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Evidence for the Presence of the Summer-Morph-Producing Hormone in the Swallowtail Butterfly, *Papilio xuthus* L. (Lepidoptera: Papilionidae)

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ABSTRACT—The swallowtail butterfly, *Papilio xuthus* L., exhibits seasonal diphenism, *i.e.*, spring and summer morphs, the development of which is determined by photoperiod and temperature imposed during the larval stage. Larvae reared under long day conditions at 25°C develop into summer morphs without pupal diapause, while larvae reared under short day conditions at 20°C develop into spring morphs after diapause development at 4°C for more than three months. To investigate the neuroendocrine mechanism regulating seasonal morph development, the activity of the summer-morph-producing hormone (SMPH) was tried to assayed by using male short day pupae of *P. xuthus* that accomplished diapause development at 4°C. The wing patterns of male butterflies shifted toward summer morphs when short day pupae were treated with the SMPH at an adequate stage. SMPH activity was detected in 2% NaCl extracts of the brain-subesophageal ganglion (Br-SG) complexes of *P. xuthus* long day pupae. Chilled male short day pupae responded to *P.xuthus* SMPH in a dose-dependent manner and lost the responsiveness within 2 days of transfer to room temperature (about 25°C). This is a report to show the existence of SMPH in butterfly species having pupal diapause.

Key words: seasonal morph, SMPH, butterfly, *Papilio*

INTRODUCTION

It is widely known that butterflies of many species show seasonal polymorphisms of wing coloration, wing form, and body size. The development of seasonal polymorphism is determined by environmental factors, such as photoperiod and temperature, during the larval and pupal stages (Müller, 1955; Hidaka and Takahashi, 1967; Shapiro, 1976). Studies of the seasonal polymorphisms of butterflies have demonstrated that the development of seasonal morphs, especially wing colorations, is regulated by neuroendocrine systems in two the butterfly species such as *Polygonia c-aureum and Lycaena phlaeas daimio* (Fukuda and Endo, 1966; Endo and Kamata, 1985). In two other butterfly species having pupal diapause, *Araschnia burejana* and *A. levana*, seasonal morph development is determined in a close association with the experience of pupal diapause. Short day pupae prevented from entering pupal diapause by the injection of 20-hydroxyecdysone developed into summer morphs (Keino and Endo, 1973; Koch and Bückmann, 1987).

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In the Asian comma butterfly, *P. c-aureum*, which has an adult diapause, summer morph development is determined by a summer-morph-producing hormone (SMPH) secreted from the brain-reterocerebral neuroendocrine system early in the pupal stage (Endo, 1984; Endo *et al*., 1988). SMPH activity is also detected in the brain extracts of young prepupae of *Precis coenia* (Starnecker *et al*., 1997). The seasonal polyphenism of *P. coenia* is regulated by the timing of ecdysteroid secretion similar to the mechanism described in the *Araschnia* species (Rountree and Nijhout, 1995).

We previously reported that the wing coloration of *Papilio xuthus* also exhibits seasonal dimorphism, spring and summer morphs. The development of spring morphs is determined coincidentally with the destination of diapause development in the pupal stage by exposure to short days (Endo and Murakami, 1985; Endo *et al*., 1985). Spring morph development is shifted toward summer morph by joining the short day pupae to long day ones. *P. xuthus* may have a humoral factor regulating seasonal morph development. Secretion of the humoral factor as well as the prothoracicotropic hormone essential for adult development is regulated by photoperiod and temperature in the larval stage (Endo and Funatsu, 1985). Therefore, we tried to confirm the existence of *P. xuthus* SMPH in the brain of long day pupa similar to that in *P. c-aureum*, but

this issue has not been examined in detail.

In the present paper, we aim to establish a bioassay method for quantifying *P. xuthus* SMPH activity and investigating the properties of SMPH from the Br-SG complexes of *P. xuthus* long day pupae.

MATERIALS AND METHODS

Insects

Adults of *P. xuthus* L. were collected near the towns of Yamaguchi and Ube, and females were allowed to oviposit on leaves of *Fagara ailanthoides*. Larvae were reared on leaves of *F. ailanthoides* under a long day (LD) photoperiod (16 hr light and 8 hr dark; 16L : 8D) at 25°C, and a short day (SD) photoperiod (8L : 16D) at 20°C. Pupae were destined to develop into summer morphs without pupal diapause (LD-pupae) under the LD conditions, or into spring morphs after diapause development (SD-pupae) under the SD conditions. LD-pupae were allowed to develop at 25°C to obtain the standard summer-morphs. SD-pupae which did not start adult development within one month after pupation were regarded as diapause pupae and were chilled at 4°C for 3 months to accomplish their diapause development. The chilled diapause pupae were allowed adult development under LD conditions at 25°C to obtain the standard spring-morphs. In addition, the chilled diapause pupae served as the test animals for SMPH bioassay.

Classification of seasonal morphs

Seasonal morphs were classified by the relative sizes of the seven pale yellow spots along the central line of the dorsal side of the posterior wings according to Endo *et al.* (1985) with a slight modification. To establish the standard criteria of spring and summer morphs, 300 male standard spring morphs and 300 male standard summer morphs were used, respectively. The product, length×width, was obtained for each spot and the relative sizes of the 4th and 5th spots on the posterior wings, hereafter referred to as CL 4 and CL 5, were obtained as the indices of seasonal morphs. Ellipses showing 99% and 95% confident lines of the spring and summer morphs were obtained from CL 4 and CL 5. Male butterflies falling under either of the ellipses of 99% confidence were classified as the respective seasonal morphs, and those eliminated from both ellipses were classified as the intermediate morphs.

Extraction of SMPH

Br-SG complexes were obtained from Day 0 LD-pupae of *P. xuthus* by dissection in 0.9% NaCl. One hundred Br-SG complexes were homogenized in 1.5 ml of ice-cold acetone, and centrifuged at 12,100 x *g* for 10 min. The resulting supernatants (referred to as acetone extract) were pooled, lyophilized, and stored at –85°C until use. The pellets were resuspended in 80% ethanol, then centrifuged in the same manner. The obtained supernatants (referred to as 80% ethanol extract) were pooled, lyophilized, and stored at –85°C until use. As the final step, the pellets were resuspended in 2% NaCl, boiled at 95°C for 3 min, cooled rapidly and centrifuged at 12,100 x *g* for 15 min. The supernatants were pooled, then loaded on a Sep-Pak plus C18 cartridge column (Waters, Milford, Mass) equilibrated with distilled water. The column was washed with distilled water to remove salt and eluted with 50% acetonitrile. The resulting eluate (referred to as 2% NaCl extract) was lyophilized and stored at –85°C until use.

Ion-exchange column chromatography

The lyophilized 2% NaCl extract prepared from 400 Br-SG complexes of LD-pupae was dissolved in 50 mM phosphate buffer (pH 6.9) and loaded on a Sep-Pak plus QMA CM double cartridge column (Waters, Milford, Mass) equilibrated with the same buffer. The column was washed with the same buffer. The non-absorbed eluate was collected. After washing, the columns were separated and each column was eluted with the same buffer containing 0.5M NaCl. The non-absorbed, QMA, and CM eluates were desalted using the Sep-Pak plus C18 cartridges as described above. The resulting three fractions (referred to as pass through, QMA fraction, and CM fraction) were lyophilized and stored at –85°C until use.

Proteinase K treatment

Lyophilized 2% NaCl extract prepared from 200 Br-SG complexes of LD-pupae was dissolved in 500 μl of 50 mM ammonium sulfate buffer (pH 6.9) containing 0.2 μg of proteinase K (Sigma, St. Louis, MO), incubated at 37°C for 90 min, and boiled at 95°C for 5 min to inactivate proteinase K. The proteinase K-treated extract was cooled on ice, and the extract was subjected to a Sep-Pak plus C18 cartridge column equilibrated with distilled water. The column was washed with distilled water to remove salt, and eluted with 50% acetonitrile solution. The resulting eluate was lyophilized and stored at –85°C until use.

Bioassay of SMPH activity

Male *P. xuthus* SD-pupae chilled at 4°C for 3 months (chilled male SD-pupae) were used as the test animals. The chilled male SD-pupae were kept at 25°C for 12 hr and were injected with 10 μl of samples. The injection was made into the dorso-ventral intersegmental region between the 6th and the 7th abdominal segments and the wound was sealed with melted paraffin. They were allowed to develop under LD conditions at 25°C. After adult emergence, the butterflies were subjected to classification of seasonal morphs (grades 0–4) according to the ellipses of spring and summer morphs. Specimens falling under the ellipses showing 99% confidence of spring and summer morphs were classified as to be grades 0 and 4, respectively. Those falling between the ellipses of 99% and 95% confidence of spring (or summer) morphs were classified as grade 1 (or grade 3), in addition to specimens of grade 2 (intermediate morphs) falling between the ellipses of 99% confidence of spring and summer morphs. An average grade score (AGS) for summer morphs was obtained from 4–14 insects.

RESULTS

Classification of seasonal morphs

Ellipses of 99% and 95% confidence of spring and summer morphs were obtained on 300 male summer morph and 300 male spring morph butterflies developed from LD-pupae and chilled SD-pupae under LD conditions at 25°C, respectively. Figure 1 shows that the 300 male butterflies from LD-pupae were classified as summer morphs (98.7%) and intermediate morphs (1.3%). On the other hand, the 300 male butterflies which had emerged from chilled SD-pupae were classified as spring morphs (99.3%) and intermediate morphs (0.7%). The results indicate that seasonal morphs of *P. xuthus* can be classified by a confidence ellipse method based on the relative size of CL 4 and CL 5 to the posterior wings.

Effect of pupal chilling on summer morph development

To know whether pupal chilling affects summer morph development in *P. xuthus*, male LD-pupae aging from 12 hr to 9 days after pupation were exposed at 4°C. Ten days later, they were transferred to 25°C and allowed adult development.

Table 1 summarizes the effects of the chilling of *Papilio* LD-pupae on the development of seasonal morphs. When LD-pupae were chilled at 4°C from 12 hr after pupation and allowed adult development at 25°C, 14% and 57% of butter-

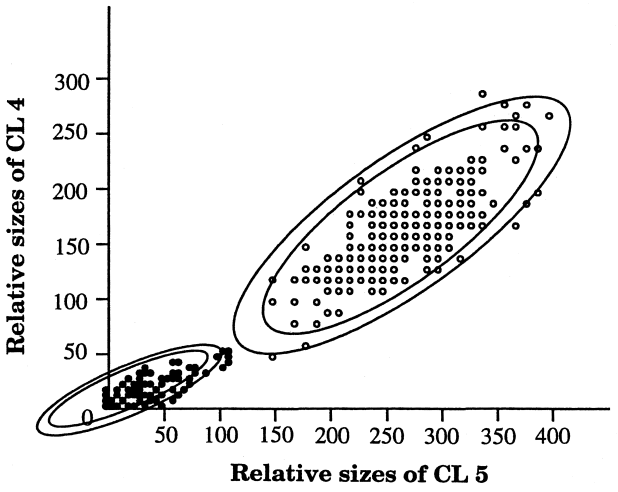

Fig. 1. Confidence ellipses obtained by 300 standard spring morph and 300 standard summer morph butterflies of *P. xuthus*. Ellipses show 95% (inside) and 99% confidence lines (outside) of spring and summer morphs. Open and solid circles show standard spring morph and standard summer morph butterflies represented on the relative sizes of CL 4 (a pale yellow spot of the central line in the 4th cells of posterior wings) and CL 5, respectively.

flies developing without pupal diapause were classified as spring and summer morphs, respectively. 29% were judged to be intermediates. In contrast, most (87%) butterflies were classified as summer morphs, and 13% as intermediate morphs, when they were chilled from 1 to 9 days after pupation. However, no pharate pupae exposed to 4°C for 10 days developed into adults. The results indicate that summer morph development is determined by LD conditions at relatively hight temperatures during the larval stage, is modified by exposing them to a low temperature (4°C) early in the pupal stage.

Extraction of *P. xuthus* SMPH from LD-pupae of *P. xuthus*

To investigate whether *P. xuthus* SMPH exists in the Br-SG complexes of LD-pupae, 200 Br-SG complexes of LD-pupae were extracted with acetone, 80% ethanol, and 2% NaCl as described in Materials and Methods. Each lyophilized extract was injected into chilled male SD-pupae for bioassay. Table 2 shows that SMPH activity was detected mainly in the 2% NaCl extract (AGS 3.3), and 77% of the recipient butterflies were classified as summer morphs. In addition, the 80% ethanol extracts showed slight SMPH activity (AGS 0.7); however, none of the recipient butterflies were classified as summer morphs. When acetone extract and distilled water were injected, all of the recipients were classified as spring morphs

Table 1. Effects of the chilling treatment of *Papilio* LD-pupae on the development of seasonal morphs

Stages of chilling treatment after pupation	No.	No. of butterflies classified into grades of summer-morphs					AGS
		0	1	2	3	4	
12 hr[*1]	14	0	2	4	2	6	2.86
1 day[*1]	10	0	0	0	1	9	3.90
2–9 days[*1]	45	0	0	7	3	35	3.62
Control[*2]	300	0	0	4	13	283	3.93

[*1] Days (or hr) were counted from pupation in LD-pupae chilled at 4°C for 10 days. Thereafter, they were developed at 25°C.
[*2] LD-pupae were allowed to develop at 25°C without chilling. They were used as the standard summer-morphs.
AGS: average grade score for summer morphs.

Table 2. Effects of the Br-SG complex extracts from *Papilio* LD-pupae on the seasonal morph development of male chilled SD-pupae

Extracts	No.	No. of butterflies classified into grades of summer-morphs					AGS
		0	1	2	3	4	
P. xuthus LD-pupae							
Acetone	10	10	0	0	0	0	0.00
80% ethanol	7	4	1	2	0	0	0.71
2% NaCl	9	0	1	1	1	6	3.33
Control							
Distilled water	5	4	1	0	0	0	0.20
LD adults*	300	0	0	4	13	283	3.93
SD adults*	300	283	15	2	0	0	0.06

* LD- and SD-adults show the standard summer and spring morphs developed at 25°C. Each recipient was injected with an extract of 20 Br-SG equivalents. AGS: average grade score for summer morph.

Table 3. Effects of the sensitive period for SMPH effectiveness on the seasonal morph development of male SD-pupae

Stages of injection after acclimation at 25°C	No.	No. of butterflies classified into grades of summer-morphs					AGS
		0	1	2	3	4	
12 hr	10	0	1	1	2	6	3.30
1 day	10	0	1	0	3	6	3.40
2 days	10	6	4	0	0	0	0.20
3 days	10	5	4	1	0	0	0.40

Ten μl of sample containing an extract of 20 Br-SG equivalents was injected into abdomens of male SD-pupae after 12 hr, 1 day, 2 days and 3 days acclimation at 25°C. AGS: average grade score for summer morphs.

Table 4. Affinity of the SMPH from Br-SG complexes of *P. xuthus* to anion- and cation-exchange columns

Eluate fractions	No.	No. of butterflies classified into grades of summer-morphs					AGS
		0	1	2	3	4	
P. xuthus LD-pupae							
QMA fraction	14	3	3	8	0	0	1.36
CM fraction	4	3	1	0	0	0	0.25
Pass-through	10	10	0	0	0	0	0.00
Control							
Distilled water	10	10	0	0	0	0	0.00

Each recipient was injected with a fraction of 20 Br-SG equivalents. AGS: average grade score for summer morphs.

(AGS 0.0 and 0.2, respectively). These results indicate that *P. xuthus* SMPH exists in the Br-SG complex of LD-pupae, and can be extracted with 2% NaCl solution.

Dose-dependence of SMPH activity

The lyophilized 2% NaCl extracts prepared from 500 Br-SG complexes of LD-pupae were injected into the abdomens of chilled male SD-pupae. As shown in Fig. 2, the recipients showed dose-dependent responses to the 2% NaCl extract. The AGS for summer morph increased with the dosage of Br-SG equivalents in 2% NaCl extract. Injections of 5 Br-SG equivalents resulted in a majority of intermediate form adults (60%; AGS 2.1), while injections of 20 Br-SG equivalents resulted in 80% summer morphs (AGS 3.4). The results indicate that in 2% NaCl extracts of *P. xuthus* LD-pupae the minimum dose required for quantifying SMPH activity by *P. xuthus* pupal assay is estimated to be 20 Br-SG equivalents.

Determination of SMPH-sensitive stage in chilled SD-pupa

To investigate the critical stage up to which chilled male SD-pupae show responsiveness to SMPH, chilled male SD-pupae were transferred at 25°C, and 20 Br-SG equivalents of 2% NaCl extracts of *P. xuthus* LD-pupae were injected into their abdomens. The injection was started 12 hr after the transfer at 25°C and continued for 3 days at one day intervals. As shown in Table 3, chilled male SD-pupae showed a response to SMPH and developed into summer morphs (AGS 3.3 and 3.4, respectively; n=10) when 2% NaCl Br-SG extracts were injected within 1 day of transfer to 25°C. But they did not show any response and development into spring morphs when the injection was made 2–3 days later. The results indicate that the sensitive stage in which chilled male SD-pupae are sensitive to SMPH may last until 2 days after transfer to 25°C.

Properties of *P. xuthus* SMPH

To examine whether SMPH is a peptide hormone, the lyophilized 2% NaCl extracts prepared from 200 Br-SG complexes of *P. xuthus* LD-pupae were treated with proteinase K. Twenty Br-SG equivalents were injected into the abdomens of chilled male SD-pupae. All male butterflies that emerged from the injected pupae (n=10) were classified as spring morphs. This result suggests that *P. xuthus* SMPH is a peptide hormone.

Table 4 shows the affinity of *P. xuthus* SMPH to ion-exchange resin. The lyophilized 2% NaCl extract prepared from 400 Br-SG complexes of *P. xuthus* LD-pupae was fractionated as described in Materials and Methods. Twenty Br-SG equivalents of QMA, CM, and pass-through fractions were injected into chilled pupae. SMPH activity was mainly detected in the QMA fraction (AGS 1.36). When the CM, pass-through fraction, and distilled water were injected, no SMPH activity was detected in any of the samples, and all recipients were classified as spring morphs.

DISCUSSION

Several species of butterflies, such as *P. c-aureum*, *L. phlaeas daimio*, and *P. coenia*, have been shown to have

cerebral factors showing SMPH activity (Endo, 1984; Endo and Kamata, 1985; Starnecker *et al*., 1997). SMPH activity was also detected in 2% NaCl extracts of the brains of *P. xuthus* pharate pupae by the *Polygonia* pupal assay, suggesting the possibility that a cerebral factor showing SMPH activity is involved in the determination of seasonal morph development of *P. xuthus* (Endo *et al*., 1988). Therefore, to certify whether the SMPH-active factor plays a significant role in the regulation of the seasonal morph development of *P. xuthus*, a method for classifying the seasonal morphs was established.

Male adults of *P. xuthus* developing from chilled LD-pupae can be classified by the confidence ellipse method in the same manner as those developing from SMPH-injected chilled SD-pupae (Fig. 1). The results suggest that summer morph development, determined by SMPH, may be shifted toward spring morph development by chilling at 4°C early in the pupal stage (Table 1) as had been demonstrated in *L. phlaeas daimio* (Endo and Kamata, 1985).

P. xuthus SMPH was extracted with 2% NaCl solution from the Br-SG complex of LD-pupae and was quantified by a *P. xuthus* pupal assay using male chilled SD-pupae (Table 2). After 2 days acclimation at room temperature, the chilled male SD-pupae did not show any response to the *P. xuthus* SMPH and developed into spring morphs (Table 3).

Physiological studies indicated that *P. xuthus* SMPH produced summer morph in a dose-dependent manner (Fig. 2). Recently, we reported that pupal-cuticle-melanizing hormone (PCMH), which produces the brown color of the pupal body in *P. xuthus*, was extracted with 2% NaCl solution from a Br-SG complex of *P. xuthus* LD-pupae (Yamanaka *et al*., 1999). We found in the present study that *P. xuthus* SMPH and PCMH could be separated from the same 2% NaCl extract by the difference in their affinities to the ion-exchange resin (Table 4).

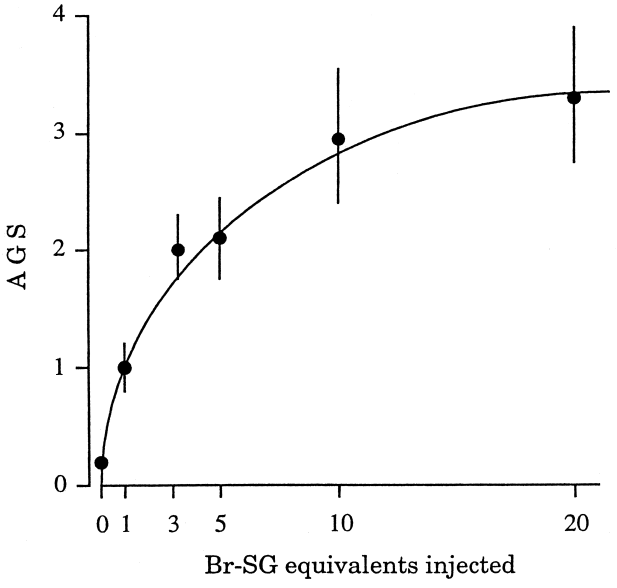

Fig. 2. Dose-dependent response curve of SMPH in 2% NaCl extracts prepared from Br-SG complexes of *P. xuthus* LD-pupae. Solid circles show the score of AGS (average grade score for summer morphs) with a standard error (n=10) shown with straight lines.

SMPH may probably exist in other swallowtail butterfly species that exhibit pupal diapause although the physiological role and molecular structure of SMPH remain to be clarified.

In *P. c-aureum* and *L. phlaeas daimio*, SMPH and ecdysone play a significant role in the determination of seasonal morph development (Endo *et al*., 1988; Endo and Kamata, 1985). This is also the case in *P. xuthus* as reported in this paper, although secretion of ecdysone as well as that of SMPH is regulated by photoperiods imposing during the larval stage. The second example is demonstrated in *P. coenia*. The seasonal polymorphisms are controlled by ecdysteroids appearing early in the pupal stage. But the function of a SMPH-active factor existing in the cerebral and thoracic ganglia of *P. coenia* young prepupae is not yet known (Rountree and Nijhout, 1995; Starnecker *et al*., 1997). The third example is reported in *A. levana* and *A. burejana* having pupal diapause. The summer morph development is thought to be determined by ecdysteroids appearing early in the pupal stage (Koch and Bückmann, 1987; Keino and Endo, 1973). However, SMPH or a SMPH-active factor are not found in these species.

Interestingly, the action of *P. xuthus* SMPH and *P. c-aureum* SMPH differs in the whole wing coloration. In *P. c-aureum*, *P.c-aureum* SMPH extracts act to change the whole wing color from dark-brown to light brown (Endo *et al*., 1988). In contrast, *P. xuthus* SMPH extracts cause a reduction of yellow spot area on the wing in *P. xuthus*. However, it is not known how SMPH acts on the wings to reduce (or stimulate) the pigmentation of the wing scales.

Further studies on SMPH may provide insight into the neuroendocrinological mechanisms underlying the control of seasonal polymorphisms in butterflies.

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REFERENCES

Endo K (1984) Neuroendocrine regulation of the development of seasonal forms of the Asian comma butterfly, *Polygonia c-aureum* L. Dev Growth Differ 26: 217–222

Endo K, Funatsu S (1985) Hormonal control of seasonal morph determination in the swallowtail butterfly, *Papilio xuthus* L. (Lepidoptera : Papilionidae). J Insect Physiol 31: 669–674

Endo K, Yamashita I, Chiba Y (1985) Effect of photoperiodic transfer and brain surgery on the photoperiodic control of pupal diapause and seasonal morphs in the swallowtail, *Papilio xuthus* L. (Lepidoptera : Papilionidae). Appl Ent Zool 20: 470–478

Endo K, Kamata Y (1985) Hormonal control of seasonal-morph determination in the small copper butterfly, *Lycaena phlaeas* daimio Seitz. J Insect Physiol 31: 701–706

Endo K, Murakami Y (1985) Photoperiodic control of determination of three different seasonal phenomena in the swallowtail butterfly, *Papilio xuthus* L. Zool Sci 2: 755–760

Endo K, Masaki T, Kumagai K (1988) Neuroendcrine regulation of development of seasonal morphs in the Asian comma butterfly, *Polygonia c-aureum*.: Difference in activity of summer-morph-producing hormone from brain-extracts of the long-day and short-day pupae. Zool Sci 5: 145–152

Fukuda S, Endo K (1966) Hormonal control of the development of seasonal forms in the butterfly, *Polygonia c-aureum* L. Proc Jap Acad 42: 1082–1087

Hidaka T, Takahashi H (1967) Temprature condition and maternal effect as modifying factor in the photoperiodic control of seasonal forms in *Polygonia c-aureum* (Lepidoptera, Nymphalidae). Annot Zool Japon 40: 200–204

Koch PB, Bückmann D (1987) Hormonal control of seasonal morphs by the timing of ecdysteroid release in *Araschnia levana* L. (Nymphalidae: Lepidoptera). J Insect Physiol 33: 823–829

Keino H, Endo K (1973) Studies of the determination of the seasonal forms in the butterfly, *Araschnia burejana* Bermer. Zool Mag 82: 48–52 (in Japanese with English summary)

Müller HJ (1955) Die Saisonformenbildung von *Araschnia levana*, ein photoperiodisch gesteuerter Diapause-Effekt. Naturwissenschaften 42: 134–135

Rountree DB, Nijhout HF (1995) Hormonal control of a seasonal polyphenism in *Precis coenia* (Lepidoptera: Nymphalidae). J Insect Physiol 41: 987–992

Shapiro AM (1976) Seasonal polyphenism. Evolutionary Biol 9: 259–333

Starnecker G, Koch PB, Matsumoto S, Endo K, Yamashita O (1997) Occurrence of four neuropeptides in the anterior central nervous system of the butterfly *Precis coenia*. Naturwissenschaften 84: 152–154

Yamanaka A, Endo K, Nishida H, Kawamura N, Hatase Y, Kong W, Kataoka H, Suzuki A (1999) Extraction and partial characterizations of pupal-cuticle-melanizing hormone (PCMH) in the swallowtail butterfly, *Papilio xuthus* L. (Lepidoptera, Papilionidae). Zool Sci 16: 261–268

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