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**Juvenile hormone esterase and correlated responses to selection:
The physiological basis of wing dimorphism in
the sand cricket, *Gryllus firmus* (Orthoptera: Gryllidae)**

Dean E. Yadlowski

A Thesis

in

The Department

of

Biology

**Presented in Partial Fulfillment of the Requirements
for the Degree of Master of Science at
Concordia University
Montreal, Quebec, Canada**

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ABSTRACT

Juvenile hormone esterase and correlated responses to selection:

**The physiological basis of wing dimorphism in
the sand cricket, *Gryllus firmus* (Orthoptera: Gryllidae).**

Dean E. Yadlowski

Previous experiments demonstrated genetic correlations among physiological, morphological, and behavioural traits determining migratory tendency in insects. It has been hypothesized that these correlations are the result of the pleiotropic effect of a single hormone, juvenile hormone, and that variation found in migratory tendency is due to underlying variation in haemolymph juvenile hormone (JH) titre. Using the wing dimorphic cricket, *Gryllus firmus*, as a model, this study investigated the role of the degradative enzyme, juvenile hormone esterase, in correlated responses to selection for wing length. Laboratory lines of *G. firmus* selected for increased and decreased percentage macroptery, as well as control lines, were assayed for haemolymph juvenile hormone esterase (JHE) activity during the last juvenile instar using an *in vitro* radiochemical assay. Degradation of JH in haemolymph samples was found to be almost exclusively due to JH-specific esterase. Activity of JHE peaked at day 5 of the last instar in all selected and control lines, and was higher in macropterous lines than in control and micropterous lines. Subsequent experiments utilizing a lower-stress rearing method

revealed lower JHE activity in micropterous lines than in control lines. In both experiments, JHE activity during the final instar was an excellent predictor of adult wing morph. Logistic regression suggested differences between selected and control lines in the level of JHE activity resulting in a molt to macropter. These experiments suggest shifts in the underlying juvenile hormone titre, as well as shifts in the juvenile hormone threshold, in response to selection for wing morphology.

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INTRODUCTION

The correlated response of traits to natural or artificial selection can be the result of pleiotropy or linkage disequilibrium. Because pleiotropic effects of genes are common (Lande, 1979; Wright, 1980; Atchley, 1984; Palmer and Dingle, 1986; Stearns et al. 1991), phenotypic and genetic correlations between traits are exhibited frequently. Genetic correlations can constrain evolution by causing co-evolution of suites of traits, and by acting to reduce the divergence of trait values (Lande and Arnold, 1983; Blouin, 1992; Hillesheim and Stearns, 1992). Pleiotropy, therefore, can have profound effects on the evolution of organisms, including maintaining maladaptive traits, retarding the evolution of traits under weak selection, and causing shifts in adaptive peaks for traits under weak selection (Lande, 1980; Price and Langen, 1992; Price et al., 1993).

Migratory tendency in insects is a complex trait, composed of a suite of correlated behavioural, physiological, and morphological traits such as flight propensity or threshold, condition of flight muscles, and wing morphology (Fairbairn and Desranleau, 1987; Fairbairn and Butler, 1990; Fairbairn and Roff, 1990). As such, it provides an excellent model for the study of correlated responses of traits to selection. Wing dimorphism is a common feature of the Homoptera, Hemiptera, Coleoptera, and Orthoptera (Harrison, 1980; Roff, 1986) and involves a macropterous or long-winged morph which may be capable of flight, and a micropterous or short-winged morph which is incapable of flight.

Roff (1986) detailed a model for wing dimorphism, based on the general threshold model for polygenic traits (Southwood, 1961; Wigglesworth, 1961; Falconer, 1981) with

levels of juvenile hormone being the underlying quantitative trait. According to Roff's (1986) model, a normal distribution of hormone titres in a given population (as would be expected for a quantitative trait such as hormone level) could result in macropters or micropters, based on whether their hormone titre was below or above the threshold.

Juvenile hormone is well known to play a role in wing dimorphism (Zera and Tiebel, 1988, 1989; Zera et al. 1989; Zera and Tobe, 1990; and reviewed in Hardie and Lees, 1985). Because juvenile hormone has also been implicated in wing muscle histolysis and flight behaviour (reviewed in Pener, 1985), Fairbairn and Roff (1990) hypothesized that its pleiotropic effects may explain the genetic correlations that have been found between physiological, behavioural, and morphological characters involved in migratory tendency (Fairbairn and Roff, 1990; Roff and Fairbairn, 1991).

The variations in haemolymph juvenile hormone titre implied by the threshold model of Roff (1986) and Fairbairn and Roff (1990) can be the result of differences in the rates of juvenile hormone synthesis, degradation, excretion, or uptake by tissues, with rates of synthesis and degradation as the most likely candidates (Feyereisen, 1985). Juvenile hormone is synthesized by the corpora allata, with changes in rates of synthesis being effected through regulation of these glands (Tobe, 1980; Gilbert et al. 1980). Degradation of juvenile hormone in the haemolymph is primarily the result of the activity of general and juvenile hormone-specific esterases (Gilbert et al. 1980; deKort and Granger, 1981). In addition, rates of degradation may be affected by the presence of juvenile hormone binding proteins, which protect hormone molecules from degradation by general esterases, but may enhance degradation by juvenile hormone-specific esterases

(Sanburg et al., 1975; Abdel-Aal and Hammock, 1988).

The roles of synthesis and degradation of juvenile hormone in the determination of wing morph have been studied in *Gryllus rubens* (Orthoptera; Gryllidae) (Zera and Tiebel, 1989; Zera et al. 1989; Zera and Tobe, 1990; Zera and Holtmeier, 1992). Zera and Tiebel (1989) found higher juvenile hormone esterase activity in individuals from laboratory lines selected for increased percentage macroptery than in individuals from lines selected for decreased percentage macroptery. This *in vitro* difference between lines was confirmed to correspond to an *in vivo* difference in juvenile hormone degradation (Zera and Holtmeier, 1992). Zera et al. (1989) discovered a corresponding difference in overall haemolymph juvenile hormone titre between presumptive macropters and micropters. No difference in juvenile hormone biosynthesis was found between the lines (Zera and Tobe, 1990), suggesting that this process was not important in causing morph-specific differences in juvenile hormone titre. While these results are highly suggestive, the study of Zera and Tiebel (1989) has several weaknesses that preclude definitive conclusions regarding the responsiveness of juvenile hormone esterase to selection. The stock lines used by Zera and Tiebel (1989) were not derived from the same source population and the short-winged stock was initiated using only eight individuals (Walker, 1987), introducing the possibility that the observed differences between short-winged and long-winged crickets were not the result of selection, but were instead the result of genetic drift or a population bottleneck. Further, no control line (line experiencing no selection for wing length) was maintained for comparison with the selected lines, and the lines were not replicated. These problems leave open the possibility that the differences

between the lines were not entirely attributable to the selection regime.

In this study, we test the hypothesis that selection for increased and decreased proportion macroptery in *G. firmus* has resulted in a correlated response in juvenile hormone esterase activity in the selected lines described in Roff (1990) and Fairbairn and Roff (1990). More specifically, we predict that lines with a high proportion of macropters will have higher esterase activity than control lines, which in turn will have higher activity than lines with a low proportion of macropters. The availability of replicated selected and control lines of *G. firmus* (Roff, 1990; Fairbairn and Roff, 1990) in which correlated responses in wing muscle histolysis, flight propensity, and wing morphology have already been demonstrated, affords an excellent opportunity to study the role of JH in these genetic correlations. If juvenile hormone titre has responded to selection for wing length, then JH titre is further implicated as the mechanism by which traits for migratory tendency are correlated, reinforcing Fairbairn and Roff's (1990) hypothesis.

MATERIALS AND METHODS

A. Study Animal

Gryllus firmus is a wing dimorphic cricket native to eastern United States from Florida to New Hampshire (Veazey et al. 1976). Crickets used in these experiments were from lines selected for increased and decreased proportion macroptery. The six lines included short winged (S), long winged (L), and control (C) lines from two concurrent but independent selection experiments (1 and 2). The selection experiments, detailed in Roff (1990) and Fairbairn and Roff (1990), were of a mass design in which each generation was started from 60-80 individuals of each sex. Only macropters were used as parents for the L lines; only micropters were used as parents for the S lines. Parents for the C lines were random in respect to wing morphology. The crickets assayed for esterase activity were in generations 32-36 of experiment 1 and generations 29-32 of experiment 2. The frequency of macroptery in these generations is reported in Table 1.

Rearing methods used in these experiments were similar to Roff (1986). Crickets were raised at 28°C with a photoperiod of 15L:9D at densities of 60 individuals per 4.5 L bucket. Each bucket contained pieces of egg carton for crickets to hide in and climb on. The diet was Purina Rabbit Chow, ground up for the first three larval instars and whole thereafter, fed *ad libitum*, and water was supplied via a cheesecloth wick to a water reservoir below the bucket.

Two different rearing methods were utilized to obtain samples of crickets of known

ages. In the first rearing method, age was determined in the following way. As crickets began molting to the penultimate juvenile instar (identified by appearance of wing buds), rearing pails were checked daily for individuals molting to last instar. Newly-molted last instar crickets were designated Day 1 and were moved to another rearing pail until they were assayed for esterase activity.

The second rearing method was used in order to reduce the effects of stress due to daily checking of the rearing pails. Crickets were raised as described above until individuals of the penultimate instar were observed. Subsequently, twenty individuals from each line were raised in pairs (one male and one female per cage to distinguish between individuals) with water vials, egg cartons, and food. Clear plastic rearing containers (28x17x13cm) with glass lids were used to facilitate observation of the crickets. The molting of individuals to last instar was recorded in order to determine age but crickets were not removed from the cages until the time of haemolymph sampling.

B. Radiochemical Assay of Pratt and Tobe (1974)

The radiochemical assay of Pratt and Tobe (1974) and a subsequent High Pressure Liquid Chromatography (HPLC) analysis were used to confirm that the hormone produced by *G. firmus* corpora allata (CA) is indeed Juvenile Hormone III, one of the forms of juvenile hormone. The assay involves the dissection of the corpora allata from subjects and their subsequent incubation in a medium containing [methyl-¹⁴C] methionine. The radiolabelled methyl group of the methionine is incorporated into products of the corpora

allata during the course of the incubation (see Appendix A).

Two last instar crickets of each sex were chilled on ice to facilitate handling. Under sterile conditions, the corpora allata were dissected under TC 199 tissue culture medium containing 2% Ficoll and 0.0555 % CaCl_2 and placed in a test tube. Cold medium was removed with a pulled pasteur pipette, and 95 μL of hot medium (TC 199 with 0.285 μCi L-[methyl- ^{14}C] methionine, 57mCi/mmol) was added to the test tube. Tubes were covered with Parafilm and incubated on a wrist shaker on low speed for three hours. CA were then removed from the tubes, and 300 μL of HPLC grade isooctane was added to the tubes. Following vortexing and centrifuging at 2000 rpm for 10 minutes, 10 μL of the isooctane was removed and evaporated to dryness under nitrogen, then resuspended in hexane. HPLC of the sample was performed along with methyl farnesoate and juvenile hormone III standards. Methyl farnesoate is the final precursor in the metabolic pathway leading to JH in Orthoptera and Dictyoptera (Schooley and Baker, 1985) and is the recipient of the methyl group from the radiolabelled methionine (see Appendix A). A solvent system of 10% ether in HPLC-grade hexane was pumped through the Spheri 5 silica column at a flow rate of 1 ml/min until a stable baseline was achieved. The hexane sample was then loaded into the sample loop and fractions were collected in 12 second intervals. These fractions were then placed in a scintillation vial with Cytoscint cocktail for scintillation counting.

This experiment is used to identify potential hormones produced by the CA. Peaks in radioactivity in certain fractions of the HPLC indicate the presence of a substance incorporating the radiolabelled methyl group from [methyl- ^{14}C] methionine. If these peaks

correspond to the retention times of the JH and methyl farnesoate standards in the HPLC column, this suggests that the CA are secreting these substances.

C. Hormone Degradation by Haemolymph *in vitro*

i. Thin-Layer Chromatography of Juvenile Hormone Metabolites

Although JH degradation by haemolymph is thought to occur via general and specific esterases (Gilbert et al. 1980; deKort and Granger, 1981), it is necessary to exclude the possibility of epoxide hydrolases having a role in JH titre regulation (Appendix B). To determine that ester hydrolysis by esterases is indeed the route of JH catabolism, thin-layer chromatography was performed in the following manner. One μL haemolymph, from a last instar cricket, was assayed for esterase activity using the radiochemical assay of Hammock and Sparks (1977) described below. Following termination of the reaction, 100 μL of chloroform was added to the sample and vortexed at high speed. The sample was then filtered through glass wool. The supernatant was evaporated to dryness under nitrogen, resuspended in diethyl ether and spotted on a plastic-backed silica-gel plate along with appropriate standards. The plate was run under a solvent system of 10% ethyl acetate in benzene at 30°C until the solvent front had moved 15 cm. The plate was allowed to dry, then run under a second system of 35% ethyl acetate in hexane with a few drops of glacial acetic acid at 30°C until the solvent front had moved 15 cm.

Based on the number of peaks on the TLC plate, thin-layer chromatography can

determine how many catabolites of JH result from the degradative enzymes in cricket haemolymph. These catabolites can then be identified based on the comigration of standards. If our hypothesis that cricket haemolymph degrades JH to JH acid by esterases is correct, then this experiment should reveal two peaks: one corresponding to intact JH, and one to JH acid (see Appendix B). The presence of other peaks would indicate the activity of other degradative enzymes, such as epoxide hydrolases. If other degradative enzymes are present in cricket haemolymph, then the radiochemical assay of Hammock and Sparks (1977) will not accurately measure JH degradation by haemolymph.

ii. Time Course Experiment

To confirm that degradation of juvenile hormone by esterases is linear over time and does not plateau within the 1 hour incubation time used in the Hammock and Sparks (1977) assay, the general radiochemical assay as described below was performed with the following modifications. From each of two last instar crickets, one μL of haemolymph was sampled and blown into 1 mL of phosphate buffer (PBS). These tubes were then subsampled into nine tubes of 100 μL each. These subsamples were used in the radiochemical assay. Reactions were terminated after 2min, 5min, 10min, 15min, 30min, 45min, 60min, 90min, and 120min of incubation.

iii. BEPAT Study of Esterase Specificity

Although both general and JH-specific esterases may be present in cricket haemolymph, only JH-specific esterase has a possible role in regulation of JH titre, as JH

is protected from the catalytic activity of general esterases by the JH binding protein *in vivo* (Sanburg *et al.*, 1975; Abdel-Aal and Hammock, 1988). To elucidate whether JH catabolism by haemolymph *in vitro* is the result of general esterase activity or JH-specific esterase activity, the following experiment was undertaken. One μL of haemolymph was taken from each of two last instar crickets and placed in 1 mL of phosphate buffer (PBS). Six 100 μL subsamples were then taken. Two tubes were placed in a boiling water bath for 10 minutes to denature any enzymes in the sample. One μL of 0.1 M *s*-benzyl-*o*-ethyl phosphoramidothiolate (BEPAT), a JH-specific esterase inhibitor (Sparks and Hammock, 1980) was added to two of the tubes. The remaining two tubes were untreated. Because BEPAT is a JH-specific esterase inhibitor but does not usually inhibit general esterases, similar activities in the BEPAT treatment and the heat treatment, as measured by the radiochemical assay of Hammock and Sparks (1977) detailed below, will indicate that the cricket haemolymph esterases that degrade JH are JH-specific esterases rather than general esterases. The hypothesis of esterases being JH-specific will be rejected if esterase activity is high in the BEPAT treatment (ie. higher than the heat treatment). The specificity of the enzyme is important to establish, as only JH-specific esterase activity differences *in vitro* will be important *in vivo* determinants of JH titre (Sanburg *et al.*, 1975; Abdel-Aal and Hammock, 1988).

iv. α -Naphthyl Acetate Competitive Inhibitor Study

To further study the specificity of the haemolymph esterases, a competitive inhibitor experiment was undertaken. Alpha-naphthyl acetate (α -NA) is a general esterase

substrate, and as such should inhibit the degradation of JH by general esterases, but not by JH-specific esterases. One μL of cricket haemolymph was collected from each of two last instar crickets as in the general procedure described below and divided into 12 subsamples (4 treatments \times 3 incubation times). These subsamples underwent 1 of 4 treatments (addition of no $\alpha\text{-NA}$, $1.32 \times 10^{-3} \mu\text{g } \alpha\text{-NA}$, $3.96 \times 10^{-3} \mu\text{g } \alpha\text{-NA}$, or $11.9 \times 10^{-3} \mu\text{g } \alpha\text{-NA}$) for 1 of 3 incubation times (10min, 60min, 160min).

D. Esterase Activity in Selected Lines

The assay of Hammock and Sparks was used to quantify juvenile hormone (JH) esterase activity in haemolymph. Insects were first cooled on ice to slow them down and facilitate haemolymph sampling. Haemolymph was taken from 20 crickets (10 males, 10 females) of each line for each of days 1,3,5,7, and 9 of the last juvenile stadium, during which wing morph is thought to be determined (Zera and Tiebel, 1988; Zera and Tiebel, 1989; Zera et al. 1989). For rearing method 2, crickets were sampled on one day only (Day 5) of the last juvenile stadium. The tip of one cercus was cut and 0.5 or 1.0 μL of haemolymph was collected using a graduated microcapillary pipette (differences in sample volume were corrected for in final calculations of esterase activity). The haemolymph was blown into 500 μL of 0.1 M phosphate buffer (PBS) (pH 7.4) with 0.01% phenylthiocarbamide to inhibit tyrosinases (Hammock and Roe, 1985). The sample was vortexed slowly and two 100 μL subsamples were taken and placed in polyethylene glycol-treated 10x75 mm glass tubes. The following procedures were performed in

duplicate.

The 100 μL subsample was allowed to warm to 30°C. One μL of juvenile hormone substrate was then added to the tube. This substrate was composed of 0.133 μg JH III, plus 35,000-40,000 DPM [$10\text{-}^3\text{H}$] JH III (17.0 Ci/mmol) in ethanol. The tube was vortexed slowly and allowed to incubate at 30°C for 1 hour. The reaction was then terminated by adding 50 μL of MeOH/H₂O/NH₃ (10:9:1) and vortexed at medium speed for 10 seconds. For phase separation, 250 μL of isooctane was added to the sample and vortexed at medium speed for 20 seconds. The sample was then centrifuged at high speed in a table-top centrifuge for 6 minutes, and the organic phase was removed with a pasteur pipette. A second extraction was performed in the same manner. Two hundred and fifty μL of isooctane was added a third time to wash the sides of the tube. The organic phase was again removed, to minimize the chance of phase mixing. Using a micropipette, 100 μL of aquatic phase was removed and placed in 5 ml of CytoScint scintillation fluid for counting.

Following haemolymph sampling, crickets were raised individually in 200 ml cages with a water vial and Purina Rabbit Chow, until the subsequent molt to adult, at which time wing morph was recorded.

RESULTS

A. Study Animal

Upon molting to adult, the morph of each cricket was recorded (Table 1). As expected, long-winged lines produced the most macropters, while lines selected for decreased percentage macroptery had the lowest percentage of macropterous individuals. There were differences between the results of rearing methods 1 and 2, however. In rearing method 1, all lines produced fewer macropters than expected based on the source stock (Table 1). Rearing method 2 resulted in long-winged lines again producing the highest percentage macropterous, although the proportion dropped in L1 and increased in L2. For control line crickets, percentage macroptery was significantly higher in rearing method 2 than in rearing method 1 (C1: $\chi^2 = 5.83$, $df=1$, $p<0.05$; C2: $\chi^2 = 8.23$, $df=1$, $p<0.01$). Crickets from L1 lines molted to micropter more often than expected, but all other lines did not differ from stock crickets in percentage macroptery (Table 1). These results indicate that rearing method plays a role in the proportion of macropterous individuals in the selected lines.

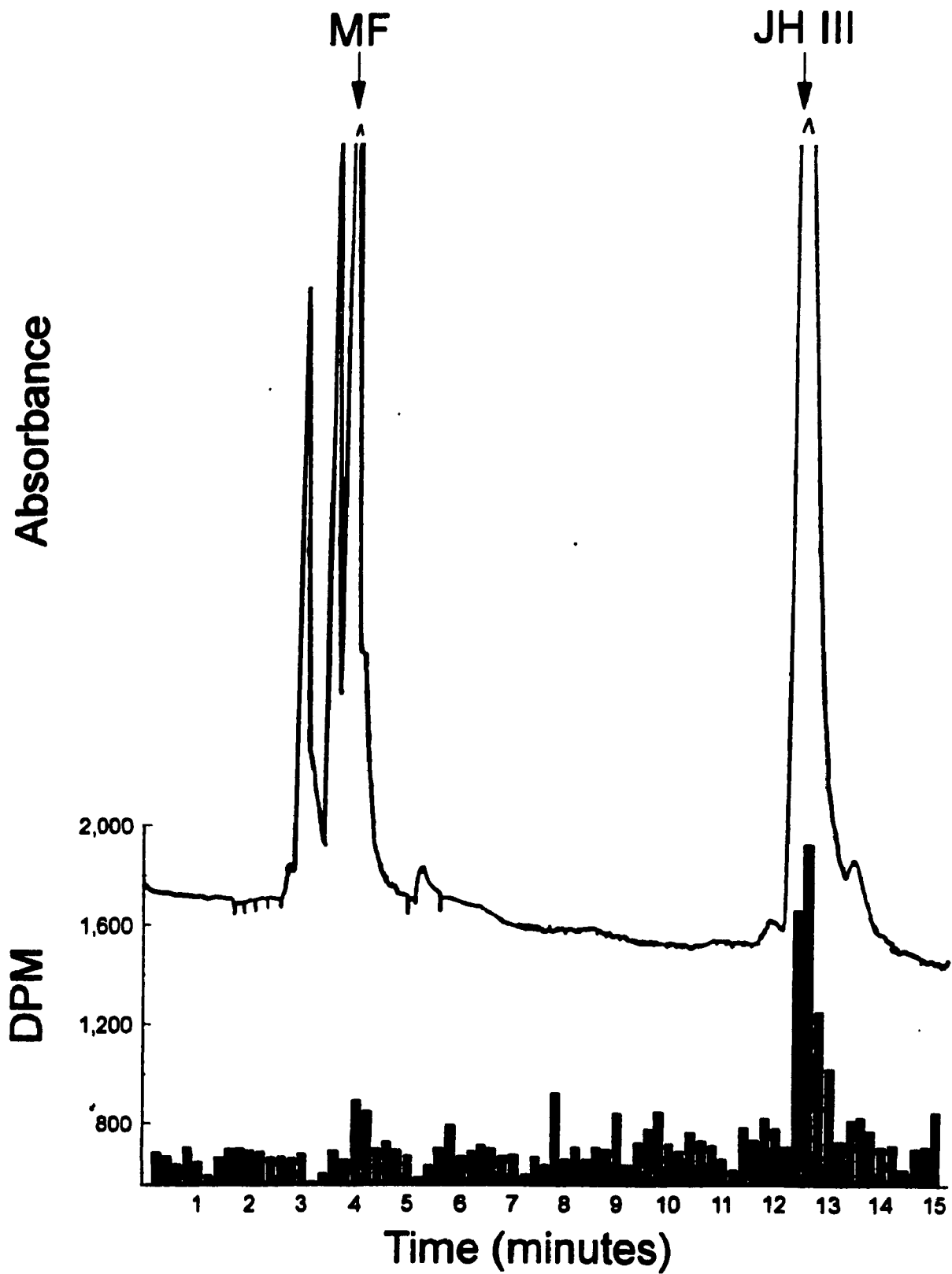
B. Radiochemical Assay of Pratt and Tobe (1974)

The methyl farnesoate standard came off the column at 3.9 minutes (Fig. 1). The JH III standards came off at 12.42 minutes ($n=2$; 12.42, 12.43 min) (Fig. 1). The iso-octane extract from the incubation medium containing female corpora allata revealed

TABLE 1. Percentage macroptery for crickets used in esterase activity experiments for both rearing methods 1 and 2, and percentage macroptery of stock lines during the experimental period. Abbreviations are as follows: L: lines selected for increased percentage macroptery; C: control lines (unselected); S: lines selected for decreased percentage macroptery; 1: selection experiment 1; 2: selection experiment 2. Sample sizes are in parentheses. Percentage macroptery for stock lines indicates typical values for selected lines during generations 32-36 of experiment 1 and 29-32 of experiment 2. Superscripts indicate significant differences in percentage macroptery between assayed crickets and stock lines, as determined by χ^2 tests (a: $p < 0.05$; b: $p < 0.025$; c: $p < 0.005$).

Line	Percentage Macroptery		
	Method 1	Method 2	Stock
L1	71.6 (67) ^c	45.5 (11) ^c	91
L2	72.5 (40) ^c	81.8 (11)	88
C1	11.4 (79) ^c	40.0 (10)	45
C2	11.1 (63) ^c	42.9 (14)	55
S1	0 (74) ^a	0 (12)	5
S2	0 (64) ^b	0 (13)	9

FIGURE 1. High pressure liquid chromatography (HPLC) of metabolites in isooctane from the radiochemical assay of female corpora allata. Time of retention (in minutes) in the HPLC column is along the x axis. Line traces are HPLC traces of absorbance of JH III and methyl farnesoate standards versus time. Methyl farnesoate peaks at 3.9 minutes (n=1); JH III peaks at 12.4 minutes (n=2). Bars represent radioactivity in disintegrations per minute (DPM) in each 12 second fraction versus time. Radioactivity indicates the presence of a substance incorporating ^{14}C -methionine during the 3 hour incubation period.

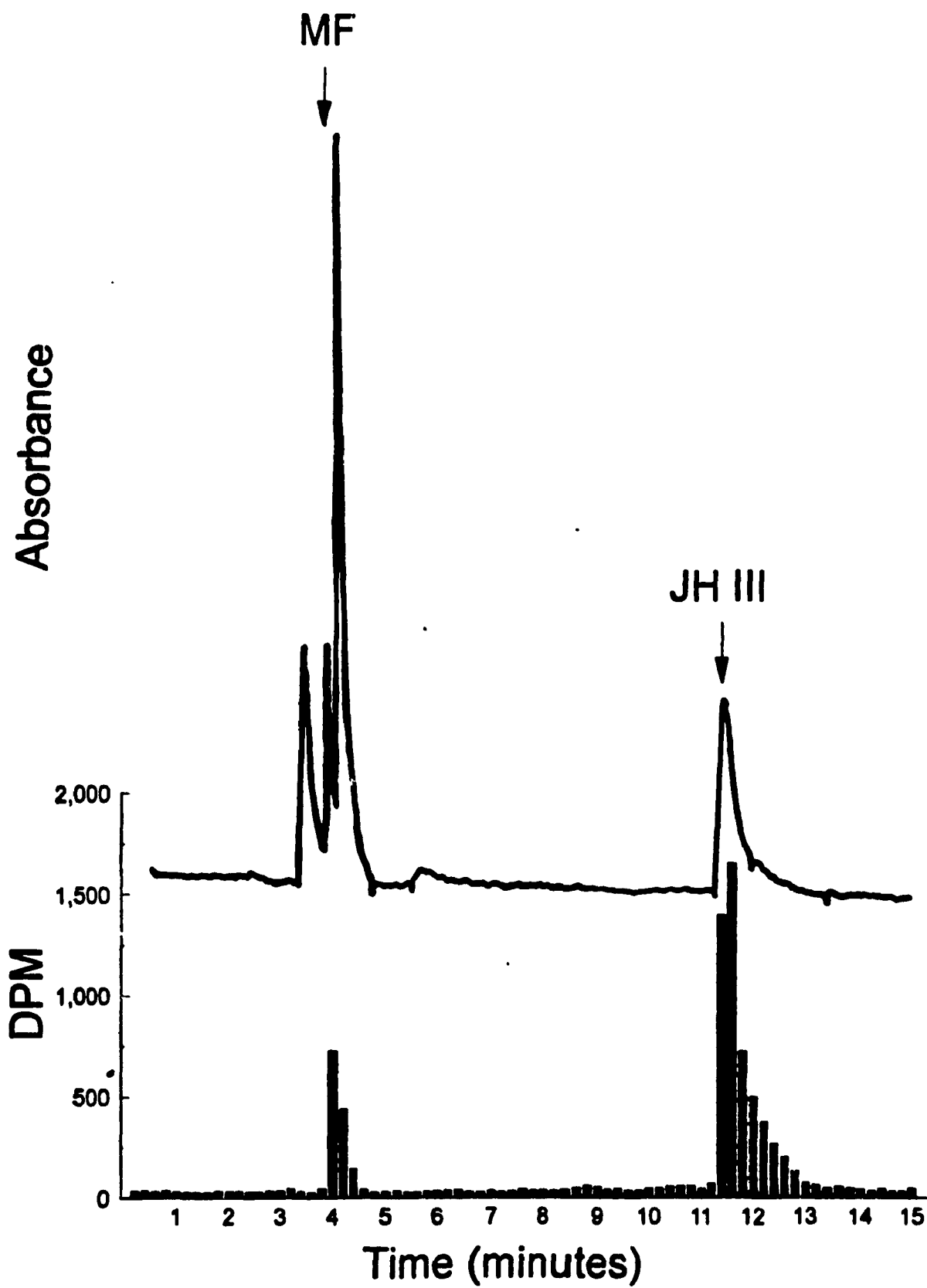


peaks on the HPLC column at 4.33, 5.62, 8.05, and 12.38 minutes (not shown in Fig. 1). Two of these peaks correspond with the standards. Following liquid scintillation counting, we found that 28.4% of the ^{14}C coeluted with these two peaks, indicating that the female corpora allata secreted methyl farnesoate and juvenile hormone III into the isooctane sample (Fig. 1).

Because high background radiation was found in all aliquots, male corpora allata extracts and standards were re-run on the HPLC on another day. The methyl farnesoate standards came off the HPLC column at 3.89 minutes ($n=3$; 3.88, 3.88, 3.90 min) (Fig. 2). The JH III standards came off at 11.33 minutes ($n=3$; 11.22, 11.35, 11.43 min) (Fig. 2). Peaks in the isooctane sample of the radiochemical assay for male corpora allata were found at 4.2, 5.43, 7.62, and 11.35 minutes (not shown in Fig. 2). Two of these peaks correspond with the standards. Following liquid scintillation counting, we found that 56.5% of ^{14}C coeluted with these two peaks, indicating that the male corpora allata secretion contained methyl farnesoate and juvenile hormone III into the isooctane sample (Fig. 2).

Because two radioactive products having the same retention time in the HPLC column as the methyl farnesoate and JH III standards were recovered from the isooctane phase, we can conclude that JH III is being produced by the CA of female and male crickets. Methyl farnesoate, the metabolic precursor of JH III in Orthopterans (Schooley and Baker, 1985), is also secreted during *in vitro* incubation, but to a lesser degree (Fig. 1, Fig. 2).

FIGURE 2. High pressure liquid chromatography (HPLC) of metabolites in isooctane from the radiochemical assay of male corpora allata. Time of retention (in minutes) in the HPLC column is along the x axis. Line traces are HPLC traces of absorbance of JH III and methyl farnesoate standards versus time. Methyl farnesoate peaks at 3.88 minutes (n=3); JH III peaks at 11.3 minutes (n=3). Bars represent radioactivity in disintegrations per minute (DPM) in each 12 second fraction versus time. Radioactivity indicates the presence of a substance incorporating ^{14}C -methionine during the 3 hour incubation period.



C. Hormone Degradation by Haemolymph *in vitro*

i. Thin-Layer Chromatography of Juvenile Hormone Metabolites

Thin-Layer Chromatography (TLC) was used to establish the number of products resulting from the breakdown of JH. Peaks in radioactivity were found in TLC slices at 6 cm and 11 cm from the origin (Table 2). These peaks correspond with the migration of the JH acid standard and the JH III standard, respectively. These results confirm that juvenile hormone III is broken down to JH acid by acid hydrolysis (see Appendix B). Peaks representing JH diol or JH acid diol are absent, indicating that esterase is the only catabolic enzyme breaking down JH III in the cricket haemolymph.

ii. Time Course Experiment

Catabolism of juvenile hormone by esterases remained linear with time and did not plateau within 120 minutes after the start of incubation, at which time 45% of radiolabelled JH III was converted to JH acid (Fig. 3).

iii. BEPAT Study of Esterase Specificity

Cricket esterase activity in haemolymph differed among treatments (ANOVA: $F=125.6$; $df=2,3$; $p<0.005$) (Fig. 4). Esterase activity in controls was found to be higher than that of heat-treated samples (Student Newman-Keuls: $p<0.005$) and higher than that of BEPAT-treated samples (SNK: $p<0.005$) (Fig. 4). BEPAT- and heat-treated samples are not significantly different (SNK: $p>0.1$). These results indicate that BEPAT is as

TABLE 2. Results of thin-layer chromatography (TLC) of juvenile hormone metabolites following incubation with last instar cricket haemolymph in PBS. The distance migrated is in cm from the origin. The DPM in the slice reflects radioactivity of the TLC plate in that region. Asterisks represent migration of the JH acid standard (*) and the JH standard ()** on the same TLC plate.

Distance Migrated	DPM in Slice	Distance Migrated	DPM in Slice
1	360	9	128
2	162	10	184
3	91	11 **	10363
4	101	12	1126
5	82	13	144
6 *	1899	14	31
7	279	15	55
8	153		

FIGURE 3. Results of the time course experiment for degradation of JH to JH acid versus time by JH esterase from last instar cricket haemolymph samples. Points (\square) represent mean values for two replicates; error bars represent one standard error. In cases for which no error bar is shown, one standard error did not exceed the symbol. Line represents results of linear regression analysis; $r^2 = 0.978$; $df = 1,7$; $p < 0.0005$.

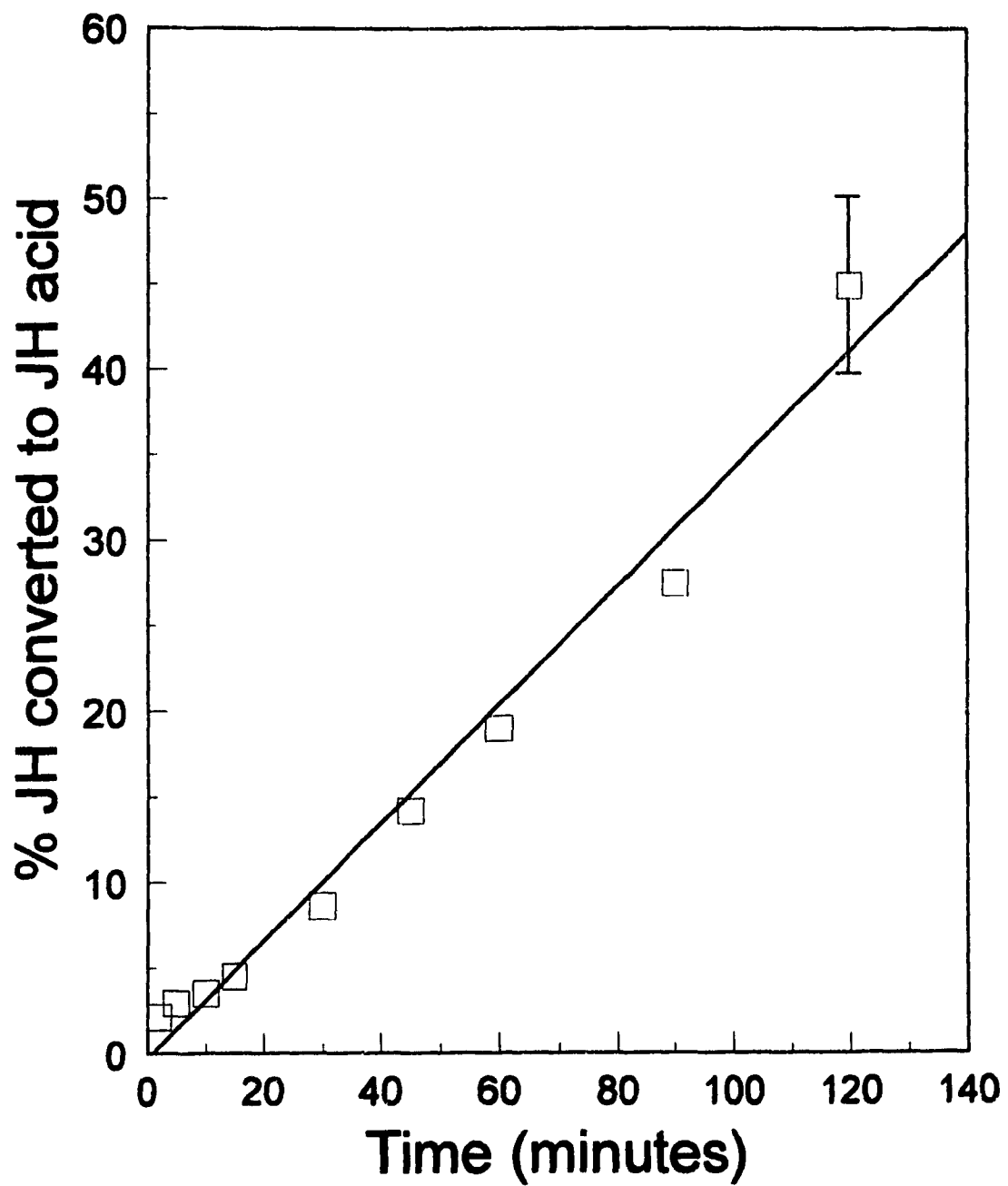
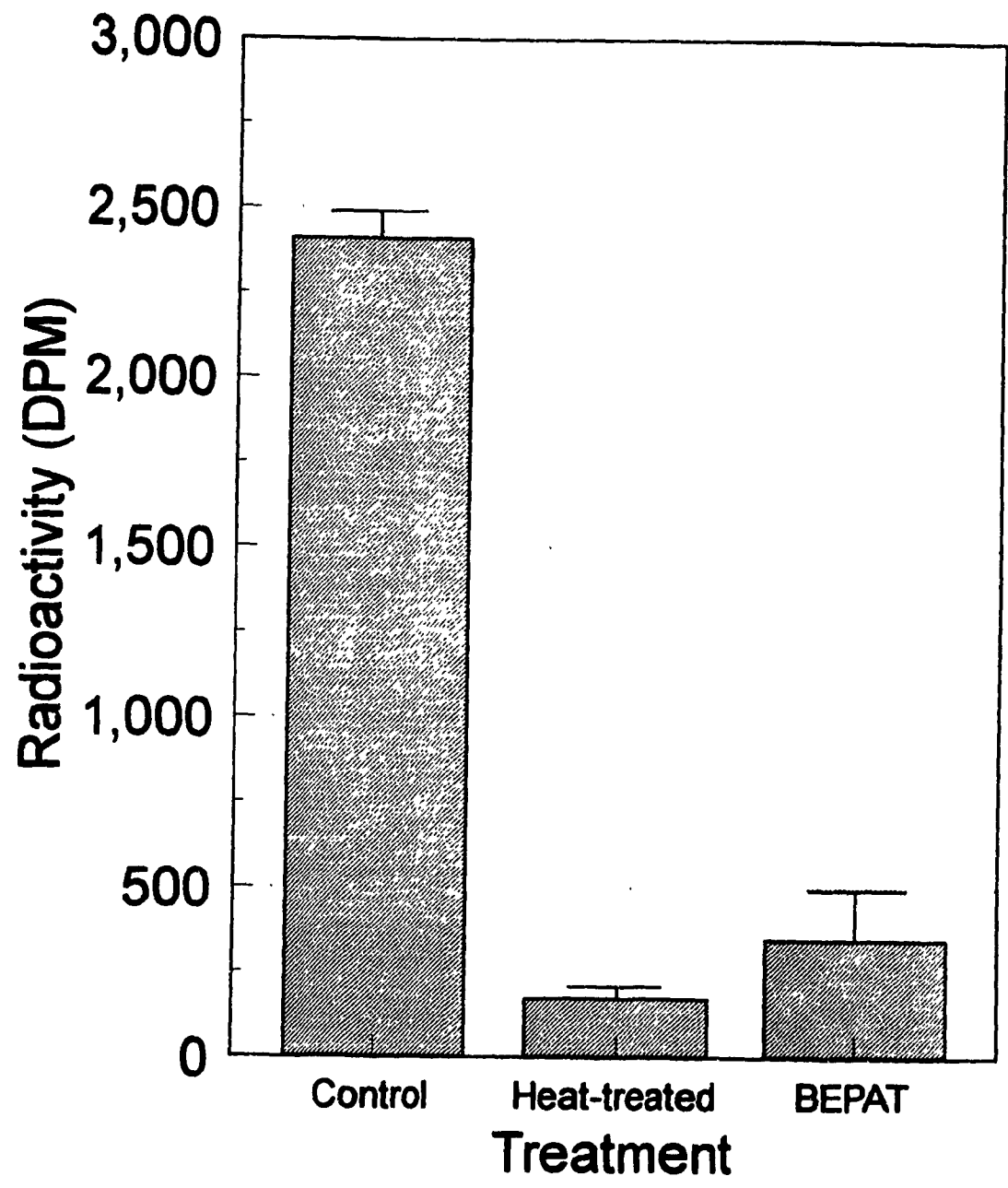


FIGURE 4. Esterase activity in haemolymph samples undergoing three treatments (see text). Bars represent mean radioactivity in disintegrations per minute (DPM) in aquatic phase for two replicates. Error bars represent one standard error. Radioactivity in the aquatic phase indicates degradation of radiolabelled JH to radiolabelled JH acid, and is therefore a measure of esterase activity in haemolymph.



effective as heat-killing in inactivating haemolymph JHEs. Since BEPAT is an inhibitor of JH-specific esterases (Sparks and Hammock, 1980), this suggests that the enzymes in haemolymph are predominantly JH-specific, rather than general esterases.

iv. α -Naphthyl Acetate Competitive Inhibitor Study

ANOVA revealed significant increase in degradation over time ($F=1293.4$; $df=2,11$; $p<0.0005$), with a treatment effect ($F=11.8$; $df=3,11$; $p<0.005$), and a significant interaction between time and treatment ($F=9.5$; $df=6,11$; $p<0.005$) (Fig. 5). These observations indicate that there are general esterases present in cricket haemolymph, as adding a competitive inhibitor resulted in lower rates of JH III degradation *in vitro*. However, when the control was excluded and ANOVA was used to see if the three α -naphthyl acetate treatments differed, no effect of increased concentration of α -naphthyl acetate was found ($F=0.036$; $df=2,8$; $p>0.9$). In addition, a higher esterase activity was only evident in the control group after 160 min of incubation. These results suggest that there is relatively little degradation of JH by general esterases *in vitro*.

D. Esterase Activity in Selected Lines

Haemolymph esterase activity varied with day, with a peak at Day 5 exhibited in all lines (Fig. 6). The effect of day was highly significant ($F=116.9$; $df=4,595$; $p<<0.0001$). ANOVA done by day and experiment revealed no significant differences in esterase activity between males and females and no interaction between sex and line when

FIGURE 5. Radioactivity (in DPM), for four haemolymph treatments with varying concentrations of α -naphthyl acetate, versus time (in minutes). Points represent means of two replicates. Error bars represent \pm one standard error. Treatments include no α -naphthyl acetate added to haemolymph sample (■); 1.32×10^{-3} μg α -naphthyl acetate added (◆); 3.96×10^{-3} μg α -naphthyl acetate added (●); and 11.9×10^{-3} μg α -naphthyl acetate added (★).

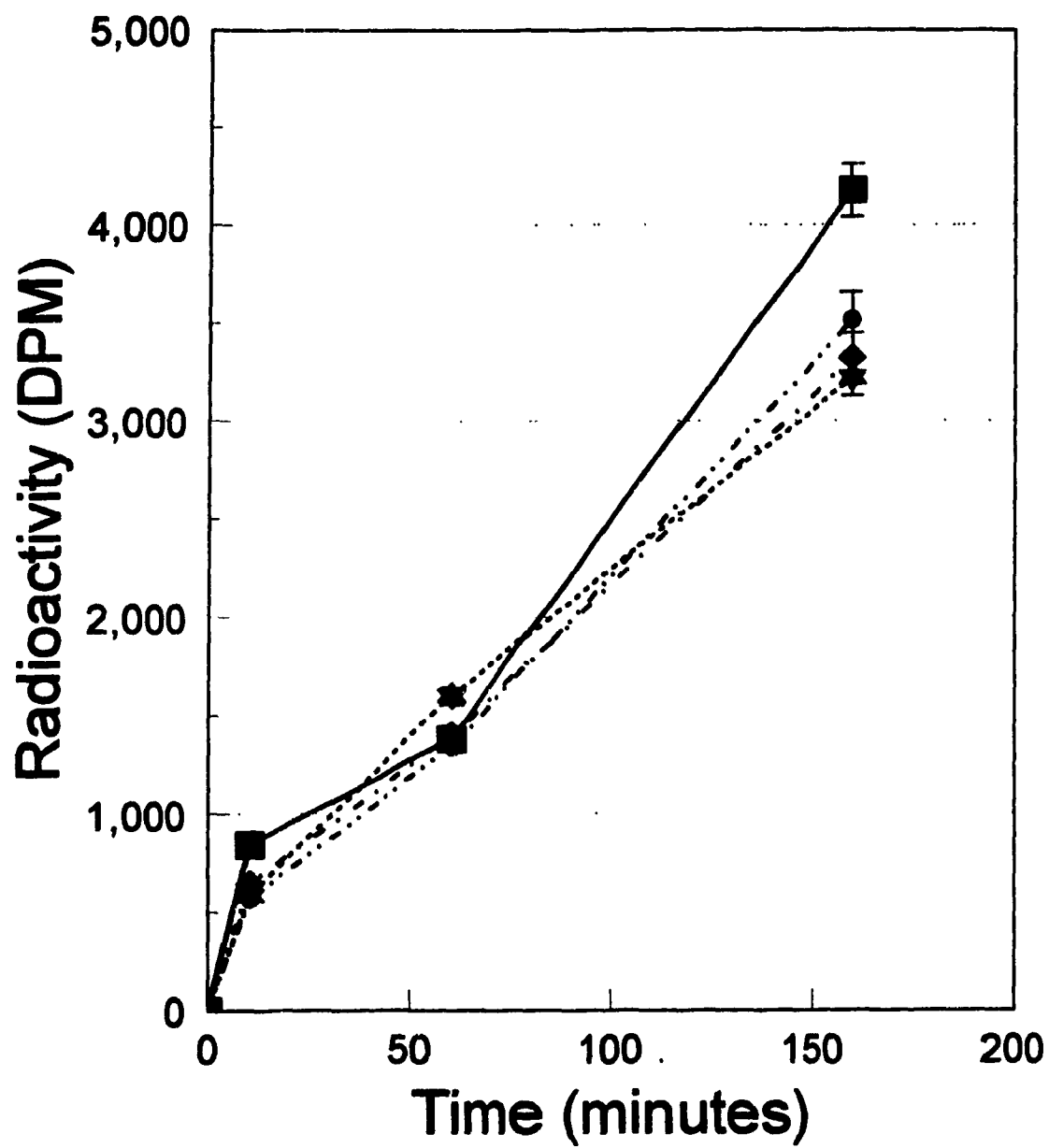
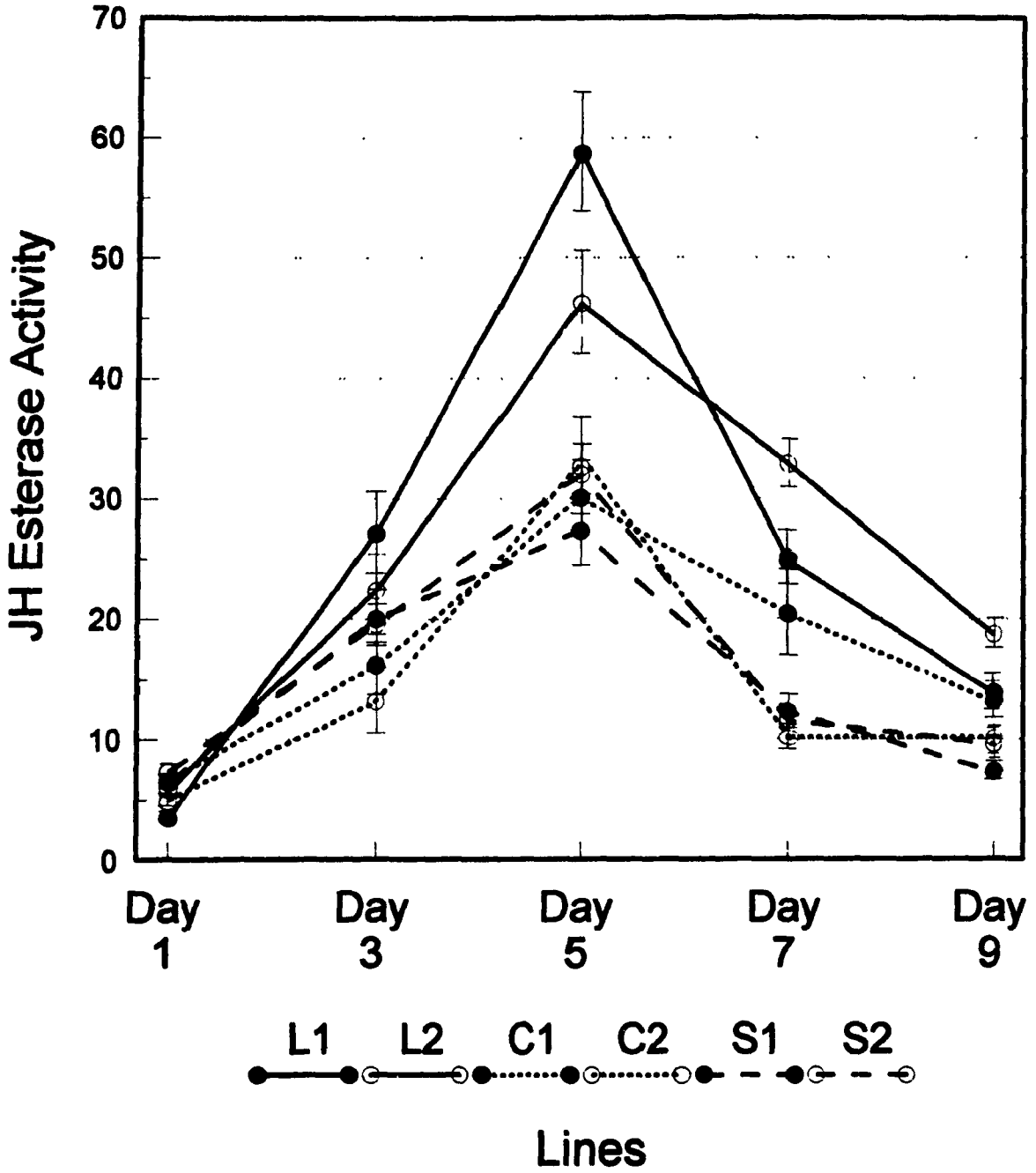


FIGURE 6. Activity of JH esterase in last instar cricket haemolymph for selected and control lines of rearing method 1 versus age. Activity is measured in nmol of JH converted to JH acid per minute per mL of haemolymph. Error bars represent \pm one standard error. Sample sizes are $n=20$ for each line for each day.



α was corrected for multiple comparisons. Data from males and females were subsequently combined in all analyses.

To establish whether the selection experiment from which the crickets came significantly affected haemolymph JHE, nested ANOVAs were done for each day. Only days 1 and 7 had significant effects of experiment within line. Data from the two selection experiments were therefore combined for days 3, 5, and 9.

ANOVA revealed a significant effect of line on all days of the last instar (Table 3). One-tailed Dunnett's tests were then performed *a posteriori* on the data, combining experiments on days that had no significant difference between experiments. It was found that L lines differed from C lines 5 of 7 times, 4 times in the hypothesized direction (Table 3). The one time when esterase activity was significantly lower in L lines than in the C lines was on day 1 for selection experiment 1. Activities for all lines on this day were low compared with esterase activities near the middle of the instar (Fig. 6), suggesting that this statistically significant difference in JHE may not have a biological significance. On days with relatively high *in vitro* degradation of JH III (days 3,5,7,9), the L lines had significantly higher esterase activity than the C lines, except day 7 experiment 1, when esterase activity was higher in L lines, but not significantly so (Table 3).

Also from the Dunnett's tests, C lines were found to differ from S lines in 3 of 7 cases, day 1 experiment 2, day 7 experiment 1, and day 9 (Table 3). The result for day 1 was again in the opposite direction of that hypothesized, and its biological significance is doubtful, since activities on this day are so low relative to other days in the instar. The

TABLE 3. Analysis of Variance and Dunnett's Test results for comparisons of esterase activity among selected and control lines of crickets. Data from the two selection experiments are combined on days 3,5,and 9 of rearing method 1, and for rearing method 2. Dunnett's tests are one-tailed. Asterisks represent significant differences in the opposite direction to that hypothesized; these tests are two-tailed.

	ANOVA Results			Dunnett's Test	
	F	df	p	L to C	C to S
Rearing Method 1					
Day 1 Exp 1	20.0	2,57	<0.0005	<0.0005*	0.496
Day 1 Exp 2	11.5	2,57	<0.0005	0.061	<0.0005*
Day 3	7.49	2,117	0.001	<0.0005	0.092
Day 5	23.3	2,117	<0.0005	<0.0005	0.431
Day 7 Exp 1	5.62	2,57	0.006	0.202	0.033
Day 7 Exp 2	78.4	2,57	<0.0005	<0.0005	0.688
Day 9	16.4	2,117	<0.0005	<0.001	0.021
Rearing Method 2					
	17.8	2,94	<0.0005	0.092	<0.0005

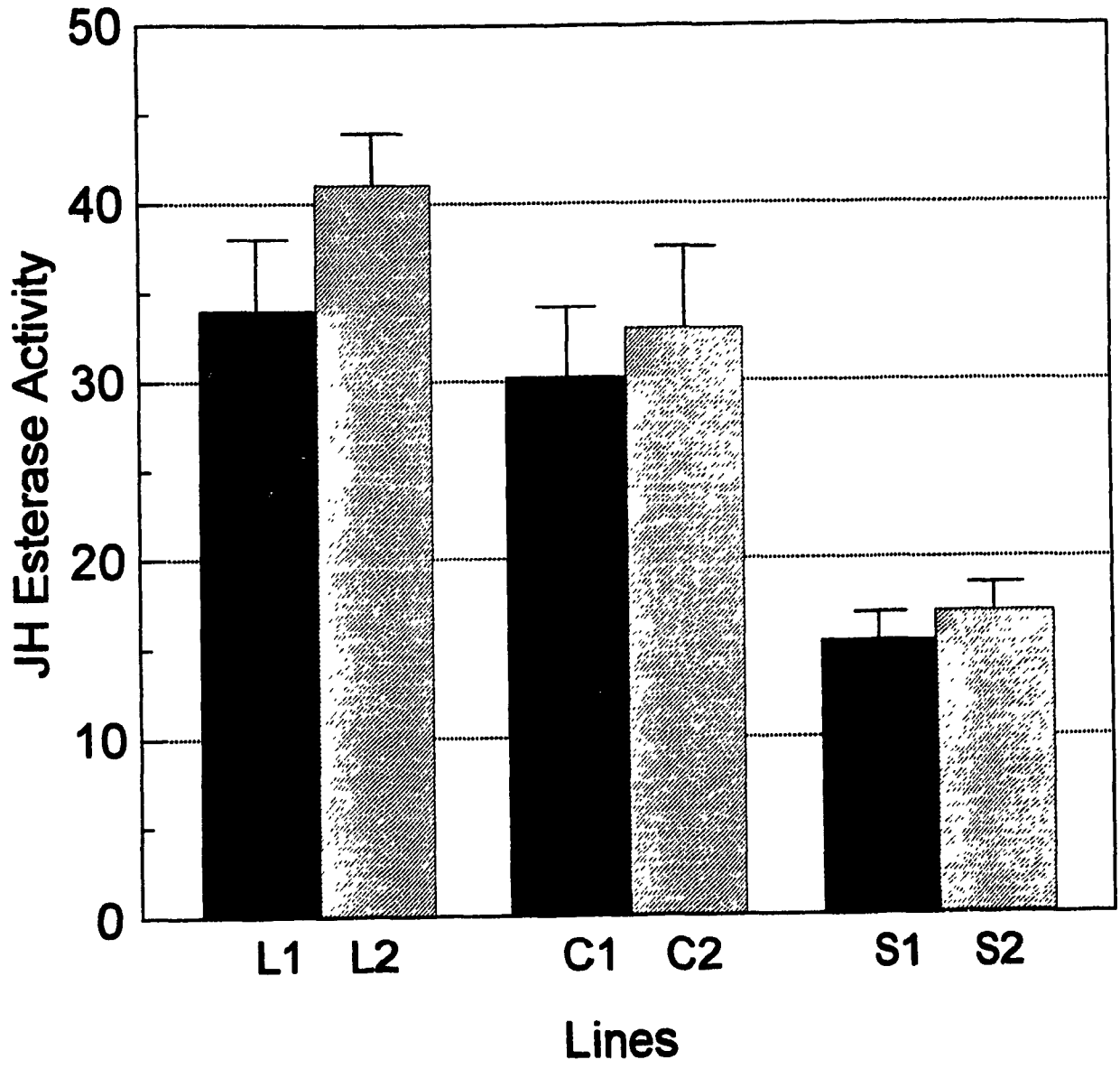
significant differences between C and S lines on day 7 experiment 1 and on day 9 were in the hypothesized direction, with S line crickets having lower esterase activity than control crickets.

The lack of difference in JHE between control lines and lines selected for decreased incidence macroptery on days 3 and 5 was unexpected. However, control lines also produced very few macropters (Table 1), which is consistent with the hypothesis that high JH titres (resulting from low JHE activity) produce micropters, but not consistent with expectations based on percentage macroptery of the source stocks. Because this experiment was conducted to investigate possible JHE differences between lines with different percentage macroptery, rearing method 2 was employed to bring the percentage macroptery back to within expectations.

Crickets raised under rearing method 2 were sampled on day 5 only, since JHE activity was highest on this day (Fig. 6). Results of this rearing method were similar to those of rearing method 1 (Fig. 7). ANOVA again found no significant difference in JH esterase activity between males and females ($F=3.175$; $df=1,91$; $p=0.078$) and no significant interaction between sex and line.

ANOVA revealed no significant effect of experiment ($F=1.49$; $df=1,95$; $p>0.2$), and these two sets of data were therefore combined for further analysis. Esterase activity differed significantly among lines (Table 3). One-tailed Dunnett's tests were employed to make multiple comparisons between selected lines and control lines (Table 3). Significant differences were found between S lines and C lines, with S lines exhibiting lower esterase activity in haemolymph samples (Table 3). However, esterase activity in

FIGURE 7. Activity of JH esterase in last instar cricket haemolymph for selected and control lines of rearing method 2. Activity is measured in nmol of JH converted to JH acid per minute per mL of haemolymph. Error bars represent one standard error. Sample sizes are as follows: L1: n=16; L2: n=15; C1: n=18; C2: n=18; S1: n=16; S2: n=14.



the L lines was not significantly higher than that of C lines (Table 3), although it was higher, as hypothesized.

Crickets were individually raised after haemolymph sampling until they molted to adult. This afforded the opportunity to investigate the esterase activity in macropters and micropters within the same line. The power of esterase activity and line in predicting molt outcome was studied using logistic regression with the statistical package SPSS 4.1.

As reported above, data for rearing method 1 included data for 5 different days of the last juvenile instar, with a significant day effect ($p < 0.0001$). The esterase data for all lines were standardized to a mean of zero and sd of 1 for each day. This procedure removes the variance in esterase activity due to day, but preserves the variance among lines, and allowed us to combine data from all five days, resulting in a sample size of 387 successfully-molting crickets. In fitting logistic regression models to the data set and using likelihood ratio tests, we evaluated the significance of standardized esterase activity and line as independent variables predicting molt outcome. Comparison of models with and without standardized esterase activity revealed a significant effect of JHE activity on molt outcome (model improvement χ^2 : 110.921, $df=1$, $p < 0.0001$). The percent correct classification of molt outcomes was 80.88%. This model was then compared to the model including line as a parameter (dummy-coded). The improvement over the previous model (the difference in the $-2 \cdot \log$ likelihood ratios) was significant (model improvement χ^2 : 155.804, $df=2$, $p < 0.0001$) indicating that the line from which the crickets came was useful in predicting molt outcome over and above the effect of JHE. The percent correct classification of molt outcomes using this new model was 92.51%. Adding interaction

terms to the model increased the percent correct classification to 93.54%, and the model improvement was again significant (improvement χ^2 : 9.519, $df=2$, $p=0.0086$). The molt outcomes as predicted by this model, plotted against standardized esterase activity for the different lines, are diagrammed in Fig. 8. The standardized esterase value at which 50% of L line crickets are predicted to molt to micropter is -0.337; the standardized esterase value at which 50% of C line crickets are predicted to molt to micropter is 2.141. The regression coefficients reduced the equation predicting probability molting to micropter to $y=1$ for the S line (due to all S line crickets molting to micropters - see Table 1). The esterase value at which 50% of S line crickets would molt to micropters must therefore be greater than 2.977 (Fig. 8). The equations predicting molt outcome are shown for L and C lines only.

For rearing method 2, with a much smaller sample size ($n=71$), we again evaluated the significance of standardized esterase activity and line in predicting molt outcome. Comparison of models with and without standardized esterase activity revealed that this parameter was significantly different from zero (model improvement χ^2 : 49.40, $df=1$, $p<0.0001$). The percent correct classification of molt outcomes was 85.92%. This model was then compared to the model including line as a parameter (dummy-coded). The improvement of the new fitted regression model over the previous model was significant (model improvement χ^2 : 6.070, $df=2$, $p=0.0481$) indicating that the line from which the crickets came was again useful in predicting molt outcome over and above the effect of JHE. The percent correct classification of molt outcomes using this model was 87.32%. Adding interaction terms to the model did not increase the percent correct

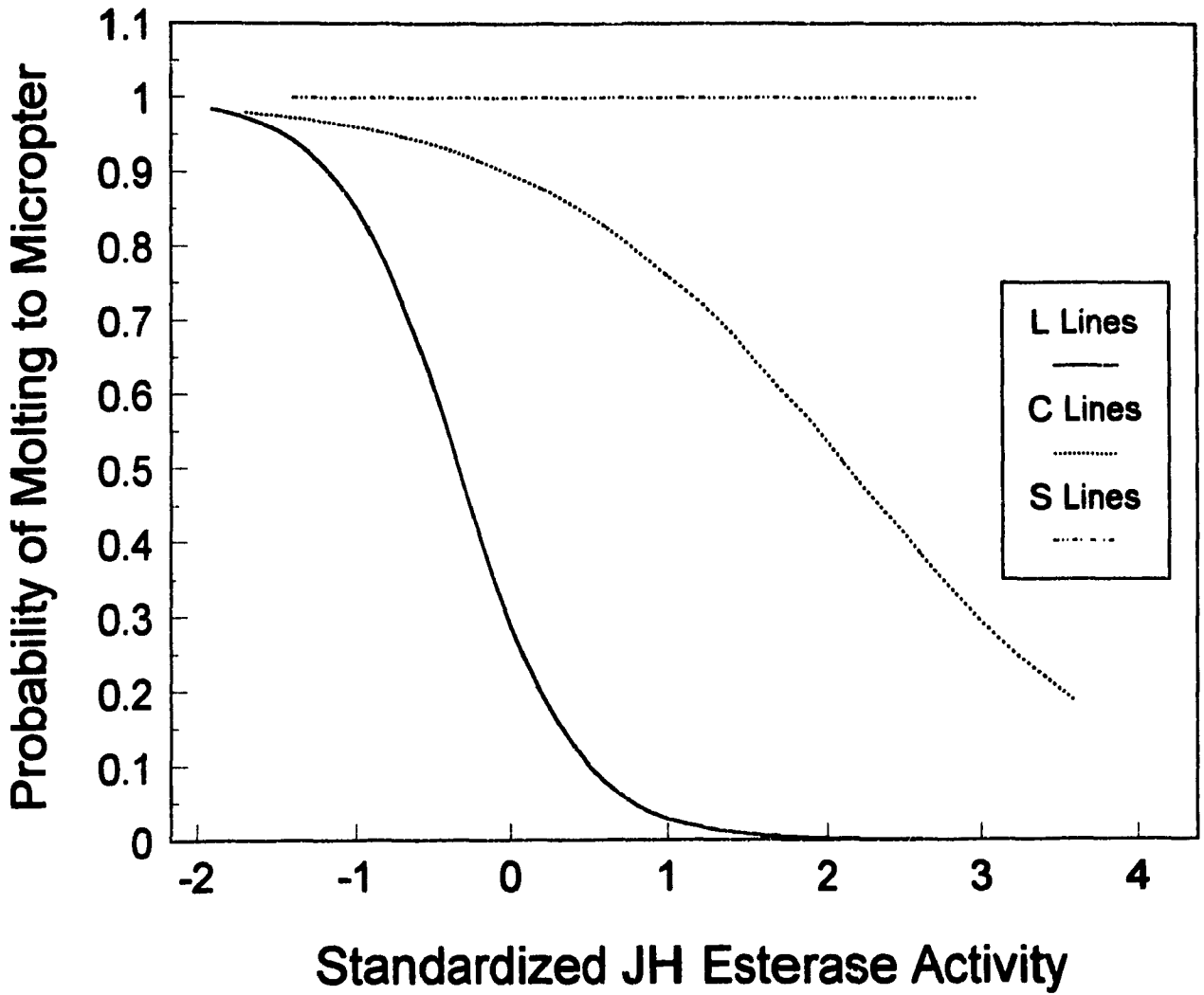
FIGURE 8. Probability of molting to micropter as a function of standardized JH esterase activity (see text) and line for rearing method 1. Curves represent the logistic regression model. Ranges and means of esterase activity were as follows: S lines: -1.414 to 2.977 (-0.186 ± 0.794 , $n=200$); C lines: -1.647 to 3.547 (-0.224 ± 0.961 , $n=200$); L lines: -1.873 to 3.922 (0.410 ± 1.088 , $n=200$). Logistic regression models were as follows:

L lines:

$$Prob_{(mic)} = \frac{1}{1 + e^{(2.6352x + 0.6873)}}$$

C lines:

$$Prob_{(mic)} = \frac{1}{1 + e^{(1.0134x - 2.1696)}}$$



classification, and the model was not significantly improved (model improvement χ^2 : 0.172, $df=2$, $p>0.9$). The molt outcomes as predicted by this model, plotted against esterase activity for the different lines, are diagrammed in Fig. 9. As in rearing method 1, the esterase value at which 50% of crickets are predicted to molt to micropter was lower for the L lines than the C lines (0.029 as compared to 0.528) (Fig. 9).

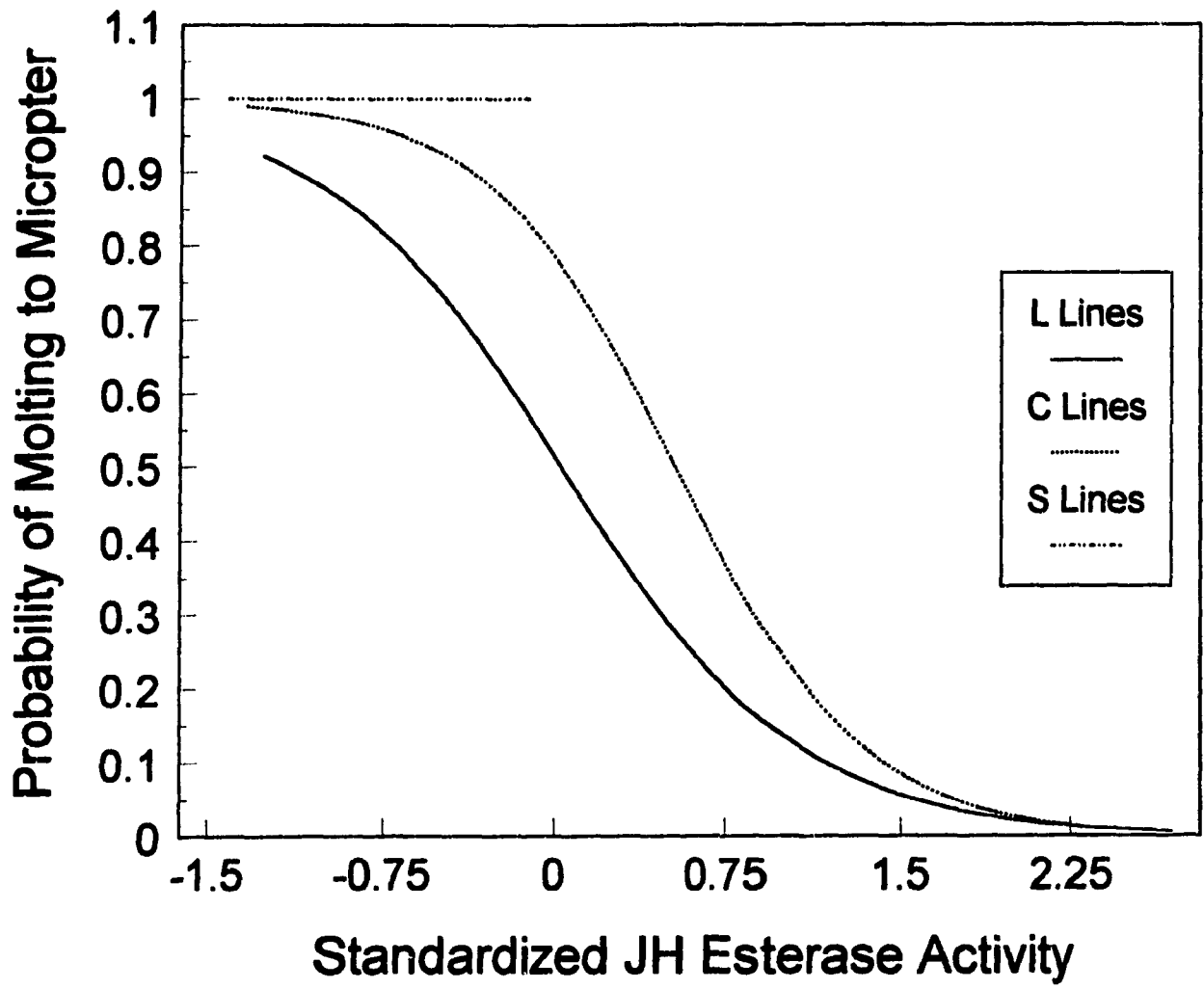
FIGURE 9. Probability of molting to micropter as a function of JH esterase activity for lines of rearing method 2 (low stress). JH esterase activity is in scores standardized to a mean of 1 and standard deviation of 0 (see text). Curves represent the logistic regression model. Ranges and means of esterase activity were as follows: S lines: -1.407 to -0.126 (-0.749 \pm 0.348, n=30); C lines: -1.342 to 2.463 (0.175 \pm 1.099, n=36); L lines: -1.255 to 2.690 (0.522 \pm 0.892, n=31). Logistic regression models were as follows:

L lines:

$$Prob_{(mic)} = \frac{1}{1 + e^{(1.9182x - 0.0564)}}$$

C lines:

$$Prob_{(mic)} = \frac{1}{1 + e^{(2.4522x - 1.2936)}}$$



DISCUSSION

The Physiology of *Gryllus firmus*

The preceding experiments have uncovered a number of general observations about the physiology of *Gryllus firmus*. First, Juvenile Hormone III (JH III) seems to be the only hormone produced by the corpora allata *in vitro*, as ascertained by the radiochemical assay of Pratt and Tobe (1974). This is in accordance with observations of non-Lepidopteran insects (Loher et al., 1983; Feyereisen, 1985; Schooley and Baker, 1985; Zera and Tobe, 1990). Second, JH has methyl farnesoate as its immediate precursor in the metabolic pathway, as shown by the HPLC of corpora allata products. Third, JH III is broken down *in vitro* in diluted haemolymph samples exclusively by ester hydrolysis during the final juvenile instar. This observation is also in accordance with studies documenting JH III degradation by haemolymph in other crickets (Renucci et al., 1984; Zera and Tiebel, 1989). Fourth, a large proportion of this ester hydrolysis is performed by juvenile hormone-specific esterases in the haemolymph. Since the presence of juvenile hormone binding proteins in the haemolymph may result in the protection of JH from general esterases (Abdel-Aal and Hammock, 1988; Sanburg et al., 1975), JH-specific esterases may be the only active catabolic enzymes to degrade JH *in vivo*. This fact, in conjunction with observations of *G. rubens* by Zera and Holtmeier (1992) that *in vitro* JH esterase activity and *in vivo* rates of JH degradation covary, suggest that the *in vitro* JH esterase activity differences found in these experiments indicate significant differences in

in vivo JH titre among the selected lines of *G. firmus* as hypothesized by Fairbairn and Roff (1990).

Responses to Selection for Wing Length

These experiments demonstrate a physiological response to artificial selection for a morphological trait. The selection for increased and decreased percentage macroptery performed by Roff (1990) and Fairbairn and Roff (1990) has resulted in increased and decreased haemolymph juvenile hormone esterase (JHE) activity *in vitro* in *G. firmus*. Selected lines differ in JHE profile throughout most of the last juvenile instar, the stage during which wing morph is thought to be determined (Zera and Tiebel, 1989). Differences are most pronounced on day 5, when activities are at their peaks in all selected and control lines. These shifts in the distribution of cricket haemolymph JHE suggest differences in the regulation of haemolymph JH titre between lines with high and low percentage macroptery during this instar. Thus, these experiments support the hypothesis of Fairbairn and Roff (1990) that JH is the common physiological factor underlying the observed genetic correlations between flight propensity, dorso-lateral flight muscle histolysis, and wing morphology in this insect species.

What is not known is if these last-instar differences in JHE can be responsible for differences among selected lines in flight propensity and degree of muscle histolysis, which are adult traits. If the developmental windows for these traits are temporally different from that of wing morphology, then shifts in JHE during the last stadium may

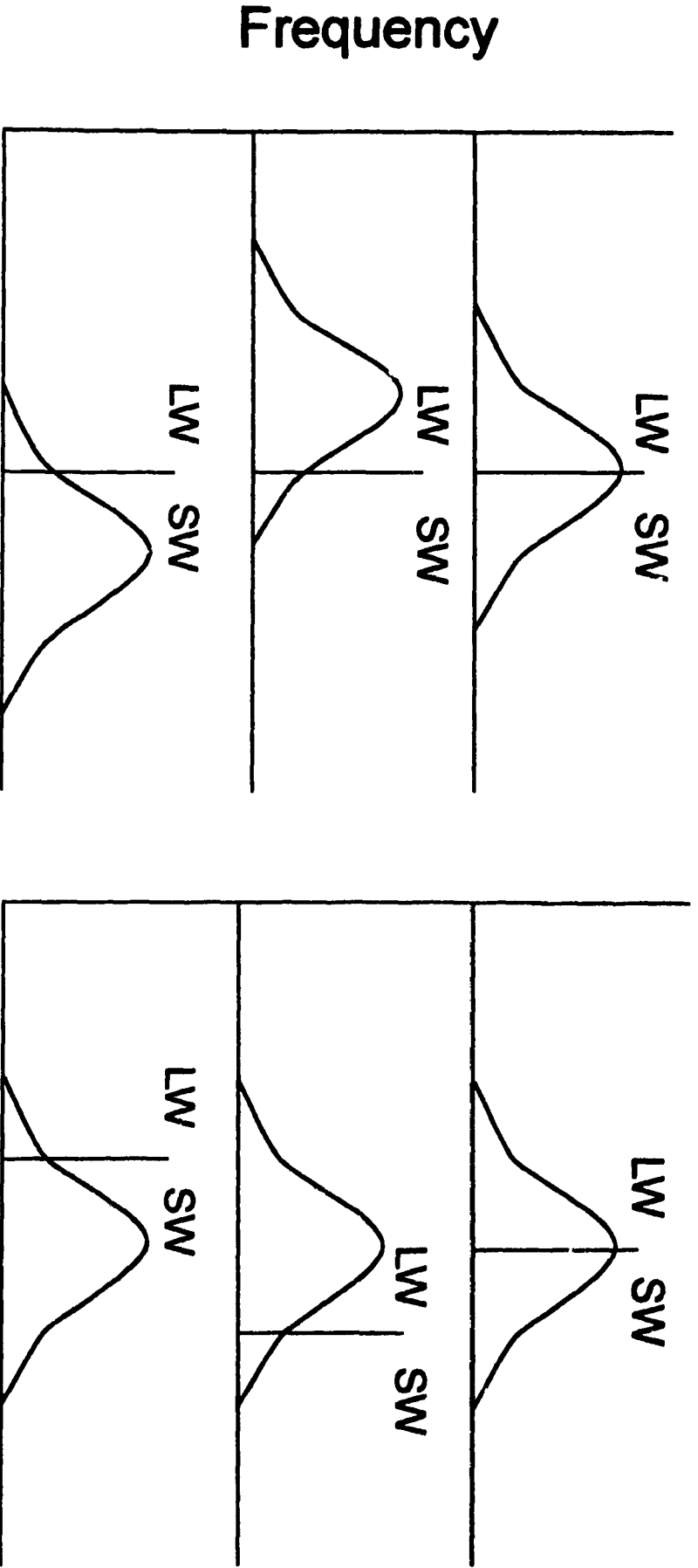
not have a bearing on the behaviour and physiology of adults. However, recent work by Zera et al. (1993) indicates that *in vivo* JH degradation as well as haemolymph JHE activity *in vitro* do not differ between adult macropters and micropters in *G. rubens*. These findings by Zera et al. (1993) support the hypothesis that JHE activity in last instar juveniles may affect adult traits determining migratory tendency. Nevertheless, more work is needed to elucidate the chronology of these types of developmental and behavioural decisions.

Shifts in JHE activity as a result of artificial selection may translate into shifts in JH titre, providing there is no concurrent change in JH synthesis as a result of selection. Work by Zera and Tobe (1990) indicates that this is the case in *G. rubens*. Thus, our experiments, showing shifts in JHE activity, suggest that the distribution of JH titres in the selected lines of crickets have shifted as shown in Figure 10A. However, an equally viable explanation could have been that the JH haemolymph titre distribution remained constant, and instead the threshold distinguishing micropters from macropters shifted upwards to produce a lower percentage microptery, or downwards to produce a higher percentage microptery (Fig. 10B) in the selected lines of Roff (1990) and Fairbairn and Roff (1990). From the logistic regression analysis we have shown that in both rearing methods, the probability of molting to a micropter as a function of esterase activity differs among the lines, such that the probability of molting to a micropter at a given esterase activity is lower for the L lines than for the control lines. In other words, the esterase activity at which half of the crickets are predicted to become macropters is lower for L lines than it is for control lines. This indicates that selection for long-winged crickets

FIGURE 10. Response to selection for changing percentage macroptery by shifting the underlying distribution of JH in the haemolymph (A) or by shifting the response threshold (B). LW indicates macropters; SW indicates micropters. A. In the shifting distribution scenario, the population begins with a percentage macroptery of approximately 50%. Selection for increased percentage macroptery leads to a downwards shift of the distribution with the threshold remaining constant, resulting in more macropters. Selection for decreased percentage macroptery leads to an upwards shift of the distribution resulting in more micropters. B. In the shifting threshold scenario, the population again begins with a percentage macroptery of approximately 50%. Selection for increased percentage macroptery leads to an upwards shift in the threshold and no response in the distribution of JH titre. Selection for decreased percentage macroptery leads to a downwards shift in the threshold and a corresponding decrease in the percentage macroptery in the population.

A. Shifting Distribution Scenario

B. Shifting Threshold Scenario



Haemolymph JH Titre

shifted the JH threshold upwards (Fig. 10B). Because no crickets from the S lines molted to macropters, the logistic regression cannot indicate if the JH threshold for these lines has shifted. However, standardized esterase scores as high as 2.5 were commonly found in S line crickets, and all crickets of these lines still molted to micropters. This suggests that the JH threshold has indeed shifted down (Fig. 10B). These two possible responses to selection (ie. shifting distribution of JH titre versus shifting macroptery threshold) are not mutually exclusive, and we have shown evidence for each.

Stress Effects

The effects of environmental stress on the endocrine systems of insects have been studied in a variety of taxa including Lepidoptera, Coleoptera, and Orthoptera (Yagi, 1976; Nakakita, 1990; Bhaskaran, 1981). Stress related to exposure to low temperatures has been shown to cause stimulation of corpora allata activity, resulting in elevated JH titres in stressed insects (Pipa, 1976; Bogus and Cymborowski, 1984). Likewise, starvation stress in Lepidoptera has been shown to induce higher haemolymph JH titres by stimulating CA activity (Bhaskaran and Jones, 1980; Bhaskaran, 1981). Yagi (1976) demonstrated that secretion of JH by the CA is greatly influenced by photoperiod, temperature, and rearing density. Crowding has been shown to cause supernumerary larval molts in *Tribolium freemani* (Nakakita, 1982; Nakakita, 1990; Kotaki et al., 1993) and in *Tenebrio molitor* larvae (Connat et al., 1991). The mechanism of this inhibition

of pupation is thought to be elevation of CA activity, resulting in relatively higher JH haemolymph titres in stressed individuals (Nakakita, 1982; Nakakita, 1990; Connat et al., 1991; Kotaki et al., 1993). Collectively, these studies indicate a common endocrine response in insects to a variety of stressors: elevation of CA activity and secretion of JH.

Crickets raised according to rearing method 1 molted to micropter more often than typically observed in the selected and control lines. The hypothesis that microptery is associated with low levels of JH esterase activity would therefore predict that crickets of rearing method 1 would have lower levels of JHE activity than would crickets of rearing method 2. In fact, esterase activities were not lower in rearing method 1. This apparent evidence against the aforementioned hypothesis, however, can be more easily understood given that rearing method 1 was a high stress environment for the crickets. If increased JH titre is a general response to stress, as the above studies would indicate, then the reduced percentage macroptery we found in all lines of rearing method 1 is more likely the result of increased JH synthesis by the CA than the result of decreased JHE activity. Alleviation of this stress, by raising crickets in rearing method 2, resulted in a restoration of percentage macroptery to normal values, presumably because of decreased JH synthesis and not because of increased degradation by JHE. Our results suggest that selection and stress may cause changes in percentage macroptery by two different mechanisms: stress may result in decreased percentage macroptery by increasing JH synthesis, while selection causes increased or decreased percentage macroptery by increasing or decreasing JH esterase activity in the haemolymph and by changing the JH thresholds for wing morphology.

In conclusion, we have found that selection for increased and decreased percentage macroptery in the sand cricket *Gryllus firmus* has resulted in endocrine changes in the lines of Roff (1990) and Fairbairn and Roff (1990). This response involves shifts in JH-specific esterase activity in the selected lines, as measured by the radiochemical assay of Hammock and Sparks (1977), and also shifts in the JH threshold for wing dimorphism. As JH is known to affect many of the physiological, morphological, and behavioural components of migratory tendency in insects, our results support the hypothesis of Fairbairn and Roff (1990) that the genetic correlations found between these components are the result of the pleiotropic action of juvenile hormone.

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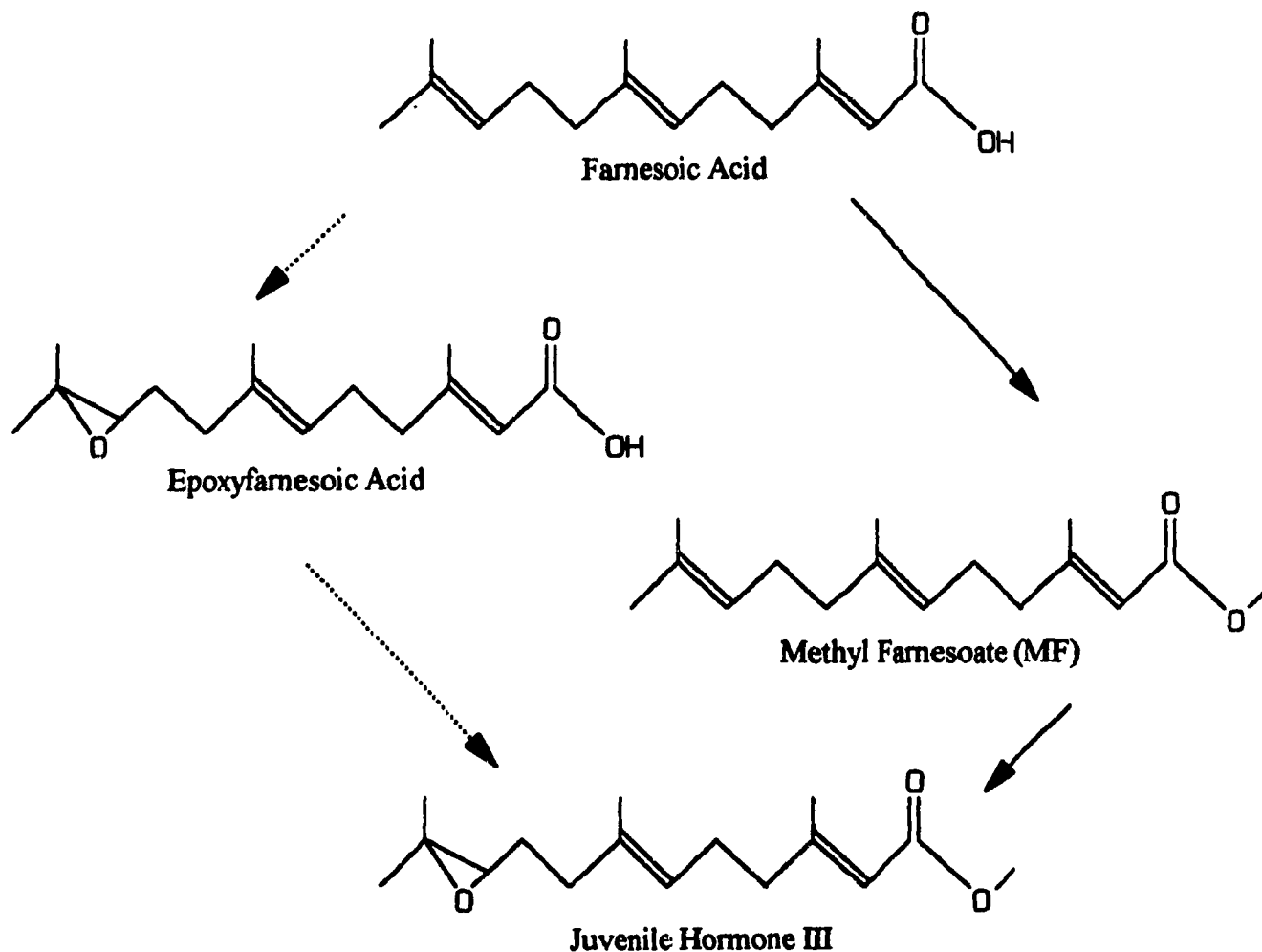
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Terminal Steps in Juvenile Hormone III Biosynthesis

from Schooley and Baker (1985)

Pathway 1. (Solid arrows). In Orthoptera and Dictyoptera, farnesoic acid has a methyl group added to it by methyl transferase to form methyl farnesoate (MF). If labelled methionine is used as a methyl donor, the MF will be radiolabelled, as will the Juvenile Hormone III formed from it. MF then undergoes epoxidation to form Juvenile Hormone III.

Pathway 2. (Dotted arrows). In other insect orders, farnesoic acid first undergoes epoxidation by epoxidase to form epoxyfarnesoic acid. It then receives a methyl group from a donor via methyl transferase to produce Juvenile Hormone III. Because the transfer of the methyl group is the final step, only one product (ie. JH III) will be radiolabelled.



Pathways of Juvenile Hormone Degradation

from King (1983)

The most common pathways of JH degradation are by ester hydrolysis, to form JH acid, and by epoxide hydrolase, to form JH diol. Each of these substances may undergo further modification to become JH acid diol.

