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Diapause intensity and ecdysiotroph: Comparisons between two *Antheraea* species (Lepidoptera: Saturniidae) having summer and winter diapause at pupae

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Photoperiodism, diapause termination, diapause intensity, ecdysteroids, catecholamines, Lepidoptera, *Antheraea* moths

Abstract. The photoperiodic and neuroendocrine regulations of diapause termination were investigated in two *Antheraea* species, *A. yamamai* and *A. pernyi* which have pupal diapause to aestivate and overwinter, respectively. The types of photoperiodic responses were inverse between the two species, a short-day type in *A. yamamai* and a long-day type in *A. pernyi*. When the diapause intensity was compared among *A. yamamai* and two strains of *A. pernyi*, the intensity in the univoltine strain of *A. pernyi* was the strongest, while that in *A. yamamai* the weakest. Although the timing of ecdysteroid release, a physiological landmark for the termination of diapause, was equivalent in *A. yamamai* and the bivoltine strain of *A. pernyi*, namely after about ten cycles of exposure to a diapause-averting photoperiod, the basal levels of ecdysteroids in pupae kept in diapause-maintaining conditions were different, higher in *A. yamamai* than in the bivoltine strain of *A. pernyi*. Therefore, one correlate was found between diapause tendency and basal ecdysteroid level: the higher was the basal ecdysteroid level, the weaker the diapause tendency.

Brain monoamine contents were next investigated in *A. yamamai* and two strains of *A. pernyi*, the bivoltine and univoltine strains. Catecholamine levels, especially that of "epinephrine" in diapause pupae were found to correlate with the general strength of diapause, since the univoltine strain of *A. pernyi* had the highest level, while *A. yamamai* the lowest and the bivoltine strain of *A. pernyi* was intermediate. The result infers that high catecholamine contents may be reflected in the refractoriness of PTTH secreting pathway or low sensitivity to PTTH.

INTRODUCTION

Antheraea yamamai and *A. pernyi* are very closely related to each other, producing partially viable offspring by interspecific hybridization. Yet, their life cycles are totally different, since the former species aestivates as diapause pupae and overwinters as diapause pharate first instar larvae. The latter, on the contrary, overwinters as diapause pupae but the eggs have no potency of diapause. Yet, two strains of *A. pernyi* exist in China, showing distinct life cycle patterns, i.e., the univoltine strain which both aestivates and overwinters as pupae and the bivoltine strain which produces both diapause and non-diapause generations, diapause under long-day conditions and non-diapause under short-day conditions. Diapause is regulated photoperiodically in both species and temperature also affects the rate of diapause development. Metabolic adaptations for aestivation and overwintering were immensely different from each other (Ohnishi et al., 1994), but no other systematic comparison has been made between the two modes of pupal diapause in the same species and between the two species. By using these moths to compare their metabolic adaptations and neuroendocrine mechanisms that regulate diapause, we can obtain clues to decipher the evolution of summer diapause and winter diapause in pupae.

Giant wild silkmoths have been used intensively for several decades for biochemical and physiological analyses of neuroendocrine mechanisms that regulate diapause and photoperiodism (Hayes, 1971; Rasenick & Berry, 1981; Truman, 1971; Williams, 1969; Williams & Adkisson, 1964), but we are still far from a comprehensive understanding of their neuroendocrine regulatory mechanisms.

The present investigation adopts a comparative approach to depict the origin and evolution of pupal diapause of *Antheraea* moths.

MATERIAL AND METHODS

Insects

A. yamamai were reared on *Quercus acutissima* in the field at Azaicho, Azai county, Shiga Prefecture, Japan (35.5°N, 135.2°E). Cocoons harvested in the second week of July, 1990 and 1994 within a week were brought into the laboratory and pupae were immediately removed from the cocoons and exposed to LD 12 : 12 or LD 16 : 8 at 30°C, 75% RH. Summer diapause had been induced in these pupae, since no emergence was observed for the subsequent 30 days at LD 16 : 8. The number of pupae were 20 for each photoperiodic treatment except for LD 12 : 12 for which 40 pupae were used.

A. pernyi pupae of the bivoltine strain were sent by air mail on October 7, 1991 from Liao-Nin Province and the univoltine strain on August 1, 1995 from Santon Province, People's Republic of China respectively, by Internationai Wild Silkmoth Research Center. Before shipment, they had been reared on various *Quercus* trees in the field. Diapause was securely instituted in these pupae, since no emergence occurred within 60 days in LD 12 : 12 at 25°C. The bivoltine strain was used for the photoperiodic investigation, exposed to LD 12 : 12 or LD 16 : 8, at 25°C. Hemolymph ecdysteroid titers were followed in these conditions. The univoltine strain was used for investigation of the development at different temperatures (20, 25 and 30°C) at LD 16 : 8.

All three stocks were used for measurement of catecholamine contents in the brain-subesophageal ganglion.

Measurement of ecdysteroids

RIA procedure employed here was as outlined in Matsumoto & Takeda (1996). Hemolymph ecdysteroids of female pupae sampled every 5 days were measured using monoclonal antibodies (2-6-D8G), donated by Prof. Y. Aizono of our institute. 30 μl hemolymph was mixed with 900 μl methanol, vortexed and left standing for 10–30 min. After centrifugation at 7,500 x g for 5 min, 50–200 μl of the supernatant was dried completely by aspiration in N_2 . 50 μl of borate buffer (PH 8.4) containing 20% rabbit serum protein was added to the tube to resuspend ecdysteroids to which 50 μl of antibodies diluted 80 times was added. The mixture was vortexed and then 10 μl ^{3}H-ecdysone (10 nCi) was added. The mixture was re-vortexed and left overnight at 4°C. 50%-saturated ammonium sulfate was added to precipitate the ecdysteroid-antibody complex and kept at 4°C for 1–2 h after vortexing. The sample was again vortexed and centrifuged at 7,500 x g for 3 min. The supernatant was washed with 300 μl of 50%-saturated ammonium sulfate to remove non-specific binding of ^{3}H-ecdysone. After centrifugated as above, 10 μl of distilled water was added to the supernatant, which was then vortexed. The radioactivity contained in this mixture was then measured by a liquid scintillation counter (Aloca model LSC3500). The standard curve was prepared for 20-hydroxyecdysone, sequentially diluted, 3 times from 1 μg/ml to $1/(3\times3\times3\times3\times3\times3)$ μg/ml. Two control treatments with no 20-hydroxyecdysone and 0.1 μg/ml 20-hydroxyecdysone were included. Ten μl standard solutions were counted twice for 2 min, as were samples.

HPLC analysis of monoamines

The procedure for HPLC analysis was as described in Natsukawa et al. (1996). The pump was replaced by a new model (IRICA-981). The brain-subesophageal ganglion (Br-SG) complex was dissected in PBS (pH 7.5, 0.9% NaCl), within 3 hours after light-on, to prevent noise due to the possible circadian fluctuation and stored at –80°C until chromatographic separation was made. Five Br-SG complexes were homogenized on ice in 200 μl of 0.1 M perchloric acid by glass-glass homogenizer. Five μl of 22 ng HVA/10 μl (internal standard) was added to 50 μl of the homogenate, which was then centrifuged

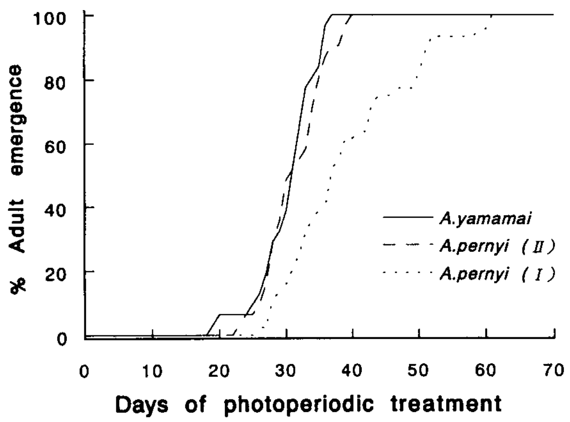

Fig. 1. Post-diapause adult emergence of three stocks of *Antheraea* moths. *A. yamamai* was placed at 30°C under LD 12 : 12, whereas *A. pernyi* at 25°C under LD 16 : 8. These photoperiods stimulate diapause development in respective stock most efficiently. (I) univoltine strain; (II) bivoltine strain.

(7,000 x g) at 4°C for 15 min. Ten μl of the supernatant was directly injected into HPLC-ECD by sample injector (IRICA-80). The protein assay was made using Bio-Rad protein reagent with BSA as standard. We believe the epinephrine-like peak represents epinephrine itself because the peak persisted after the sample was passed through aluminium oxide column; epinephrine was collected in the eluate.

RESULTS

Post-diapause adult emergence was compared among *A. yamamai* at 30°C and two strains of *A. pernyi* at 25°C (Fig 1). *A. yamamai* was exposed to LD 12 : 12, while *A. pernyi* to LD 16 : 8. All individuals completed adult emergence within 40 days both in *A. yamamai* (32.5 ± 6.1 days N = 32) and the bivoltine strain of *A. pernyi* (31.6 ± 4.4 days, N = 31). However, when *A. yamamai* was exposed to LD 15 : 9 which is the strongest diapause-maintaining photoperiod, adults emerged in 87.1 ± 24.9 days, whilst no emergence occurred in the bivoltine strain of *A. pernyi* even after 3 months (Matsumoto & Takeda, 1996).

The univoltine strain of *A. pernyi* both overwinters and aestivates as diapause pupae. Fig. 2 shows the developmental retardation of this strain at 30°C. Development at LD 16 : 8, 25°C was faster than those at LD 16 : 8, 30°C and 20°C. This retardation may reflect the nature of aestival diapause.

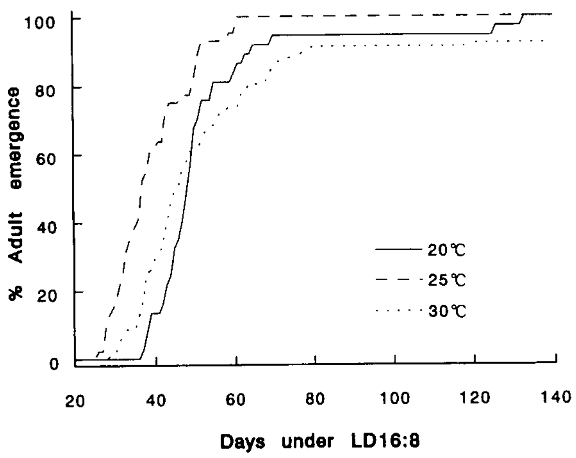

Fig. 2. Post-diapause adult emergence of the univoltine strain of *A. pernyi* under LD 16 : 8 at 20, 25 and 30°C.

Fig. 3 summarizes the relative strength of diapause of these stocks. Diapause tendency was strongest in the univoltine strain of *A. pernyi*, weakest in *A. yamamai*, and the bivoltine strain of *A. pernyi* was intermediate.

Fig. 4 shows the fluctuations of the hemolymph ecdysteroid titer in the bivoltine strain of *A. pernyi*, which was exposed to LD 12 : 12 or LD 16 : 8, at 25°C and that in *A. yamamai* placed at 30°C. In *A. pernyi*, the highest count (1,609 ng/ml hemolymph) was observed in an individual which had been exposed to LD 16 : 8 for 20 days whereas the lowest count was 0.0 ng/ml in an individual which had been exposed to LD 12 : 12 for 15 days. The basal level of hemolymph ecdysteroids (ng/ml hemolymph) was 13.73 ± 9.97 (N = 21), much lower than that in *A. yamamai* kept in a diapause maintaining condition, LD 16 : 8 at 30°C; 77.15 ± 52.22 (N = 17), (Fig. 5). The difference between the two species was highly significant (t = 5.46). The highest count in *A. yamamai* was also higher (2,320 ng/ml in an individual which had been kept at LD 12 : 12 for 20 days) than that in *A. pernyi*.

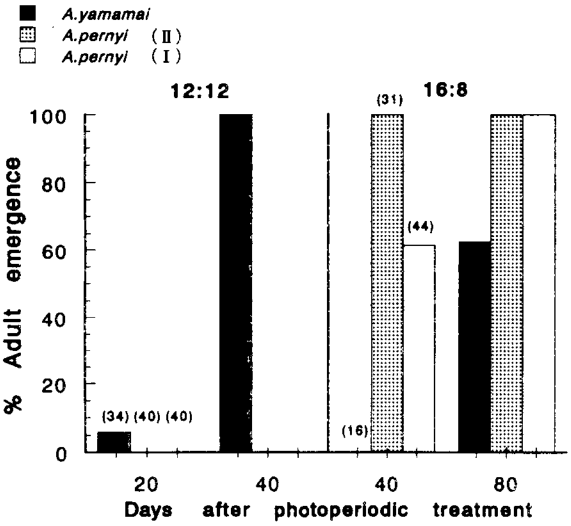

Fig. 3. Post-diapause adult emergence in three stocks of *Antheraea* moths under LD 12 : 12 and LD 16 : 8. *A. yamamai* was placed at 30°C, while *A. pernyi* at 25°C. Brackets – the number of insect used.

Fig. 6 compares the contents of catecholamines in the brain-subesophageal ganglion complex from *A. yamamai* and *A. pernyi*, of both the univoltine and bivoltine strains at times when diapause pupae still retain the larval brain morphology. We considered the possibility that some of these monoamines might be involved in the upstream regulation of the prothoracicotropic hormone release into the hemocoel.

The result shows that the levels of catecholamines are different between both the species and the strains. There is a correlation between the diapause intensity and the content of catecholamines, especially the level of "epinephrine" among these stocks. The highest level was in the univoltine strain of *A. pernyi*, and the lowest level in *A. yamamai*.

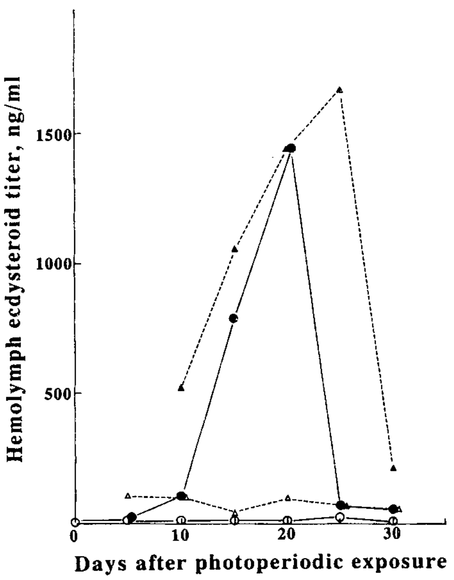

Fig. 4. Hemolymph ecdysteroid titers of *A. yamamai* (dotted line) and the bivoltine strain of *A. pernyi* (solid line) under LD 12 : 12 and LD 16 : 8. Solid triangles – LD 16 : 8 at 30°C; open triangles – LD 12 : 12 at 30°C; solid circles – LD 16 : 8 at 25°C; open circles – LD 12 : 12 at 25°C.

DISCUSSION

Developmental systems that involve photoperiodism and diapause at the pupal stage are extremely complex and causal

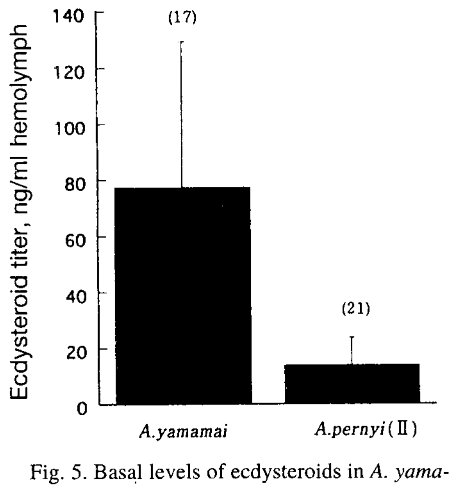

Fig. 5. Basal levels of ecdysteroids in *A. yamamai* and the bivoltine strain of *A. pernyi*. Mean ± SD.

relationships among different elements, nervous or neuroendocrine or metabolic, are still beyond speculation at present (Denlinger, 1985). We have already shown striking differences in the hemolymph free amino acid composition between *A. yamamai* and *A. pernyi* (Ohnishi et al., 1994, vs Mansingh, 1967). As mentioned before, these moths are very closely related and produce partially viable hybrid offspring. The difference in the hemolymph amino acid composition could well be attributed to the different natures of their pupal diapause and related adaptations. Interestingly, Mr. Tokichi Tsuji of Azai Wild Silkmoth Production Group told us that if eggs of *A. yamamai* were forced to hatch in September, the resulting pupae overwintered at this developmental stage! The regulatory mechanism for the diapause in the univoltine strain of *A. pernyi* is also intriguing. Since the univoltine strain of *A. pernyi* completes its larval development before summer, it must have a potency of aestivation as well as overwintering. The present result confirmed this field life cycle, since diapause termination in the univoltine strain was retarded by a high temperature, 30°C. Yet, diapause termination was not accelerated by 20°C as was the case in many aestivating species (e.g. Nakai & Takeda, 1995). Are adaptations for raestivation and overwintering alike and interchangeable with each other, even though photoperiodic response curves and hemolymph free aminoacid profiles are grossly different? The *Antheraea* system (*A. yamamai* and the univoltine and the bivoltine strains of *A. pernyi*) seemed to be ideal to investigate the evolutionary derivation of insect life cycle adaptations involving diapause from both neuroendocrine and metabolic point of view.

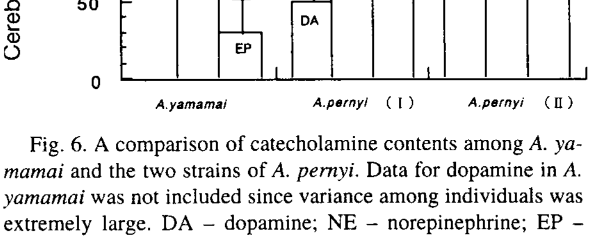

Fig. 6. A comparison of catecholamine contents among *A. yamamai* and the two strains of *A. pernyi*. Data for dopamine in *A. yamamai* was not included since variance among individuals was extremely large. DA – dopamine; NE – norepinephrine; EP – epinephrine. Mean ± SD.

We compared, in this investigation, 1) general tendencies and intensities of diapause, 2) hemolymph ecdysteroid levels and 3) catecholamine levels of brain-subesophageal ganglion complex of three stocks of *Antheraea* moths. Termination mechanisms for aestival diapause in the univoltine strain are, however, not well-understood. Summer diapause of *A. yamamai* is the weakest of the three stocks and even mechanical shaking alone terminates diapause (Kato & Sakate, 1981). The difference in diapause intensity may be a reflection of the basal level of hemolymph ecdysteroids, since the level was much higher in *A. yamamai* than in the bivoltine strain of *A. pernyi* under diapause maintaining conditions.

Catecholamine levels, especially of "epinephrine", in the brain-subesophageal ganglion complex of diapause pupae is highest in the univoltine strain of *A. pernyi* which has the strongest diapause tendency of the three stocks. The bivoltine strain has an intermediate intensity of diapause. Whether these monoamines are directly involved in the regulation of ecdysiotroph requires further study. A correlation still exists both between diapause intensity and ecdysisteroid and between diapause intensity and monoamine levels. If the correlation is real, "epinephrine" could be the ecdysiotropin-inhibitory factor that creates the diapause condition. Diapause is characterized typically by the arrest of morphogenesis. However, summer and winter diapauses are not metabolically the same (Ohnishi et al., 1994). Can the release of ecdysteroids alone control all the physiological states, nondiapause, aestival diapause and hibernal diapause? The upstream regulatory mechanism of the PTTH release must be complex since two ecdysiotropins are known to occur in the saturniid moths. Different monoamine mechanisms may regulate these ecdysiotropins. As L'Helias et al. (1995) has demonstrated that 5HT accelerates diapause termination in *Pieris brassicae*, we are tempted to propose a dual regulatory system of diapause, where 5HT accelerates and catecholamines inhibit PTTH release which terminates diapause.

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REFERENCES

DENLINGER D.L. 1985: Hormonal control of diapause. In Kerkut G.A. & Gilbert L.I. (eds): *Comprehensive Insect Physiology, Biochemistry and Pharmacology. Vol. 8.* Pergamon Press, Oxford, pp. 353–412.

HAYES D.K. 1971: Action spectra for breaking diapause and absorption spectra of insect brain tissue. In Menaker M. (ed.): *Biochronometry.* Symp. Friday Harbor. Natl. Acad. Sci., Washington, pp. 392–402.

KATO Y. & SAKATE S. 1981: Early termination of summer diapause by mechanical shaking in pupae of Antheraea yamamai (Lepidoptera: Saturniidae). *Appl. Entomol. Zool.* **18**: 441–443.

L'HELIAS C., GALLIBERT J., BEAUDRY P. & LAUNAY J.M. 1995: N-acetyl transferase activity during the photoperiodic-dependent Pieris brassicae development. *J. Insect Physiol.* **41**: 827–835.

MANSINGH A. 1967: Changes in the free amino acids of the haemolymph of Antheraea pernyi during induction and termination of diapause. *J. Insect Physiol.* **13**: 1645–1655.

MATSUMOTO M. & TAKEDA M. 1996: Photoperiodic and neuroendocrine regulation of the ecdysiotroph in Antheraea yamamai (Lepidoptera, Saturniidae). *Int. J. Wild Silkmoths Silk* (in press).

NAKAI T. & TAKEDA 1995: Temperature and photoperiodic regulation of summer diapause and reproduction in Pyrrhalta humeralis (Coleoptera: Chrysomelidae). *Appl. Entomol. Zool.* **30**: 295–301.

NATSUKAWA T., TANIGAWA T. & TAKEDA M. 1996: Circadian fluctuation of catecholamines in the cockroach brain-subesophageal ganglion complex detected by HPLC-ECD. *Biol. Rhythm Res.* (in press).

OHNISHI H., MATSUMOTO M. & TAKEDA M. 1994: Hemolymph amino acid pool of the giant wild silkmoth, Antheraea yamamai (Lepidoptera: Saturniidae), during estival diapause and subsequent morphogenesis. *Int. J. Wild Silkmoths Silk* **1**: 36–49.

RASENIK M.M. & BERRY S.J. 1981: Regulation of brain adenylate cyclase and termination of silkmoth diapause. *Insect Biochem.* **11**: 387–395.

Truman J.W. 1971: The role of the brain in the ecdysis rhythm of silkmoths: Comparison with the photoperiodic termination of diapause. In Menaker M. (ed.): *Biochronometry*. Symp. Friday Harbor. Natl. Acad. Sci., Washington, pp. 483–501.

Williams C.M. 1969: Photoperiodism and the endocrine aspect of insect diapause. *Symp. Soc. Exp. Biol.* **23**: 285–300.

Williams C.M. & Adkisson P.L. 1964: Physiology of insect diapause. XIV. An endocrine mechanism for the photoperiodic control of pupal diapause in the oak silkmoth Antheraea pernyi. *Biol. Bull.* **127**: 511–525.

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