalyzes tyrosine to DOPA as a cofactor during the cuticle sclerotization and melanization (O'Donnell et al., 1989; Neckameyer and White, 1993; Piedrafita et al., 1994). In accordance with these results in a dipteran insect, our results in *Precis* suggest that BH<sub>4</sub> is an essential cofactor for hydroxylation of tyrosine during sclerotization and melanization in insects. Melanization and sclerotization in insects may have the same mechanism as biosynthesis of neurotransmitters in the mammalian nerve cells. Further investigation will be required to clarify the relation between the synthesis of melanin and BH<sub>4</sub>.

In the tobacco hornworm, *Manduca sexta*, it has been shown that wing pigmentation in the late pupa is delayed by infusion of 20E and that the declining ecdysteroid titer regulates the late metamorphic development (Schwartz and Truman, 1983). Koch (1995) reported the same delay of pigmentation by treatment with 20E in wings of *P. coenia* which corresponded to a delay of the DOPA decarboxylase activity involved in melanin synthesis. Accordingly, in the present paper we studied the effect of 20E on the expression of *GTP-CH I* mRNA and the enzyme activity in developing wings of *P. coenia*. The strongest positive signal of *GTP-CH I* was shifted from day 6 to day 7 corresponding to the delay of adult emergence (Fig. 4). The time point of increasing enzyme activity was also delayed as shown in Fig. 3. These results strongly suggest that the *GTP-CH I* gene expression is controlled by the declining ecdysteroid titer and that the changes in GTP-CH I enzyme activity are regulated mainly at the level of transcription during pupal wing development. Furthermore, in the experiment involving 20E injection, we recognized that the expression of two kinds of *GTP-CH I* mRNA, which were approximately 1.0 and 2.7 kb, showed a differentiated response in time. The expression of the 1.0 kb mRNA showed a fast response with a strong signal after 20E treatment. In contrast, expression of the 2.7 kb mRNA was weaker on day 6 compared to the control and showed a delayed signal compared to the 1.0 kb mRNA (Fig. 4). The expression of the two kinds of mRNA may be controlled by different kinds of transcription factors. Further studies on the gene expression mechanism in relation to hemolymph ecdysteroid titer and ecdysteroid receptor levels are under way.

In whole-mount in situ hybridization, we observed specific signals on the wings of *P. coenia*. The specific signals corresponded to the prospective eyespot focus (center) and the prospective white ring around the eyespot (Fig. 5). The expression of the *GTP-CH I* gene at

these positions may be responsible for pteridine synthesis, because both positions will become white in color on the adult wing. However, at present we have no data on what kind of pteridine is localizing at the white areas in the wing. Investigations of the pteridines in *P. coenia* will clarify whether GTP-CH I has a pattern-specific and a dual role in serving the synthesis of white pigments as well as for a cofactor of melanin synthesis.

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## References

- Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acidguanidium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.* 162, 156–159.
- Gütlich, M., Schott, K., Werner, T., Bacher, A., Ziegler, I., 1992. Species and tissue specificity of mammalian GTP cyclohydrolase I messenger RNA. *Biochim. Biophys. Acta* 1171, 133–140.
- Hatakeyama, K., Inoue, Y., Harada, T., Kagamiyama, H., 1991. Cloning and sequencing of cDNA encoding rat GTP cyclohydrolase I. The first enzyme of the tetrahydrobiopterin biosynthetic pathway. *J. Biol. Chem.* 266, 765–769.
- Hearing, V.J., 2000. The melanosome: The perfect model for cellular responses to the environment. *Pigment Cell Res.* 13 (Suppl. 8), 23–34.
- Iino, T., Dohke, K., Tsusue, M., 1992. The purification and characterization of sepiapterin reductase from fat body of the silkworm *Bombyx mori*. *Zool. Sci.* 9, 119–125.
- Katzenmeier, G., Schmid, C., Kellermann, J., Lottspeich, F., Bacher, A., 1991. Biosynthesis of tetrahydrofolate. Sequence of GTP cyclohydrolase I from *Escherichia coli*. *Biol. Chem. Hoppe–Seyler* 372, 991–997.
- Kaufman, S., 1986. The metabolic role of tetrahydrobiopterin. In: Cooper, B.A., Whitehead, V.M. (Eds.) *Chemistry and Biology of Pteridines*. Walter de Gruyter, Berlin, New York, pp. 185–200.
- Koch, P.B., 1992. Seasonal polyphenism in butterflies: A hormonally controlled phenomenon of pattern formation. *Zool. Jahrb. Physiol.* 96, 227–240.
- Koch, P.B., 1995. Colour pattern specific melanin synthesis is controlled by ecdysteroids via dopa decarboxylase in wings of *Precis coenia* (Lepidoptera: Nymphalidae). *Eur. J. Entomol.* 92, 161–167.
- Koch, P.B., 2000. Color pattern formation in lepidopteran wings and ecophysiological adaptations. *Mitt. Dtsch. Ges. Allg. Angew. Entomol.* 12, 559–570.
- Koch, P.B., Behnecke, B., Ffrench-Constant, R.H., 2000a. The molecular basis of melanism and mimicry in a swallowtail butterfly. *Curr. Biol.* 10, 591–594.
- Koch, P.B., Weigmann-Lenz, M., Behnecke, B., Ffrench-Constant, R.H., 2000b. Insect pigmentation: Activities of  $\beta$ -alanyl dopamine synthase in wing color patterns of wild-type and melanic mutant swallowtail butterfly *Papilio glaucus*. *Pigment Cell Res.* 13 (Suppl. 8), 54–84.
- Koch, P.B., Kaufmann, N., 1995. Pattern specific melanin synthesis and DOPA decarboxylase activity in a butterfly wing of *Precis coenia* Hübner. *Insect Biochem. Mol. Biol.* 25, 73–82.
- Koch, P.B., Keys, D.N., Rocheleau, T., Aronstein, K., Blackburn, M., Carroll, S.B. et al., 1998. Regulation of dopa decarboxylase expression during colour pattern formation in wild-type and melanic tiger swallowtail butterflies. *Development* 125, 2303–2313.
- Maier, J., Witter, K., Gütlich, M., Ziegler, I., Werner, T., Ninnemann, H., 1995. Homology cloning of GTP-cyclohydrolase I from various unrelated eukaryotes by reverse-transcription polymerase chain reaction using a general set of degenerate primers. *Biochem. Biophys. Res. Commun.* 212, 705–711.
- Marletta, M.A., 1993. Nitric oxide synthase structure and mechanism. *J. Biol. Chem.* 268, 12231–12234.
- McLean, J.R., Krishnakumar, S., O'Donnell, J.M., 1993. Multiple mRNAs from the *Punch* locus of *Drosophila melanogaster* encode isoforms of GTP cyclohydrolase I with distinct N-terminal domains. *J. Biol. Chem.* 268, 2910–2917.
- Nakagoshi, M., Masada, M., Tsusue, M., 1984. The nature of the seasonal colour dimorphism in the scorpion fly, *Panorpa japonica* Thunberg. *Insect Biochem.* 14, 615–618.
- Nakagoshi, M., Takikawa, S., Tsusue, M., 1992. Relationship between seasonal colour dimorphism and pteridines in the scorpion fly, *Panorpa japonica*, Thunberg. *Pteridines* 3, 69–70.
- Neckameyer, W.S., White, K., 1993. *Drosophila* tyrosine hydroxylase is encoded by the *pale* locus. *J. Neurogenet.* 8, 189–199.
- Nijhout, H.F., 1980a. Ontogeny of the color pattern on the wings of *Precis coenia* (Lepidoptera: Nymphalidae). *Dev. Biol.* 80, 275–288.
- Nijhout, H.F., 1980b. Pattern formation on lepidopteran wings: Determination of an eyespot. *Dev. Biol.* 80, 267–274.
- Nijhout, H.F., 1985. The developmental physiology of color patterns in Lepidoptera. *Adv. Insect Physiol.* 18, 181–247.
- Nijhout, H.F., 1997. Ommochrome pigmentation of the linea and rosa seasonal forms of *Precis coenia* (Lepidoptera: Nymphalidae). *Arch. Insect Biochem. Physiol.* 36, 215–222.
- Nijhout, H.F., Koch, P.B., 1991. The distribution of radiolabeled pigment precursors in the wing patterns of nymphalid butterflies. *J. Res. Lepid.* 30, 1–13.
- Nomura, T., Ichinose, H., Sumi-Ichinose, C., Nomura, H., Hagino, Y., Fujita, K. et al., 1993. Cloning and sequencing of cDNA encoding mouse GTP cyclohydrolase I. *Biochem. Biophys. Res. Commun.* 191, 523–527.
- O'Donnell, J.M., McLean, J.R., Reynolds, E.R., 1989. Molecular and developmental genetics of the *Punch* locus, a pterin biosynthesis gene in *Drosophila melanogaster*. *Dev. Genet.* 10, 273–286.
- Ohashi, M., Tsusue, M., Yoshitake, N., Sakate, S., Kiguchi, K., 1983. Epidermal pigments affecting the larval coloration of the silkworm, *Bombyx mori*. *J. Sericult. Sci.* 52, 498–504.
- Piedrafito, A.C., Martínez-Ramírez, A.C., Silva, F.J., 1994. A genetic analysis of aromatic amino acid hydroxylase involvement in DOPA synthesis during *Drosophila* adult development. *Insect Biochem. Mol. Biol.* 24, 581–588.
- Pinna, L.A., 1990. Casein kinase 2: an eminence grise in cellular regulation. *Biochim. Biophys. Acta* 1054, 267–284.
- Riley, P.A., 1997. Molecules in focus. Melanin. *Int. J. Biochem. Cell Biol.* 29, 1235–1239.
- Rountree, D.B., Nijhout, H.F., 1995. Hormonal control of a seasonal polyphenism in *Precis coenia* (Lepidoptera: Nymphalidae). *J. Insect Physiol.* 41, 987–992.
- Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Sawada, H., Kanekatsu, M., Nakagoshi, M., Dohke, K., Iino, T., Taki-

- kawa, S., 1998. Purification and characterization of sepiapterin deaminase from silkworm, *Bombyx mori*. *Pteridines* 9, 18–21.
- Sawada, H., Nakagoshi, M., Mase, K., Yamamoto, T., 2000. Occurrence of ommochrome-containing pigment granules in the central nervous system of the silkworm, *Bombyx mori*. *Comp. Biochem. Physiol.* 125B, 421–428.
- Sawada, H., Tsusúé, M., Iino, T., 1994. Identification of ommin in the integument of the silkworm, *Bombyx mori*. *Biol. Chem. Hoppe–Seyler* 375, 425–427.
- Sawada, H., Tsusúé, M., Yamamoto, T., Sakurai, S., 1990. Occurrence of xanthommatin containing pigment granules in the epidermal cells of the silkworm, *Bombyx mori*. *Insect Biochem.* 20, 785–792.
- Schwartz, L.M., Truman, J.W., 1983. Hormonal control of rates of metamorphic development in the tobacco hornworm *Manduca sexta*. *Dev. Biol.* 99, 103–114.
- Stuehr, D.J., 1997. Structure–function aspects in the nitric oxide synthases. *Annu. Rev. Pharmacol. Toxicol.* 37, 339–359.
- Takikawa, S., Curtius, H.C., Redweik, U., Leimbacher, W., Ghisla, S., 1986. Biosynthesis of tetrahydrobiopterin. Purification and characterization of 6-pyruvoyl-tetrahydropterin synthase from human liver. *Eur. J. Biochem.* 161, 295–302.
- Takikawa, S., Nakagoshi, M., 1994. Developmental changes in pteridine biosynthesis in the toad, *Bufo vulgaris*. *Zool. Sci.* 11, 413–421.
- Thöny, B., Auerbach, G., Blau, N., 2000. Tetrahydrobiopterin biosynthesis, regeneration and functions. *Biochem. J.* 347, 1–16.
- Togari, A., Ichinose, H., Matsumoto, S., Fujita, K., Nagatsu, T., 1992. Multiple mRNA forms of human GTP cyclohydrolase I. *Biochem. Biophys. Res. Commun.* 187, 359–365.
- Tsusúé, M., Kuroda, S., Sawada, H., 1990. Localization of sepiapterin deaminase and pteridines in the granules in epidermal cells of the silkworm, *Bombyx mori*. *Pteridines* 2, 175–182.
- Witter, K., Cahill, D.J., Werner, T., Ziegler, I., Rödl, W., Bacher, A. et al., 1996. Molecular cloning of a cDNA coding for GTP cyclohydrolase I from *Dictyostelium discoideum*. *Biochem. J.* 319, 27–32.
- Woodgett, J.R., Gould, K.L., Hunter, T., 1986. Substrate specificity of protein kinase C. Use of synthetic peptides corresponding to physiological sites as probes for substrate recognition requirements. *Eur. J. Biochem.* 161, 177–184.
- Ziegler, I., McDonaldo, T., Hesslinger, C., Pelletier, I., Boyle, P., 2000. Development of the pteridine pathway in the zebrafish, *Danio rerio*. *J. Biol. Chem.* 275, 18926–18932.