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Endogenous 20-hydroxyecdysone levels in the haemolymph of non-diapause-destined and diapause-destined generations of tasar silkworm, *Antheraea mylitta* (Lepidoptera: Saturniidae) and associated developmental changes

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Abstract. A complete profile of the 20-hydroxyecdysone (20-HE) titer, development and endocrine events from 1st instar to pupation of the larvae of non-diapause-destined (NDD) and diapause-destined (DD) tasar silkworm, *Antheraea mylitta* Drury (Lepidoptera: Saturniidae) was studied. Diapause is induced by short days of 11 hr photophase coupled with ≤ 24°C prevailing in September-November. Diapausing pupae produce adults in July (≥ 12h light, ≥ 26°C) and one generation is completed by August. The growth rate during the course of development of larval instars decreases and instar durations are inversely related to the body weight at the time of initiation of a larval instar. A growth compensation mechanism operates during the development of the larval instars. The growth rate was higher in early instars (1st to 4th) in both generations. The DD larvae complete the final instar in 16 days followed by a spinning stage of 13 days. The NDD larvae complete the final larval instar in 9 days followed by spinning stage of 6 days and spend 14 days in the pupal stage. The signal to release the prothoracicotropic hormone (PTTH) is related to critical body weight of larvae. From 1st to 4th instar, pre-ecdysial peaks of 20-HE were recorded in both NDD and DD generations. The programme for undergoing diapause was initiated during 3rd instar and induced by a sudden decrease in the level of 20-HE in the DD generation. Two peaks of 20-HE are required for the larval-pupal transformation, first at the wandering stage and the second at cuticle formation.

INTRODUCTION

Diapause is an arrest in development accompanied by a major shutdown of metabolic activities, which is genetically programmed and occurs at a specific stage in each species (Denlinger et al., 2004). The ability to pass through adverse periods in diapause helps insects to exploit seasonally fluctuating resources (Kostal, 2006). The induction and termination phases of diapause are influenced by environmental factors such as photoperiod, temperature, moisture and diet, of which photoperiod is the major factor (Trimble, 1994; Denlinger, 2000, 2002; Denlinger et al., 2004; Saunders et al., 2004).

All the life stages of non-diapause-destined (NDD) *Diatraea grandiosella* are larger or heavier than diapause-destined (DD) individuals (Kikukawa et al., 1984). In the NDD larvae of *Antheraea mylitta*, the growth rates during the first three instars and the first half of each instar are higher than later in development (Pradeep et al., 1995).

For pupal diapause, it is presumed that the external stimuli are stored in the brain-retro-cerebral complex during larval-pupal development and diapause behaviour expressed during the pupal stage is due to the cessation of prothoracicotropic hormone (PTTH) release (Bowen et al., 1985), which stops the activation and release of ecdysone from prothoracic gland into the haemolymph that induces moulting. This is confirmed by the ecdysone titer assays of several insect species viz., *Galleria melonella* (Cymborowski et al., 1991); *Manduca sexta* (Bowen et al., 1985); *Sarcophaga argyrostoma* (Richards et al., 1987) and *Bombyx mori* (Sakurai et al., 1998). There are differences in titer patterns in diapause-bound and non-diapause-bound larvae as well as diapausing and non-diapausing pupae of *Heliothis virescens* (Loeb, 1982).

Indian tropical tasar silkworm, *Antheraea mylitta* Drury (Lepidoptera: Saturniidae) is a sericigenous insect that produces silk (tasar silk) of high commercial importance. It is wild and polyphagous and feeds mainly on forest trees viz., Arjun, *Terminalia arjuna* Bed., Asan, *T. tomentosa* W. & A. and Sal, *Shorea robusta* Gaertn. Efforts to cultivate this species by feeding it on detached host leaves under controlled laboratory conditions have been unsuccessful due to poor survival (20–24%). Daba ecorace is one of the most commercially exploited races of the 44 ecoraces of *A. mylitta*. It is distributed from latitude 16°N to 24°N and longitude 80°E to 89°E (Suryanarayana & Srivastava, 2005) and undergoes a pupal diapause from November to May. Voltinism in *A. mylitta* is dependent on abiotic factors and habitat. Erratic emergence due to weak by regulated voltinism and unseasonal emergence in late May or early June adversely affect eggs production. There are few studies, however, on the hor-

monal control of diapause and determination of voltinism in *A. mylitta*. Recently the timing of the release of PTTH from the brain and ecdysone from the prothoracic glands during 4th and 5th larval instar of non-diapause-destined generations was studied (Pradeep et al., 1997, 1998). The growth patterns of non-diapause-destined and diapause-destined larvae differ in *A. mylitta* (Dinesh Kumar et al., 2000). There are significant differences in the timing of the increase in larval weight and endocrine events in fourth-instar non-diapause-destined and diapause-destined larvae of *A. mylitta* (Dinesh Kumar et al., 2007). Knowledge of the factors that determine the voltinism of this wild species will contribute to a higher yield of silk. Therefore, a proper understanding of the role of brain and related endocrine events, which control larval growth, development, metamorphosis, moulting and the induction and maintenance as well as termination of pupal diapause, is essential. The present study elucidates the role of critical body weights, PTTH release and 20-hydroxy-ecdysone titers and attempts to understand, in a holistic manner, the physiology of the diapause behaviour of the Daba ecorace of *A. mylitta* by comparing data on the above parameters obtained from non-diapausing and diapause bound generations.

MATERIAL AND METHODS

Host trees

The farm and laboratory of the Central Tasar Research and Training Institute, Ranchi, are located at 23°.21′N, 85°.20′E and an altitude of 652 m a.s.l., in the state of Jharkhand, India. Foliage from 25 year old plantation of the primary host tree Asan, *Terminalia tomentosa*, maintained under standard agronomic practices viz., pruning, manure and fertilizer application was used as the food source. The plantation produces annually flushes of fresh foliage from June onwards. The bivoltine larvae of the Daba ecorace of *Antheraea mylitta* Drury (Lepidoptera: Saturniidae) were used in this study.

Experimental insects

Thousands of cocoons (pupae) that entered diapause in the previous season (December – May) were kept in a hanging position in wire-mesh cages (6 cu ft) near the field laboratory. The freshly eclosed moths were paired inside cages and decoupled after mating (8 h). A single moth was kept for egg laying in nylon net bags (6 ’ × 7’). Eggs were collected daily and kept separately. The larvae were reared in situ on a host tree. The experimental larvae were weighed in field laboratory located adjacent to the plantation. Each batch of larvae, which all hatched on the same day were reared on the same host tree with several such batches on different trees. The total number reared was in excess of that required for the experiments viz., larval growth, ligation and RIA. This procedure ensured uniform growth and synchronized moulting of the larvae. Non-diapause-destined (NDD) stock was reared during July to August, under a natural decreasing 13 h 43 m to 13 h 12 m day length regime at 26 ± 2°C and 48 to 85% relative humidity. Diapause-destined (DD) generation larvae were reared in September to October under a natural photoperiod of 12 h 18 m to 11 h 19 m, 22 ± 1°C mean temperature and 54 to 91% relative humidity (Singh et al., 2005). Synchronously hatched and moulted larvae of each instar of NDD and DD generations were used in the experiments.

Chemicals

20-hydroxyecdysone (20-HE) and ecdysone were purchased from Sigma Chemical Co. USA. Tritium labelled ecdysone (NET 621, ecdysone α-[23, 24-^{3}H (N)], specific activity 50–110 Ci/mmol) was obtained from M/s. Perkin Elmer Life science, 549 Albany Street, Boston, MA 02118 USA. The ecdysteroid antiserum “2A” used in this study was produced by Prof. W.E. Bollenbacher, University of North Carolina, Chapel Hill and distributed by Prof. E.S. Chang, Bodega Marine Laboratory, Bodega Bay, California. All other chemicals are of analytical grade.

Larval growth

Age of a newly hatched larva was classified as day 0 in 1st instar and in subsequent instars, the day of ecdysis was day 0. Thirty larvae from each treatment were weighed daily (n = 30). The growth of larvae was expressed in terms of daily mean weight and daily weight gain calculated using the formula of Waldbauer (1968). The same larvae were weighed daily from neonate stage (0 day) to when they started spinning a cocoon for pupation.

Endocrine events

In the 1st to 3rd larval instars, ecdysis was used as an indicator of endocrine events. For recording the endocrine events in 4th and 5th instar larvae of the non-diapausing generation, larvae that were at day 0 to day 6 in the 4th instar and day 0 to day 10 in the 5th instar of NDD and day 0 to day 6 in the 4th instar and day 0 to day 16 in the 5th instar in the DD generation (n = 150 for each age group), were weighed individually before ligation. To determine the timing of release of the prothoracicotropic hormone (PTTH) in the fourth and fifth-instar, the larvae of each group were neck-ligated on a particular day. The ligated larvae were kept in the laboratory at 24 ± 2°C and a RH of 75–80%. Simultaneously, 4th and 5th instar ligated and non-ligated (control) larvae were examined daily in the morning and evening for moulting / gut purging symptoms and only mortality recorded. In the 4th instar, PTTH release was indicated by moulting whereas in the 5th instar the first PTTH release was indicated by gut purging and the second by pupation. Head critical period (HCP) denotes the PTTH release time in terms of the day after which ligation did not affect the larval-larval moult.

Radioimmunoassay (RIA)

Haemolymph samples were collected from NDD and DD larvae at 24 h intervals, at dawn from 1st to 3rd instar and spinning 5th instar larvae, and from the 4th & 5th instar larvae samples were collected at both dusk and dawn. In the first instar, haemolymph from 5–6 larvae was pooled to obtain a sample of 10–20 µl. From the second instar onwards, haemolymph from one larva was sufficient for a sample of 10–20 µl. Haemolymph was collected on chilled parafilm by puncturing the tip of a proleg of larvae and then using a calibrated capillary tube a 10/20 µl volume was transferred into pre-chilled Eppendorf tubes containing 1 ml methanol. The samples were stored at –70°C till used for RIA. Samples were centrifuged at 5,000 rpm at 4°C for 10 min and 500 µl supernatant were transferred in to pre-chilled Eppendorf tubes and used for RIA after evaporating methanol in a stream of nitrogen. All RIA steps were completed in the same tube, following the protocols of Borst & O’Connor (1972). A standard curve was developed under identical incubation condition for 20-HE in the range of 50–20,000 pg and haemolymph hormone titers were determined by a log-logit transformation and regression equation (Josephrajkumar & Subrahmanyam, 1999). At least six samples of haemolymph

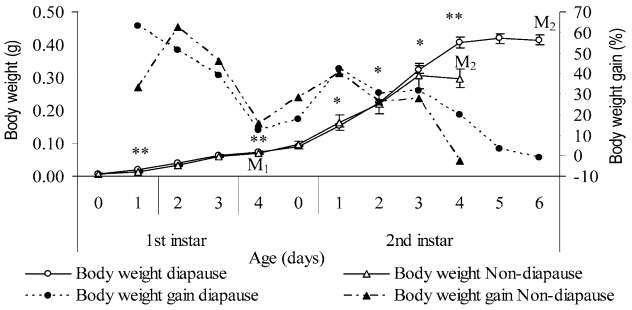

Fig. 1. Growth curve and percentage weight gain/loss of diapause-destined and non-diapause-destined 1st and 2nd instar larvae, M_1–M_2: larval moult, vertical bars denote SE (n = 30). Body weight difference in NDD and DD were significant at $p < 0.01$ (**) and at $p < 0.05$ (*).

from six synchronously developing larvae of identical age were used for each determination. The results were statistically analyzed using Student's *t* test.

RESULTS

Growth of non-diapause and diapause-destined larvae

Daily changes in mean body weight of *A. mylitta* larvae from 1st instar to pupation of both NDD and DD generations are illustrated in Figs 1–3. The body weight gains were higher in early instars in both the generations (10–65% in 1st to 3rd instar). In the 5th instar of NDD generation, the weight gain starts declining from 7th day (–5 to –25%). However, in DD generation, decline in growth rate of 5th instar larvae showed a fluctuating trend with peaks on days 2, 5, 10 and 12 (+2 to 20%). After 6th–7th day, weight gains remained more or less similar (with at least two minor peaks) untill 15th–16th day. The larvae stopped feeding on 10th day in NDD and on 16th day in DD generations. The interval between cessation of feeding and the start of spinning was 24 h in the NDD and 36 h in the DD generation, respectively. During this period, gut purging occurred in both generations. After gut purging, the larvae searched for a suitable place to start spinning. This may be equivalent to the wandering stage in Diptera. In the NDD generation the negative rate of growth remained constant untill pupation (≤ 15%), but

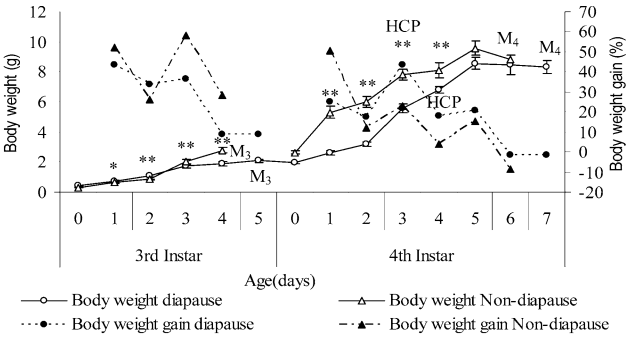

Fig. 2. Growth curve and percentage weight gain/loss of diapause-destined and non-diapause-destined 3rd and 4th instar larvae, M_3–M_4: larval moult, HCP: Head Critical Period, Vertical bars denote SE (n = 30). Body weight difference in NDD and DD were significant at $p < 0.01$ (**) and at $p < 0.05$ (*).

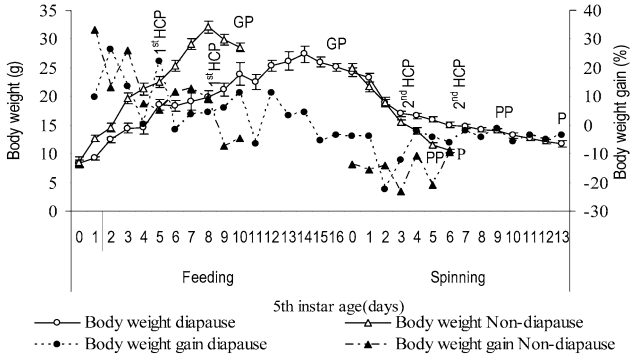

Fig. 3. Growth curve and percentage weight gain/loss of diapause-destined and non-diapause-destined 5th instar larvae up to pupation. HCP: Head Critical Period, PP: Pre Pupa, P: Pupa, Vertical bars denote SE (n = 30). Difference of body weight of NDD and DD larvae were significant at $p < 0.01$ in all age group.

in the DD generation the negative rate of growth increased initially (–10 to –20%) and then remained constant (≤ 5%) from day 5 untill pupation. The head capsule widths of DD and NDD larvae did not differ significantly (data not presented).

Endocrine events in non-diapause and diapause-destined larvae

In the 4th instar NDD larvae, the release of PTTH occurred on day 3 and in DD larvae on day 4, as 13.68 and 27.27%, respectively of the ligated larvae moulted. In the fourth instar, mean body weight ranged from 2.618 to 9.560 g in NDD and from 1.946 to 8.427 g in DD larvae. Highly significant differences were observed between the mean larval weights of NDD and DD larvae of the same age. As the larvae grew older, larval weight and weight difference also increased in both generations. Larvae attaining the weight of 6.798 g and above in the DD and 7.795 g and above in the NDD, moulted to the next instar (Table 1).

The timing of the release of PTTH in the 5th instar NDD larvae was synchronized and occurred maximally on days 8 (58.44%) and 9 (76.67%). A second release was

TABLE 1. Relationship between the larval weight and timing of PTTH release in non-diapause-destined and diapause-destined 4th instar larvae of *A. mylitta*.

Age (days)	Larval weight (g) Mean ± SE		*Z*-test	Larvae moulted after ligation (%)	
	NDD	DD		NDD	DD
0	2.618 ± 0.061	1.946 ± 0.039	–5.8197**	–	–
1	4.289 ± 0.083	2.599 ± 0.033	–23.2959**	–	–
2	6.023 ± 0.105	3.149 ± 0.059	–24.8814**	–	–
3	7.795 ± 0.112	5.562 ± 0.079	–19.3364**	13.68	–
4	8.099 ± 0.089	6.798 ± 0.123	–11.2658**	59.55	27.27
5	9.560 ± 0.087	7.566 ± 0.119	–17.2703**	79.55	55.17
6	8.786 ± 0.115	8.427 ± 0.106	–3.1099**	89.22	73.00
7	–	8.313 ± 0.125	–	–	78.33

n = 150 for each day of observation; ** – significant at $p < 0.01$.

TABLE 2. Relationship between the larval weight and timing of PTTH release in non-diapause-destined and diapause-destined 5th instar larvae of *A. mylitta.*

Age (days)	Larval weight (g) Mean ± SE		*Z*-test	% larvae gut purged / pupated after ligation	
	NDD	DD		NDD	DD
Feeding					
0	8.505 ± 0.151	8.296 ± 0.204	5.8154**	–	–
1	12.708 ± 0.239	9.194 ± 0.177	7.2701**	–	–
2	14.630 ± 0.204	12.470 ± 0.203	16.3157**	–	–
3	19.733 ± 0.212	14.418 ± 0.311	9.0187**	–	–
4	21.312 ± 0.401	14.484 ± 0.305	25.3768**	–	–
5	22.476 ± 0.392	18.606 ± 0.306	24.5822**	12.00	–
6	25.420 ± 0.376	18.323 ± 0.354	24.9460**	11.82	–
7	29.126 ± 0.361	19.050 ± 0.654	11.0813**	37.14	–
8	32.072 ± 0.591	19.954 ± 0.564	10.4173**	58.44	–
9	29.896± 0.508	21.199 ± 0.480	35.7015**	76.67	8.16
10	28.547 ± 0.393	23.923 ± 0.456	58.3260**	89.41	7.69
11	–	22.469 ± 0.474	–	–	26.92
12	–	25.344 ± 0.464	–	–	38.00
13	–	26.177 ± 0.442	–	–	24.82
14	–	27.343 ± 0.445	–	–	35.00
15	–	25.956 ± 0.536	–	–	44.00
16	–	25.094 ± 0.508	–	–	72.38
Post feeding (spinning)					
0	25.063 ± 0.657	24.197 ± 0.727	12.1046**	–	–
1	21.665 ± 0.842	23.290 ± 0.731	–2.9686**	–	–
2	19.007 ± 0.848	19.002 ± 0.552	12.0170**	–	–
3	15.441 ± 0.512	16.955 ± 0.449	16.0285**	46.00	–
4	13.946 ± 0.555	16.630 ± 0.487	10.2687**	77.78	–
5	11.526 ± 0.391	15.932 ± 0.480	12.9591**	98.00	–
6	10.534 ± 0.298	15.033 ± 0.468	8.8785**	98.00	–
7	–	14.754 ± 0.372	–	–	–
8	–	14.150 ± 0.350	–	–	10.00
9	–	13.961 ± 0.361	–	–	33.34
10	–	13.183 ± 0.457	–	–	60.00
11	–	12.721 ± 0.452	–	–	55.54
12	–	12.118 ± 0.438	–	–	73.00
13	–	11.721 ± 0.505	–	–	83.00

n = 150 for each day of observation; ** – significant at p < 0.01.

recorded on the 3rd day of the post feeding stage. Most of the larvae aged 8 days and above and weighing 28.547 g to 32.072 g, purged their guts. PTTH release in the majority of NDD 5th instar larvae ceased on days 8 or 9. In DD larvae, first release was on days 11 to 16 and the second on days 8 to 13 of the post feeding phase. None of the DD larvae ligated up to day 8 purged their guts. Gut purging gradually increased with age. 26.92 to 72.38 per cent of the larvae ligated within the weight range 22.469 g to 25.094 g purged their guts (Table 2).

Titer of 20-hydroxyecdysone in non-diapause and diapause-destined larvae

The level of 20-hydroxyecdysone (20-HE) in µg ml^{-1} of haemolymph of NDD and DD larvae of 1st to 3rd instar are shown in Figs 4–5. The 20-HE level increases with age at

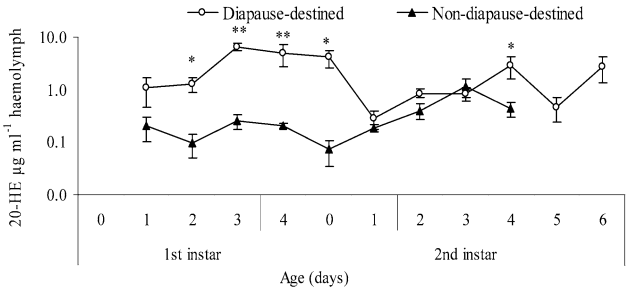

Fig. 4. Haemolymph 20-hydroxyecdysone titer (20-HE) in non-diapause-destined and diapause-destined generations of *A. mylitta* larvae in 1st and 2nd instar, vertical bars denote SE (n = 6). 20-HE difference in NDD and DD were significant at p < 0.01 (**) and at p < 0.05 (*).

the beginning of 1st and 2nd instar in both NDD and DD generations. A single broad peak in 20-HE was observed in the first instar, one day prior to moulting in both the DD (6.65 ± 1.06 µg ml^{-1}) and NDD (0.26 ± 0.08 µg ml^{-1}) larvae. In 2nd instar, two peaks of ecdysone were recorded in DD larvae on day 4 (2.85 ± 1.26 µg ml^{-1}) and day 6 (2.78 ± 1.43 µg ml^{-1}) and only a single peak in NDD larvae on day 3 (1.15 ± 0.45 µg ml^{-1}). The high standard deviations indicate large variation in the samples derived from the large population growing under natural conditions. Significant differences were recorded during 1st instar for NDD and DD larvae on day 2 to day 4. In the 2nd instar larvae of the NDD and DD generations the only significant differences were on day 0 and day 4 (p < 0.05). In the 3rd instar DD larvae there was an insignificant downward trend in 20-HE concentration up to day 2 and then it increased. This trend was not noticed in the NDD 3rd instar larvae where the level of 20-HE increased continuously and attained a peak (3.48 ± 1.21 µg ml^{-1}) on day 4 significantly higher than in the DD generation (0.48 ± 0.15 µg ml^{-1}). Significant differences between the two generation were observed only on day 1 (p < 0.01) and day 4 (p < 0.05). The 20-HE levels in the third instar NDD larvae were 7.16 and 32.76 times higher than the levels in the corresponding DD larvae.

Significant variation was observed in the 20-HE profiles of 4th and 5th instar larvae in both the NDD and DD

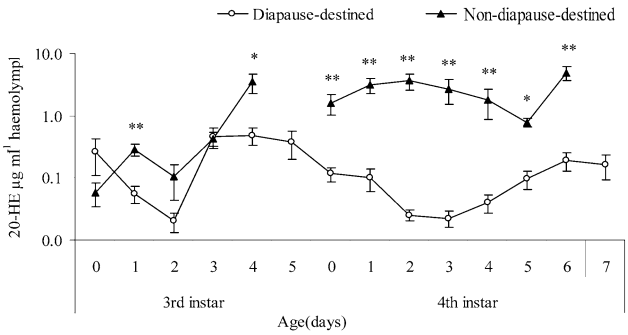

Fig. 5. Haemolymph 20-hydroxyecdysone titer (20-HE) in non-diapause-destined and diapause-destined generations of *A. mylitta* 3rd and 4th instar larvae. Vertical bars denote SE (n = 6). 20-HE difference in NDD and DD were significant at p < 0.01 (**) and at p < 0.05 (*).

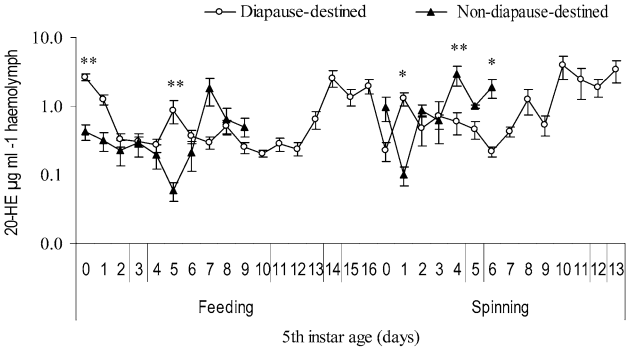

Fig. 6. Haemolymph 20-hydroxyecdysone titer (20-HE) in non-diapause-destined and diapause-destined generations of *A. mylitta* 5th instar larvae at feeding and post feeding (spinning) stages. Vertical bars denote SE (n = 6). 20-HE difference in NDD and DD were significant at $p < 0.01$ (**) and at $p < 0.05$ (*).

generations (Figs 5–6). In 4th instar DD larvae, there was a very low concentration of 20-HE on day 3 (0.02 ± 0.01 µg ml^{-1}), thereafter the level increased up to 0.19 ± 0.06 µg ml^{-1} on day 6. In NDD larvae, the 20-HE level was very high (≈2.50 µg ml^{-1}) throughout the 4th instar except on day 5 (0.79 ± 0.13 µg ml^{-1}), which resulted in two peaks, the first on day 2 (prior to HCP) and the second of 4.87 ± 1.23 µg ml^{-1}at the moult (day 6). The differences in the level of 20-HE were highly significantly different ($P < 0.01$) in the NDD and DD larvae on day 0 to day 4 and day 6 ($p < 0.01$) and significant on day 5 ($P < 0.05$) (Fig. 5). 20-HE level in fourth instar larvae was higher at dusk than at dawn in NDD larvae. No significant difference was observed in the levels at dusk and dawn in DD larvae except on day 2 ($p < 0.05$) (Fig. 7).

In 5th instar NDD larvae, the 20-HE level decreased considerably initially and was 0.42 ± 0.11 µg ml^{-1} on Day 0 (Fig. 6). This downward trend continued untill day 5 (0.06 ± 0.02 µg ml^{-1}) and thereafter increased up to day 7 (1.78 ± 0.76 µg ml^{-1}). This increase in 20-HE level is well supported by the results of the ligation experiments,

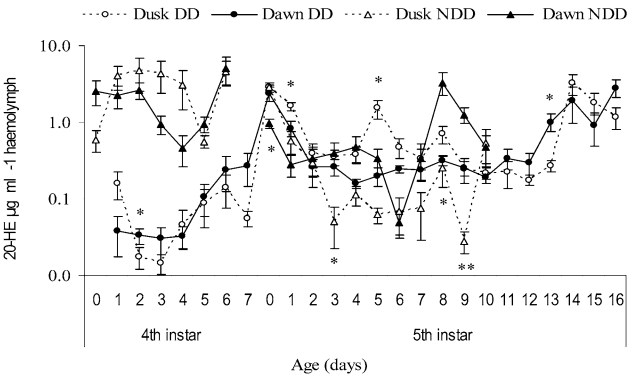

Fig. 7. Haemolymph 20-hydroxyecdysone titer (20-HE) in non-diapause-destined and diapause-destined generations of *A. mylitta* of 4th and 5th instar feeding larvae at dusk and dawn. Vertical bars denote SE (n = 6). 20-HE difference in NDD (below) and DD (above) were significant at $p < 0.01$ (**) and at $p < 0.05$ (*).

where the first head critical period was noticed on day 7, which resulted in the activation of prothoracic gland, followed by gut purging. The decrease in the level of 20-HE after day 7 untill the wandering stage of larvae (0.05 ± 0.15 µg ml^{-1}) suggests that a single peak of ecdysone on day 7 (1.78 ± 0.76 µg ml^{-1}) triggers gut purging. In contrast to the single peak in NDD larvae, in DD larvae there were four 20-HE peaks in the fifth instar. The first, small peak occurred on day 5 (0.86 ± 0.32 µg ml^{-1}) and bigger peak on day 14 (2.56 ± 0.68 µg ml^{-1}) at the first head critical period, followed by another small peak on day 16 during the wandering stage (1.98 ± 0.48 µg ml^{-1}) and another still smaller peak on day 8 (0.51 ± 0.11 µg ml^{-1}). Significant differences between NDD and DD larvae were observed only on day 0 and day 5 (Fig. 6).

In the 5th instar, the level of 20-HE was higher at dawn in NDD larvae, with two peaks, a small peak on day 4 and higher peak on day 8. The 20-HE level was higher at dusk in DD larvae with three peaks at dusk and two at dawn. These differences in hormone levels at dawn and dusk may be a characteristic of NDD and DD generation larvae (Fig. 7).

In the 5th instar NDD spinning (post feeding) larvae, the ecdysteroid level dropped just before the initiation of the second head critical period on day 1 (0.96 ± 0.37 µg ml^{-1}) followed by two peaks, the first on day 4 at the pharate pupal stage (2.92 ± 0.95 µg ml^{-1}) and the second on day 6 at the formation of the pupa (1.86 ± 0.58 µg ml^{-1}). In DD post feeding stage, the second head critical period occurred from day 7 onwards, which was associated with a smaller peak of the hormone on day 8 (1.23 ± 0.49 µg ml^{-1}). The level decreased on day 9 (0.53 ± 0.17 µg ml^{-1}) followed by a strong upsurge on day 10 (3.90 ± 1.43 µg ml^{-1}), which coincided with the pharate pupal stage and again on day 13 (3.38 ± 1.19 µg ml^{-1}) at the time of pupa formation. The levels of 20-HE in DD larvae on day 4 ($p < 0.01$), day 1 and day 6 ($p < 0.05$) were significantly higher than in NDD larvae during post feeding (Fig. 6).

DISCUSSION

Larval growth

In the bivoltine ecorace of tasar silkworm *A. mylitta*, the fresh larval body weight gain was higher in NDD than DD individuals. In each instar, the weight gain was higher during the first half in both NDD and DD larvae, except in fifth instar DD larvae. Earlier, it was reported that NDD larvae of *A. mylitta* attain a higher fresh body weight than DD larvae (Pradeep et al., 1995). Decrease in growth rate may be due to either a reduction in feeding rate or decrease in body weight (Carne, 1966; Delvi & Pandian, 1971, Sehnal, 1985). A positive relation between body weight and timing of endocrine events is evident in *A. mylitta* because in each instar the larvae first increase in weight and then show a decline in weight. The weight gained in each instar enables larvae to program themselves for the next moult as recorded by Nijhout (1975) and Bowen et al. (1985) for *Manduca sexta*. Weight gain, in terms of both wet and dry weight, by DD generation larvae shows several peaks which may be

attributed to endocrine release being dependent not only on attaining a critical weight but also on periodical declines in growth rate, which do not occur in NDD larvae (Denlinger, 1972; Nijhout & Williams, 1974). In the present study, the NDD larvae attained higher wet weights than DD larvae.

Endocrine events

Neck ligation confirmed that the brain of penultimate instar larvae becomes competent to release PTTH and evoke a larval moult once they attain a weight of 7.6 g or more on day 3 in NDD and day 4 in DD larvae. Attainment of a critical weight for moult initiation in larvae and PTTH release is also reported for several species of Lepidoptera (Safranek & Williams, 1984; Sehnal, 1985), Similar observations on the timing of ecdysis of larvae are reported for *Samia cynthia ricini* kept under various light-dark conditions (Fujishita & Ishizaki, 1981). Larval moulting is induced by a single peak of ecdysone (Sakurai, 1983, 1984; Okuda et al., 1985, Gilbert et al., 1996). This is confirmed by the present study, because prior to moulting, the ecdysone titer peaked in penultimate instars of both NDD and DD larvae.

Gut purging, which precedes a moult, occurred in a staggered pattern in DD larvae which indicates that PTTH release occurred after cessation of active growth. Symptoms of the onset of moulting, such as cessation of feeding, gut purging, wandering behaviour etc., in the final instar are also reported for other lepidopteran larvae (Agui & Hiruma, 1982; Fujishita et al., 1982). With increase in age and weight, the timing of PTTH release varied although the time span was shorter in NDD than DD larvae. None of the larvae ligated during the feeding period pupated but did show signs of moulting in the form of a gut purge, as reported for other lepidopteran larvae (Dean et al., 1980). Further, DD larvae receive environmental cues well in advance of the onset of diapause and delay the release of PTTH (Bowen et al., 1985). Similar factors may be responsible for the staggered pattern of PTTH release observed in 5th instar DD larvae of *A. mylitta*. Temperature is known to influence the photoperiodic control of diapause (Masaki, 1980). Different effects of short photoperiod and temperature on DD compared to NDD larvae may be one of the reasons for the observed staggered pattern in PTTH release and prolonged larval duration. Fast growing larvae release PTTH more promptly than slow growing larvae. Therefore, the total time spent in the instar plays no direct role in the process that renders a larva competent to release PTTH (Nijhout & Williams, 1974). This also occurred in *A. mylitta* 5th instar larvae in the present investigation.

20-hydroxyecdysone titer

The level of ecdysone secreted by the prothoracic glands plays a crucial role in ecdysis and metamorphosis (Bollenbacher et al., 1975; Hiruma et al., 1979). The larval moults including apolysis, epidermal proliferation and cuticle secretions are induced by a single peak of ecdysteroid (Hsiao & Hsiao, 1977; Sehnal et al., 1981; Richards et al., 1987). The levels of 20-HE in the 1st and 2nd instar were much higher in DD than NDD larvae. But, the trend was reversed in the 3rd instar of NDD larvae. Such differences suggest that the shift to programming for diapause occurs in the 3rd instar of *A. mylitta.* Reduction in 20-HE titers indicates diapause determination in the 3rd instar, which has not been previously reported and hence needs further study.

During the penultimate and last larval instars in other Lepidoptera, the 20-HE titer in the haemolymph or in whole body extracts is reported to vary (Bollenbacher et al., 1975; Calvez et al., 1976; Agui & Hiruma, 1982; Okuda et al., 1985). Before the onset of wandering in the ultimate instar, a large peak in the 20-HE titer is reported (Fujishita & Ishizaki, 1982; Richards et al., 1987) and PTTH has to be released for pupation to occur on schedule (Truman & Riddiford, 1974; Gilbert et al., 1981). Each PTTH release is followed by a corresponding increase in 20-HE titer (Bollenbacher et al., 1975; Sakurai, 1984) and second release of PTTH is required for the larval-pupal transformation (Safranek & Williams, 1980; Sakurai, 1984). Similar results were also obtained in the present study as the two large peaks of 20-HE were recorded in 5th instar larvae on day 8 of the feeding period and day 4 of post-feeding period in NDD and on day 14 of feeding period of DD larvae in *A. mylitta* which confirms the findings of the above authors for other insects. There appears to be little difference in the 20-HE titer at the time of the larval-pupal transformation or formation of the pupal cuticle in larvae of non-diapausing and diapausing generations of *A. mylitta.*

It is also confirmed that the requirement for ecdysone in the haemolymph is much greater in the NDD than the DD generation due to their shorter instar duration and faster growth. The higher level of ecdysone at dawn in NDD and at dusk in DD generation larvae indicates the requirement of specific circadian events in the two different types of generations in *A. mylitta.*

Dominic & Truman (1984) have shown that the onset of wandering in *Manduca sexta* is a circadian controlled event to which the larvae are committed by the release of PTTH and the accompanying elevation in ecdysteroid level. Further, the onset of wandering is also a gated event (Bollenbacher et al., 1981; Gilbert et al., 1981; Richards et al., 1986). The shorter wandering period coupled with early pupation of non-diapausing generations of insects is attributed to a higher average 20-HE titer (Richards et al., 1987). In our study, a relatively high 20-HE level was observed in NDD generation larvae on the last day of the 5th instar (day 9) and at gut purge compared to the same stages of DD generation larvae. The synchronized and distributed pattern of PTTH release in NDD and DD 4th and 5th instar larvae during their feeding period indicates that these events take place in a gated manner under the influence of environmental conditions.

From the above study, it can be inferred that in *A. mylitta*, there is a decrease in growth rate during the course of development of each larval instar, and instar durations are inversely related to the weight at the time of initiation of a larval instar. A growth compensation mechanism operates

during the course of development of the larval instars. The release of PTTH is related to the larvae attaining a critical body weight. In the first four instars larval ecdysis is characterized by an increase in 20-HE titer preceding the ecdysis, both in NDD and DD generations. Decrease in the level of the 20-HE ecdysone titer in the 3rd instar of the DD generation is evident. Two peaks in the 20-HE titer are required for the larval-pupal transformation, the first at the time of wandering and second at the time of formation of the new cuticle.

Results of this study can be summarized as: (a) highly significant differences were observed between the mean larval weights of NDD and DD larvae of the same age; (b) the final instar was completed in 10 days by the non-diapausing generation while the diapausing generation took 16 days; (c) PTTH release in 5th instar NDD larvae occurred synchronously on days 8 and 9 when the critical body weight was 28.547 to 32.072 g, whereas in DD larvae, the first release occurred on days 11 to 16 and the second release on days 8 to 13. The critical body weights for gut purging were 22.476 g (NDD) and 21.199 g (DD); (d) the 20-HE levels in 4th instar DD generation larvae were one order of magnitude lower (< 100 μg ml^{-1}) compared to those in NDD generation larvae (> 1000 μg ml^{-1}) during the first five days suggesting that physiological events leading to diapause start early in the 4th instar.

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