

increase in the beta<sub>2</sub>-receptors. Apart from this, the significant somatic changes seen in rats treated simultaneously with L-thyroxine and atenolol, specially the heart weight and T<sub>3</sub> levels, may have played some vital role in the decrease of the affinity (pD<sub>2</sub> value) of adrenoceptors to noradrenaline.

It is concluded that the beneficial effects of atenolol in hypertension and hyperthyroidism may be related to the reduction in the number of beta<sub>1</sub>-adrenoceptors in heart.

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## Octopamine titer in the circulating fluid of tropical tasar silkworm, *Antheraea mylitta* Drury (Lepidoptera: Saturniidae) and its response to injected estrogen during critical phase of diapause termination

Anathbandhu Chaudhuri\*, N. Krishnan\*<sup>†</sup>, Pradip Kumar Sarkar<sup>§</sup>, Ashok Kumar Sinha\*\*<sup>†</sup>, S. S. Sinha\*\* and Arun Kumar Ray<sup>§</sup>

\*Central Sericultural Research and Training Institute, Berhampore 742 101, India

<sup>§</sup>Bose Institute, P/12 CIT Scheme, VII-M, Kankurgachi, Calcutta 700 054, India

\*\*Central Tasar Research and Training Institute, Ranchi 835 303, India

The presence and role of the biogenic amine, octopamine has been demonstrated in *Antheraea mylitta* during pupal diapause. For elucidation of estrogen-induced responsiveness of insects, three consecutive injections of estradiol-17- $\beta$  (E<sub>2</sub>) at doses of 1, 5, 10 and 50  $\mu$ g/pupa on days 130, 135, and 140 of pupal age were injected to both male and female pupae of *A. mylitta* during diapause. E<sub>2</sub> treatment caused a significant enhancement in plasma octopamine concentration of haemolymph on day 150 (except in males with 50  $\mu$ g dose) and reduction on day 165 in both the male and female *A. mylitta*. On the contrary, plasma protein concentration was found to be higher only on day 150 when treated with E<sub>2</sub> between 1 and 50  $\mu$ g doses. Octopamine titer in haemolymph plasma always remained higher in male than its female counterpart while in case of plasma protein titer it was found to be just reverse in control animals. E<sub>2</sub>, @1-50  $\mu$ g/pupa caused a significant reduction in pupal duration inducing early moth eclosion. Egg production increased at lower doses and decreased at higher doses of this hormone. E<sub>2</sub> at the dose by 0.5  $\mu$ g/pupa remained ineffective in all the cases except in elevating the female plasma protein titer and egg production. Hence, diapausing pupae of tropical tasar silkworm, *A. mylitta* is physiologically responsive to vertebrate estrogen, E<sub>2</sub>.

PUPAL diapause is a common phenomenon in wild tropical tasar silkworm *Antheraea mylitta* Drury, which continues up to 200-210 days depending on the ambient environmental conditions<sup>1</sup>. In Lepidoptera, pupal diapause occurs because the pupal brain stops secreting the peptidic prothoracicotropic hormone (PTTH) in response to diapause programming signals (mainly short day photoperiod) received in the larval stage. According to Denlinger<sup>2</sup>, since PTTH is necessary for maintaining

<sup>†</sup>For correspondence.



the activity of prothoracic glands, these also stop producing ecdysone, thereby interrupting the development as diapause. Studies by Puiroux *et al.*<sup>3</sup> have indicated that certain brain mechanisms, particularly biogenic amines, could be directly involved in the perception of variations in photoperiod and could mediate some physiological effects particular to dormancy.

Octopamine is one of the most abundant biogenic amines found in insect nervous system which functions as a neurotransmitter, neuromodulator and neurohormone<sup>4-7</sup>. However, no information is available on the controlling influence of octopamine on pupal diapause in *A. mylitta* nor even its presence in the circulating fluid of this insect having economic importance. An aminergic-peptidergic relationship may exist behind the events of induction and termination of pupal diapause of *A. mylitta*, which in turn is controlled by the environment. Hence, the present study was undertaken to examine the concentration of circulating octopamine together with the effect of vertebrate 17- $\beta$ -estradiol ( $E_2$ ) during critical phase of diapause termination in *A. mylitta* and their possible role in diapause physiology. It should be mentioned here that metabolic effect of estrogen has been reported in silkworms by Ogiso and Ohnishi<sup>8</sup>, as well as Das and Ray<sup>9</sup>.

Healthy diapausing pupae of tropical tasar silkworm, *Antheraea mylitta* Drury, of each sex were selected at random from the bivoltine brood at the time of pupation (0 day pupae) and subjected to hormonal treatment in the same physiological condition for each dose. Body weight of the pupae ranged from 9 to 10 g in male and 15 to 16 g in female during the time of experiment.

Three consecutive injections of  $E_2$  (Sigma, USA) were given to the male and female pupae of diapause-destined *A. mylitta* on days 130, 135 and 140 respectively. Injected hormonal doses were 0.5, 1, 5, 10 and 50  $\mu$ g per pupa. The control animals received an equal volume of the vehicle (absolute alcohol: 0.65% saline mixture, 1:1). The insects were sacrificed on days 150 and 165 (critical phase) of pupal age for biochemical assay.

The technique used for assay of octopamine is a modification of a simple, rapid and sensitive procedure for measuring dopamine  $\beta$ -hydroxylase activity in human blood first described by Nagatsu and Udenfriend<sup>10</sup>. The assay is based on the enzymatic conversion of tyramine to octopamine[1-(*p*-hydroxyphenyl)2-aminomethanol] which is then oxidized to *p*-hydroxybenzaldehyde and determined photometrically (Abs at 330 nm). Thus, the oxidized product of octopamine was assayed. Sufficient precautions were taken to ensure that interfering components/compounds during the assay procedure were reduced to a minimum. Pure octopamine @ 2  $\mu$ mol/ml at the concentration of 5–15 nanomoles (Sigma, USA) was used for preparation of standard calibration curve. Spectral overlay studies also ensured that absorption maxima of interfering com-

pounds was different from that of *p*-hydroxybenzaldehyde at 330 nm.

Wing margins of the pupae were punctured to collect the haemolymph in a phenyl-thiourea coated microfuge (eppendorf) tubes. The fluid was then centrifuged at 3000 rpm (700 g) to precipitate the haemocytes. One hundred microliter of haemolymph plasma was taken for assay of octopamine concentration. 0.4 ml TCA was added to the measured amount of plasma to precipitate the plasma proteins. Supernatant was then transferred to a small column of Dowex-50<sup>(H+)</sup> (200–400 mesh). Before loading the sample, the column was repeatedly (2–3 times) washed with double-distilled water (DDW) followed with 5(N) HCl solution and finally with DDW 2–3 times. The octopamine in the sample is absorbed with the Dowex-50<sup>(H+)</sup>. The column was then washed with DDW repeatedly to remove the TCA from the column. Finally the octopamine was eluted from the column by applying a measured amount (1 ml) of 4(M)  $NH_4OH$ . To the eluted material 0.1 ml (20 g/l) of  $NaIO_4$  was added. After about 10 min, excess periodate was reduced by adding  $Na_2S_2O_5$ . Absorbance was read in a UV-spectrophotometer (Shimadzu, Japan, Model No. 168) at 330 nm.

The plasma protein was assayed by the method of Vera<sup>11</sup> using bovine serum albumin as standard.

Emergence duration (from 0 day pupa to adult eclosion) after injection of different doses of estrogen was recorded sex-wise. Total egg production was noted by counting the mature eggs laid per female for three consecutive days and the unlaidd mature chorionated eggs by dissecting out the abdomen after complete oviposition.

Results are expressed as the mean  $\pm$  standard error of at least three individuals pooled for single replication and each mean value is the average of five replications for octopamine and protein. Experimental results were analysed by the Student's *t* test in all the cases.

This paper first reports the presence of octopamine in the circulating fluid haemolymph of tropical tasar silkworm, *A. mylitta*, besides estrogen-induced alterations in its concentration. The circulating octopamine concentration in haemolymph (nanomoles/ml plasma) was found to be sharply reduced (32.03% in female and 29.28% in male) in control lots on day 165 compared with day 150 of diapausing pupae. Male pupae always showed higher octopamine titer (37.51% to 147.13%) than their female counterparts.

$E_2$  at the doses of 1, 5, 10 and 50  $\mu$ g/pupa caused a significant enhancement in plasma octopamine concentration level over control at 150 days with a single exception in case of male at 50  $\mu$ g dose where a significant reduction was noted. By contrast, this hormone (1–50  $\mu$ g) significantly reduced the level of octopamine on day 165 in diapausing pupae in both the sexes when compared with control animals. Lower dose of estrogen (0.5  $\mu$ g) remained ineffective in altering the plasma



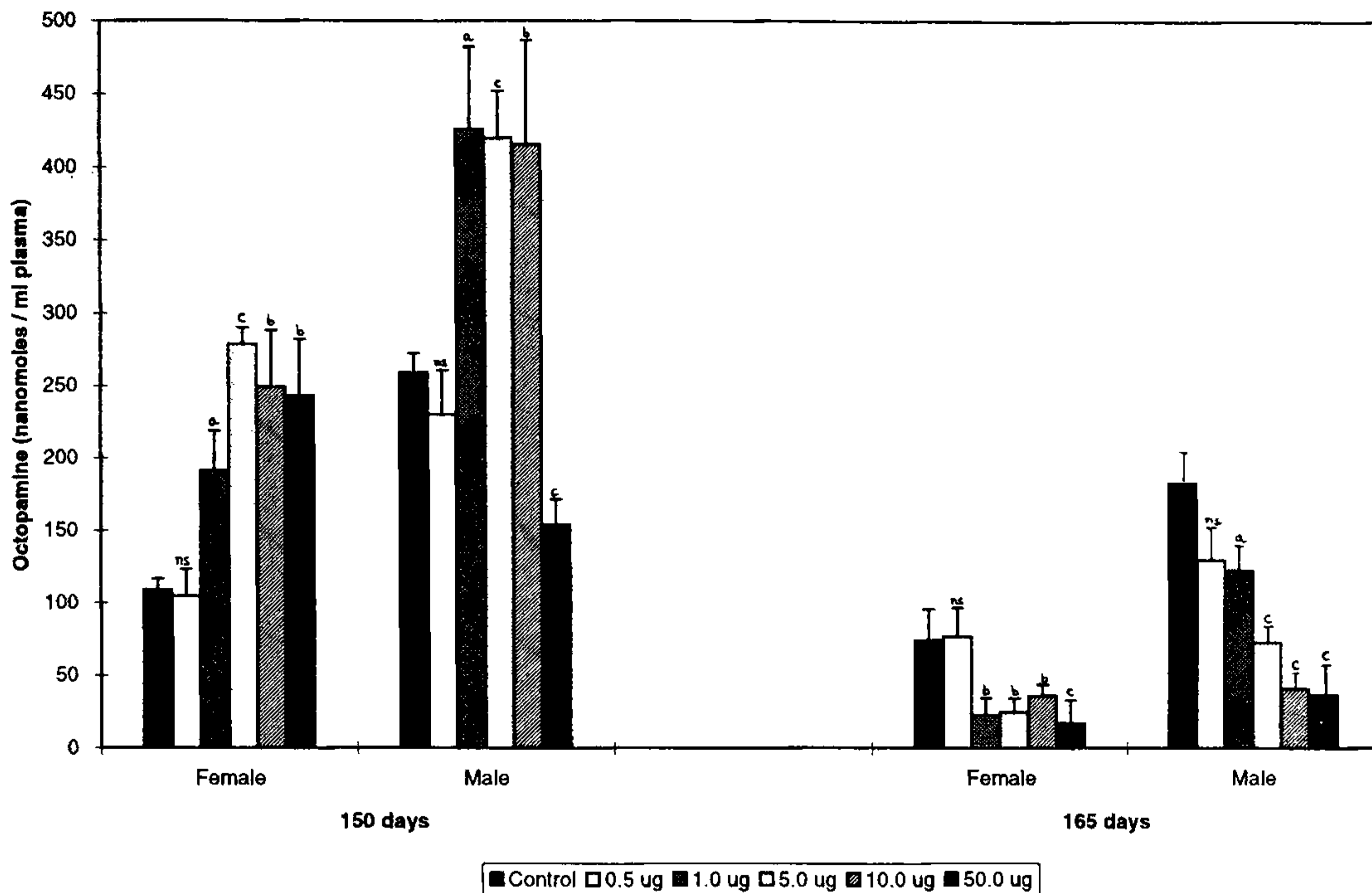


Figure 1. Effect of estradiol-17 $\beta$  on plasma octopamine titre during critical phase of pupal diapause in *A. mylitta*. Three consecutive injections were given to 130, 135 and 140 day pupae. Vertical bars represent the standard error of the mean (ns, not significant; a,  $P < 0.05$ ; b,  $P < 0.01$ ; c,  $P < 0.001$ ).

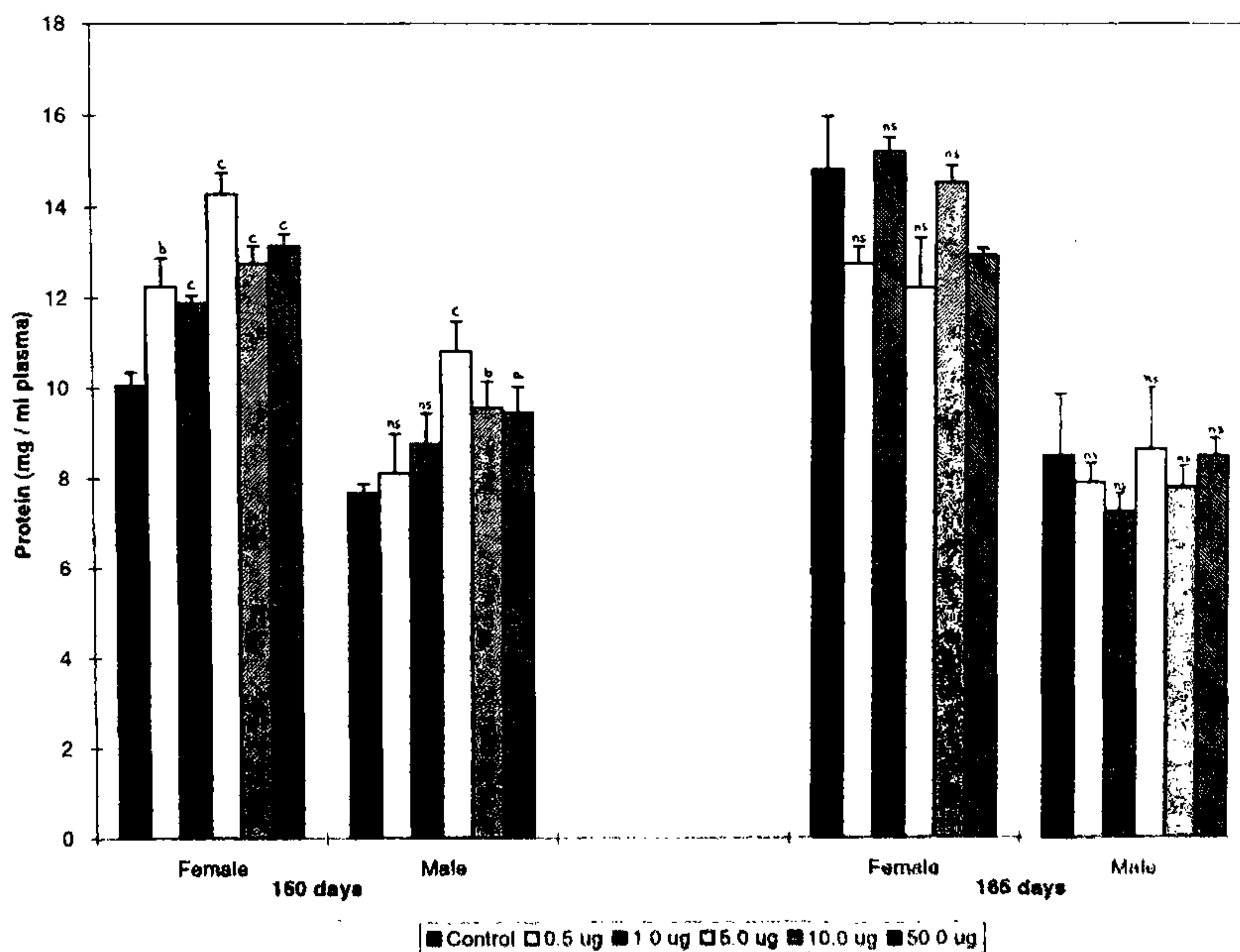


Figure 2. Effect of estradiol-17 $\beta$  on plasma protein concentration during critical phase of pupal diapause in *A. mylitta*. Three consecutive injections were given to 130, 135 and 140 day of pupal age. Vertical bars represent the standard error of the mean (ns, not significant; a,  $P < 0.05$ ; b,  $P < 0.01$ ; c,  $P < 0.001$ ).

octopamine titer on both the days under study during pupal age (Figure 1).

Protein content of plasma increased with all the doses of estrogen (with exception at 0.5 and 1 µg doses in male) on day 150 while the same remained unchanged on day 165 during pupal development. Protein concentration was found to be higher on day 165 than day 150 in both the sexes, and female contained more protein than male in control group of insects (Figure 2).

Significant fall in circulating octopamine titer together with concomitant rise in plasma protein concentrations between 150 and 165 days of pupal age in both the sexes of control silkworm during pupal diapause further reveal a strong possibility of the beginning of diapause termination after 150 days, which is in agreement with our earlier findings on age-dependent, tissue-specific fluctuations in carbohydrate metabolism during pupal diapause having physiological significance<sup>12</sup>. Plasma octopamine may act as a neurohormone in these insects to initiate the pupal diapause between 150 and 165 days by triggering the onset of tissue metabolism. This may evidently be caused by a significant rise in plasma protein level on day 165 of pupal age in *A. mylitta*. The haemolymph composition may be an indicator of the metabolic changes in some vital organs<sup>13,14</sup>. Changes in haemolymph octopamine levels have been reported under a number of species circumstances performing several biochemical changes controlling short term lipid and carbohydrate metabolism on being released as a part of an arousal mechanism in response to stressful circumstances<sup>15,16</sup>. These observations corroborate our present findings.

Control male and female moths emerged on an average of 202 and 215 days respectively after availing pupal diapause. Estrogen at the doses of 1–50 µg/pupa caused a significant reduction in emergence duration in a dose-dependent manner (Table 1). 50 µg of estrogen was found to be more effective in inducing the early eclosion behaviour of moth than the other doses of the hormone applied to the pupae. E<sub>2</sub> at the dose of 0.5 µg

remained ineffective in this respect. Egg production (laid + unlaidd) by female moth was significantly enhanced with the doses of 0.5–5.0 µg while 10 and 50 µg of E<sub>2</sub> caused significant reduction in egg production (Table 1).

Male haemolymph plasma contains more octopamine than females which may be one of the important physiological causes for early eclosion of male moth by accelerating the rate of development for pupal–adult transformation. In general, males of tasar moth emerge earlier and in proportionately greater frequency than females<sup>1</sup>. Such protandry fits the model of natural selection for optimum reproductive strategy for males<sup>17</sup>.

Estrogen-enriched octopamine titer on day 150 in the haemolymph with all the doses so far used in both the sexes may be due to more synthesis of this neurohormone by the neurosecretory cells (aminergic neurones) which may have caused earlier emergence of tasar moth in a dose-dependent fashion (Table 1). Steroid (20-OH ecdysone)-induced effect on aminergic neurones in *Rhodnius prolixus* has also been reported by Orchard *et al.*<sup>18</sup>. Higher doses (10–50 µg) of hormone (E<sub>2</sub>) exerted their negative influence on egg production in *A. mylitta*. The formation of less fecund functional adults may be due to E<sub>2</sub> enhanced early maturation of female reproductive system which results in more advanced moth eclosion than in other doses. Under normal circumstances, functional female for diapause destined generation requires 190–220 days (depending upon the prevailing environmental conditions) for optimum reproductive maturation<sup>1,19</sup>. Estrogen-enhanced moth eclosion may, however, interfere with the normal developmental pattern of the reproductive system, thereby giving rise to a less fecund female as indicated in the present study. E<sub>2</sub> elevated egg production over control at lesser doses (<10 µg) could possibly be due to the anabolic effect of this hormone on oogenesis. Estradiol-17β stimulation of production of egg-specific protein vitellogenin, oocyte maturation and embryogenesis has been reported<sup>8,9</sup>.

**Table 1.** Estrogen-induced effect on emergence duration (days) and egg production (number) of diapause destined generation of *A. mylitta*. Three consecutive injections were given to 130, 135 and 140-day pupae during diapause. (*n* = 10–15 individuals)

Dose	Emergence (Mean ± SE)		Egg production (Mean ± SE)		
	Male	Female	Laid	Unlaid	Total
Control	202 ± 3	215 ± 2	162 ± 3	23 ± 2	185 ± 4
0.5 µg/pupa	205 ± 2 (NS)	209 ± 3 (NS)	195 ± 4 (b)	17 ± 2 (b)	212 ± 3
1.0 µg/pupa	190 ± 3 (a)	195 ± 2 (b)	232 ± 6 (b)	29 ± 3 (b)	261 ± 5
5.0 µg/pupa	184 ± 2 (b)	192 ± 2 (b)	222 ± 5 (b)	43 ± 3 (b)	265 ± 2
10.0 µg/pupa	173 ± 3 (b)	178 ± 3 (b)	142 ± 8 (b)	18 ± 1 (b)	160 ± 7
50.0 µg/pupa	170 ± 2 (b)	173 ± 3 (b)	128 ± 6 (b)	20 ± 2 (b)	148 ± 8

SE, Standard error; *t*, test probability differences between control and treated lots; NS, Not significant; *a*, *P* < 0.020; *b*, *P* < 0.001.



Thus, it emerges that octopaminergic neurones are physiologically responsive to 17- $\beta$ -estradiol which is actively involved in diapause regulatory mechanism by triggering tissue metabolism in advance in tropical tasar silkworm *A. mylitta* D.

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## Micropropagation of *Eremostachys superba* – An endangered, endemic species from India

V. G. Sunnichan and K. R. Shivanna\*

Department of Botany, University of Delhi, Delhi 110 007, India

*Eremostachys superba* Royle ex Benth. is an endangered species of potential ornamental value. In the natural habitat, seed set is poor and germination does not occur. Seed viability lasts for only five months. A protocol has been developed to micropropagate *E. superba*. Seeds failed to germinate even *in vitro*. Excised embryos could be germinated on Murashige and Skoog's (MS) basal medium. Excision of roots was necessary to promote growth of the plumule. Multiple shoots could be induced in cultures of shoot tips as well as nodal segments on MS medium containing 6-benzylaminopurine and indole-3-acetic acid. The shoots were rooted on MS medium containing indole-3-butyric acid, and the plantlets were hardened and transferred to soil in the open.

*EREMOSTACHYS SUPERBA* Royle ex Benth. (Lamiaceae) is a herbaceous ornamental, perennating by means of a thick, deep penetrating tuber. The species is endemic to Garhwal Himalayas and has been declared endangered<sup>1</sup>. The species was reported to be reduced to a single population with only 25 individuals at Mohand, near Dehra Dun (UP)<sup>2</sup>. Browsing by herbivores, collection of flower twigs by travellers, poor seed set and lack of seed germination are responsible for its diminishing

population size<sup>2</sup>. According to Rao and Garg<sup>2</sup>, the multiplication of the species through tissue culture techniques is urgently needed for enhancing the population size to counteract genetic stochasticity<sup>3</sup>.

Plant tissue culture is an effective means for rapid multiplication of endangered species in which conventional methods present limitations<sup>4,5</sup>. Establishment of aseptic cultures and development of an efficient protocol for regeneration and multiplication of plants are required for developing *in vitro* strategies for conservation. Attempts have been made to micropropagate endangered species of ornamental or medicinal value such as *Picrorhiza kurroa*<sup>6</sup>, *Coleus forskohlii*<sup>7</sup>, *Rauvolfia serpentina*<sup>8</sup>, *Coptis teeta*<sup>9</sup> and *Meconopsis simplicifolia*<sup>10</sup>. In this communication we report an efficient micropropagation protocol for *E. superba* using shoot tips and nodal segments from seedlings raised from excised embryos.

Detailed studies were made using seeds collected during April 1995 and 1996 from the natural population near Dehra Dun. The seeds were stored at room temperature and used for experiments during July–August 1995 and 1996. As the work was in progress, the authors were informed that a few more populations of *E. superba* have been located near Jammu also (A.K. Koul, personal communication). The results obtained with Dehra Dun population were subsequently confirmed with seeds collected from the Jammu population. Attempts made to germinate seeds in soil as well as on petri plates lined with moist filter paper were unsuccessful. To achieve germination *in vitro*, the seeds were washed in 1% Teepol (a commercial bleach) for 20 min and kept in running tap water for 1 h. They were surface-sterilized with 0.1% (w/v) mercuric chloride for 5

\*For correspondence.