INSECT DEVELOPMENT IN ALTERED GRAVITATIONAL ENVIRONMENT

FINAL REPORT

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ABSTRACT

When tobacco hornworm (*Manduca sexta*) larvae burrow underground (25-30 cm) to pupate, they reorient themselves to a relatively horizontal position indicating an ability to sense gravity. To evaluate their sensitivity to gravitational environment during metamorphosis, *Manduca* (pharate adults) were placed in a vertical (head-up) position. Distinct morphological changes, each one reflecting ensuing phases, were used to follow adult development. Five days after pupation, the vertical group showed accelerated ($P < 0.05$) development and were nearly 4 phases ahead ($P < 0.0001$) after 10 days. Differences in development in the vertical group were characterized further by increased (7-48%) hemolymph concentrations of 13 amino acids, but a decrease in cys and pro and no change in arg, his, met and val (trp, undetectable). Decreased (36%) turnover of injected ^{3}H-phenylalanine suggested slower utilization of amino acids contributed, at least partly, to the increased concentrations. Vertically-oriented *Manduca* also exhibited a greater (20%, $P < 0.001$) protein content in their flight muscles near the end of development. Analysis of hemolymph sugar levels showed a redistribution of sugars from the monosaccharide glucose to the disaccharide trehalose. Since injection of 20-hydroxyecdysone decreased (49%) turnover of ^{3}H-phenylalanine in pharate adults and since ecdysteroids are known to increase flight muscle size and control adult development, these results are consistent with our measuring a greater (+80%, $P < 0.05$) ecdysteroid titer in the vertically-oriented insects. These results suggest that gravity environment influences ecdysone output by the pharate adult. When we evaluated hemolymph flow in the head-up and control positions, we found that injected ^{14}C-inulin was distributed somewhat more rapidly in the head-up group irrespective of the sight of injection (head or abdomen) likely because in the head-up position flow of the hemolymph is facilitated throughout the animal. Other experiments showed that an intact prothoracic gland is needed for the response. Hence vertical, head-up orientation affects release of ecdysone from the prothoracic gland.

INTRODUCTION

Life on Earth has evolved under the constancy of a 1 G environment. It is not astonishing that life forms have developed means of sensing this gravitational force. Likewise it is plausible that development of terrestrial organisms, which have remained subject to the force and vector of gravity, evolved in a manner making them dependent in some fashion on this environmental parameter. Examining how organisms develop under conditions of an altered gravity environment may reveal significant findings concerning the influence of gravity on evolutionary processes or at the very least on development of the organism. An important goal of gravitational biology has been to identify stages of development which are particularly sensitive to an altered gravity field, achieved by use of weightlessness, clinorotation, and hypergravity. Developmental studies have largely focused on vertebrate systems both pre- and post-natally (1-4,9,13,19-21,26,28,29,33,36), although some invertebrate studies have been conducted (10,17,18,25,27).

Metamorphosis provides a unique condition for studying the role of gravity in development. Formation of new organs in a previously existing organism requires a highly active period of turnover of amino acids and proteins, and of changes in the endocrine profile. Furthermore, metamorphosis offers the advantage of studying a self-contained biological system. Since mammalian studies have shown that endocrine cells of rats can be influenced by microgravity (14), it is not unreasonable to consider whether the insect endocrine system may also be affected.

The tobacco hornworm, *Manduca sexta*, provided a suitable species to study the effect of altered gravitational environment on invertebrate development. This species has been one of the most thoroughly investigated organisms in a variety of aspects of insect biology. *M. sexta* pharate adults can provide significant amounts of material with which to work, thus facilitating the study of metabolic aspects of adult development.

METHODS

Insect care. *Manduca sexta* larvae were derived from eggs provided by Drs J.P. Reinecke and J. Buckner, United Sates Department of Agriculture, Fargo, N.D. Larvae were reared on an artificial wheat germ diet (22) in a Rheem environmental chamber (26°C, 55%-65% humidity, photoperiod 17 h light:7 h dark). Diet components were obtained from United States Biochemical Corp. (Cleveland, OH) or ICN Biomedical (Aurora, OH). The first three larval instars were maintained in plastic trays and thereafter were transferred to 9 oz plastic cups with perforated lids. After wandering, individual insects were placed into a new cup to undergo pupation. Following pupation, insects were transferred to a 50 ml perforated plastic, screw-top centrifuge tube and maintained in either a horizontal or vertical position.

Morphology. To be able to follow adult development, distinct changes were identified in the developing adult. Each successive phase describes one or more characteristics for the normal pharate adult covering the full period of development from pupation (phase 1) to adult eclosion (phase 37) (Table 1). The first 5 phases are distinguished by changes in the cuticle and the disappearance of larval leg remnants. Phases 6 to 14 feature appearance and development of the adult legs. Beginning with phase 15 through 19, development of the eye provides discrete details for comparison. During phases 21 through 23 spines and claws appear on one set of legs. Subsequently (phases 24 to 31) there are distinct changes in the characteristics of the wing. The final phases are defined by changes in the body color and texture up to phase 37.

Amino acid turnover. Pharate adults at the desired phase of development were selected and weighed. They were injected, in the eighth abdominal segment just under the spiracle using a 100 μl Hamilton syringe, with 2.5 μCi [^{3}H]phenylalanine contained in 50 μl of insect medium (32). The wound was sealed with a drop of cyanoacrylate, and insects were maintained at 26°C. At 30, 60 or 90 minutes after injection, the insect was cut into 3 or 4 segments and placed in a plastic cup containing 20 ml of 2% perchloric acid and placed on ice. Total free phenylalanine and total radioactivity remaining in the free amino acid pool was extracted by pulverizing the segments with a pestle. An aliquot of extracted total body sample was centrifuged at 8000 g for 15 minutes. A portion (1 ml) of the supernatant solution was added to 0.5 ml of saturated tripotassium citrate to precipitate the perchlorate anion as $KClO_4$ (11,15). After centrifugation at 8000 g for 15 minutes, 1 ml of supernatant solution was taken for analysis of phenylalanine. The phenylalanine turnover rate (K_s) was determined (7) from the slope derived by plotting $(-a)\ln(q_t/q_0)$ vs time, where a = total body free phenylalanine (μmol/insect); q_0 = radioactivity of injected [^{3}H]phenylalanine (dpm); q_t = radioactivity remaining in the phenylalanine pool at time t (dpm/insect).

Analyses. Measurement of phenylalanine activity was modified from Garlick et al (11). Aliquots (1 ml) from the above procedure were combined with 0.5 ml of 0.5 M sodium citrate (pH 6.3) containing 0.35 unit of L-tyrosine decarboxylase (Sigma Chemical Co., St. Louis, MO) and 0.25 mg pyridoxal phosphate, and then incubated overnight at 50°C to convert phenylalanine to phenethylamine. The latter was separated from any contaminating

tyramine (derived from tyrosine) by extraction into 10 ml of chloroform:n-heptane (1:3, v/v) after adding 1 ml of 3 N NaOH. The organic layer was then added to 5 ml of chloroform and 4 ml of 10 mM H_2SO_4 and shaken. Phenethylamine in the aqueous acidic layer was analyzed for radioactivity and total phenethylamine. For radioisotope counting, 0.2 ml sample was added to 5 ml liquid scintillant (Ecolume, ICN Biomedical) and analyzed in a Beckman LS250 liquid scintillation counter. Total phenethylamine was assayed fluorometrically (11).

For amino acid analyses, hemolymph samples (200 μl) were collected by puncturing the head with a 25 gauge needle. The samples were treated with 20 μl 50% sulfosalicylic acid. A 5 μl aliquot of the supernatant solution was diluted to 500 μl with analyzer buffer and 50 μl was then analyzed on a Beckman 7300 automated amino acid analyzer. Tryptophan was not detectable and data for aspartate and asparagine or for glutamate and glutamine are reported as a total value.

Protein content of the dorsolongitudinal flight muscle was analyzed using the Lowry procedure (16) after excising the muscle as described previously (25).

Ecdysteroid was extracted from 50 μl hemolymph using methanol (450 μl). The extract (35 μl) was further diluted with 1 ml of methanol. An aliquot (5 μl) was analyzed for ecdysteroid by enzyme immunoassay using a kit from Cayman Chemical Co. (Ann Arbor, MI). Sample spiking to test for quenching was found to be unnecessary for these samples.

Hemolymph sugars were analyzed by high pressure liquid chromatography.

Data are presented as the mean $\pm$ SEM. In most experiments differences between control and vertical groups were tested using the unpaired Student's t-test with $P<0.05$ being considered statistically significant. Exceptions are noted in the legends.

RESULTS AND DISCUSSION

Gravity orientation and adult development. During wandering, the period immediately following cessation of larval feeding, the larva burrows into the soil to form a pupation chamber (8). To ascertain whether larvae could sense the gravity vector underground, we monitored their orientation 1 week subsequent to formation of their chambers. Fifteen wandering larvae were allowed to burrow into a mixture of moist sand and potting soil. Despite burrowing down 25 to 30 cm, the insects had reoriented themselves to a slightly head-up ($10 \pm 1°$) position. Since light and temperature were not factors in this process, the larvae must have sensed the gravity vector.

To assess whether developing adults might be sensitive to their gravitational environment, pupae were oriented in a vertical head-up position within 24 h after pupal ecdysis. The extent of development was monitored using a detailed set of characteristic phases which were derived from morphological changes for defining the entire period of adult development (Table 1). By day 5 after pupation, the vertical group was slightly, but significantly ($P<0.05$), ahead in development with the difference increasing to nearly five phases by day 10 (Table 2). On the basis of time, this phase difference represented about 3 days of development; total adult development requiring about 19-21 days normally. With further development, the difference between the groups decreased so that there was no significant difference in the time to adult eclosion (i.e., emergence of the moth), although the vertical group tended to eclose about one day earlier. It is noteworthy that the pupa must be vertically oriented within the first 4 days in order to elicit accelerated development. When reorientation occurred later than 96 hours, no difference in development was noted. This finding is significant since it is after day 4 that the hemolymph ecdysteroid titer begins to rise rapidly, doubling between days 4 and 5 (5).

Amino acid metabolism. The rapid turnover of tissues during adult development is associated with rapid metabolism of the free amino acid pool. Fractional phenylalanine and alanine turnover may be as high as 50% to 60% of total body phenylalanine or alanine per hour (31,34). Therefore, it is possible that an altered rate of development might affect the pool of free amino acids. In a preliminary study, whole body analysis of acid-extracted phenylalanine showed a higher (+36%) content in the head-up than in the control group (1.02 ± 0.13 vs. 0.75 ± 0.07 μmol/insect; $P<0.05$) on day 10 of adult development. Analysis of alanine content also showed it to be elevated (+21%; 3.55 ± 0.10 vs 2.94 ± 0.09 μmol/insect; $P<0.05$).

Based on these preliminary findings, we measured the hemolymph concentration of all the amino acids of 7 day pharate adults (Table 3). The total amino acid concentration in controls was about 70 mM and covered a broad range from about 40 μM for aspartate and asparagine combined to greater than 10 mM for lysine. When oriented in a vertical, head-up position, the total amino acid concentration was nearly 76 mM. In addition to alanine and phenylalanine, nine other amino acids showed a significant increase in concentration ranging from 12 to 48% and 2 others, gly and thr, showing a marginal increase. Therefore 13 of the

19 measurable amino acids (tryptophan excluded) increased in amount with the change in gravitational orientation. Of the remaining amino acids, four were unaffected and two decreased in response to the vertical orientation. Of the six amino acids which did not increase, the group included both sulfur-containing amino acids, and two amino acids with basic side groups. The reason why not all amino acids showed an increase is unclear.

To evaluate the potential reason for the rise in concentration of most of the amino acids, we tested the effect of head-up orientation on rate of turnover (disappearance) of injected trace amounts of ^{3}H-phenylalanine. In both groups, the rate of turnover was linear with strong correlation coefficients (Fig. 1). The turnover rate was slower (-36%) in the vertical group (0.67 ± 0.08 μmol/insect/h) compared to the control group (1.04 ± 0.13), with a significant difference at each time point. These results suggest that amino acid concentrations increase with vertical, head-up orientation at least in part as a consequence of decreased turnover. This finding does not exclude a contribution from increased protein breakdown of pre-existing tissues. Since development of the vertical group is more rapid, it is very likely that accelerated protein breakdown of larval tissues adds to the amino acid pool.

Sugar metabolism. The principal hemolymph sugar is the disaccharide trehalose ($\approx$55%), with glucose accounting for most of the remainder of the total sugar (Fig. 2). We also evaluated whether head-up orientation might affect sugar metabolism. Indeed there was a shift towards a greater amount of trehalose. These results imply that either there is a diminution in trehalase activity or an increased formation of trehalose from glucose. Since trehalose is largely formed from glycogen via glucose-1-phosphate and UDP-glucose the more likely result is a decline in trehalase activity.

Role of ecdysteroid. Adult development of *M. sexta* is controlled largely by ecdysone. The hemolymph titer of ecdysone increases markedly after day 4 following pupation. It increases nearly 4-fold reaching a peak (3.5 μg/g fresh wt) between days 7 and 9 and declining rapidly thereafter to less than 1 μg/g fresh wt by day 11 (5). Since a previous study from our laboratory showed differential rates of phenylalanine turnover between the middle (days 6-10) and end of adult development (31,35), we considered whether such rates might correlate with changes in the ecdysteroid titer. Therefore, we followed the time course for rates of ^{3}H-phenylalanine turnover during adult development for comparison with the published ecdysteroid profile (Fig. 3). These results suggest an inverse relationship exists between ecdysteroid concentration in the hemolymph and the rate of phenylalanine turnover. Alanine turnover also showed a similar, though less dramatic, inverse relationship (34). Therefore, ecdysteroid levels may control the rate of amino acid turnover in *M. sexta* pharate adults.

The potential effect of ecdysteroid on amino acid turnover was evaluated by injecting 20-hydroxyecdysone into well-developed (day 16) pharate adults, which normally have a very low ecdysteroid titer (5, see Fig. 3). A similar approach was used to show that ecdysteroid injection could restore rapid growth of the dorsolongitudinal flight muscle (30, 31). Injection of a dose which was about 67% of the normal peak amount lowered the rate of ^{3}H-phenylalanine turnover by 49% ($P<0.001$) (Table 4). This rate (0.64) was similar to that

observed on days 5 and 10 of development (Fig. 3), when the ecdysteroid titer was similar to the amount injected.

As noted above, another parameter which is sensitive to the ecdysteroid titer is growth of the dorsolongitudinal flight muscle during adult development. Injection of 20-hydroxyecdysone on day 16 of development led to a significant increase in size of this muscle within 24 hours, by slowing protein degradation (31). Since the data here show that other ecdysteroid-controlled processes are enhanced by head-up orientation, we measured the protein content of the flight muscle near the end of development (day 20). These results showed a 20% greater ($P < 0.001$) content of protein in this muscle in the vertical (6.8 ± 0.3) than in the control (5.6 ± 0.2) group.

These various findings are all consistent with an elevated ecdysteroid titer being the cause of the observed changes namely: a) accelerated development occurred especially during and after the peak period of ecdysteroid titer (Table 2) and was only observed if the insects were reoriented during the first 4 days prior to the large increase of the ecdysteroid titer; b) concentrations of most amino acids increased (Table 3); c) turnover of phenylalanine decreased (Figs 1,2; Table 4); and d) the flight muscle was enlarged. To determine the validity of this hypothesis, we measured (Table 5) the ecdysteroid titer on day 7 of development when the hormone peak is normally first reached (5). The measured control value (4.2 μg/g fresh wt) was within one SEM of the peak value (≈ 3.5 μg/g fresh wt) reported previously (5). In keeping with the developmental and biochemical data, the vertical group had an 80% greater amount of ecdysteroid.

We conclude from these findings that altering *M. sexta* gravitational orientation during the initial 4 days of adult development in some manner increases ecdysteroid output which in turn modifies a number of events related to development. While most production of ecdysteroid occurs in the prothoracic gland, there is evidence for its production by other tissues (6). In the absence of prothoracic glands, adult development of *M. sexta* is delayed but can still occur as long as the brain is intact (23,24). Therefore increased production and/or release of ecdysone could have occurred in the prothoracic gland or in other tissues such as the integument and epidermis (6). When *M. sexta* are placed in a vertical, but head-down, position, they do not show accelerated development. Therefore, brain function of some sort must be critical to the response since a simple direct effect on abdominal tissues would not be differentially affected by a head-up versus a head-down orientation.

In considering plausible mechanisms it is important to consider the potential effect of gravity on the insect's hemolymph circulatory system. As an "open" system in which the organs are bathed, gravity would facilitate downhill flow but restrict uphill flow (12). Therefore, placing the pupa in a vertical, head-up position would facilitate flow of hemolymph away from the brain possibly increasing the efficiency of movement of regulatory factor(s) from the head region. To test this possibility, we measured the distribution of ^{14}C-inulin injected into either the head or abdominal region of control or head-up insects (Figs 4,5). When ^{14}C-inulin was injected in the head there was a tendency for a more rapid initial distribution in the head-up

position (Fig 4). When inulin was injected in the abdomen the vertical position clearly facilitated distribution at the early time points. Since the insect hearts pump blood towards the head, the likely scenario is that in the head-up position, hemolymph flow is facilitated towards the abdomen thus increasing the cycling of the hemolymph. It is curious, however, that the head-down position did not impede development suggesting that the flow of any factor from the head region may be normally so slow as to not be adversely affected by the uphill flow in this condition.

Finally we established whether the phenomenon of the head-up position accelerating development required an intact prothoracic gland from which the ecdysone is released. This was tested by surgically removing the gland from wanderers immediately prior to pupation. Without injection of ecdysteroid development ceased at phase 6. Pharate adults were injected daily for 7 days with 20-hydroxyecdysone at a concentration which produced a total development which approximated that of insects with intact glands. Normally after 7 days, the phase of development averages 13.8 (Table 1). Insects from which the gland was removed and which were maintained in a normal orientation and injected in either the head or abdomen achieved an average of 13.3 and did not differ in development from normal insects. Therefore the site of injection had no affect on the extent of development. When placed in a vertical position, normally insects would develop to an average of nearly 18 phases after 7 days (Table 2). However, without intact glands development did not differ from hormone-treated controls. Therefore the effect of altered gravitational orientation depends on an intact prothoracic gland ruling out the contribution of other sites of ecdysteroid production for this effect of vertical orientation.

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Figure Legends

Fig. 1. Vertical, head-up orientation decreases turnover of injected ^{3}H-phenylalanine. The rate of turnover for controls (open circles) is 1.04 $\pm$ 0.13 μmol/insect/h (r^2 = 0.67) compared to 0.67 $\pm$ 0.08 μmol/insect/h (r^2 = 0.59) for the head-up group (filled squares). Pupae were selected and maintained as described in Table 2. After 7 days, pharate adults were injected with ^{3}H-phenylalanine to determine the rate of turnover by making measurements at 30, 60 and 90 min after injection. Each time point is the mean $\pm$ SEM for 12 to 18 pharate adults. At each time point the head-up value is significantly ($P < 0.001$) lower than the control value as determined by Analysis of Variance (ANOVA).

Fig. 2. Distribution of saccharides in the hemolymph. Sugar concentrations were measured using high pressure liquid chromoatography. Data are results from 10 insects.

Fig. 3. Phenylalanine turnover and ecdysteroid titer are inversely related during adult development. Phenylalanine turnover (filled circles) was measured on the days of adult development indicated as described in Fig. 1. Ecdysone titer data (open squares) were taken from a previous publication (5) with about half the daily points excluded to simplify the plot.

Fig. 4. Distribution of ^{14}C-inulin after injection into the head region. Approximately 1 μCi of ^{14}C-inulin was injected into control and head-up insects. Samples (5 μl) were collected at the indicated time intervals from the nicked proboscis of each of 10 pharate adults at 7 days after pupation. Data are expressed as a percent of the maximal activity measured at the outset. Values decrease with time as the radioactivity is distributed away from the head region.

Fig. 5. Distribution of ^{14}C-inulin after injection into the abdomen. Approximately 1 μCi of ^{14}C-inulin was injected into the space between abdominal segments in control and head-up 7-day pharate adults. Samples (5 μl) were collected at the indicated time intervals from the nicked proboscis of each of 8 pharate adults. Data are expressed as a percent of the maximal activity measured at the end of the experiment. Values increase with time as the radioactivity is distributed towards the head region.

Table 1. *Characteristics of developmental stages*

Phase 1 - Partly green with a very soft cuticle
Phase 2 - Darker brown cuticle without green; soft cuticle; larval leg remnants
Phase 3 - Dark brown, soft cuticle; proboscis, eye area, lower body much darker; leg remnants remain
Phase 4 - Cuticle becomes firm
Phase 5 - Leg remnants no longer evident
Phase 6 - Inner leg formation of adult begins; dark outline of leg visible around tracheole
Phase 7 - Inner leg easily visible with slight segmentation; dimpled in
Phase 8 - Inner leg lightly segmented
Phase 9 - Entire outer leg visible, but without segmentation
Phase 10 - Moderate segmentation on inner leg; differences in density on outer leg that mark future segmentation
Phase 11 - Full segmentation of inner leg
Phase 12 - Segmentation on lower portion of outer leg
Phase 13 - More extensive segmentation on lower portion of outer leg; upper portion of leg beginning to dimple.
Phase 14 - Full segmentation of outer leg
Phase 15 - Eye contains a slightly dark shadow when pharate adult is in a flat position
Phase 16 - Legs are fading back and eye is much more defined
Phase 17 - Legs fade further and eye is now completely dark and uniform in color
Phase 18 - The circumference of the eye becomes much darker than the center as the eye gets lighter towards the center
Phase 19 - Eye is very dark around the circumference; ring of dark instead of gradual fading
Phase 20 - Buds (beginning of spines) are visible on the inner leg
Phase 21 - Very dark eyes which lighten towards the center; spines now evident on inner leg
Phase 22 - Claw visible but transparent on inner leg
Phase 23 - Very dark claw on inner leg
Phase 24 - A few flecks on the wings are noticeable
Phase 25 - Many flecks apparent on wings in striped pattern
Phase 26 - Three lines run down center of back
Phase 27 - Thick, dark line down back with lighter circles on both sides of the line
Phase 28 - Wings developing a pattern on interior and exterior; stripes of flecks evident
Phase 29 - Definitive pattern of dark markings on wings
Phase 30 - More than 50% of wing has turned dark
Phase 31 - Wing at least 90% dark
Phase 32 - Bottom half of insect dark; same color front and back sides
Phase 33 - Entire body of animal very dark
Phase 34 - Accumulation of fluid in upper body causes softness
Phase 35 - Upper body mushy to the touch
Phase 36 - Upper and lower body very, very mushy

Table 2. *Vertical, head-up orientation accelerates adult development*

	Phase of development				
Day after pupation:	4	5	7	10	14
Control	5.8±0.1	6.4±0.2	13.8±0.2	23.0±0.3	29.3±0.3
Vertical	6.0±0.1	7.2±0.2	17.8±0.2	27.7±0.3	32.1±0.2
P	NS	<0.05	<0.0001	<0.0001	<0.05

Immediately following pupation twenty *M. sexta* pupae were assigned alternately to the control or vertical groups. Phase of development is the series of descriptions for the normal pharate adult which characterize the full period of development from pupation (phase 1) to adult eclosion (phase 37) (Table 1).

Table 3. *Vertical, head-up orientation modifies amino acid concentrations (mM) in the hemolymph*

	Amino acids significantly increased								
	Ala	Asp+ asn	Glu+ gln	Ile	Leu	Lys	Phe	Ser	Tyr
Control	1.77 ±.11	0.037 ±.003	7.14 ±.38	1.31 ±.05	1.70 ±.06	12.28 ±.68	0.63 ±.02	3.54 ±.21	3.67 ±.11
Vertical	2.14 ±.19	0.046 ±.003	8.45 ±.42	1.46 ±.07	1.98 ±.06	13.96 ±.65	0.81 ±.04	5.24 ±.24	4.16 ±.14
Change (%)	+20	+25	+18	+12	+17	+14	+28	+48	+13
P	<0.02	<0.05	<0.02	<0.05	<0.002	<0.05	<0.001	<0.001	<0.01

	Other amino acids							
	Gly	Thr	Cys	Pro	Arg	His	Met	Val
Control	3.27 ±.14	3.41 ±.14	1.73 ±.11	6.04 ±.21	7.50 ±.27	9.92 ±.80	0.58 ±.07	4.79 ±.20
Vertical	3.50 ±.08	3.67 ±.10	1.35 ±.08	5.31 ±.29	8.24 ±.47	9.96 ±.51	0.63 ±.09	4.82 ±.27
Change (%)	+ 7	+ 8	-22	-12				
P	<0.08	<0.08	<0.005	<0.05	NS	NS	NS	NS

Twenty *M. sexta* pupae were selected and maintained for 7 days. Hemolymph samples were collected and after processing were analyzed for physiological amino acids. Tryptophan was not measurable. P values below 0.1 and above 0.05 are only marginally significant.

Table 4. *Ecdysteroid injection decreases turnover of ^{3}H-phenylalanine*

	^{3}H-phenylalanine turnover (μmol/insect/h)	
Treatment:	Saline	20-Hydroxyecdysone
	1.26±0.21	0.64±0.13

Pharate adults on Day 16 of their development were injected with saline or 2 μg 20-hydroxyecdysone/g insect. The next day, phenylalanine turnover was determined as in Fig. 1. The number of insects per time point was 10 to 12. Data are given as means ± SEM with the correlation (r^2) value given in parentheses.

Table 5. *Vertical, head-up orientation increases hemolymph ecdysteroid titer*

	Ecdysteroid titer (μg/g insect)
Control	4.19 $\pm$ 0.80
Vertical	7.55 $\pm$ 1.11
	$P < 0.05$

Twenty pupae were selected and maintained. Ecdysteroid was extracted from 50 μl hemolymph and was analyzed for ecdysteroid using an enzyme immunoassay kit.

Table 6. *Development in normal and vertical pharate adults lacking prothoracic glands*

Orientation	Injection site	# Insects	Phase of Development
Normal	none	9	6.2 ± 0.5
Normal	head	9	13.3 ± 0.2
Normal	abdomen	12	13.2 ± 0.3
Vertical	head	10	13.3 ± 0.2
Vertical	abdomen	13	12.9 ± 0.3

Prothoracic glands were excised from wanderers approximately 1 day prior to pupation after allowing sufficient time for the prepupal ecdysone titer to be released. Pharate adults were then injected at the outset of each day with insect saline (none) or with the following schedule of 20-hydroxyecdysone (μg/g body wt) contained in 50 μl saline: days 1-2, 0.6; days 3-4, 1.32; days 5-6, 2.52; day 7, 3.72. The ideal injected amounts were determined by testing different injection schedules until normal development was obtained. Initial amounts to be injected were estimated from published results for hemolymph ecdysone concentrations (5).

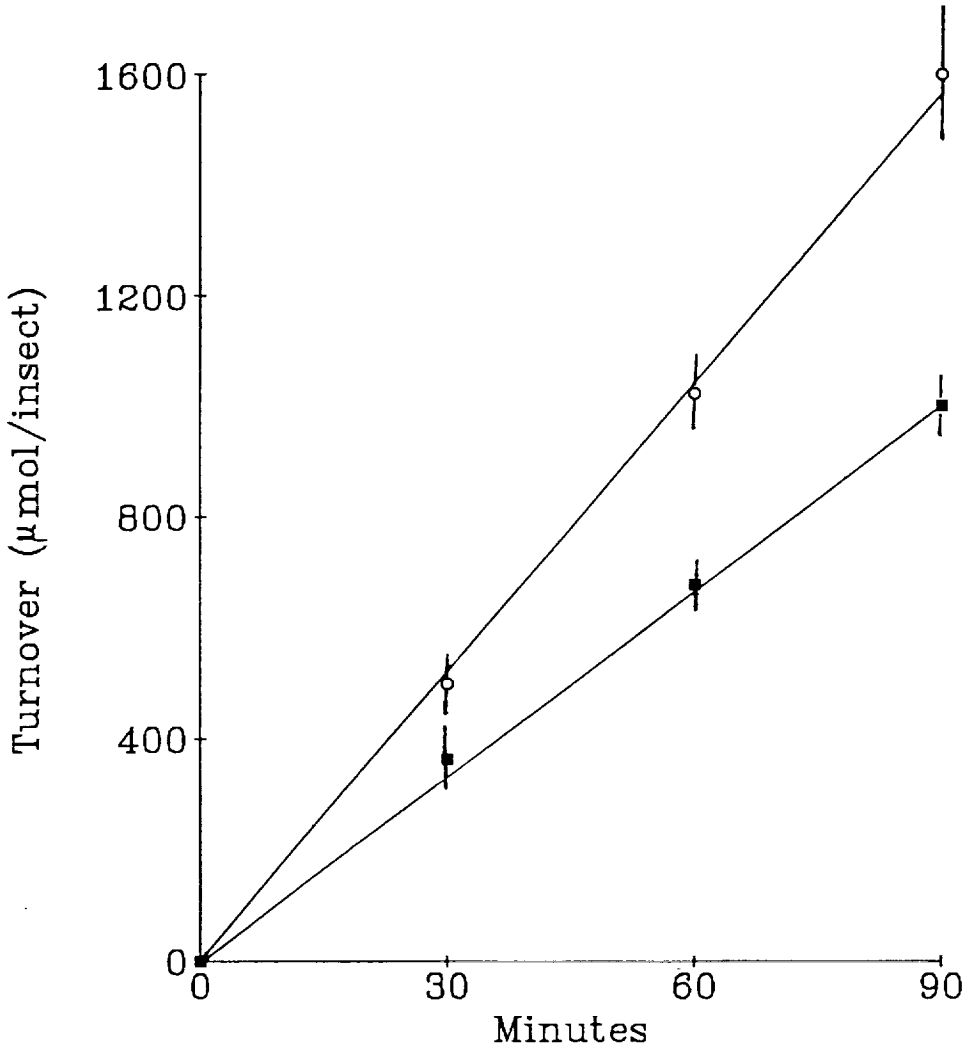

FIG. 1

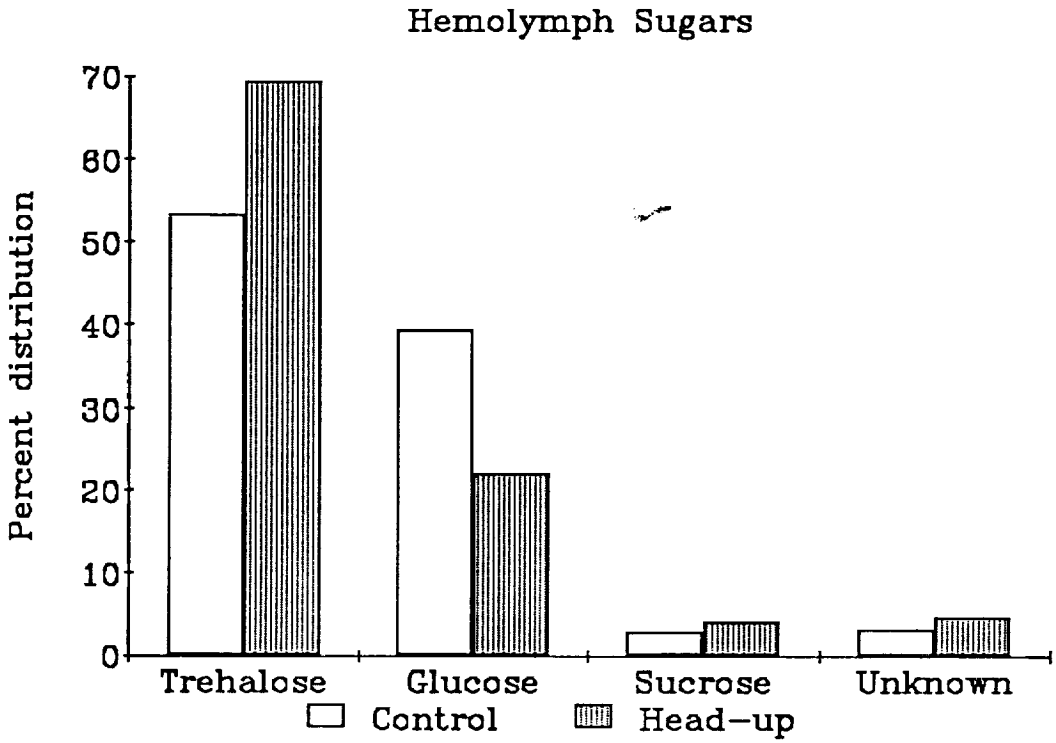
Hemolymph Sugars
Percent distribution
70
60
50
40
30
20
10
0
Trehalose
Glucose
Sucrose
Unknown
Control
Head-up

FIG. 2

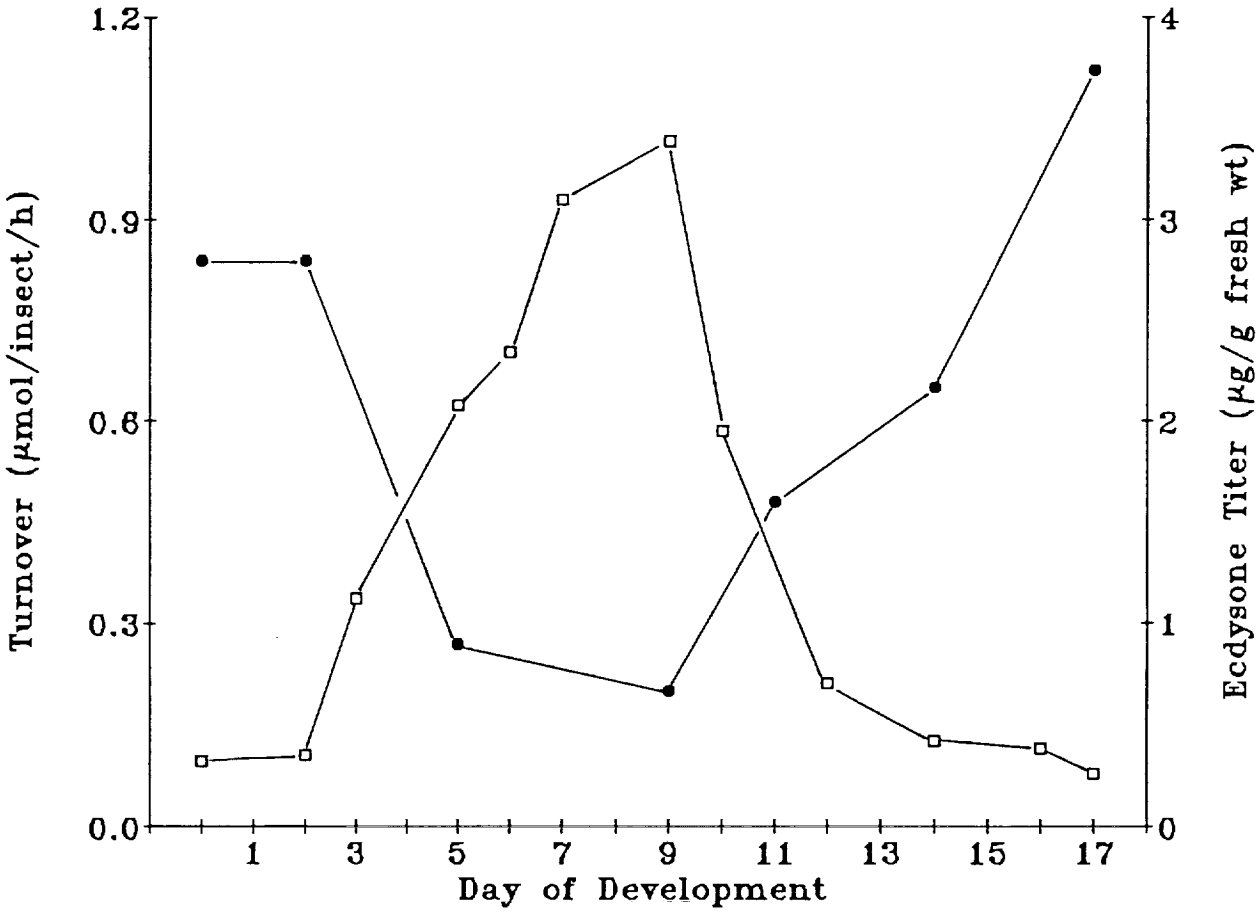
1.2
0.9
0.6
0.3
0.0
Turnover (μmol/insect/h)
4
3
2
1
0
Ecdysone Titer (μg/g fresh wt)
1
3
5
7
9
11
13
15
17
Day of Development

FIG 3

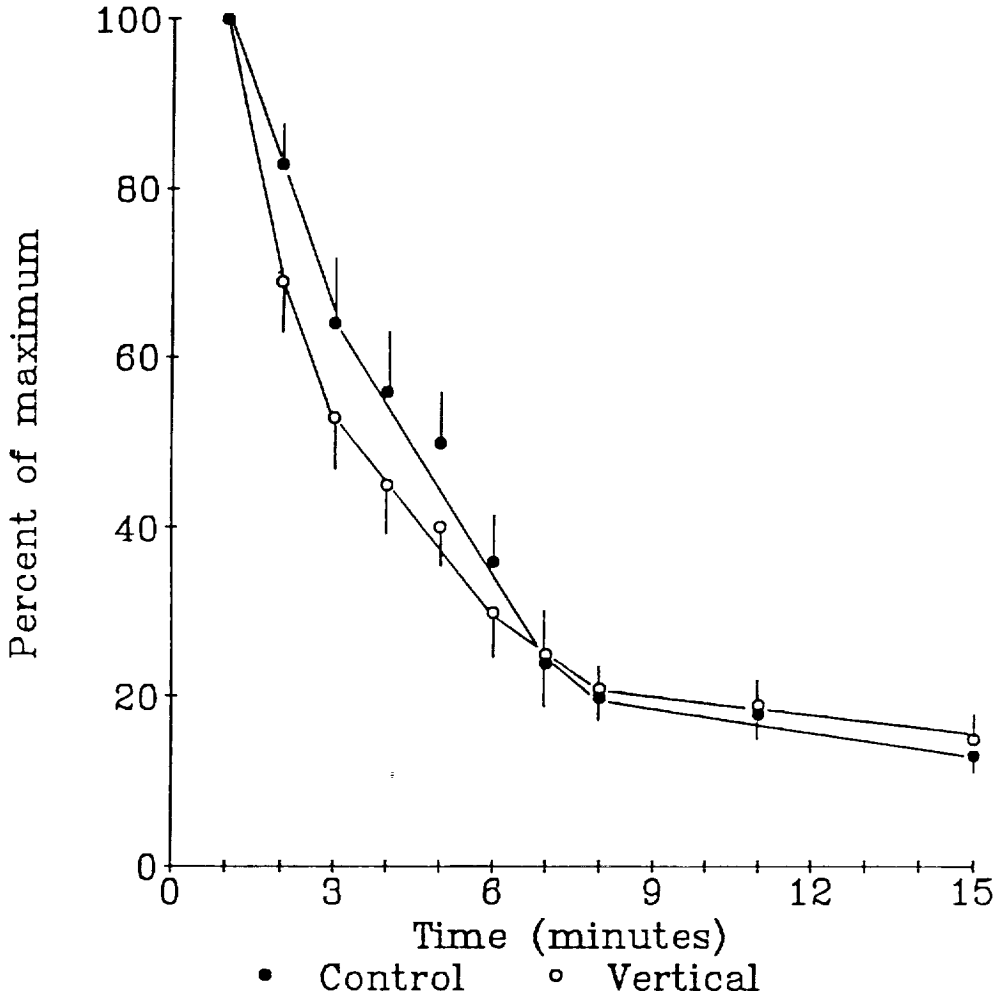
100
80
60
40
20
0
Percent of maximum
0
3
6
9
12
15
Time (minutes)
• Control
○ Vertical

FIG 4

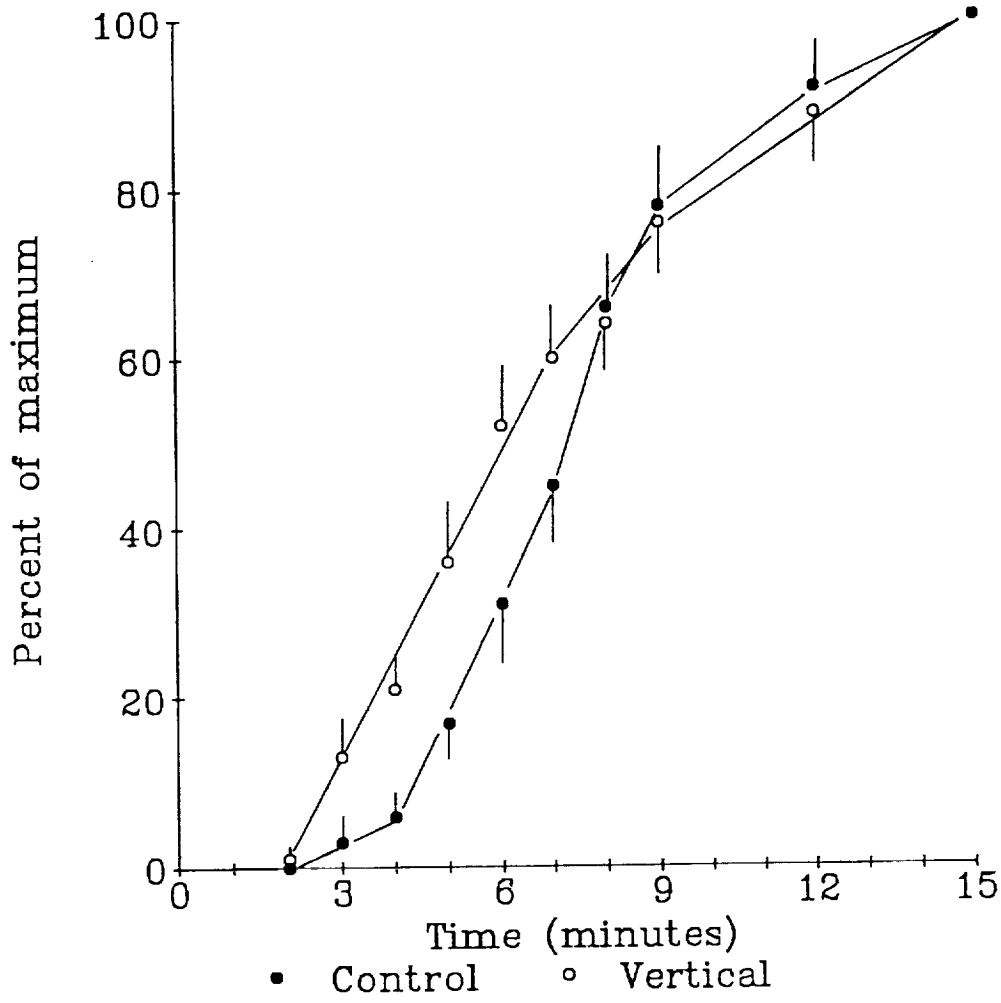
100
80
60
40
20
0
Percent of maximum
0
3
6
9
12
15
Time (minutes)
• Control
○ Vertical

FIG 5