Extrinsic and intrinsic control of diapause termination in the Colorado potato beetle

Promotor : dr. L.M.Schoonhoven, hoogleraar in de entomologie, in het bijzonder de fysiologie der insekten

Co-promotor: dr. C.A.D. de Kort, universitair hoofddocent bij de vakgroep entomologie

K.S.Lefevere

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Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
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Lay-out: Gon van Laar en Kathy Lefevere
Omslagontwerp: Frederik von Planta
Druk: Pudoc, Wageningen

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STELLINGEN

1. De verbreking van de diapauze bij de Coloradokever wordt door de temperatuur, de vochtigheid van de grond en de aanwezigheid van voedsel bepaald.

   Dit proefschrift.

2. Het concept dat de adulte diapauze voornamelijk door juveniel hormoon gereguleerd wordt, doet onvoldoende recht aan de gecompliceerdheid van het diapauze syndroom.

   Dit proefschrift.

3. De bewering, dat denervering van corpora allata tot hoge synthese van juveniel hormoon leidt in diapauze individuen van Locusta migratoria, is op basis van de door Baehr et al. gebruikte methode onbetrouwbaar.

   Baehr,J.C.; Caruelle,J.P. and Poras,M. (1986) Int. J. Invertebrate Reprod. Dev. 10,143-150.

4. De uit het vetlichaam afkomstige factor, die de verbreking van de pupale diapauze in Heliothis zea veroorzaakt, zou dezelfde kunnen zijn als het ecdyson-synthese stimulerende eiwit, geïsoleerd uit Manduca sexta larven.

   Gray,R.; Meola,R. and Holman,G.M. (1987) J.Insect Physiol. 33, 325-331.

   Watson,R.D.; Williams,T.K. and Bollenbacher,W.E. (1987) J.exp. Biol. 128,159-173.

5. De conclusie van Koch and Bückmann, dat seizoens dimorfisme van de vlinder Araschnia levana alleen door het tijdstip van ecdysteroïden afgifte wordt gecontroleerd, volgt geenzins uit de resultaten van hun experimenten.

   Koch,P.B. and Bückmann,D. (1987) J.Insect Physiol. 33,823-829.

   Endo, K.; Yamashita,I. and Chiba,Y. (1985) Appl. Ent. Zool. 20, 470-478.

6. De relevantie van veranderingen in het gewicht van de mannelijke geslachtsklieren van Danaus plexippus om uitspraken over duur en intensiteit van de diapauze te doen is discutabel.

   Herman,W.S. (1981) Biol. Bull. 160,89-106.

7. De toepassing van neuropeptiden-biotechnologie ten behoeve van de insektenbestrijding is een gevaar voor de Vertebraten.

8. De hogere doeltreffendheid van het ingrijpen op neurohormonaal niveau ten opzichte van dat op juveniel hormoon en ecdysteroïden niveaus is discutabel met betrekking tot destabilisering van het neuroendocrien systeem.

Keeley,L.L. and Hayes,T.K. (1987) Insect Biochem. 17,639-651.

9. De aanbeveling om nieuwe, d.w.z. niet door evolutie ontstane predator/prooi associaties, bij voorkeur te gebruiken in biologische bestrijding systemen van plagen kan bedreigend worden voor het behoud van insekten soorten.

Hokkanen,H. and Pimentel,D. (1984) Can. Ent. 116,1109-1121.

10. Het belang van internationale uitwisselingen ter bevordering van wetenschappelijk onderzoek kan niet genoeg worden benadrukt, wanneer men beseft hoe diep de manier van denken door de eigen cultuur wordt bepaald.

11. De wijze waarop insekten gepresenteerd worden in sommige kinderboekjes kan onbewust insektenvrees óf later overmatig gebruik van insektendodende= middelen doen ontstaan, afhankelijk van de persoonlijkheid van het kind.

Vanetti,G. (1977) Un petit trou dans une pomme. Editions Fernand Nathan, Paris, 1980.

12. Het Nigeriaanse spreekwoord: "vrijwillig werken is erger dan slavernij" is heel toepasselijk bij de huidige economische situatie, waar vrijwillig in feite zonder loon betekent.

Proefschrift van K.S.Lefevere.
Extrinsic and intrinsic control of diapause termination in the Colorado potato beetle.
Wageningen, 12 januari 1988.

A la mémoire de mon père

A ma mère

Extrinsic and intrinsic control of diapause termination in the Colorado potato beetle.

ABSTRACT

The adult Leptinotarsa decemlineata enters a winter diapause, which is mainly induced by short photoperiod. The effects of environmental factors (such as temperature, humidity, daylength and food) on the behaviour and the metabolism of some haemolymph components, as for example the juvenile hormone, were analysed in diapausing and post-diapausing females.
During diapause three consecutive phases could be distinguished, based on changing response to temperature: 'diapause development' or true diapause, followed by a quiescence which is facultative, and a transient phase of post-diapause development leading to emergence from the soil (or diapause termination). After diapause, high metabolic rates are resumed and post-diapause development culminates in reproduction. The endocrine control of the termination of diapause was investigated by means of hormonal injections. Only the combined injections of 20-hydroxyecdysone and juvenile caused a temporary break of diapause only, lasting as long as the JH titre in haemolymph was high. 20-Hydroxyecdysone (20-HE) injected before JH suppresses the effects of exogenous JH on the endogenous metabolism of JH. It is suggested that 20-HE could be responsible for triggering the programme for diapause termination by the brain. Allatectomy experiments after diapause confirm the role of JH in reproduction and reveal the endocrine control of the induction of a second diapause. The presented results corroborate the theory that the brain is the primary organ controlling diapause.

CONTENTS

All chapters will be submitted for publication.

Voorwoord

Ik wil hier allen danken, die door hun medewerking hebben bijgedragen aan het totstandkomen van deze dissertatie.
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GENERAL INTRODUCTION

The concept of diapause: definitions and historical perspective

Dormancy is an arrest in development that enables living organisms to synchronize their life cycle with favourable seasonal conditions, and to avoid or survive unfavourable periods.

Two kinds of dormancies are distinguished in insects:

i) quiescence, which is a direct response to exposure to environmental extremes (e.g. temperature) and which ends immediately at resumption of favourable conditions.

ii) diapause, which is an active mechanism for adaptation to seasonal cycles (predictable conditions). This adaptive mechanism separates (at a more or less high degree) the sensitive stage (induction) from the responsive stage (diapause) and enables insects to anticipate the unfavourable period. The termination of diapause is in turn regulated by an intrinsic mechanism, allowing a delay of response.

Diapause thus implies the capacity of an insect to perceive environmental cues (or tokens; Lees, 1955) and to transduce them into chemical signals via their neurohormonal system, eventually leading to the diapause commitment (Williams, 1946). This developmental programme can be stored and retrieved much later in the life cycle (see Denlinger, 1985).

Daylength appears to be the prevailing cue for diapause induction in most insect species (Danilevskii, 1965; Saunders, 1982), although other environmental factors such as temperature, food and water may also interfere (see Beck, 1980; Behrens, 1985). Termination of diapause is also affected by environmental factors, often non-token stimuli (e.g. temperature; Tauber and Tauber, 1976; Hodek, 1983).

Diapause can intervene in any season (Masaki, 1980) and at any stage of the development, but its sensitive and responsive stages are genetically determined and species-specific. Several attempts to classify all types of diapause prove that we are dealing with a multi-faceted physiological mechanism, showing a great diversity in insects (Müller, 1970; Mansingh, 1971; Thiele, 1973; Ushatinskaya, 1976). These classifications have been criticized by Beck (1980), Tauber et al. (1986) and Danks (1987).

Ecologists and physiologists seem to have reached a general consensus of the diapause concept. That is: diapause is a neuro-hormonally mediated

dynamic state of low metabolic activity, involving several phases throughout the seasons (Tauber and Tauber, 1976). Diapause is generally associated with increased resistance to environmental extremes. At the onset of diapause, insects enter a phase of 'diapause development' (Andrewartha, 1952, or physiogenesis), which is followed by a post-diapause quiescence and a post-diapause development (see Tauber et al., 1986). Danks (1987) listed the synonyms used for the different phases by several authors.

The term 'diapause syndrome' was coined by De Wilde (1959). It is a general term for the species-specific set of behavioural and physiological symptoms of diapause, referring initially to pre-diapause preparation for the future seasonal conditions. This concept of 'diapause syndrome' has recently been enlarged to include all pre-diapause, diapause and post-diapause processes related to seasonal changes (Tauber et al., 1986).

The Colorado potato beetle case

The adult Colorado potato beetle enters a winter diapause, which is induced mainly by short daylength (De Wilde, 1955). The various behavioural and physiological aspects of diapausing beetles have been well documented by De Wilde and collaborators. Beetles induced to enter diapause stop feeding ten days after adult emergence, become positively geotactic and burrow into the soil (De Wilde, 1958). Sexual activity ceases and the ovaries fail to mature (De Wilde, 1962). Flight muscles degenerate (De Kort, 1969). Lipids, carbohydrates and proteins accumulate (De Loof and De Wilde, 1970a; see also Ushatinskaya, 1956). The metabolic rate is suppressed (El-Ibrashy, 1965). The neurosecretory cells of the brain and the retrocerebral glands (corpora cardiaca (CC) and corpora allata (CA)) show signs of inactivity (Schooneveld, 1970).

The pioneering experiments of De Wilde and collaborators have uncovered the endocrine control of the induction of adult diapause (De Wilde and Stegwee, 1958; De Wilde et al., 1959; De Wilde and De Boer, 1961, 1969; De Wilde et al., 1968). The juvenile hormone (JH) deficiency (a hormone produced by the corpora allata) has long been advocated as the central cause of adult diapause. More recent studies suggested that ecdysteroid hormones participate in diapause induction as well (Briers and De Loof, 1981; Briers et al., 1982).

Colorado potato beetles of both sexes enter diapause. The suppression

of reproduction is a major characteristic of adult diapause, due to an arrest in development of the primary reproductive organs of females (ovaries) and secondary reproductive glands of males (accessory reproductive glands; De Loof and Lagasse, 1972). In females, diapause termination culminates in the production of fertile eggs. The resumption of reproduction after diapause is obviously more difficult to diagnose in males (histology and electron microscopy). Therefore, the present study concentrates on female diapause.

Objective

In previous studies on Colorado potato beetles, emphasis has been given to the mechanism of diapause induction and its endocrine control. So far little attention has been paid to diapause termination and the post-diapause period.

This study aims to place the Colorado potato beetle case-history in the context of the present concept of diapause and to unveil the environmental and endocrinological regulation of diapause termination.

The environmental factors affecting diapause development and termination were studied in the laboratory, in combination with field studies on the seasonal progress of diapause (Chapter 1). Metabolic changes as reflected in haemolymph composition during and after diapause were investigated and their significance is discussed in chapter 2. The role of environmental factors on JH metabolism during and after diapause has been analysed in chapter 3.

Chapter 4 discusses the endocrine control of diapause termination. Finally, chapter 5 focuses on post-diapause development. The effects of extrinsic (photoperiod) and intrinsic (CA) factors were investigated on the development of the ovaries. The sensitivity to photoperiod after diapause was also analysed in view of the induction of a second diapause.

Chapter 1

Adult diapause in the Colorado potato beetle, Leptinotarsa decemlineata: effects of external factors on maintenance, termination and post-diapause development.

ABSTRACT

This study tests the existence of distinct phases during hibernation of Leptinotarsa decemlineata (Say) (Coleoptera: Chrysomelidae). Experiments were conducted with beetles diapausing under natural winter conditions or at constant temperatures in the laboratory, to determine the effects of temperature, water, food and photoperiod on diapause termination (emergence from the soil). Behavioural and physiological criteria, such as oviposition and corpus allatum activity were used to characterize the various phases of diapause. Three successive phases can be distinguished during hibernation: 'diapause development' or true diapause, 'post-diapause quiescence' or diapause maintenance and finally, a transient phase of post-diapause development leading to emergence from the soil. Diapause development is completed within 3 months in the field and its duration depends on temperature. During this phase, beetles although they are buried in the soil, remain sensitive to photoperiod. They do not terminate diapause when exposed to higher temperatures. Thereafter, beetles stay in a quiescent state maintained by low temperature, low humidity or lack of food. The response to temperature changes during hibernation. Movements in the soil start when soil temperature reaches 4 to 5°C. However, this temperature is too low to permit post-diapause development. The transient phase has a temperature threshold between 8 and 10°C, whereas emergence (diapause termination) occurs only when the temperature exceeds 11°C. Post-diapause development is strongly influenced by temperature and humidity. After emergence, post-diapause development leads eventually to reproduction. Food is essential for reproduction after diapause termination, whereas photoperiod plays no further role.

INTRODUCTION

Diapause is a developmental phase in an insect's life cycle, allowing the animal to survive adverse environmental conditions. This phase is characterized physiologically by low metabolism, suppression of development or reproduction, and often by a high degree of resistance to cold. Ecologically, diapause is defined as a dynamic process in which responses to environmental factors change in the course of time (Tauber and Tauber, 1976). Two phases have been distinguished on the basis of the sensitivity to diapause-inducing factors:

i) 'diapause development' (Andrewartha, 1952) during which insects are still sensitive to inducing factors, but do not respond to diapause-breaking factors. This phase is a very complex phenomenon, during which the insect prepares itself for active resumption of morphogenesis or reproduction. 'Diapause development' is difficult to define and to quantify in physiological terms, as several factors interact concurrently or sequentially (Hodek, 1983).

ii) 'post-diapause quiescence' during which insects are responsive to breaking factors, but remain in a dormant state (diapause maintenance) as long as adverse environmental conditions (i.e. low temperature, lack of food, etc.) persist. When these conditions change, dormancy is promptly terminated (Tauber and Tauber, 1976).

'Diapause' is thus a period of dormancy consisting of a first phase of 'true diapause', followed by a second phase resembling 'quiescence'. As soon as the phase of 'diapause development' is completed, 'post-diapause development' can start. An activation phase in which the insect prepares itself for the onset of post-diapause activities, leads to 'diapause termination' (Hodek, 1983). Post-diapause development results eventually in reproduction (see review by Behrens, 1985).

The first aim of this study is to test the existence of these different phases in the hibernation of Leptinotarsa decemlineata (Say) (Col.: Chrysomelidae), in which diapause is induced mainly by short daylength (De Wilde, 1955 and 1962, De Wilde et al., 1959).
Secondly, since each of these phases is characterized by a set of species-specific responses to environmental cues (Tauber and Tauber, 1973), we investigate the effects of temperature, water, food and photoperiod on the different phases of hibernation.

Some peculiarities concerning diapause in Leptinotarsa should be

noted. Under Dutch climatic conditions Colorado potato beetles dig into the soil and enter diapause in early September. The adults hibernate in the soil (up to 60 cm deep) for a period of 8 to 9 months and emerge in the field at the end of May. In what follows, 'diapause termination' refers to emergence from the soil or loss of positive geotaxis.
The duration of 'diapause development' is determined by photoperiod, temperature, humidity, sensory and dietary factors (reviewed by Beck, 1980). As diapausing beetles are not directly exposed to light, photoperiod is unlikely to affect diapause completion in nature.
Furthermore, potato foliage, which constitutes the main food-plant of Colorado potato beetles in the Netherlands, is not available during the diapause period. Food is thus unlikely to affect the duration of 'diapause development'. On the other hand, soil humidity may well affect 'diapause development'. Unfortunately, this depends on many factors (i.e. rainfall, underground water level, soil composition, etc.). In our experimental conditions, moisture is difficult to control because of its dependence on temperature. For these reasons, this study concentrates mostly on responses to temperature during hibernation.

MATERIALS AND METHODS

Beetles were taken from the laboratory stock (Wageningen, the Netherlands) and reared on fresh potato leaves. Diapause was induced in the laboratory by rearing the beetles from the egg stage onwards under short-day (SD) conditions: 10 hrs light/14 hrs darkness, at 25°C and R.H. = 60-70%. Young pre-diapause adults feed actively for 10 days, but do not oviposit. On day 11, when beetles normally leave the plant to enter the soil, groups of 35 females and 5 males were placed in plastic boxes (9x9x7 cm) on moistened sand (5 cm deep) for further use in laboratory experiments.
For experiments in the field, groups of 50 females and 10 males were allowed to bury themselves in a 6 cm layer of moistened sand, in plastic boxes (25 x 15 x 9 cm) with perforated lid and bottom for gas/liquid exchange. Two days after burial, the boxes were transferred to appropriate diapause conditions for further experiments. The treatments were as follows:

I) Beetles diapausing outdoors, under natural winter conditions, were buried in the soil at a depth of 50-60 cm. This is the maximum burrowing

depth in sand for natural populations in a temperate-cold climate (Ushatinskaya, 1978). Soil temperature at a depth of 50 cm, was recorded daily throughout the year, at a meteorological station situated about 500 m from the experimental field. No mortality occurred under these conditions.

II) Beetles diapausing in the laboratory, were kept in continuous darkness, at the following constant temperatures: 4°C, 12°C and 25°C. At 12 and 25°C, a filter paper was placed on the box lid and moistened regularly to compensate for evaporation. Mortality during the experiment was normally less than 10%, at 12 and 25°C. At 4°C, mortality increased after 3 months (15%) and was relatively high (50-70%) after 6 to 7 months at 4°C; dead beetles were not included in the calculation of the results.

To check for beetles moving upwards in the field, the boxes were dug out at regular intervals during winter and spring. Care was taken to disturb diapausing beetles as little as possible. Beetles wandering on the sand surface were counted and removed. The boxes were buried again.

To study changes in response to temperature and sensitivity to photoperiod during hibernation, boxes were transferred from the field to the laboratory (at 25°C) throughout winter and spring. In the laboratory, boxes were transferred from 4°C to 12° or 25°C after various periods. To prevent any other external stimulation, boxes were kept in continuous darkness and no food was supplied. Emergence from the sand was recorded daily after transfer. Emerged females were kept individually in glass-jars (150 ml) containing moistened sand, food and active males, under SD conditions. Criteria for diapause termination were: emergence or lack of digging behaviour, feeding and oviposition.

To determine reproductive capacity and sensitivity to photoperiod, females diapausing under field conditions were removed from the sand and subjected to SD (see above) or long-day (LD) conditions (16 hrs photophase/8 hrs scotophase) at 25°C and kept individually in glass-jars, as above. The pre-oviposition period was defined as the time from removal from the sand to production of the first batch of eggs. Eggs were counted daily for 3 weeks. The rate of oviposition was defined as the average number of eggs laid daily per ovipositing female.

Juvenile hormone (JH) homeostasis

The rate of JH synthesis was measured by the in vitro radiochemical assay for corpus allatum (CA) activity (Tobe and Pratt, 1974) using ^{14}C-methyl-

methionine (final specific activity: 37 mCi/mmol.; Amersham, England). Details of the procedure are described by Khan et al. (1982a).
The JH titre in the haemolymph was determined by radioimmunoassay using antiserum prepared by A. and C. Strambi, Marseille, France (Strambi et al., 1981) as described for Leptinotarsa by De Kort et al. (1985).

Statistical analysis

The following non-parametric tests were used for analysis of data: Chi-square (Chi^2), Spearman's rank correlation (r_S); and for paired data: Sign test, Wilcoxon-Mann-Whitney test.

RESULTS

DIAPAUSE DEVELOPMENT AND MAINTENANCE IN THE FIELD

Figure 1 shows that during the winters of 1983/84 and 1984/1985 the soil temperature was never below 0°C at a depth of 50 cm. Beetles do not move upwards in the soil as long as soil temperature is below 5°C. Movements first occurred in mid-April, and increased in frequency until the end of June. The soil temperature threshold for upward movements is between 5 and 8°C (fig. 1).

Diapausing beetles transferred to the laboratory (25°) emerge from the soil and terminate diapause even under continuous darkness (fig. 2). In addition, diapausing beetles buried in the field in mid-November (late season) emerged after transfer at 10 weeks diapause (at the beginning of February) in the laboratory. The rate of emergence was similar to that of beetles transferred at the same time, but after 4 months in diapause (see fig. 2). This indicates that soil temperature determines the emergence rate after completion of 'diapause development'.

The plateaus in the emergence curves in figure 2 may be due to desiccation of the sand at 25°C, which leads to an arrest of emergence. The different levels of these plateaus reflect increasing rates of emergence after transfer, as diapause time increases. Sprinkling the sand with water results in emergence of the remaining beetles. This indicates the important role of water in determining post-diapause emergence.

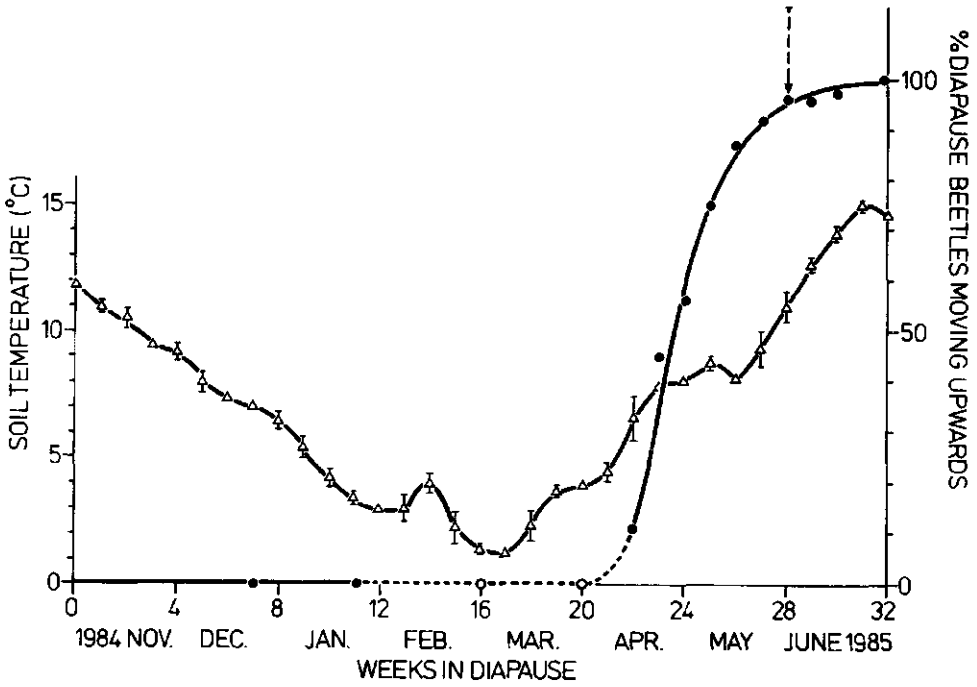

Fig. 1: Upward movements of diapausing beetles buried under natural winter conditions in relation to soil temperature.
Soil temperature at a depth of 50 cm was measured daily but is presented here as the weekly averages (± S.D.). The arrow indicates the time of first emergence in the field.
NB: Open circles were estimated by interpolation, as deep frost prevented us from digging.

EFFECT OF TEMPERATURE ON THE DURATION OF DIAPAUSE

As shown in figure 3, post-diapause emergence is virtually absent at constant 4°C. At a constant temperature of 12°C the first beetles emerged after 12 weeks. The duration of diapause at 12°C in this population is 21.4 weeks ± 3.8 (standard deviation). At 25°C, emergence starts after only two weeks of diapause. Diapause duration at 25°C is 14.5 ± 4.5 weeks (fig. 3). Diapause duration is thus reduced by an increase in ambient temperature. The possibility of a differential action of temperature on 'diapause development' and on emergence rate of 'quiescent' beetles will be examined in a following section.

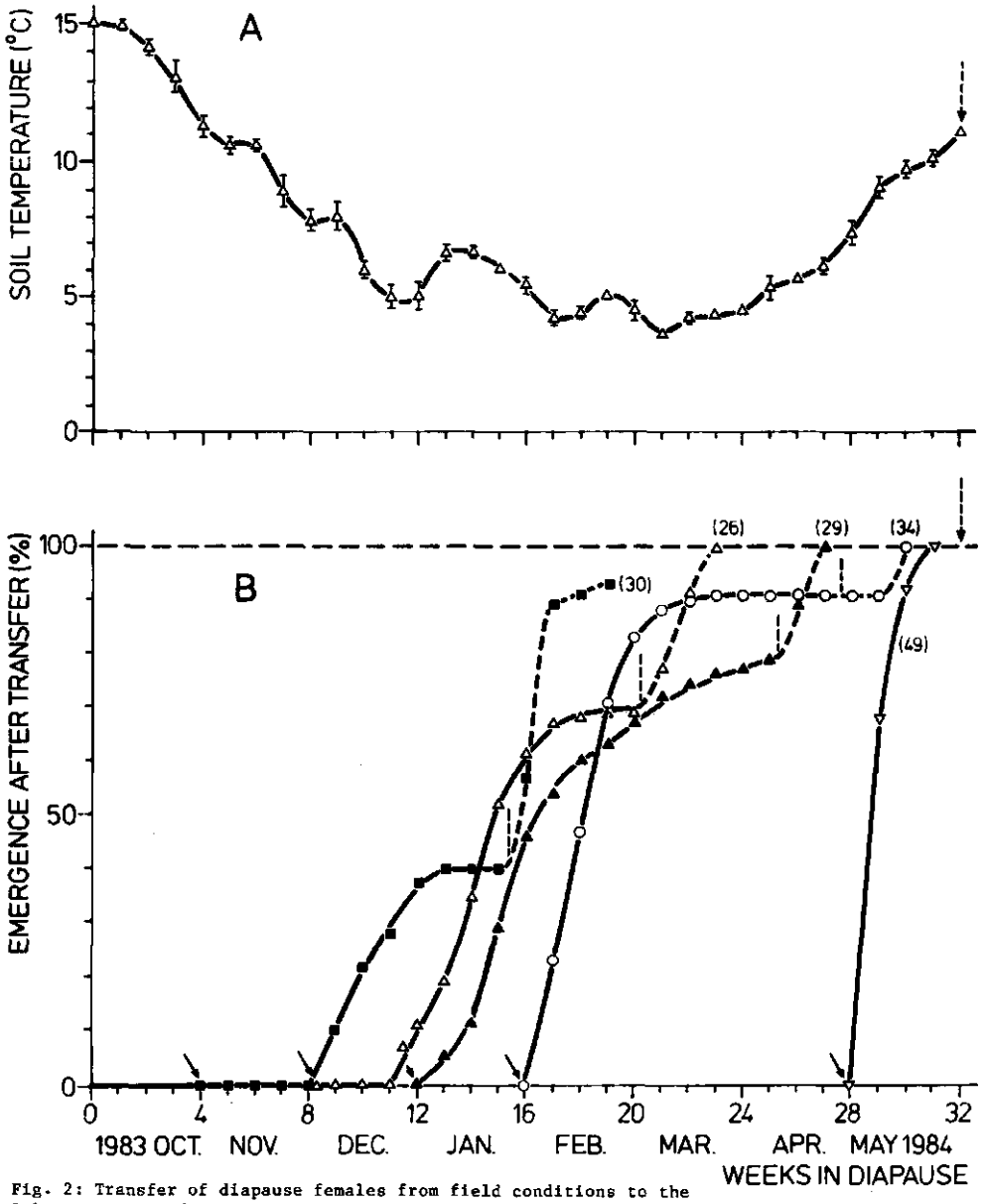

Fig. 2: Transfer of diapause females from field conditions to the laboratory at different times throughout the year.
A: Soil temperature at a depth of 50 cm (weekly means ± S.D.)
B: Spontaneous emergence from moistened sand after transfer from the field to the laboratory and kept at 25°C. X-axis gives the effective diapause time (in weeks) and the time of year (months).
N is given between brackets. Symbols: closed squares: transfer after 1 month diapause; open triangles: after 2 months; closed triangles: after 3 months; open circles: after 4 months; reversed triangles: after 7 months diapause. Water sprinkling on the sand surface is represented by dashed bars and subsequent emergence by dashed curves. Arrows indicate time of transfer to the laboratory. Broken arrows indicate time of first emergence in field populations in Wageningen.

DOES DIAPAUSE DEVELOPMENT OCCUR AT 4°C?

If diapause were a single developmental process uniformly dependent on temperature, the results shown in figure 3 should mean that no development occurs at 4°C, so the time spent at 4°C should have no effect on percentage emergence. In fact, however, the proportion of beetles emerging within 3 weeks at 12°C (fig. 4A) and at 25°C (fig. 4B) increases significantly with the time spent at 4°C (Chi^2=1,492; d.f.=7; $p < 0.01$). Since emergence from the soil is affected by the time spent at 4°C, diapause development must be able to proceed at 4°C.

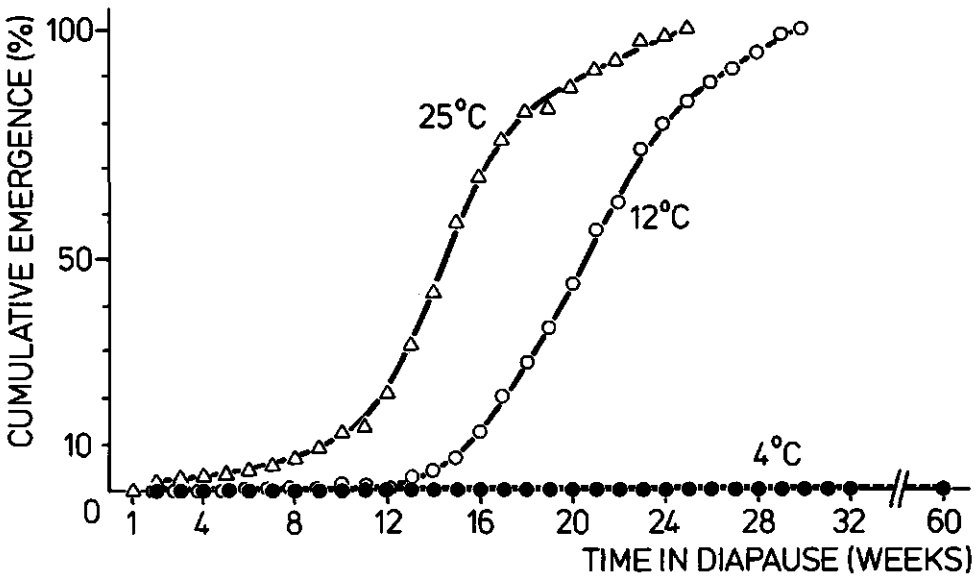

Fig. 3: Emergence of diapausing females at various constant temperatures. Diapause beetles were kept in moistened sand, in permanent darkness without food supply. Daily recordings were made of spontaneous emergence. N was 1,383, 460 and 1,080 females for constant 25°C, 12°C and 4°C respectively.

EFFECTS OF WATER, PHOTOPERIOD AND FOOD ON BEETLES IN 'DIAPAUSE DEVELOPMENT'

Sprinkling water on the sand surface had no effect on emergence when beetles had been in diapause at 12°C for 1 or 2 months.

To assess the role of photoperiod during 'diapause development', females in diapause for 2 months at 25°C (n=20 in each group) were removed from the sand and exposed to LD photoregime for one to three days, without food or sand. They were subsequently placed on moistened sand (without food) under SD conditions and their behaviour was checked daily. Within three days, all beetles exposed to a single LD-cycle had burrowed and 90% of those that had received three LD photocycles returned into the sand. However, 12 days later 45% of these beetles re-emerged, whereas only 10% of the beetles that had received one LD-cycle did so. This percentage is in the same range as for beetles diapausing at constant 25°C in continuous darkness (see fig. 3). Diapausing beetles are thus still sensitive to photoperiod during the phase of 'diapause development' and need at least three LD photocycles to break diapause.

The effect of food was studied in the following experiment: a group of beetles was removed from the sand and kept continuously under SD conditions in the presence of food. Within 12 days, 50% of the beetles had re-entered diapause. In a second series of experiments, groups of beetles removed from the sand were kept under SD photoregime with a supply of food, but were prevented from digging for various periods. No beetles tried to return into the soil after nine days of this treatment. Food can thus be another important trigger for diapause termination, but food alone is not sufficient to break diapause.

EFFECTS OF TEMPERATURE, WATER AND FOOD ON EMERGENCE OR DIAPAUSE TERMINATION

1) Temperature

Figure 4 illustrates the rates of emergence after various periods in diapause in the laboratory and subsequent transfer to a higher temperature.

The effect of temperature on emergence rate can be studied only after 'diapause development' is completed (min. 12 weeks). The time to emergence (in days; mean $\pm$ standard deviation) for females diapausing at 4°C for 16, 20, 24 and 28 weeks before transfer to 25°C were: 11.3 $\pm$ 6.8, 7.4 $\pm$ 4.2, 6.8 $\pm$ 2.4, 5.2 $\pm$ 2.5 days, respectively. The time to emergence at 25°C decreases significantly with increasing time at 4°C (r_S = -0.288, t = -5.06, 283 d.f., $p < 0.001$).

The time to emergence at 12°C, after a period of 24 weeks diapause at 4°C, was 25.8 $\pm$ 12.9 days. This time is significantly greater than that at 25°C

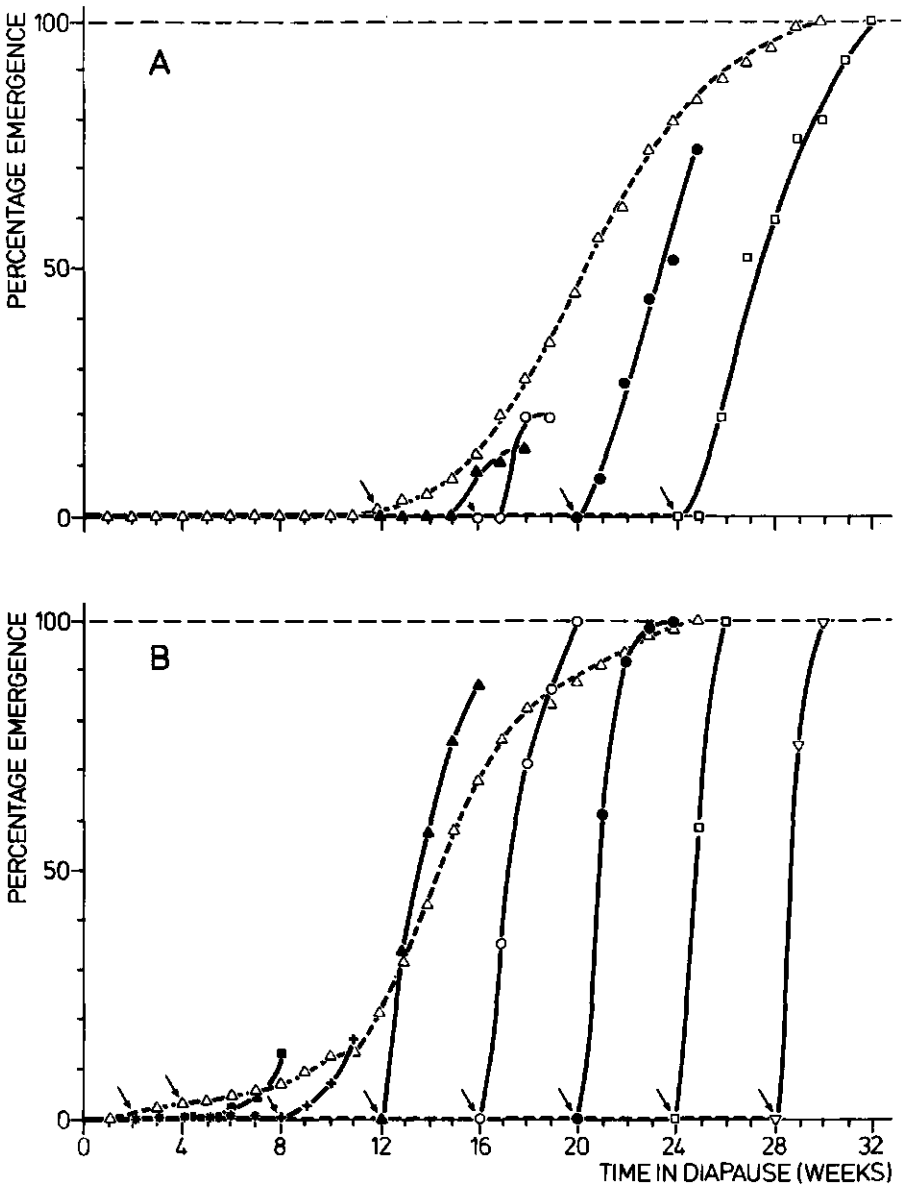

Fig. 4: Post-diapause emergence after transfer of diapausing females from 4°C to 12°C (A) or 25°C (B) at various times after the onset of diapause. Dashed line represent spontaneous emergence under constant temperature conditions (no transfer). Arrows indicate time of transfer. Emergence was checked daily after transfer, in continuous darkness for a minimum period of 4 weeks.
Symbols: stars: transfer after 2 weeks diapause (N_B=20); closed squares: after 1 month (N_B=127). crosses: after 2 months (N_B=65); closed triangles: after 3 months (N_A= 22, N_B=97); open circles: after 4 months (N_A=23; N_B=58); closed circles: after 5 months (N_A=27, N_B=103); open squares: after 6 months diapause (N_A=25, N_B=36); reversed triangles after 7 months (N_B=88).

(see above).

It should be noted that to minimize disturbance of diapausing beetles, we could not assess mortality until the end of the experiment. Dead beetles were discarded in calculations. Mortality should not, however, be an important source of bias in the results, since the assumption can be made that most of these beetles were dead before transfer (start of the experiment; see materials and methods).

2) Water

When the sand surface is sprinkled with water at the time of temperature transfer, the emergence rate is significantly accelerated (Table I: Sign test, $p < 0.01$). This confirms the role of water in

Table I: Effect of water sprinkling on emergence of beetles transferred from 4°C to a higher temperature after various times spent in diapause.

Weeks in diapause at 4°C before transfer	n	Time (days) to 50% emergence after transfer to 12°C	
		water sprinkling	without water sprinkling
16	24	22	28
24	24	16	19
		after transfer to 25°C	
12	23	8	13
16	57	6	11
20	27	6	7
24	16	5	7
28	25	4	5

determining emergence after 'diapause development' (see fig. 2).

3) Food

Experiments with diapausing beetles (2 months at 25°C; n = 20 females) in continuous darkness, with or without fresh potato foliage on the sand surface, show that spontaneous emergence and diapause termination is significantly higher when food is available (Table II).
Lack of food seems thus to be a major factor in maintenance of diapause at 25°C. However, we observed regular emergence and reburial of beetles

Table II:

A: Effect of presence of food on the sand surface on spontaneous emergence of diapausing beetles (2 months at 25°C) in continuous darkness.

	+ FOOD	WITHOUT FOOD
after 3 days	25%	10%
after 10 days	50% oviposition on day 5	10% no oviposition

B: Effect of food on diapause termination when diapausing beetles (2 months at 25°C) are removed from the sand and placed on freshly moistened sand, under continuous darkness.

	+ FOOD	WITHOUT FOOD
after 3 days	60%	5%
after 12 days	75% oviposition on day 6	10% no oviposition

N = 20 females in each sample.

diapausing at constant 25°C, even when no food was provided. It is noteworthy that feeding and oviposition occurred in constant darkness.

DOES TEMPERATURE AFFECT THE DURATION OF 'DIAPAUSE DEVELOPMENT'?

The total diapause duration is determined by the 'diapause development' time and the time required for emergence (transient phase). After 20 weeks in diapause, extra time at 4°C does not significantly affect the rate of emergence (see previous section, "effects of temperature on emergence": mean time to emergence at 25°C). This suggests that 'diapause development' is completed within 20 weeks at 4°C. The effect of temperature on 'diapause development' time can be estimated from the results summarized in Table III. The rate of 'diapause development' thus increases with temperature.

Table III: Effect of temperature on the duration (in weeks) of the different phases of adult diapause in Leptinotarsa females.

	4°C	12°C	25°C
(total) diapause	60[a]	21	14.5
transient phase	40	4.5[b]	1[b]
diapause development	20	16	13.5

a: No individuals had emerged at 60 weeks of diapause.

b: Estimated from the emergence rates after transfer from 4°C at 20 weeks of diapause.

Figure 5 shows that females diapausing under field conditions and exposed to SD or LD photoregime after removal from the sand have higher rates of oviposition as the season proceeds (LD: r_S= 0.47, t= 4.05, 58 d.f., p < 0.001; SD:r_S= 0.71, t= 8.66, 74 d.f., p < 0.001), and the pre-oviposition period declines significantly over time (LD: r_S= -0.46, t= -3.94, 58 d.f., p < 0.001 and SD:r_S= -0.64, t= -7.16, 74 d.f., p< 0.001).

This increase in reproduction is not affected by the photoregime after diapause. However, a quantitative effect of photoperiod is observed when

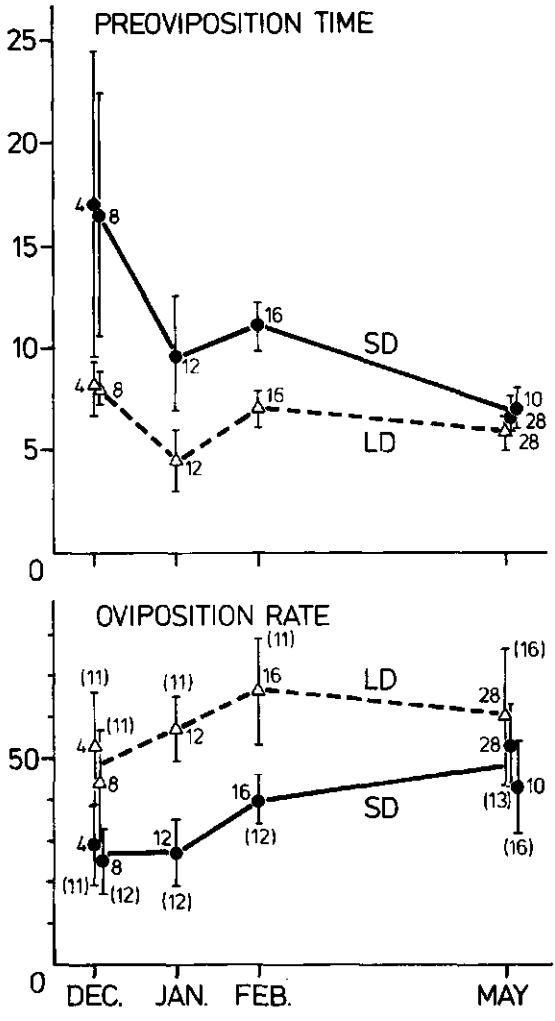

Fig. 5: Reproduction of post-diapause females after transfer from field conditions into the laboratory (at 25°C), at various times throughout the season, for the different photoregimes.
A: Mean length of the pre-oviposition period (in days ± S.D.).
B: Mean rate of oviposition: number of eggs laid daily per ovipositing female (± S.D.).
SD = short days: 10 hrs photophase/14 hrs scotophase.
LD = long days: 16 hrs photophase/8 hrs scotophase.
Numbers indicates the time (in weeks) spent in the field before transfer to the laboratory.
N is given between brackets for each group of females.

beetles are transferred to the laboratory before March. The pre-oviposition time is significantly longer (Wilcoxon, $p < 0.01$) and the rate of oviposition lower (Wilcoxon, $p < 0.01$) in females exposed to SD photoregime than in LD-females. Beetles transferred to the laboratory in May no longer displayed any response to photoperiod. This indicates that 'diapause development' is completed and 'post-diapause quiescence' has started after February.

It should be noted that diapause duration (see fig. 5: actual number of weeks spent in diapause) does not significantly affect the rate of reproduction, which depends only on the time of year.

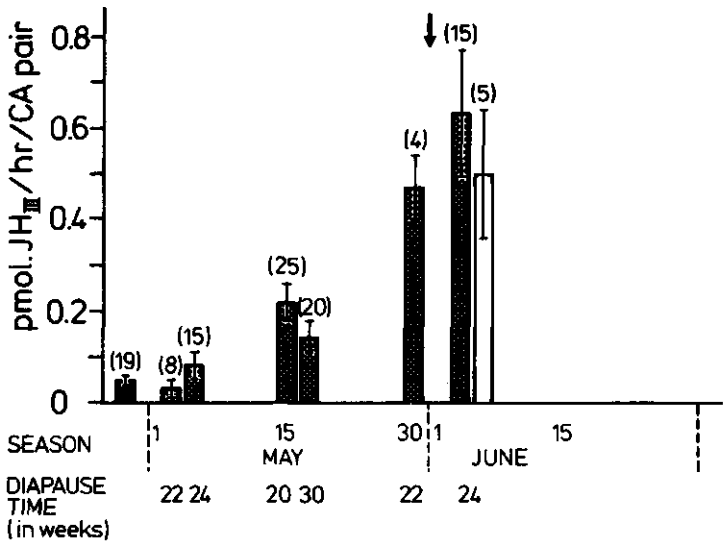

Fig. 6: Rates of in vitro JH III synthesis by female corpora allata (CA) during diapause under natural winter conditions.
Time in diapause differs as all groups of beetles were not induced in diapause at the same time. Soil temperature as in figure 1.
Number of CA pairs incubated individually is given between brackets.
Columns represent means ± S.E.M. (vertical bars). Shaded columns represent results for diapausing females that have shown some upward movement (under the lid). Open column is for diapausing females still buried in the sand and showing no tendency to emerge. Black column represents control CA activity of females after diapausing for 24 weeks at constant 4°C in the laboratory. The rate of JH synthesis in females after 28 weeks of diapause at 4°C was 0.7 pmoles JH III/hr/CA pair ± 1.2 (S.D., n = 16) at spontaneous emergence from the sand at 25°C.
The arrow indicates first emergence above the soil in the field population.

RATES OF JH-SYNTHESIS BY CORPORA ALLATA TAKEN FROM FEMALES DIAPAUSING IN THE FIELD

Figure 6 shows that before emergence from the soil (at the end of May), the rate of JH-synthesis increases progressively in 'quiescent' females under field conditions (r_S = 0.64, t= 4.44, 90 d.f., $p < 0.001$). The JH titre in haemolymph was then 20.85 $\pm$ 1.85 ng JH III equivalent/ml haemolymph. Interestingly, 'quiescent' beetles of the same age kept in the laboratory at constant temperatures did not display this increase in JH concentration towards the end of diapause (see Chapter 3).
In addition, CA activity does not increase with diapause duration (see fig. 6). Soil temperature, which increases until the end of June, seems thus to affect CA activity in the transient phase of diapause termination.

DISCUSSION

The finding of several temperature thresholds during diapause is evidence for changes in response to temperature, corresponding to distinct physiological states, as has been emphasized by Le Berre (1965), Tauber and Tauber (1973) and Hodek (1978).
The results show that three phases can be distinguished during hibernation of the Colorado potato beetle: i) 'diapause development', or true diapause, ii) 'post-diapause quiescence' or maintenance, and iii) a transient phase of post-diapause development, which leads to emergence from the soil.

DIAPAUSE DEVELOPMENT

Evidence for development during this phase is provided by the increase in the rate of reproduction in diapausing beetles artificially exposed to photoperiod, as 'diapause development' proceeds.

Another important result is that 'diapause development' occurs at 4°, 12° and 25°C. Since this development can be completed at a temperature as low as 4°C, its temperature threshold is lower than that for other phases of diapause. Furthermore, it should be noted that no chilling period is required for diapause termination in the Colorado potato beetle, in contrast to some other species (Williams, 1956).

The duration of 'diapause development', estimated as approx. 12 weeks

in this population, can vary greatly among individuals (fig. 2: 8 to 16 weeks; see also: Ushatinskaya, 1978 and Hoy, 1978). This duration is in agreement with earlier work showing that 'true diapause' lasts for 16 weeks in field populations of Colorado potato beetles (Ushatinskaya, 1958; Le Berre, 1965). Similar conclusions have been reached recently for other beetles diapausing in the adult stage (Ashida and Kanehisa, 1981; Gehrken, 1985).

Our results indicate that increasing temperature increases the rate of 'diapause development'. Food alone is not sufficient to break diapause, but since beetles check regularly while diapausing at high temperatures and remain above the sand when food is available, food does play a role in its completion. The perception of food by beetles still underground remains, however, to be investigated. It is unlikely that a change in humidity due to the presence of fresh foliage on the sand would terminate 'diapause development', since beetles do not respond to water sprinkled on the sand during this phase.

The sensitivity to photoperiod is retained during 'diapause development'. Beetles are sensitive to photoperiod until at least February, as indicated by the effects on reproduction. Diapause can be terminated by three LD photocycles during this phase. This number corresponds exactly to that required to reverse the induction of diapause in pre-diapause adults (De Kort and Khan, 1984).

POST-DIAPAUSE DEVELOPMENT

By definition post-diapause development starts as soon as the first phase of true diapause is terminated. Our results indicate that environmental factors can delay its occurrence. We have shown that diapause in Colorado potato beetles is prolonged by low temperature, desiccation or lack of food. This second phase of diapause lasts until environmental conditions are favourable for resumption of activity or reproduction; it can therefore be called 'quiescence'.

No major progress in ovary maturation occurs during quiescence, since absolute time in diapause does not affect the pre-oviposition time or the rate of oviposition. Ovarian maturation is probably completed during 'post-diapause development' (see Chapters 2, 3 and 5).

Unless temperature is sufficiently high, neither water nor a supply of food lead to emergence of 'quiescent' beetles. Therefore, the main factor

controlling 'post-diapause development' is temperature; higher temperatures increase the rate of emergence. Water sprinkling, however, also accelerates emergence. This confirms the essential role of moisture in the completion of the transient phase, as has been emphasized in studies of several other insect species (Church, 1955; Beck, 1967, 1980; Behrens, 1985). Since light and food are unlikely to be perceived in the soil, temperature and water should be regarded as the main environmental factors synchronizing diapause termination in nature (see also: De Wilde, 1969).

Our laboratory experiments show that a temperature of 4°C does not allow emergence of beetles, even after they have completed their 'true diapause' phase. This low temperature does not prevent movements (unpublished observations); 4°C must thus be below the threshold for the transient phase of post-diapause development.

The transient phase seems to occur when the soil temperature is greater than 8-10°C, as indicated by the slight increase in CA activity from mid-May, which coincides with a slightly increased rate of O_2 consumption before emergence of the beetles (Schröder, 1957; Le Berre, 1965).

A temperature threshold of 9°C for upward movements has been proposed by Le Berre (1965). As upward movements start when soil temperature reaches 4-5°C, it should be emphasized that upward movements are distinct from emergence moves occurring only after a transient phase of post-diapause development. Upward moves should not, therefore, be used as criterion for diapause termination. Our temperature threshold of 8-10°C for the transient phase corresponds to the 9°C of Le Berre (1965) and the 9.88°C threshold (for activity after diapause) estimated by Lashomb and coauthors (1984).

Emergence occurs in the field only when soil temperature is above 11°C. This agrees with values published earlier: 12°C (Le Berre, 1965) and 14°C (Wegorek, 1959) taking soil type and climatic variations into account (see Lashomb et al., 1984).

After diapause termination (emergence), post-diapause development continues and results in reproduction. The temperature threshold for oviposition is 15°C, but maximal fecundity is reached at 25°C (Grison, 1950).

Interestingly, our results indicate that light is not necessary for oviposition after diapause. On the other hand, food is essential for reproduction as has been shown also in LD-females which do not diapause (Khan et al., 1982b).

Finally, the reproductive rate no longer depends on photoperiod after 'diapause development' is completed. This confirms previous results on rate of oviposition in females exposed to LD or SD photoregimes after diapause in the laboratory (at constant 25°C) (Lefevere and De Wilde, 1984).

Chapter 2

Changes in the concentrations of metabolites in haemolymph during and after diapause in female Colorado potato beetle, Leptinotarsa decemlineata (Say).

ABSTRACT

Haemolymph concentrations of proline and other free amino acids, glucose, total lipids and total proteins, including the typical diapause proteins and vitellogenins, were measured at various stages in the diapause of adult Colorado potato beetles. The concentration of the various metabolites is much higher in the haemolymph of diapausing females than in that of non-diapause females. The specific haemolymph composition of diapausing Leptinotarsa decemlineata displays similarities with that of other insect species diapausing as larvae or pupae. The change from 'diapause development' (3 months) to post-diapause quiescence is reflected in alterations in the levels of total haemolymph proteins. Results show that most of the reserves accumulated during the pre-diapause period are used immediately after diapause termination for resumption of activity and start of reproduction. Thus the 'diapause proteins' seem to have no important function during diapause, but may have a role in termination of diapause. Comparison with results obtained from females diapausing at higher temperature indicates that specific metabolic changes arise in response to cold in diapausing beetles. Diapausing beetles are better able to resist frost damage than non-diapause active adults.
Finally, proline appears to have a cryoprotective function, rather than an energetic one, during diapause.

INTRODUCTION

Under natural conditions (in the Netherlands) Colorado potato beetles enter diapause in late August and emerge from the soil in the following spring (late May). In this way these insects avoid frost and lack of food. Two periods of increased cold-resistance have been observed during hibernation : one at the beginning of diapause and a second one in mid-Winter (Minder and Chesnek, 1970). Diapause also enables the beetles to synchronize reproduction with favourable environmental conditions for development (De Wilde, 1969).

Diapause is a dynamic developmental process consisting of three successive phases: (1) 'diapause development', or true diapause, during which insects are still sensitive to inducing factors and insensitive to terminating factors; (2) 'post-diapause quiescence', or diapause maintenance (Tauber and Tauber, 1976), in which they are responsive to diapause-terminating factors, but remain in diapause as long as unfavourable environmental conditions persist; (3) a transient phase of post-diapause development leading to emergence from the soil or diapause termination. In this phase, activating (or 'tachytelic': Hodek, 1983) processes associated with temperature increase occur (Chapter 1).

During the pre-diapause period total body content of lipids, proteins and carbohydrates increases (Salem, 1984; De Loof and De Wilde, 1970a; Grison and Le Berre, 1953; El-Ibrashy, 1965) and diapause proteins accumulate in the haemolymph (De Loof and De Wilde, 1970a; Dortland, 1978; Peferoen et al., 1982).

Diapause is characterized by low metabolic rates and gradual cessation of all anabolic processes, such as flight muscle development (De Kort, 1969) and synthesis of protein and JH (Dortland and De Kort, 1978; Chapter 3). Therefore energy demands by tissues are greatly reduced (Harvey, 1962). Since the quantity of reserves decreases only slightly during diapause (Busnel and Drilhon, 1937; De Wilde, 1954; Ushatinskaya, 1956; De Loof and De Wilde, 1970b) some authors have suggested that reserves are destined mainly for use after diapause termination, such as migration and reproduction.

Another feature of diapausing insects, including Leptinotarsa, is the exceptionally high concentration of free amino acids in the haemolymph (Duchâteau and Florkin, 1958; Florkin and Jeuniaux,.1974; Jungreis, 1980). The physiological role of this accumulation remains unclear. Proline,

however, has been found to be a cryoprotective agent in plant cells (Heber and Santarius, 1976; Withers and King, 1979) and to protect membranes against desiccation and toxic solutes (Levitt, 1980; Kandpal and Rao, 1984). Since proline concentration has been shown to increase dramatically in insects exposed to low temperature (Hanec and Beck, 1960; Mansingh, 1967; Veimer, 1972; Storey et al., 1981; Morgan and Chippendale, 1983), it has been suggested that proline could have a cryoprotective function in insects too. On the other hand, it is well-documented that proline is an energy substrate for flight in Leptinotarsa (Mordue and De Kort, 1978; Khan and De Kort, 1978; Weeda, 1981).

The aim of this study is to assess the role of proline and other metabolites accumulated in the haemolymph during diapause. We consider the following questions. Are metabolic changes detectable in the haemolymph of diapausing females? Do they reflect a dynamic process of 'diapause development' or a transition to 'quiescence', the phase of diapause maintenance? Are these reserves utilized throughout diapause or only after its termination? If the latter, do they have another function during diapause, e.g. improving resistance to cold?

MATERIALS AND METHODS

Colorado potato beetles (Leptinotarsa decemlineata (Say)) were obtained from the laboratory strain (Wageningen, the Netherlands) and fed on fresh potato leaves. Adult diapause was induced by rearing the beetles ab ovo under a short-day (SD) photoregime (=10 hrs photophase/14 hrs scotophase) at 25°C and R.H. = 70%. Young adults fed actively, building up reserves, but did not reproduce. When adults left the plant to dig into the soil (11 days after emergence), groups of 35 females and 5 males were placed on moistened sand (5 cm deep) in plastic boxes (9 x 9 x 7 cm). The beetles buried themselves within two days, after which the boxes were transferred to permanent darkness at constant 4°C. Other groups were kept at constant 25°C under permanent darkness. 'Diapause development' lasts for 5 months at 4°C and approx. 3 months at 25°C (Chapter 1). Only females were used for further experimentation.

Haemolymph was sampled after various times spent in diapause and after diapause termination. Haemolymph was collected in glass-capillary micropipettes, after clipping off the wings or hindlegs from ten females (in melting ice). After 1 min. centrifugation in an Eppendorf centrifuge,

the pooled haemolymph was stored at -20°C. Later, the concentrations of free amino acids, glucose, total lipids and total proteins were measured in all samples.

For post-diapause determinations, samples were collected from females that had been removed from the sand (artificial termination of diapause) or from emerged females after the boxes were transferred from 4 to 25°C (spontaneous termination of diapause). Spontaneous emergence was checked daily. After diapause termination, females were placed under either long-day photoregime (LD = 16 hrs photophase/8 hrs scotophase) or SD conditions (see above), at 25°C and R.H. = 70%, with a food supply and active males.

To determine survival of beetles at sub-zero temperatures, transparent plastic boxes containing groups of 21 to 30 females were placed in the freezing compartment of an ordinary refrigerator at -4° $\pm$ 1°C (continuous darkness). The boxes were removed after various periods: 2, 6, 24, 48 or 72 hours and transferred to LD conditions (see above) at 25°C. Survival, feeding behaviour and oviposition were checked daily.

Determinations in haemolymph

Concentrations of free amino acids (FAA) were analysed by ion exchange-HPLC, using ionic strength gradient elution, with post-column fluorescence detection (Pfeifer et al., 1983). Each analysis was performed twice. Prior to injection into the column, haemolymph proteins were precipitated using 5% trichloroacetic acid (TCA) and the sample was passed through a SEP-PAK C_{18} Cartridge, discarding lipids and high molecular weight proteins. The sample was finally collected in 0.1% trifluoroacetic acid (TFA) in water: methanol (70:30). Proline concentration is corrected by a factor 0.35 for imperfect response to orthophthaldehyde (OPA) in the fluorescent detection reaction (Pfeifer et al., 1983) and is then of the same order as concentration measured by colorimetry.

Proline was determined colorimetrically (Bergman and Loxley, 1970); toluene was used to extract the ninhydrin complex. Glucose was quantified enzymatically (GLUCO-QUANT Kit, Boehringer-Mannheim GmbH, Diagnostica) using glucose hexokinase combined with G-6-P-dehydrogenase. Total lipids (vanillin reactive material) were quantified using the method described by Goldsworthy et al. (1972).

The total quantity of proteins was measured colorimetrically (at λ = 595 mm) using Coomassie Blue G - 250 reagent (Bradford, 1976), against a

reagent blank. Bovine serum albumin (1 mg/ml) was used as a standard. The proportions of diapause proteins (DP) and vitellogenins (Vg) in the haemolymph were quantified by densitometry after agarose-gel electrophoresis (with ready-for-use plates: Pfizer Pol-E film system). The electrophoresis buffer was 0.05 M barbital in 0.035% Na-EDTA (pH=8.6). Aliquots of 0.6 µl of haemolymph were applied and subjected to 90 volts (± 5%) for 45 mins. The thin-gel agarose films were subsequently stained for proteins with a 0.2% Amido Black solution in 5% acetic acid, for 15 mins; briefly washed in 5% acetic acid and dried at 70°C for 30 mins. The plates were finally cleared by 3 rinses of 5% acetic acid and dried at 70°C. The concentration of the protein bands was recorded by scanning through a densitometer (at λ = 550 mm) and the relative percentage of diapause proteins (DP_1, DP_2, DP_3 according to Peferoen et al., 1982) and vitellogenins were calculated.

In vitro proline synthesis by fat body

The dorso-abdominal cuticle with attached fat tissue was dissected under ice-cold Leptinotarsa saline. Groups of 5 fat bodies were incubated at 25°C (for 2 hrs) in saline with alanine substrate (4 mM), as described by Weeda et al. (1980a). Each incubation was repeated twice. After addition of tricholoroacetic acid (final concentration: 8.3% TCA) and 4 mins. centrifugation in an Eppendorf centrifuge, the synthesized proline was assayed colorimetrically, as above (Bergman and Loxley, 1970). Spontaneous release of proline by the fat bodies was quantified by control incubations (without addition of alanine) and subtracted from the results. The 5 fat bodies were subsequently homogenized by ultrasound. After addition of 10% TCA, samples were allowed to stand at 4°C for 12 hrs; they were then centrifuged for 4 mins., in an Eppendorf centrifuge and processed for quantification of total protein content (as above: Bradford, 1976). Proline synthesis is expressed in µg proline/hr/mg protein.

RESULTS

Proline and other free amino acids in haemolymph

Figure 7 shows that proline is the main amino acid in the haemolymph of diapausing and 'post-diapause' beetles. At the onset of diapause,

proline accounts for 77% of the total amino acids in haemolymph. This percentage decreases progressively throughout diapause as the concentrations of the other free amino acids increase. However, the concentration of proline remains high and relatively constant throughout diapause, as compared with that in non-diapause beetles (Tables IV and V).

Proline oxidation is accompanied by changes in alanine and glutamate concentrations (Khan and De Kort, 1978; Weeda et al., 1980a and b). Alanine concentration increases in the first months of 'diapause development', while glutamate concentration decreases (Table IV). This reflects the decreasing rate of *in vitro* proline synthesis by the fat body (Table V),

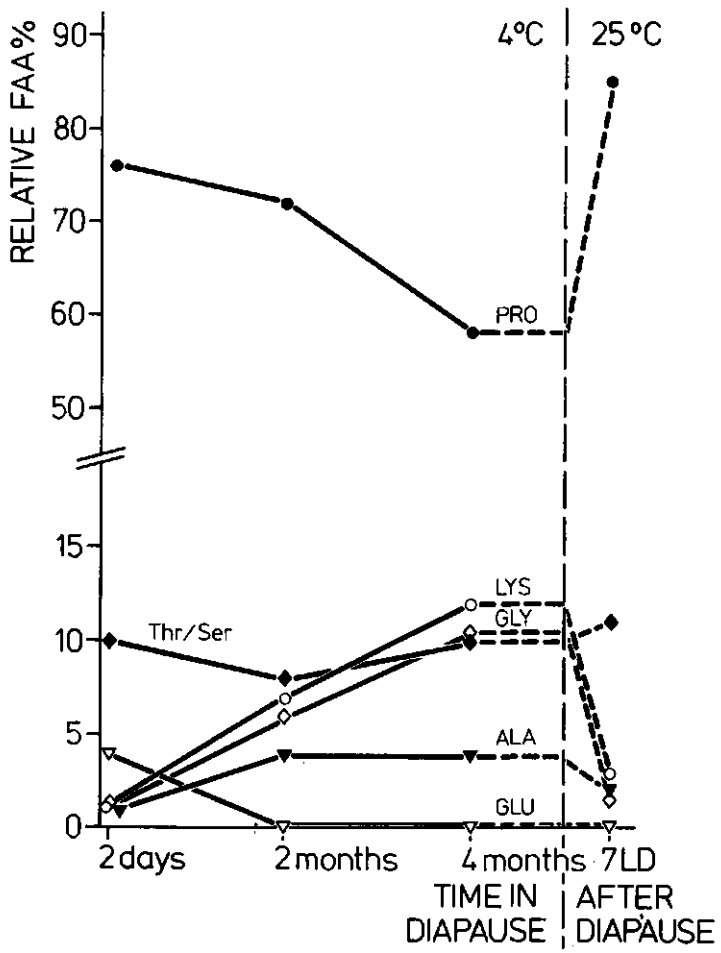

Fig. 7: Concentrations of main free amino acids (FAA) as a percentage of total FAA in haemolymph of *L. decemlineata* females, during diapause and after artificial termination of diapause (5 months).

Table IV: FAA concentrations (in mM) in haemolymph of L. decemlineata females, throughout diapause at 4°C and after artificial diapause termination (5 months)

	DIAPAUSE DURATION:			AFTER DIAPAUSE	AFTER DIAPAUSE	WITHOUT
	2 days	2 months	4 months	at 4°C (7 LD)	at 25°C (7 LD)	DIAPAUSE (8LD at 25°C)
Asp	0.05 ± 0.05	0.06 ± 0.03	N	0.11 ± 0.03	N	N
Thr/Ser	9.90 ± 0	10.37 ± 0.33	13.28 ± 0.59 bc	3.98 ± 0.59 de	7.80 f	6.03 ± 0.47
Glu	4.34 ± 0.01	0.12 ± 0.05 a	0.09 ± 0.03 c	N e	7.02 f	11.34 ± 0.03 g
Pro	78.86 ± 0	97.68 ± 2.20 a	77.31 ± 3.69 b	79.51 ± 0.21	41.49 f	38.69 ± 2.23
Gly	1.82 ± 0.01	7.61 ± 0.06 a	12.71 ± 0.29 bc	1.97 ± 0.05 d	1.98	2.81 ± 0.23
Ala	1.65 ± 0.01	5.82 ± 0 a	5.75 ± 0.35 c	0.48 ± 0.03 de	1.05 f	0.57 ± 0.03 g
Cys	N	0.12 ± 0.12	N	N	N	N
Val	1.11 ± 0	1.79 ± 0.11 a	2.52 ± 0.27 bc	0.63 ± 0.06 de	1.83 f	1.47 ± 0.12
Met	N	N	N	N	N	N
Ile	0.18 ± 0	0.35 ± 0.05 a	0.66 ± 0.03 bc	0.05 ± 0.01 de	0.21 f	0.20 ± 0.02
Leu	0.11 ± 0.01	0.14 ± 0	0.27 ± 0.03 bc	0.12 ± 0.06 d	0.24	0.24 ± 0
Tyr	0.99 ± 0.03	1.25 ± 0.03 a	1.82 ± 0.26 bc	0.55 ± 0.02 de	0.84 f	1.22 ± 0.11
Phe	0.09 ± 0	0.14 ± 0.02	0.25 ± 0.01 bc	0.22 ± 0.01 e	1.02 f	0.93 ± 0.06
His	1.92 ± 0.03	1.10 ± 0.08 a	3.12 ± 0.15 bc	2.90 ± 0 e	2.97	3.62 ± 0.26
Trp	0.03 ± 0	0.26 ± 0.02 a	0.32 ± 0.01 c	0.10 ± 0.0	N	N
Lys	2.37 ± 0.03	9.11 ± 0.02 a	15.68 ± 0.80 bc	2.46 ± 0.03 e	5.34 f	3.83 ± 0.35
Arg	N	N	N	N	N	N
T.	103.4	135.9	133.8	93.1	71.8	71.0

Legend to Table IV:

Each sample was pooled haemolymph of 10 females. Concentrations represent means of two independent analyses ± S.D. T = total free amino acid titre in haemolymph. LD = long-day photoregime (16 hrs light/8 hrs dark). Glu and Asp stand for glutamine and asparagine respectively, together with their acid form. N = undetected in this sample. Least significant differences (5%) are given by letters. a: 2 days and 2 months at 4°C; b: 2 months and 4 months at 4°C; c: 2 days and 4 months at 4°C; d: 4 months diapause 4°and after diapause; e: 2 days diapause 4°C and after diapause; f: after diapause at 4°C and 25°C; g: after diapause at 25°C and control without diapause (LD).

Table V: Proline synthesis of fat body (in vitro) and the concentrations of different metabolites in haemolymph (in µg/µl), throughout diapause and after artificial diapause termination (5 months at 4°C) in Leptinotarsa females.

TIME AFTER START OF DIAPAUSE	PROLINE SYNTHESIS (in µg pro/hr/mg proteins)	PROLINE	GLUCOSE	TOTAL LIPIDS
2 days	31.0 ± 4.9	6.4 ± 0.7	1.42 ± 0.02	11.0 ± 0.3
2 months	23.3 ± 2.9	6.2 ± 0.1	0.28 ± 0.01	12.0 ± 1.2
4 months	17.9 ± 0.6	5.1 ± 0.2	0.06 ± 0.01	9.9 ± 0.6
After diapause-break (7LD)	42.3 ± 2.2	4.7 ± 0.3	0.05 ± 0.01	8.5 ± 0.3
Control (without diapause)	54.6 ± 2.7	4.3 ± 0.2	0.30 ± 0.10*	7.5 ± 0.3*

Each sample was pooled haemolymph of 10 females.
Results are means of two independent determinations ± S.D.
LD = long-day photoregime (16 hrs light/8 hrs dark).
Data with asterisks are from Weeda et al. (1979).

causing the fall in the rate of alanine utilization as diapause continues.

Apart from these three amino acids, the most abundant amino acids in the haemolymph of diapausing females are: Thr/Ser, Gly, His and Lys (fig. 7). Their concentrations increase progressively during diapause. This results in an increase in the total concentration of free amino acids during the first months of 'diapause development' (Table IV).

After diapause termination, most of the free amino acids concentrations drop to their initial level (at diapause onset) or lower (fig. 7 and Tables IV and V). The capacity of the fat body to synthesize proline in vitro is fully restored after diapause (Table V).

Total haemolymph proteins

Figure 8 shows that the total concentration of haemolymph proteins is initially high and remains so during 'diapause development'. However, it decreases, slowly but significantly during the post-diapause quiescence (after 5 months; Spearman's rank correlation coefficient: $r_S = -0.49$, $t = -2.8$, 25 d.f., $p < 0.01$).

Specific diapause proteins constitute about one third of the total haemolymph proteins (33.5% $\pm$ 1.7) and remain in this proportion throughout diapause (fig. 8). No vitellogenin was detected in haemolymph during diapause.

Figure 9A shows that the total concentration of proteins continues to decline during the transient phase of post-diapause development (during the first 2 days after temperature transfer before emergence). The concentration of diapause proteins decreases as well during the transient phase leading to emergence. On day 5 (i.e. 3 days after spontaneous termination of diapause) the total protein concentration increases as a result of the sudden accumulation of vitellogenin in haemolymph (28% of total proteins in agarose-gel, fig. 9A). Feeding, which starts two days after emergence, probably also accounts for this increase in haemolymph proteins. At the time of emergence from the soil, diapause proteins represent only 18% of the total amount of protein. Within 8 days of post-diapause, diapause proteins amount to less than 2% of total haemolymph proteins.

Figure 9B indicates that the decrease in diapause proteins and the accumulation of vitellogenin are slower under SD conditions than in LD-post-diapause females.

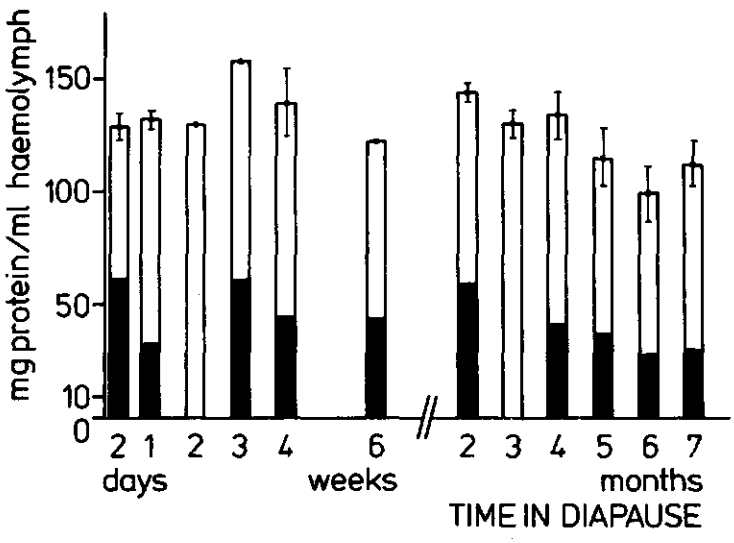

Fig. 8: Total concentrations of protein in the haemolymph of female L. decemlineata at various times during diapause at 4°C.
Each sample consisted of pooled haemolymph of 10 females. Vertical bars represent ± S.E.M. ($n \geq 3$). Black areas represent concentration of diapause proteins (when measured).

Energy-rich components of the haemolymph

Total carbohydrate concentration is low and difficult to detect in haemolymph of diapausing Leptinotarsa adults. However, the concentration of glucose at the onset of diapause (at 4°C) is much higher than in non-diapause controls. It decreases progressively throughout diapause and becomes almost undetectable after diapause termination (Table V).

Total lipid concentration in the haemolymph does not seem to vary significantly during diapause, but is higher than in 'non-diapause' females. After diapause is terminated, it decreases significantly (Table V).

Comparison with data obtained from females diapausing at 25°C

To separate the effects of diapause and low temperature, we analysed the haemolymph of beetles diapausing at 25°C. The methods used were the same as those described above.

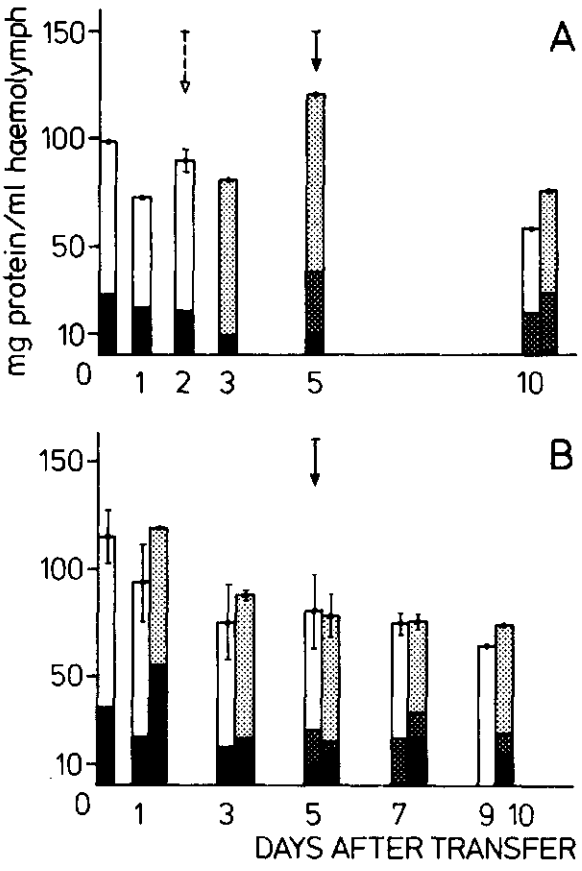

Fig. 9. Total haemolymph proteins concentrations in L. decemlineata females.
A: after spontaneous termination of diapause (7 months at 4°C) after transfer to 25°C.
B: after artificial diapause break (5 months at 4°C) by removal from the sand and exposure to long or short day conditions at 25°C.
Each sample was pooled haemolymph of 10 females. Open columns represent mean concentrations under long day (LD) conditions, stippled columns, under short day (SD) conditions. Bars represent ± S.D. Black areas represent concentration of diapause proteins. Shaded areas represent vitellogenin concentration as a percentage of total proteins. Dotted arrow indicates time of spontaneous outcome of all beetles. Plain arrows indicate first accumulation of vitellogenin in haemolymph.

The concentrations of haemolymph metabolites in females diapausing at 25°C were different of those in non-diapausing females at 25°C. The accumulation in haemolymph of metabolites such as total FAA (including proline), total proteins (including diapause proteins) and total lipids, typical for diapause, was recorded in diapausing females at 25°C. The glucose concentration was at this high temperature significantly lower in diapause than in non-diapausing females. The in vitro synthesis of proline by the fat body declined during diapause at 25°C. The specific haemolymph composition of diapausing females was thus present at 25°C as well.

The effects of low temperature (4°C) on haemolymph metabolites are

summarized in Table VI. It should be noted that neither tryptophane nor glucose was detected in haemolymph at 25°C.

In addition, although the total protein concentration did not differ significantly during 'diapause development', it decreased more rapidly at 25°C than at 4°C. Finally, the diapause proteins represented only 18% of total haemolymph proteins in post-diapause quiescence at 25°C. This percentage is similar to that reached during the transient phase leading to emergence at 25°C (see fig. 9A). Low temperature did not affect the *in vitro* synthesis of proline by the fat bodies.

Table VI: Effects of low temperature (4°C) on concentrations of haemolymph metabolites during diapause in *Leptinotarsa* females.

	-	0	+
Free amino acids (FAA)	Glu	Pro total FAA	Ala Gly Thr/Ser Tryp.
Other metabolites		lipids	glucose total proteins* diapause proteins*

Significant differences were tested by the Wilcoxon-Mann-Whitney test ($p < 0.05$).

- : concentration at 4°C lower than that at 25°C.

0 : no difference between 4 and 25°C

+ : higher concentrations at 4°C than at 25°C

Asterisks indicate when this effect is observed only during 'quiescence' (diapause maintenance phase).

In conclusion, both proline and glucose metabolisms are affected by temperature in diapause. Low temperature results in the accumulation of Ala, Gly and Thr/Ser in the haemolymph of diapausing females.

Resistance to freezing

The lethal minimum temperature for adult Leptinotarsa reported in the literature ranges from -4 to -5°C (Klein-Krautheim, 1950; Leib, 1951). Figure 10 shows clearly that resistance to frost is much higher in diapause.

Active 'non-diapause' females could not survive for longer than 6 hours at -4°C; 60% died within two hours of exposure to frost and indications of cell damage were observed (i.e. beetles were black and soft after thawing). In contrast diapausing females were still able to feed, and even oviposited, after having spent a period of 24 hrs at -4°C, although their haemolymph had frozen. Cells of diapausing females were damaged only after 48 hrs of frost. It should be noted that all damage could have occurred during either the freezing or thawing processes.

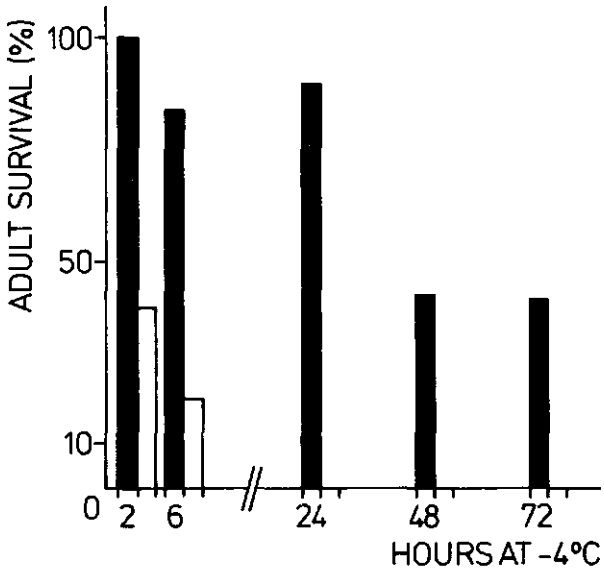

Fig. 10: Adult survival in L. decemlineata after exposure to sub-zero temperature.
Data presented on this graph are survival on day 7 after treatment. Black columns represent diapausing females (3 months at 4°C); open columns represent LD active females (5 days old).

DISCUSSION

Clearly, the composition of haemolymph in diapausing females differs from that in non-diapause beetles. Haemolymph metabolites such as free amino acids, proteins, glucose and lipids are in much higher concentrations at the onset of diapause than in non-diapause beetles.

Proline is the most abundant amino acid in the haemolymph during and after diapause, as in total body extract of non-diapause beetles (De Kort and Kramer, 1976). Proline concentration is higher during diapause than in active beetles. Our measurements of *in vitro* synthesis by the fat body, and the low alanine concentrations, suggest that proline is synthesized at the beginning of diapause. This conclusion is supported by the fact that the total pool of proline in pre-diapause beetles does not differ significantly from that in active adults (De Kort and Kramer, 1976).

The other important free amino acids (i.e. Thr/Ser, Lys, Gly, Ala, Glu) in haemolymph of diapausing females correspond almost exactly to those found in other diapausing insect species (Mansingh, 1967; Veimer, 1972; Morgan and Chippendale, 1983). The only major differences are that arginine is absent in the haemolymph of *L. decemlineata* and the concentration of histidine is lower than in the other species.

Our results indicate that the major haemolymph proteins in diapause are the 'diapause proteins' (34%), which do not seem to be used during 'diapause development'. Their name suggests they have a function in diapause, but our results show they do not. Moreover, some diapause proteins have been reported in non-diapause developmental stages of *L. decemlineata* (i.e. larvae and pupae, De Loof, 1972; Peferoen et al., 1982). Although their structure remains to be identified, we propose to use the term 'storage proteins' instead of 'diapause proteins'. The precise function of storage proteins remains to be assessed (see Levenbook, 1985).

The composition of haemolymph varies throughout diapause, reflecting changes in metabolism. During the phase of 'diapause development', the total titre of free amino acids in haemolymph increases (during the first two months of diapause) whereas total protein and lipid concentrations remain constant.

On the other hand, it is well-documented that lipids stored in the fat body are used mainly during the first phase of diapause (Fink, 1925; Busnel and Drilhon, 1937; Ushatinskaya, 1956). As this turn-over is not reflected in haemolymph concentrations, the total lipid concentration is not an

adequate indicator of lipid metabolism during diapause.

After completion of 'diapause development', haemolymph proteins, including diapause proteins, seem to be utilized at an increasing rate. These data agree with values published earlier (De Loof and De Wilde, 1970b). During post-diapause quiescence at 25°C, the concentration of diapause proteins is similar to that in the transient phase occurring after transfer from 4 to 25°C. This indicates that beetles in post-diapause quiescence are more responsive to temperature changes than in the 'diapause development' phase. Interestingly, Adedokun and Denlinger (1985) found that the end of diapause development in pupae of the flesh fly is marked by a transition from lipid utilization to carbohydrate or protein utilization. Our data suggest a similar metabolic transition in adult diapause of Leptinotarsa.

We can conclude that the reserves accumulated before diapause are scarcely used during diapause. Most of them are used after diapause termination, when a high metabolic rate is resumed during the pre-oviposition period. The largest quantity of haemolymph proteins is used after diapause. In addition, the supplies of lipids and glycogen stored in the fat body are also exhausted in the nine days following emergence (Busnel and Drilhon, 1937; Grison and Le Berre, 1953; Ushatinskaya, 1956). This exhaustion of reserves after diapause termination is probably related to the rapid redevelopment of flight muscles (de Kort, 1969) and to the resumption of ovarian maturation.

Therefore it should be emphasized that diapause termination is not initiated by either a drop of reserves during diapause or a lack of reserves after a certain period in diapause.

Since reserves seem to play a minor role during diapause, do they have any function in diapause? We showed that diapausing females are more frost-tolerant than active (LD) females, and that they have a different haemolymph composition. Moreover, the haemolymph composition in diapause is strongly affected by low temperature; concentrations of Thr/Ser are much higher from the onset of diapause than in non-diapause females, while Ala and Gly accumulate only during diapause at low temperature. Similar accumulations in haemolymph of Ala, Ser and Gly (Somme, 1966; Osanai and Yonezawa, 1984) and of Thr, Ser and Gly (Storey et al., 1986) have been reported in other insect species, in non-diapause developmental phases in response to low temperature. This phenomenon is thus related directly to cold acclimatization and is not related specifically to diapause.

In other diapausing insect species, the accumulation of alanine is always associated with low temperature (Somme, 1967; Veimer, 1972; Hansen and Viik, 1975; Morgan and Chippendale, 1983). Colorado potato beetles, diapausing at the adult stage, seem thus to respond to cold in the same way as other species.

It is well-documented that glycerol, free amino acids, proteins and other substances accumulated in haemolymph are cryoprotectants in cold-hardy insect species (see reviews: Baust, 1981; Duman and Horwath, 1983).In diapausing Colorado potato beetles, glycerol formation increases after exposure to low temperature (Marzusch, 1952). In addition, the high concentration of metabolites in the haemolymph of diapausing beetles suggests that the various solutes may increase tolerance of sub-zero temperatures. Cell membranes of diapausing Leptinotarsa seem better protected against freezing than those of non-diapausing beetles. Several mechanisms of cryoprotection may be involved: e.g. cellular bound water (Storey, 1983), or a non-specific colligative dilution of 'membrane-toxic' solutes (Heber and Santarius, 1976). Proline, which is in a very high concentration in the haemolymph of diapausing beetles regardless of temperature, might fulfil this role and have in this way a cryoprotective function. A certain balance in amino acids concentrations has to be reached to avoid cell membranes damage in freezing conditions (Heber and Santarius, 1976). Proline may thus also be a source of alanine during diapause at low temperature, since the concentration of alanine increases significantly in diapause at low temperature, whereas it did not at 25°C. Proline is unlikely to be an energetic substrate in diapause of Colorado potato beetles, since energy demands are reduced during diapause (see Downer, 1981).

Chapter 3

Juvenile hormone metabolism during and after diapause in the female Colorado potato beetle, Leptinotarsa decemlineata.

ABSTRACT

The corpus allatum (CA) activity, juvenile hormone (JH) III titre and JH-esterase activity in the haemolymph were examined throughout and after adult diapause in Leptinotarsa decemlineata. The influence of external factors (i.e. temperature, photoperiod and food supply) during and after diapause on JH metabolism and post-diapause reproduction was studied.

During diapause the JH titre, in good correlation with CA activity, remains at a constant low level regardless of temperature or duration of diapause. The transition from 'diapause development' to post-diapause quiescence is undetected with respect to JH titre. JH synthesis rate becomes undetectable soon after the onset of diapause. A gradual inactivation of CA may be one of the physiological traits of 'diapause development'. Shortly before diapause termination (emergence from the soil), a slight increase in the rate of JH synthesis occurs in relation to the increase in temperature.

The CA become completely activated only after emergence. The reactivation of CA is thus a consequence of diapause termination, rather than its cause. Although exposure to light may affect CA activation, food does not seem to play any role. After diapause, the JH titre increases progressively. JH-esterase activity in haemolymph is low throughout and after diapause, irrespective of temperature and photoperiod.

Sensitivity to photoperiod with regard to rate of oviposition is lost after 'diapause development' completion. A reduced responsiveness is, however, retained in post-diapause for JH synthesis rates.

INTRODUCTION

The adult Colorado potato beetle Leptinotarsa decemlineata (Say) enters a winter diapause in response to seasonal changes. The main cue is short photoperiod (SD) (De Wilde, 1955). Although adults feed actively during pre-diapause, no reproduction occurs and corpus allatum (CA) activity decreases gradually (Kramer, 1978a; Khan et al., 1982a). This leads to a low juvenile hormone (JH) titre in haemolymph (De Wilde et al., 1968; De Kort et al., 1981; De Kort et al., 1985). The onset of diapause is characterized by burrowing behaviour.

Under natural conditions, diapausing beetles remain in the soil, at a depth of up to 60 cm, from September to May. Under our climatological conditions, soil temperature in wintertime normally ranges between 1°C and 15°C at a depth of 50 cm. Around the end of May, diapause is terminated and beetles emerge from the soil.

Diapause is a dynamic process involving three successive phases:

1) diapause development, during which insects are insensitive to diapause-terminating stimuli. The sensitivity to diapause-inducing factors (e.g. short photoperiod) is still present. In Leptinotarsa, this phase lasts for 3 months under natural winter conditions. Its duration is reduced at high temperatures (Chapter 1), as has also been observed in several other species (Tauber and Tauber, 1976).

2) post-diapause quiescence: after diapause development is completed, the beetles maintain diapause, owing to unfavourable environmental conditions; they become active as soon as favourable conditions (e.g. temperature, food) for development and reproduction are restored.

3) transient phase of post-diapause development, in which activating processes occur in correlation with temperature increase and lead to emergence from the soil.

After diapause termination (or emergence), post-diapause development continues and leads eventually to reproduction. Under long-day conditions (LD = 16 hrs light/8 hrs darkness), CA activity is fully resumed within 5 to 7 days (Kramer, 1978a; Khan et al., 1982a). Vitellogenesis in Leptinotarsa decemlineata requires the presence of JH, as well as a cerebral factor (De Loof and De Wilde, 1970b). Thus the increase in JH titre soon leads to oviposition.

The central role of the CA in diapause induction has been demonstrated in Leptinotarsa by allatectomy (De Wilde and De Boer, 1961). The question

arises, whether or not JH is also involved in diapause termination. Towards the end of diapause in the field, CA activity increases slightly with soil temperature (Chapter 1).

Implantations of active CA do not terminate diapause (De Wilde and De Boer, 1961). Applications of JH I and various JH analogues during diapause break diapause only temporarily (De Wilde and Lutke-Schipholt, 1974; Sehnal and Skuhravy, 1976; Schooneveld et al., 1977).

In order to study the regulation of diapause termination, it is necessary to examine the JH metabolism during and after diapause. The JH titre in haemolymph is determined by the secreting activity of the CA (Pratt et al., 1975; Khan et al., 1982a) and the break-down activity of specific JH-esterases in the haemolymph (De Kort et al., 1978; see review De Kort and Granger, 1981). Therefore, JH titres, CA activities and JH-esterase activity in haemolymph were studied throughout diapause and after its termination. Moreover, we analysed the effects of external factors, such as temperature, photoperiod and food on JH metabolism.

MATERIALS AND METHODS

The Colorado potato beetles were from the laboratory strain (Wageningen, the Netherlands) that has been reared for 30 years on fresh potato leaves (De Wilde, 1957).
Experimental conditions were short day (SD) = 10 hrs photophase/14 hrs scotophase; long day (LD) = 16 hrs photophase/8 hrs scotophase. The temperature (unless stated otherwise) was 25°C and the relative humidity 60-70%.

Adult diapause was induced by rearing from the egg stage onwards under SD conditions. Groups of 30 to 35 females and 5 males (11 days old) were allowed to burrow into moistened sand (5 cm deep) in plastic boxes (9 x 9 x 7 cm). Two days later, these boxes were transferred to permanent darkness either at 4°C or at 25°C. 'Diapause development' lasts for 20 weeks at constant 4°C and for 13.5 weeks at constant 25°C (Chapter 1). To examine diapause under field conditions, groups of 50 females and 10 males were allowed to burrow into moistened sand (6 cm deep) in plastic boxes (25 x 15 x 9 cm). The boxes were then buried outdoors at a depth of 60 cm in the soil. Only females were used for further experimentation.

To assess the effect of temperature on JH metabolism during diapause, two groups of beetles were studied: one group underwent diapause at 4°C and the other at 25°C.

To discriminate between the two processes involved in diapause completion (i.e. 'diapause development' and activation), two different experimental procedures were used for measurements in post-diapause beetles:

1) artificial termination of diapause: diapausing females were removed from the sand and immediately exposed to LD or SD conditions in the presence of a food supply and active males. The influence of external factors can thus be evaluated, before the end of 'diapause development'.
2) spontaneous termination: boxes containing beetles in diapause were transferred to a higher temperature (25°C) under SD conditions and spontaneous emergence from the soil was recorded daily. The active beetles were used for further experiments.

JH metabolism measurements

At different times throughout diapause, groups of 10 females were carefully dug out for each sample. Haemolymph was collected at 0°C in glass-capillary micropipettes after clipping off the wings or hindlegs, centrifuged for one minute in an Eppendorf centrifuge, and stored at -20°C. Females were then immediately decapitated and the retrocerebral complex removed for determination of CA activity.

The in vitro radiochemical assay (RCA) for measuring JH synthesis by the CA (Tobe and Pratt, 1974) was performed according to the method described by Khan et al. (1982a). The final specific activity of ^{14}C-methyl-methionine (Amersham, England) was 37 mCi/mmol. The sensitivity of the RCA used is 0.1 pmoles JH (Tobe and Feyereisen, 1983).

The JH titres in the haemolymph were determined by the radio-immunoassay for juvenile hormones developed by Strambi et al. (1981), using antiserum prepared by A. and C. Strambi, Marseille, France. The procedure followed was described in detail for Leptinotarsa by De Kort et al. (1985).

The specific JH-esterase activity was measured by the method developed by Sanburg et al. (1975). JH hydrolysis in the haemolymph was determined by measuring the release of tritiated methanol from JH labelled on the methylester group (specific activity: 20.7 mCi/mmol.). Free ^{3}H-JH III was

removed by adsorption on activated charcoal. The procedure used was as described by Kramer and De Kort (1976).

Statistical differences were evaluated using the Wilcoxon-Mann-Whitney test, unless otherwise mentioned.

RESULTS

JH titre in the haemolymph

At the onset of diapause, JH titres were very low (below 6 ng JH III

Table VII: JH titres in the haemolymph of L. decemlineata females during diapause, measured by RIA and expressed in ng JH III - equivalent/ml haemolymph.

	4°C		25°C	
DIAPAUSE DURATION	Average	Range	Average	Range
2 days	2.7	(2.5-3.1)	5.6	(3 -8.1)
1 week	3	(2.5-3.0)	-	-
2 weeks	-	-	5.0	-
3 weeks	3	-	3.3	-
4 weeks	7.4	(6.2-8.5)	3	-
6 weeks	3.7	(3 -4.3)	12.1	(7.6-17.2)
2 months	7.3	(5.2-9.4)	5.1	-
3 months	-	-	4.6	(3 - 7.3)
4 months	5.1	(3 -8.2)	5.3	-
5 months	3	-	5.4	(3 - 7.8)
6 months	3	-	11.1	(7.9-14.2)
7 months	3	-	5.5	(4.3- 6.7)

Haemolymph from 10 females was pooled. Measurements were carried out in duplicate or triplicate, except when no range is shown.

equiv./ml haemolymph) (see Table VII). They remained constant throughout diapause regardless of the ambient temperature (Wilcoxon matched-pairs signed-ranks test). At 25°C, JH titres above 10 ng/ml were sometimes recorded, but this was never the case at 4°C.

Within 24 hrs after 'artificial' termination of diapause, the JH titre had risen (see Table VIII), and the titre increased rapidly under both photoregimes, until day 5. Subsequently, the JH titre decreased gradually (Table VIII). It is noteworthy that under SD conditions, the JH titre in the haemolymph increased at slower rate than under LD conditions, and did not reach such a high level.

The rate of oviposition was also significantly lower in SD-females

Table VIII: JH titres in the haemolymph of L. decemlineata females, after diapause (5 months at 4°C) had been broken by digging from the soil and transferred to 25°C, under long or short-day conditions (in ng JH III-equivalent/ml haemolymph).

DAYS AFTER DIAPAUSE BREAK	LD (oviposition rate = 47.9 eggs/day/♀ ± 2.4. n=38)		SD (oviposition rate = 36.7 eggs/day/♀ ± 1.8. n=39)	
	Average	Range	Average	Range
1	20.7	(14.4-27.5)	23.5	(21.3-25.6)
3	41.7	(37.9-45.4)	20.7	(11.8-29.6)
5	99.6	-	42.1	-
7	76.8	-	29.4	(24.9-33.8)
9	39.4	-	21.3	-

Each sample was pooled haemolymph from 10 females. Measurements were carried out in duplicate, except when no range is shown. Rates of oviposition are significantly different (Mann-Whitney U test, $p < 0.05$).
LD = 16 hrs photophase/8 hrs scotophase; SD = 10 hrs photophase/14 hrs scotophase.

(Mann-Whitney U test, $p < 0.05$; see Table VIII). The mean ($\pm$ standard deviation) pre-oviposition period was significantly prolonged under SD conditions: 8.4 days $\pm$ 0.9 ($p < 0.0001$); whereas under LD conditions, females started ovipositing 6.6 days $\pm$ 1.0 after diapause break.

Rates of JH synthesis during diapause

Figure 11 shows that at the onset of diapause, CA activity is low,

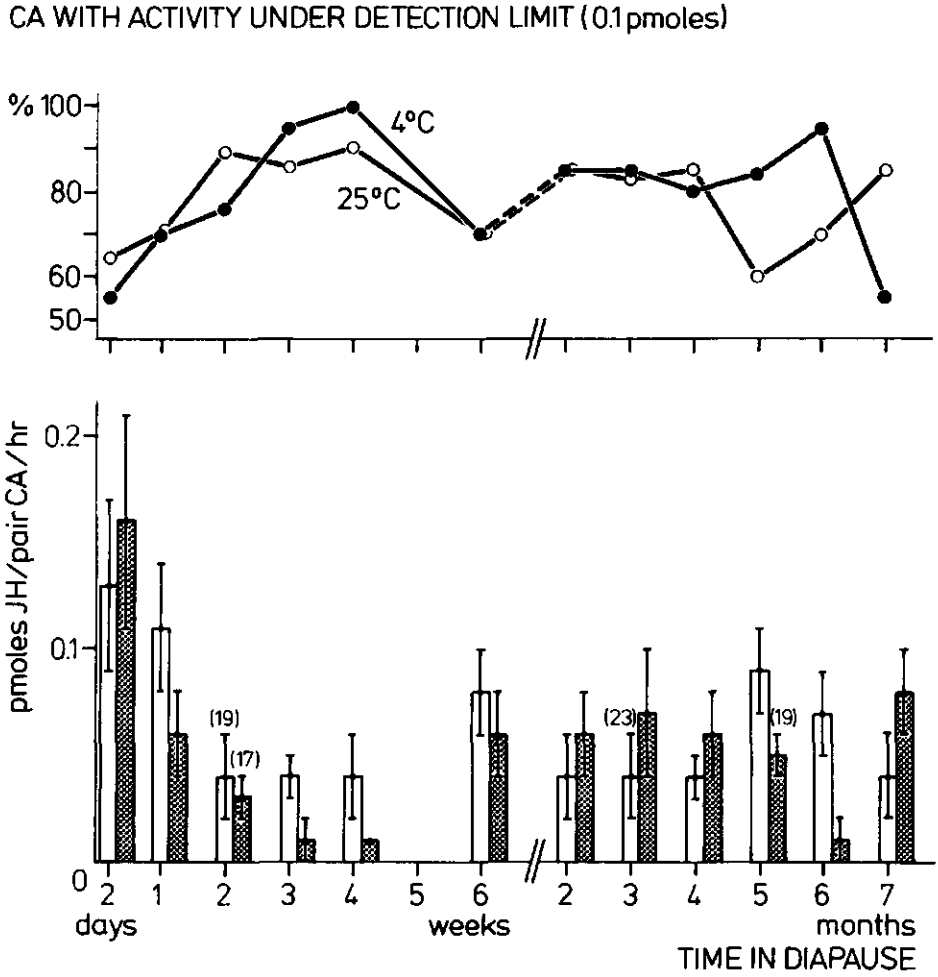

Fig.11: Mean rates of JH synthesis throughout diapause of L. decemlineata adult females (expressed in pmoles JH III synthesized/hr/CA pair). Open columns: diapause at 25°C; shaded columns: diapause at 4°C. Unless otherwise mentioned in brackets, n=20 in each column. Bars represent $\pm$ S.E.M.

ranging between 0.1 and 0.3 pmoles JH III synthesized/hr/CA pair. Relatively large individual variations in CA activity were recorded during the first month of diapause (ranging from 0 to 0.71 pmoles/hr/CA pair). The rates of JH synthesis decrease during the first month after burrowing (Spearman's rank correlation coefficient, at 4°C: $r_S = -0.25$, $t = -2.43$, 95 d.f., $p < 0.01$; at 25°C: $r_S = -0.19$, $t = -1.94$, 97 d.f., $p < 0.05$).

It should be emphasized that the decline in the rate of JH synthesis in the first month of diapause results from the fact that some individuals retain a detectable CA activity for a longer period than others after burrowing (fig. 11).

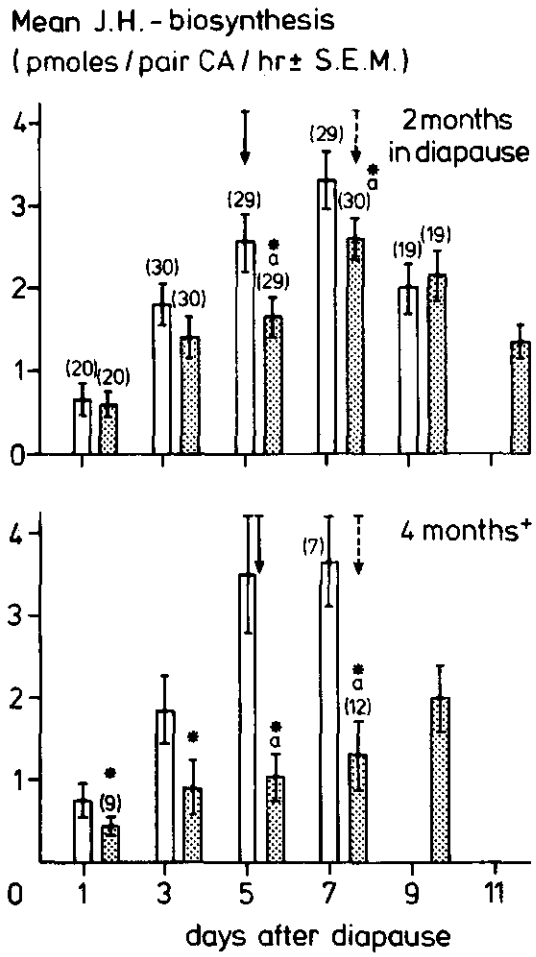

Fig. 12: Effect of photoperiod on mean rates of JH-synthesis in L. decemlineata females (in pmoles JH III/hr/CA pair), after diapause at 25°C was broken artificially. Open columns represent synthesis rates under LD conditions; stippled columns, under SD conditions. Unless otherwise mentioned in brackets, n=10 in each column. Bars represent ± S.E.M. Arrows indicate oviposition start in LD conditions; dotted arrows in SD photoregime. Asterisks indicate significant difference between photoregimes; a indicates differences between diapause duration (Wilcoxon-Mann-Whitney test: $p < 0.05$).
+ = data published in Lefevere and De Wilde, 1984.

Sensitivity to photoperiod and CA activity

During 'diapause development' the beetles are still sensitive to diapause-inducing factors, such as short photoperiod. When diapause is broken by exposure to photoregime (see Chapter 1) before completion of 'diapause development', the sensitivity to photoperiod is reflected in the rates of JH synthesis and in the pre-oviposition periods (fig. 12 and 13). Under SD conditions, CA activity remains significantly lower than under LD conditions, regardless of temperature (fig. 12 and 13). This agrees well with quantitative differences in JH titres after diapause (see Table VIII).

Short photoperiods also prolong the pre-oviposition period (fig. 12 and 13). The length of this period appears thus to be closely related to the rate of JH synthesis and JH titre in haemolymph.

It should be noted that absolute values of CA activity cannot be compared between different groups of experiments (i.e. fig. 12 to 15) since levels of rates of JH synthesis vary greatly throughout the year.

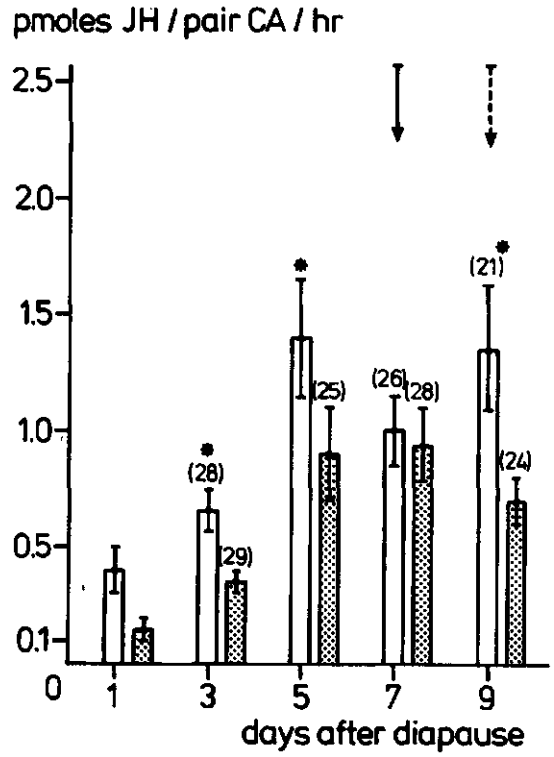

Fig. 13: Effect of photoperiod on rates of JH synthesis in L. decemlineata females, after diapause (4 months) at 4°C broken artificially.
Open columns represent mean synthesis rates under LD conditions; stippled columns, under SD photoregime. Unless otherwise mentioned in brackets, n = 20 in each column. Bars represent ± S.E.M. Arrow indicates start of oviposition in LD photoregime; dotted arrow, in SD conditions. Asterisks indicate significant difference between photoregimes (Wilcoxon-Mann-Whitney test, $p < 0.05$).

CA reactivation at the end of diapause

Emergence after transfer from 4° to 25°C does not occur before three days. Moreover, it should be noted that a lapse of 16 hrs (time between two observations) may exist after emergence. In what follows, this time is referred to, as time at emergence.

Figure 14 shows that at the time of spontaneous emergence from the soil, the CA are already fully active. The reactivation of the glands occurs thus within a period of 88 hours after transfer to 25°C. A full reactivation of CA is, however, not necessary for emergence, as shown after

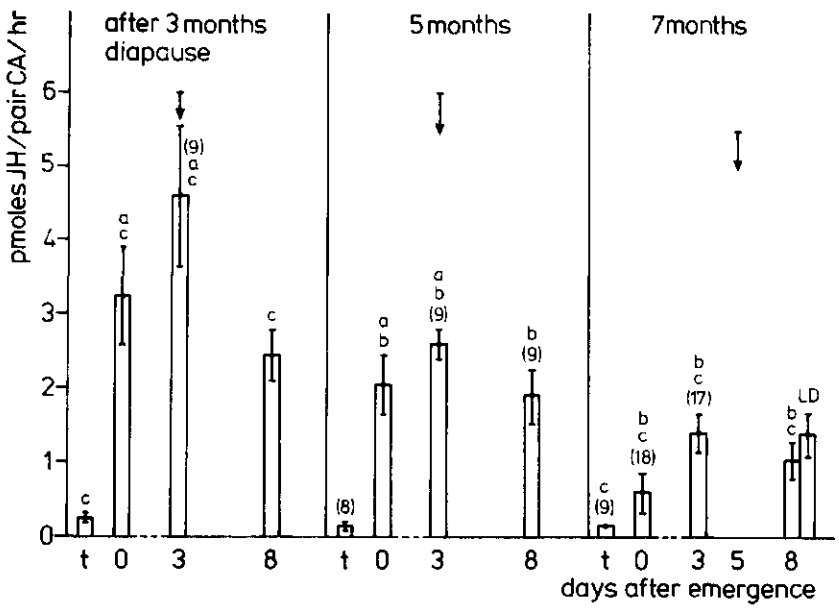

Fig. 14: Mean rates of JH synthesis after spontaneous termination of diapause, after diapausing L. decemlineata females were transferred from 4°C to 25°C under SD conditions (in pmoles JH III/hr/CA pair). Unless otherwise mentioned in brackets, n=10 in each column. Bars represent ± S.E.M. t=time of temperature transfer. day 0 = less than 16 hours after spontaneous emergence from the soil. LD = long days. Arrows indicate oviposition start. Significant diapause duration effect ($p < 0.05$) is given by a,b,c. a: between 3 and 5 months diapause; b: between 5 and 7 months; c: between 3 and 7 months.

7 months of diapause (fig. 14).

To determine the timing of CA reactivation more accurately, CA activity was measured at regular intervals of 24 hrs after transfer to 25°C. Table IX indicates that the glands become slightly active only 48 hrs after transfer to high temperature and retain a very low activity up till 72 hrs. This corresponds to the transient phase of post-diapause development (activating processes). Clearly, the full reactivation of CA observed at emergence (see fig. 14) must occur within 16 hrs of emergence from the sand.

Rates of JH synthesis increase rapidly after diapause termination, even when beetles are exposed to SD conditions (figures 12 to 15). The level of CA activity after diapause decreases with increasing time spent in diapause (fig. 14). This effect of diapause duration on CA activity is also found in females diapausing at 25°C and exposed to SD conditions after

Table IX: Reactivation of the CA after diapausing L. decemlineata females (6 months at 4°C) were transferred to 25°C, in continuous darkness and without food.

Time after transfer (in hours)	Mean JH synthesis rate (in pmoles/hr/CA pair) ± S.E.M.	Number of females with detectable CA activity (> 0.1)
0	0	0
24	0	0
48	0.08 ± 0.05	2
72	0.17 ± 0.03	7

N = 10 females in each sample. Note that spontaneous emergence occurs only 3 days after transfer.

diapause (fig. 12).

Effects of temperature, light and food on CA reactivation

The reactivation of CA is dependent on temperature increase, since a time-lag of 48 hrs was observed before activation occurred (Table IX). Similarly, the pre-oviposition period is prolonged for 2 days after diapause at 4°C as compared to beetles diapausing at 25°C (fig. 13 and 12, respectively).

Figure 15 shows the CA activity of beetles diapausing in the field, exposed to a soil temperature of 15°C (in mid-June), and transferred to the laboratory at 25°C. A temperature increase of 10°C in continuous darkness does not result in increased CA activity even after 5 days. On the other hand, the rate of JH synthesis increased significantly within 48 hrs of transfer and exposure to SD photoperiods (fig. 15). Exposure to light (or

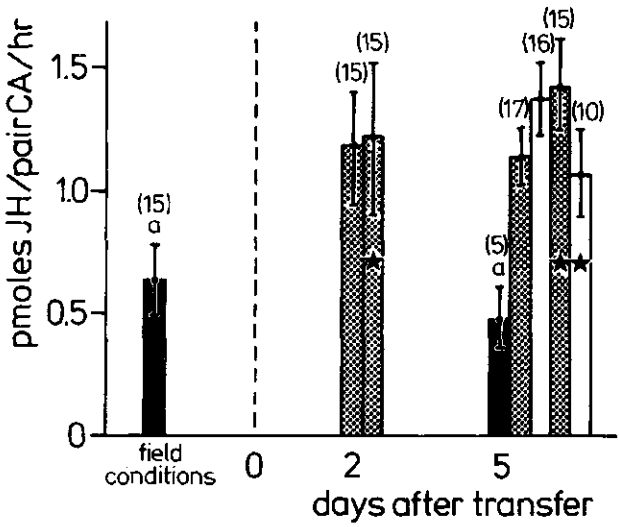

Fig. 15: Mean rates of JH synthesis after L. decemlineata females diapausing under natural conditions (7 months) were transferred in mid-June to the laboratory (25°C) and exposed to different photoregimes, with or without food.
CA activity is expressed in pmoles of JH synthetized/hr/CA pair. N is given between brackets. Vertical bars represent ± S.E.M. Plain columns represent beetles kept in permanent darkness; open columns represent those in LD conditions and shaded columns those in SD conditions. Stars indicate beetles which had food supplied. a indicates significant differences as compared with beetles exposed to light (Wilcoxon-Mann-Whitney test, $p < 0.05$).

possibly photoperiod) seems thus to be necessary for a full reactivation of CA. The lack of food does not affect the reactivation of CA within 5 days after diapause termination (fig. 15).

JH-esterase activity in haemolymph

JH-esterase activity in the haemolymph of diapausing females is relatively low (5.6 μmoles of JH-acid produced/hr/ml haemolymph) at the onset of diapause. This activity does not vary significantly throughout diapause (range: 3.3 to 8.7 μmoles), regardless of temperature.

After artificial termination of diapause (5 months at 4°C) the JH-esterase activity remained at the same level as during diapause (range: 2.9 - 8.0 μmoles/hr/ml haemolymph) during 7 days. Thereafter, the JH-esterase activity decreased to a very low level (range: 0.9 - 2.1 μmoles/hr/ml haemolymph), similar to that in young LD-females (without diapause). Similar changes in JH-esterase activity were observed after spontaneous emergence at 25°C (after 7 months diapause at 4°C). During the first 9 days after diapause termination, no effect of photoperiod could be detected on JH-esterase activity.

DISCUSSION

JH metabolism throughout diapause

Our results show that during diapause JH titres, the rate of JH synthesis and JH-esterase activity in the haemolymph are maintained at a constant low level from the onset of diapause up to 7 months, irrespective of temperature. In view of the fact that all previous data on diapausing beetles were obtained in the laboratory at a temperature of 25°C, rather unnatural conditions for diapause, it is noteworthy that JH metabolism is not affected by temperature during diapause.

A gradual inactivation of the CA occurs during the first month of diapause, as shown by the increasing number of glands with undetectable activity. This progressive inactivation of the CA could be a physiological trait of 'diapause development' (= physiogenesis). However, the physiological significance of this phenomenon is unknown.

No differences between the two successive phases of 'diapause development' and post-diapause quiescence could be detected in JH titres,

CA activity or JH-esterase activity in the haemolymph.

The sensitivity to photoperiod changes during diapause. During 'diapause development', transfer to higher temperature and exposure to photoperiod induced reproduction in LD and SD females, but there were quantitative differences in fecundity (i.e. rate of oviposition and pre-oviposition period). During post-diapause quiescence, the photoperiodic effect on fecundity is lost (see Chapter 1 and 5), but photoperiod still affects the rate of JH synthesis by the CA. A change in response to photoperiod intervenes thus at the transition between 'diapause development' and post-diapause quiescence.

Our results are in good agreement with the few published determinations of CA activity during diapause (Kramer, 1978a; Khan et al., 1982a), JH titre in diapausing beetles (De Wilde et al., 1968; De Kort et al., 1982) and JH-esterase activity in their haemolymph (Kramer and De Kort, 1976). The low JH titre during diapause correlates well with low CA activities and is similar to that of pre-diapause beetles (De Kort et al., 1981 and 1985).

The JH-esterase activity in the haemolymph remains constantly low throughout diapause, irrespective of temperature.

JH metabolism after diapause termination

Is CA reactivation the cause or a consequence of diapause termination? Our results clearly show that the CA are reactivated in the first 16 hrs after emergence from the soil. CA reactivation is thus a consequence of diapause termination. In addition, fully active CA are not required for diapause termination, as spontaneous emergence occurred after 7 months diapause, while CA activity was still low (fig. 14).

Although reactivation of the CA occurs after a time-lag of 48 hrs from transfer to 25°C, temperature increase alone does not induce the post-emergence activation of CA. Exposure to light seems necessary for full reactivation of the CA, whereas food does not affect their reactivation after diapause (Table IX and fig. 15). Khan et al. (1982b) found that CA activity increases significantly within 4 hrs of adult emergence from the soil and suggested that emergence itself was the triggering factor for CA activation in young-LD beetles. The CA were active irrespective of a food supply. In young non-diapausing adults, however, a starvation effect restraining the JH synthesis was observed after 24 hrs (Khan et al.,

1982b). We found no starvation effect on CA activity after diapause. This could be explained by the fact that reserves accumulated in the haemolymph before diapause, such as lipids and diapause proteins, are rapidly broken down after post-diapause emergence (Chapter 2). Moreover, a similar decline of reserves in the fat body has been reported during the first nine days following diapause (e.g. Ushatinskaya, 1956). Young non-diapausing beetles on the contrary have no reserves to be used after emergence.

Photoperiod still affects rates of JH synthesis after diapause termination, whereas rates of oviposition become independent of photoperiod in post-diapause quiescence (see Chapters 1 and 5; De Wilde et al., 1959; Lefevere and De Wilde, 1984).

Our results demonstrate that the JH titre after diapause is correlated with the length of the pre-oviposition period. A delay in CA reactivation either by low temperature or short photoperiods, always resulted in a delay in oviposition. This suggests a significant role of JH in post-diapause reproduction of Leptinotarsa decemlineata.

Finally, it is clear that after diapause the JH titre is mainly affected by CA activity and that the JH-esterases in the haemolymph play only a minor role.

We have shown that the rise in JH titre after diapause is not due to a decrease in JH-esterase activity, but to an increased rate of JH synthesis. The JH titre increases significantly within 24 hrs of diapause break, in close correlation with CA activity, and reaches values similar to those in young LD-beetles (De Kort et al., 1981 and 1985; Khan et al., 1982b and 1983).

On the other hand, the ecdysteroid content, which progressively increased during diapause, decreased simultaneously within 24 hrs of diapause break in both sexes (Briers and De Loof, 1981). Therefore, the balance between ecdysteroids and JH is reversed after diapause, as compared with pre-diapause adults (Briers et al., 1982). Although it is not yet known how these two hormones interact in Leptinotarsa, this observation indicates drastic changes in the neuro-endocrine system at post-diapause emergence.

Chapter 4

Endocrine control of diapause termination in the adult female Colorado potato beetle, Leptinotarsa decemlineata (Say).

ABSTRACT

To examine the possible involvement of endocrines in the termination of diapause, single doses of juvenile hormone III (JH) and 20-hydroxyecdysone (20-HE) were injected either alone or in combination, after various periods spent in diapause. 20-HE alone failed to terminate diapause, whereas JH III alone resulted in a temporary break of diapause. Only the combined injections of both hormones caused permanent termination of diapause. Hormonal administration seems most effective during the first month of diapause. Hormonal injections in this period failed, however, to induce reproduction. This change in response to exogenous hormones occurring after one month suggests a change in the neuro-endocrine system during the 'diapause development' phase.

The endogenous JH metabolism in diapause is not affected by 20-HE when this hormone is injected alone or following an injection of JH III. In contrast, exogenous JH III inhibited the rate of JH synthesis and induced high JH-esterase activity in haemolymph. These effects were retained throughout diapause, suggesting a continuous control of JH metabolism by the brain throughout diapause.

The effects of JH III on JH metabolism were suppressed by an early injection of 20-HE. The present results suggest that 20-HE plays a key role in modifying the regulation of JH metabolism in diapause. It is suggested that 20-HE switches off the 'diapause' programming of the brain at the end of diapause.

INTRODUCTION

The adult Colorado potato beetle, Leptinotarsa decemlineata (Say) enters a winter reproductive diapause under a temperate climate. This diapause is induced mainly by short photoperiods (SD) (De Wilde et al., 1959) resulting in the inactivation of the corpora allata (CA) (De Wilde and De Boer, 1961; Kramer, 1978a; Khan et al., 1982a). Juvenile hormone (JH) synthesis by the CA is controlled by nervous pathways, as well as by humoral factors (De Wilde and De Boer, 1969; Schooneveld et al., 1979; Khan et al., 1983 and 1986). The finding of a peak of ecdysteroids in pre-diapause adults of both sexes has led to the suggestion that diapause induction results from a change in hormonal balance between JH and ecdysteroids rather than from a depletion of JH alone (Briers and De Loof, 1981; Briers et al., 1982).

The endocrine control of diapause termination , however, is less well understood. Diapause could be broken by implantations of brains from active beetles (Grison, 1949). Grison noted that this operation was less effective in beetles that had just entered diapause than in the ones that had spent two months in diapause. On the other hand, implantations of active CA in diapausing beetles remained ineffective (De Wilde and De Boer, 1961). Topical applications of high doses of JH I or different juvenile hormone analogues (JHA) resulted only in a temporary break of diapause (De Wilde and Lutke-Schipholt, 1974; Sehnal, 1976; Schooneveld et al., 1977). The endogenous JH in the Colorado potato beetle was later identified as JH III (De Kort et al., 1982).

A reappraisal of hormonal control of adult diapause seems appropriate. JH-biosynthesis by the CA is low throughout the whole diapause period and the CA activity is fully resumed only after emergence from the soil (Chapter 3). This suggests that JH is not the primary factor for diapause termination. Ecdysteroid levels slowly increase throughout diapause (Briers et al., 1982). The role of 20-hydroxyecdysone (20-HE) and JH in the control of diapause termination is investigated in this chapter, by injection of these hormones, either singly or in combination, in diapausing females. A preliminary model of the neuro-endocrine control of adult diapause termination is proposed.

MATERIALS AND METHODS

Insects

Colorado potato beetles (Leptinotarsa decemlineata (Say)), were from the laboratory stock, reared on fresh potato foliage. Diapausing adults were obtained by ab ovo rearing under short day (SD) photoperiod (10 hrs photophase/14 hrs scotophase; at 25°C and 60-70% relative humidity). Young pre-diapause beetles fed actively during 10 days. No reproduction occurred and digging behaviour was observed on day 12.

Groups of 35-40 females and 5 males were allowed to dig in moistened sand (5 cm. deep) in ventilated plastic boxes (7 x 9 x 9 cm.). Two days later, the boxes were transferred to continuous darkness at 4°C. Diapausing beetles were kept under these conditions until further use. Only females were used for experiments.

To ensure treated females were still in the first phase of diapause (diapause development, Chapter 1), no experiment was performed later than 2 months after the onset of diapause.

Hormone administration

The boxes were transferred to 25°C 48 hrs prior to injection. Diapausing females were removed from the sand and anaesthetized with carbon dioxide for 4 mins. Hormones were injected into the dorso-lateral region of the second abdominal segment, using a Hamilton microsyringe, operated by means of a repeating dispenser.

Hormones were injected singly (JH III or 20-HE) or in combination (JH III followed by 20-HE, or vice-versa) at various times during diapause: 2,4,6 and 8 weeks. When two hormones were injected in combination, 48 hrs elapsed between two injections.

A single dose of 15 µg of a racemic mixture of JH III (Calbiochem) dissolved in 2 µl olive oil, was administered per female. This dose was based on JH titres measured by radioimmunoassay after injection of JH III (De Kort et al., 1985). Within 2 days the JH titre is similar to that of long day (LD) active females and remains so for 5 to 6 days. 20-HE (a gift from Organon, Oss, the Netherlands) was dissolved in ethanol and then diluted with distilled water to yield solutions in 2% ethanol. The single dose of 1 µg/female (in 2 µl)was chosen because this is biologically active

in Leptinotarsa larvae (De Wilde et al., 1980). Moreover, this dose is far in excess to the ecdysteroid titres found in active adult females (Briers et al., 1982: 15 ng/ml haemolymph). Control beetles received either olive oil or 2% ethanol (2 µl). Untreated controls were also transferred from 4°C to 25°C.

After recovering from anaesthesia, the injected females were buried in freshly moistened sand. Beetles were kept at 25°C in groups of 25 to 35 in the dark for 35 days. Mortality after injection ranged between 0 and 15%. Emergence was checked daily and emerged females were transferred to individual glass-jars (150 ml), provided with moist sand, fresh food and an active male, and exposed to SD conditions. Their digging behaviour and oviposition were recorded daily for another 3 weeks.

Measurements of JH biosynthesis and JH-esterase activity.

The effects of hormone injection on CA and haemolymph JH-esterase activity were studied as follows: groups of 10 injected females were removed from the sand 48 hrs after the (last) injection. Haemolymph samples were collected in glass-capillary pipettes after clipping off the wings and were pooled at 0°C. After 1 min. centrifuging in an Eppendorf centrifuge, the samples were stored at -20°C until further analysis. After bleeding the females were decapitated and the retro-cerebral complexes removed for determination of the rate of JH-synthesis, using the in vitro radiochemical assay (Tobe and Pratt, 1974).

The procedure using ^{14}C-methyl-methionine (Amersham, England; final specific activity: 37 mCi/mmole) was followed as described by Khan et al. (1982a).

JH-esterase activity in haemolymph was determined by an assay using isooctane for extraction of intact JH (Hammock and Sparks, 1977). Aliquots of 1000 and 2000 times diluted haemolymph in 0.1 M phosphate buffer (pH: 7.5) were incubated for 15 mins. at ambient temperature (22°C) with tritiated JH III (Radiochemical Centre, Amersham, England; final specific activity: 26-30 mCi/mmole; final concentration: 2.5×10^{-6} M). The reaction was stopped by addition of pure ethanol (final concentration: 16%). Undegraded JH was removed by addition of 2 volumes isooctane and intense shaking on a whirl-mix for 2 mins. After 10 mins. centrifuging at 3000 rpm, the aqueous phase was collected with glass micropipettes and counted for radioactivity in a liquid scintillation counter. The amount of JH-acid

formed was calculated after subtraction of the counts for a blank (without haemolymph) from the specific activity of the JH solution. The measurements of JH-esterase activity in haemolymph of active LD-females were similar to these obtained by the activated charcoal adsorption method used in previous work (i.e. Chapter 3; Kramer and De Kort, 1976).

Statistical treatment

A Chi-square test was used to assess significant differences in emergence between hormone treated beetles and controls ($p < 0.001$). The Wilcoxon-Mann-Whitney test was used to determine significant differences ($p < 0.05$) between mean rates of JH synthesis by the CA.

RESULTS

Behavioural criteria for diapause termination.

After injection, females showed several types of behaviour under SD photoregime:

1) No emergence within 5 weeks after injection.
2) Emergence the following day, no feeding during a period of 7 to 10 days and eventually return into the sand.
3) Emergence within a week, but no feeding or oviposition for 3-4 weeks.
4) Emergence within a week, start of feeding and oviposition after a pre-oviposition period.

In the following sections diapause termination will refer to beetles emerged from the sand (behaviour 3 and 4). Temporary emergence (as in behaviour 2) will be considered as failure to terminate diapause.

Percentages of beetles terminating diapause were calculated from the number of beetles emerged and the total number of survivors 35 days after treatment.

Since a high proportion of emerged beetles after JH treatment returned into the sand after 8 days, the percentage of beetles emerged within a week after injection was chosen, in all treatments, as an index of the immediate effects of hormones on diapause.

Diapause termination in control beetles.

Control injections always resulted in lower emergence than hormone injections. In the control groups, the average percentage of diapause termination one week after injection was 5 $\pm$ 4.8% (mean $\pm$ standard deviation; n = 16). The number of beetles in each group varied between 46 and 65.

Five weeks after control injections at 4 or 6 weeks in diapause, emergence was 25% and upto 50% when beetles were injected at 8 weeks. A similar increase in percentage emergence was recorded in untreated controls transferred from 4° to 25°C after 4, 6 or 8 weeks of diapause. Emergence after control injections never exceeded 4 $\pm$ 3.8% of that recorded in untreated controls transferred to higher temperature.

Control emergence has been subtracted from percentages of emergence after hormonal treatment.

Diapause termination by exogenous hormones.

Figure 16 shows that single injections of 20-HE do not affect diapause termination significantly. However, when the injection of 20-HE is followed by an injection of JH III, diapause is terminated as compared with control injections (Chi^2= 21.6, 4 d.f., $p < 0.001$).

When JH III is injected singly, only a temporary (although significant, Chi^2 = 17.9, 3 d.f., $p < 0.001$) break of diapause is recorded (fig. 17). Two weeks after the JH injection, all beetles treated at 2 or 4 weeks had returned into the sand and remained in diapause.

When the JH injection is followed by a single injection of 20-HE, diapause termination is also significantly enhanced (fig. 17, below: Chi^2 = 41.2, 4 d.f., $p < 0.001$). The combined injections in this sequence seem most effective when administered 2 weeks after the onset of diapause (fig. 17).

In conclusion, only the combined treatments with both hormones produce a durable termination of diapause. When injected at 2 weeks in diapause, JH and 20-HE have a synergistic effect on emergence: 22% of treated beetles terminated diapause, whereas the sum of emergence after single hormone injections is only 5% (see fig. 16 and 17: one week after injection). It should be stressed that the immediate response to hormone injections changes throughout 'diapause development'. Before one month, exogenous

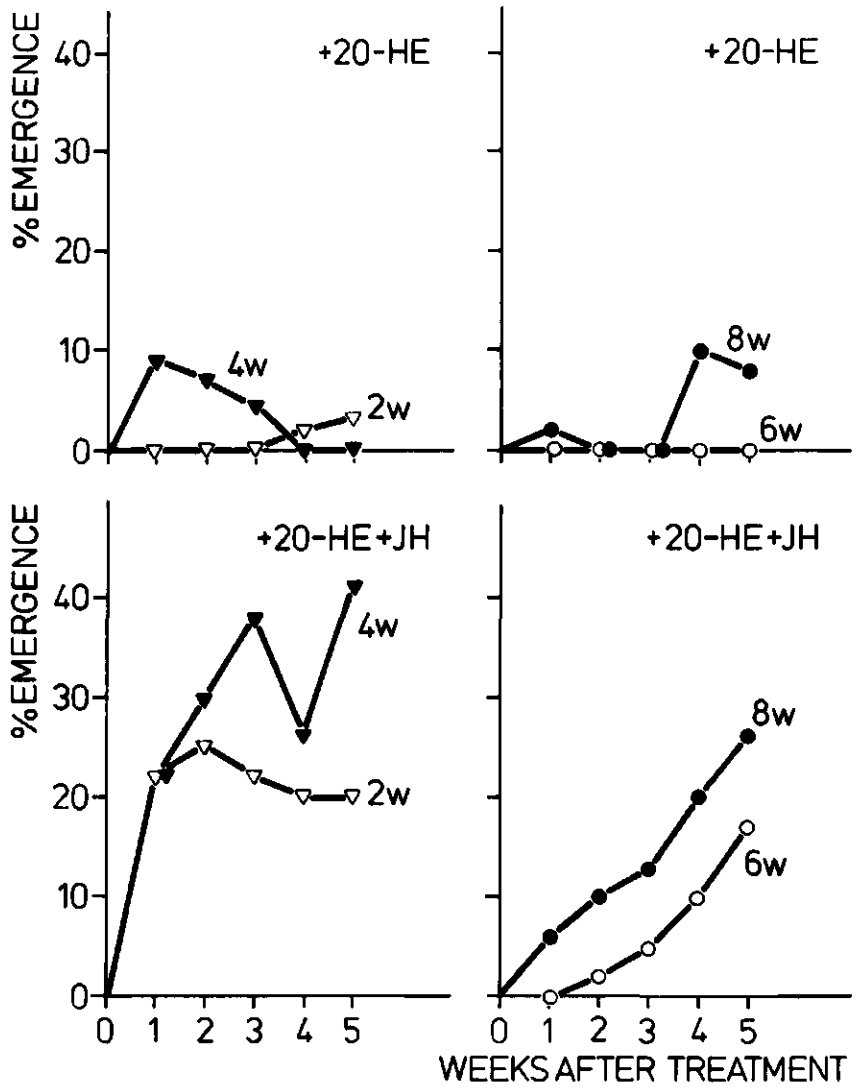

Fig. 16: Effect of 20-hydroxyecdysone (20-HE) injected singly or followed by a single injection of juvenile hormone III (JH) on diapause termination of L. decemlineata females (at 25°C) after various times in diapause (at 4°C).
For each treatment, N was between 31 and 66 individuals. Percentages of emergence given here result from the subtraction of control emergence. Open triangles represent beetles injected at 2 weeks of diapause; solid triangles: beetles treated at 4 weeks in diapause; open circles: 6 weeks in diapause; solid circles: 8 weeks in diapause. Doses injected were of 1 µg 20-HE in 2% ethanol and 15 µg JH III in olive oil per female. Combined hormone injections were made at 48 hrs intervals.

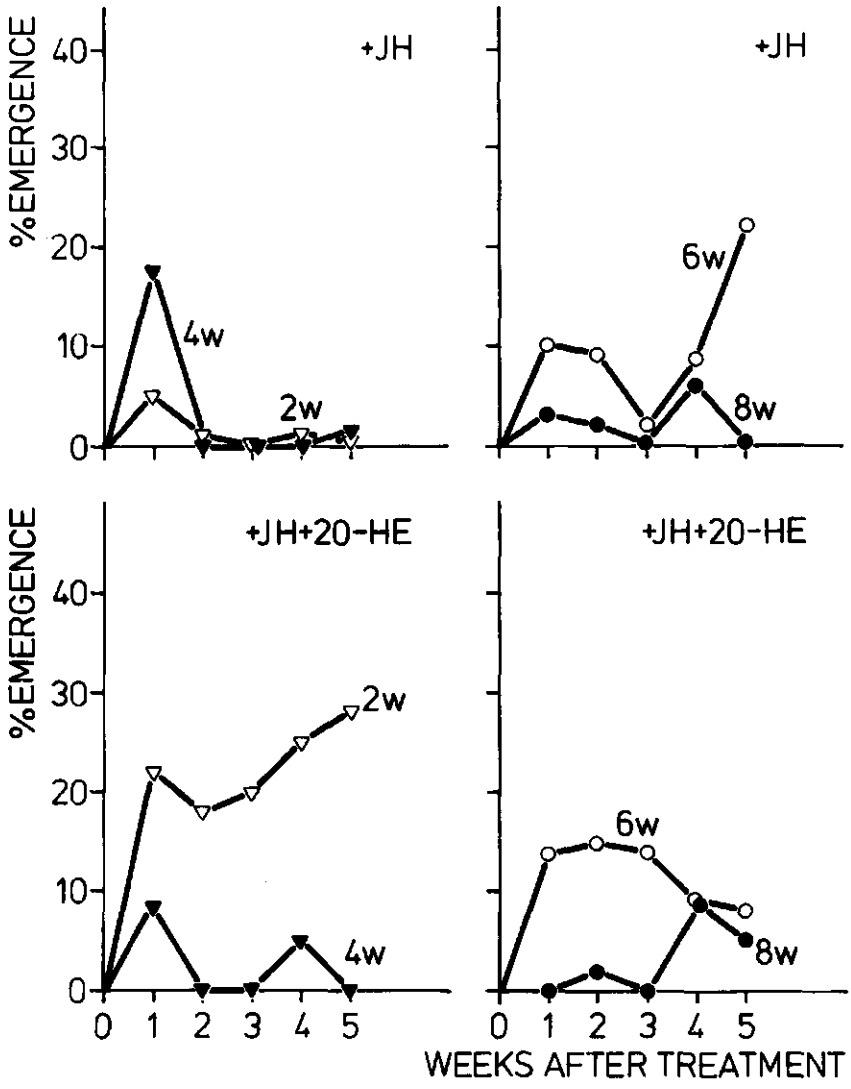

Fig. 17: Effect of JH III injected singly or followed by a single injection of 20-HE on diapause termination of L. decemlineata females (at 25°C) after various times in diapause (at 4°C).
See figure 16, for legend.

hormones seem to trigger the neuro-endocrine system to terminate diapause. After one month exogenous hormones have no immediate (or little) effect.

When treatments with both hormones in a different sequence are compared (fig. 16 and 17, below), it appears that the particular sequence (20-HE + JH III) is more effective in terminating diapause than the reverse

sequence: percentage emergence increases with age at injection (see 2 or 4 weeks and 6 or 8 weeks, fig. 16). This progression is not recorded when the reverse sequence (JH III + 20-HE) is used (fig. 17).

Effects of hormone injections on JH metabolism in diapausing beetles.

Degradation of JH III in haemolymph was determined by measuring the specific JH-esterase activity. Table X shows that single injections of 20-HE do not affect JH-esterase activity in haemolymph. On the other hand, single injections of JH III into diapausing females evoke a drastic increase of JH-esterase activity (Wilcoxon, $p < 0.01$). Interestingly, when the injection of JH III is preceded by an injection of 20-HE, the JH-esterase activity does not increase within 48 hrs (Table X).

Table X: JH-esterase activity in haemolymph of diapausing females of L. decemlineata, transferred from 4° to 25°C and within 48 hrs after injection (expressed in µmoles JH-acid produced/hr/ml haemolymph).

	Time in diapause at injection (in weeks)				
Treatment	2	4	6	8	20
+20-HE	3.3	2.7	2.6	1.2-7.2	-
+Water	-	2.8	5.8	4.1	-
+JH III*	24.2	17.7	22.8-27.5	16.9-20.6	17.2
+Olive oil	2.1-8.9	1.1-2.4-6.6	7.4	5.1	3.2-4.6
+20-HE + JH III	7.5	2.7	6.6	9.8	-
+Water + Olive oil	1.0	5.2	4.8	4.9	-
Untreated controls	1.6-1.9	1.5-1.8	1.5	-	-

Doses injected were as in figure 16. Haemolymph of 10 females was pooled for each sample. Asterisk indicates significant induction of JH-esterase activity after hormone injections as compared to injected controls (Wilcoxon-Mann-Whitney test: $p < 0.01$).

The synthesis of JH III by the CA was measured in vitro. Figure 18 indicates that 20-HE either injected alone or followed by an injection of JH III does not affect the rate of JH synthesis 48 hrs after the (last) injection. In contrast, JH III injected alone or followed by an injection of 20-HE significantly inhibits the CA activity, as compared with controls (fig. 18, Wilcoxon: $p < 0.05$).

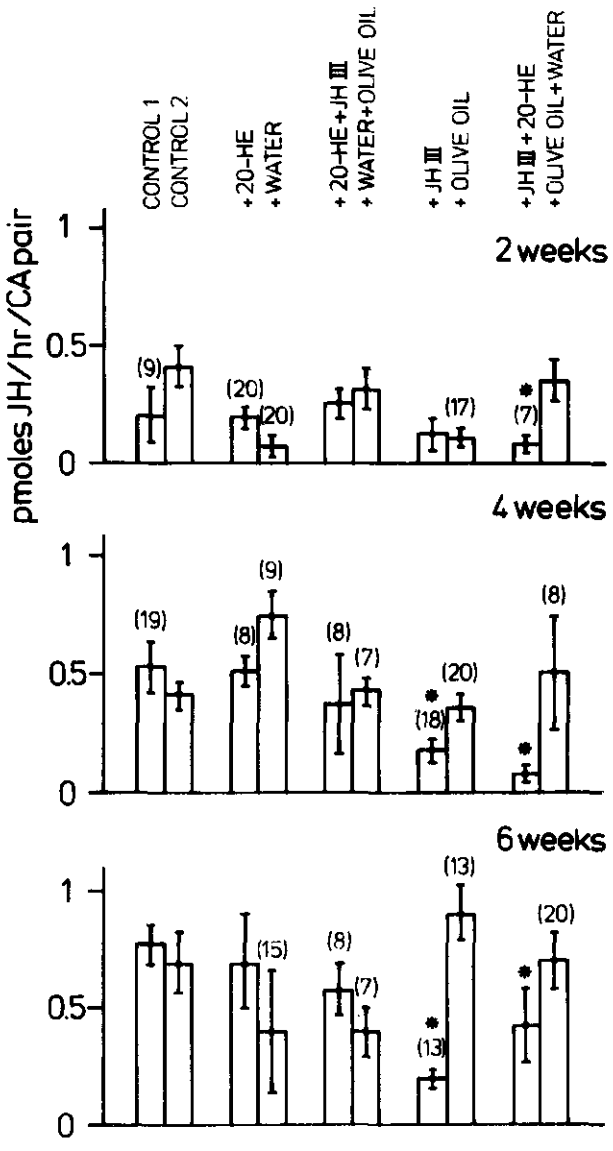

Fig. 18: Mean rates of JH synthesis after single or combined injections of 20-HE and/or JH III in diapausing L. decemlineata females (in pmoles JH III/hr/pair CA ± S.E.M.) at different times. Beetles diapausing at 4°C were transferred to 25°C 48 hrs prior to injection. Untreated controls were assayed at the same time as injected beetles. Hormonal doses injected were as in figure 16. Groups of beetles were injected with control solutions. The CA activity was determined 48 hrs after the (last) injection. N for each group was 10, unless otherwise mentioned between brackets. Asterisks indicate significant difference ($p < 0.05$) of JH-synthesis rate as compared to controls (Wilcoxon-Mann-Whitney test).

When exogenous 20-HE precedes the injection of JH III, JH does not produce the same effect as when injected singly or when the administration of 20-HE follows the JH injection.

Rates of JH synthesis in untreated controls that were transferred from 4° to 25°C increased significantly with time spent in diapause (fig. 18; Spearman's rank correlation test: $r_S = 0.65$, $t = 5.1$, 36 d.f., $p < 0.001$).

Effects of exogenous hormones on reproduction after diapause break.

Exogenous hormones by interfering with the endogenous neuro-endocrine system might induce premature emergence, but do not necessarily trigger reproduction. The reproductive behaviour of beetles after diapause break may thus provide an additional criterion of effectiveness of the treatment.

As mentioned above, hormones injected singly are generally ineffective in breaking diapause. However, of the 25 beetles that did emerge and started feeding, 94% also oviposited, irrespective of time of injection. On the other hand, combined hormone injections resulted in higher percentages of emergence. As for diapause termination, the response to combined injections of 20-HE and JH III changed at one month with respect to oviposition. Fewer females injected after 2 or 4 weeks of diapause with 20-HE followed by a JH III injection reproduced. Only 14% of emerged females oviposited compared with 94% of the females that received the same hormone combination after 6 or 8 weeks of diapause.

Injections in the reverse sequence (JH III + 20-HE) at 2 or 4 weeks did not enable emerged females to oviposit at all; whereas 95% of the females treated at 6 or 8 weeks oviposited.

DISCUSSION

The endocrine control of diapause termination appears to depend on a complex neuro-endocrine mechanism involving the interaction of 20-HE and JH III. Combined injections of both hormones are necessary to evoke a permanent termination of diapause. The administration of 20-HE alone does not evoke diapause termination. Injections of JH III alone evoke only a temporary break of diapause. Diapause is resumed as soon as the JH titre in haemolymph has returned to values similar to those found in pre-diapause or diapausing females (see De Kort et al., 1985). This confirms earlier findings of Schooneveld et al. (1977) obtained with high doses of JH I. The

isomeric mixture of JH III injected alone does not terminate diapause permanently in Leptinotarsa.

A change in immediate response to exogenous hormones occurs throughout the 'diapause development' phase. A synergism of 20-HE and JH on diapause termination (one week after treatment) was observed mainly in the first month. Hormones administered later had little or no immmediate effect on emergence. On the other hand, hormones injected in the first month of diapause were unable to evoke full ovarian maturation; whereas hormones injected later resulted in oviposition. This change in response to hormones after one month in diapause still remains to be explained. Interestingly, this change coincides with the time when activity of CA is at its lowest in diapausing females (Chapter 3). Schooneveld (1970) reported a peak of neurosecretion release from the medial neurosecretory cells (type A) of the brain at one month of diapause. As the cells of the pars intercerebralis are essential for reproduction in females (De Wilde and De Boer, 1969), this peak could be responsible for the occurrence of the change enabling beetles that have spent one month in diapause to oviposit after hormonal treatment. This is understandable in view of the fact that ovarian maturation requires both JH and a neuro-endocrine factor from the brain (De Loof, 1969; De Loof and De Wilde, 1970b). This cerebral factor appears thus to be absent during the first month of diapause. Grison (1949) observed that active brains implanted in diapausing beetles were more effective in terminating diapause in beetles that had spent two months in diapause than in those that had just entered diapause.

The present observations suggest that the intensity of diapause is highest after one month of diapause: the neuro-endocrine system may become completely inactive at this time. The cerebral factor essential for reproduction seems to be totally absent in the first month, while the activity of the CA seems lowest after a period of one month. A small quantity of the cerebral factor could be released after one month. An alternative interpretation may be that reproductive organs are not yet responsive to hormonal factors before one month of diapause. In either case, 'diapause development' seems to be a dynamic phase with respect to the neuro-endocrine system which goes through different states. Changes in responsiveness to hormones during diapause have also been reported in diapausing pupae (Bodnaryk, 1977) and larvae (De Loof et al., 1979), indicating that diapause development is also a dynamic process in other species.

The effects of hormonal injections on JH synthesis and its degradation give insight in the endocrine mechanism controlling diapause and its termination. Exogenous JH III, either alone or followed by an injection of 20-HE, generates a negative feed-back regulation of JH synthesis by the CA. This is in agreement with earlier findings in pre-diapause beetles treated with JH I (Schooneveld et al., 1979). The inhibition of CA activity by elevated JH titres occurs via humoral as well as neural pathways (Khan et al., 1982c), the latter originating from the lateral neurosecretory cells in the brain (Khan et al., 1983, 1986).

In addition, exogenous JH III induces an increased JH-esterase activity in haemolymph. This induction is also mediated by the cerebral neuro-endocrine system (Kramer, 1978b).

Interestingly, these effects of JH III on the regulation of endogenous JH metabolism are retained during the entire diapause period. This suggests that JH-homeostasis is under continuous control of the neuro-endocrine system throughout diapause. A cerebral inhibition of CA activity has been demonstrated in several insect species during adult diapause (Hodková, 1977; Panov and Kryuchkova, 1977; Poras, 1982; Poras et al., 1983; Baehr et al., 1986). Whether the CA are continuously inhibited by the lateral neurosecretory cells of the brain during the entire diapause duration remains, however, to be tested in Leptinotarsa. The finding that spontaneous electrical activity of brains in diapausing beetles does not differ from that of active beetles (Schoonhoven, 1963) suggests the participation of the brain.

Combined injections of both hormones in a different sequence reveal that the effect of exogenous JH III on JH metabolism no longer exists when an injection of 20-HE precedes the one of JH III. Since 20-HE alone did not affect CA activity or the JH-esterase activity, it is suggested that 20-HE acts on the neuro-endocrine control of JH metabolism during diapause. A brain-mediated regulation of JH synthesis by 20-HE has recently been reported in Manduca sexta larvae (Whisenton et al., 1985; Watson et al., 1986). However, 20-HE alone did not stimulate the CA activity in Leptinotarsa, in contrast to Manduca.

In addition, our experiments do not enable us to distinguish whether 20-HE acts on the brain on its own or in combination with JH III. Present results, however, suggest a key role of 20-HE in modifying the regulation of JH metabolism in diapause, and perhaps to switch off the 'diapause' programming of the brain.

It would be interesting to investigate whether the progressive increase of ecdysteroids throughout diapause (Briers and De Loof, 1981), eventually reaching a threshold level, would trigger the brain or switch it off for the full reactivation of CA after diapause termination.

Chapter 5

Effects of photoperiod and allatectomy on post-diapausing females of the Colorado potato beetle*.

ABSTRACT

Sensitivity to different photoperiods was examined in female Colorado potato beetles after diapause termination. Daylength does not influence the rate of oviposition after diapause. With regard to vitellogenesis and ovarian maturation, however, a quantitative effect of short daylengths is retained after diapause. Short daylength slows the vitellogenic phase of ovarian development down.

After diapause, photoperiod is not used as a cue factor for the induction of a second diapause. This loss of sensitivity to photoperiod is only temporary and is restored after three weeks in part of the population. Integument injury immediately restores the responsiveness to photoperiod in part of the population.

Post-diapause reproduction is controlled by the same endocrine factors as in young adults. Allatectomy experiments confirm the essential role of JH for vitellogenesis and oviposition. In addition, ovarian maturation is positively correlated with the rates of JH synthesis by the corpora allata (CA).

A second diapause could be induced by allatectomy, but only if the post-diapause beetles were allowed an initial period of feeding prior to the operation. Since diapause does not occur unless the photoperiodic clock has been reset by feeding, its induction is not caused solely by the inactivity of CA. It is suggested that the primary control of diapause induction is exerted by the brain.

* Some of the results have been published with the late Professor Dr. J. de Wilde in Int. J. Invertebr. Reprod. Dev. (1984), 7, 69-72.

INTRODUCTION

The life cycle of the Colorado potato beetle is strongly influenced by daylength. A short photoperiod (less than 13 hrs photophase) prevents reproduction and induces adult diapause (De Wilde et al., 1959). Under these conditions, the juvenile hormone (JH) III titre decreases to extremely low levels, whereas under long-day (LD) conditions the hormone titre increases until oviposition starts (De Wilde et al., 1968; De Kort et al., 1982, 1985). The JH titres are regulated mainly by the rate of JH synthesis by the corpora allata (CA), which in turn are controlled by neurally and humorally mediated factors (Kramer, 1978a; Khan et al., 1983). Allatectomy of young LD-adults induces diapause (De Wilde and De Boer, 1961), indicating the central role played by the CA in the induction of diapause. Reproduction, on the other hand, occurs under LD conditions, as the presence of active CA and a cerebral factor are required for vitellogenesis (De Loof and De Wilde, 1970b).

We have demonstrated that after diapause the CA are fully reactivated within a few days under both photoregimes (Chapter 3). After diapause, photoperiod no longer influences reproduction (De Wilde et al., 1959). The pre-oviposition period and rate of oviposition are no longer affected by daylength (Chapter 1). Some sensitivity to photoperiod is, however, retained with regard to JH metabolism (Chapter 3). In the present chapter, we will investigate whether the insect resumes its full sensitivity to photoperiod with regard to the induction of a second period of diapause.

The second aim of this study is to assess the role of the CA in post-diapause reproduction. Dortland (1979) observed that females allatectomized during diapause synthesized vitellogenins in the absence of JH and even oviposited. We report here the effects of allatectomy during and after diapause on post-diapause reproduction and determine to what extent these effects depend on photoperiod. The effects of allatectomy on the induction of a second diapause will also be discussed.

MATERIALS AND METHODS

Adult Leptinotarsa decemlineata (Say) were taken from the laboratory stock (Wageningen, the Netherlands). Diapause was obtained by rearing the beetles ab ovo under short-day conditions (SD= 10 hrs photophase/14 hrs scotophase, at 25°C; relative humidity: 60-70%) on fresh potato foliage.

On day 11 after adult emergence, when beetles left the plant to burrow into the soil, groups of 25 females and 5 males were placed on moistened sand (6 cm. deep) in plastic boxes (9 x 9 x 7 cm.). Two days later, the boxes were transferred to diapause conditions: i.e. constant darkness at 25°C. Although the 'diapause development' phase lasts for approx. 3 months under these conditions, diapause can be broken in most cases after 2 months. Beetles that had spent 2 months in diapause were removed from the sand and exposed to either LD conditions (16 hrs photophase/8 hrs scotophase) or SD conditions (see above).

Post-diapause females were kept individually in ventilated glass-jars, provided with moistened sand and active males. Fresh food was supplied daily. Diapause behaviour (burrowing) and oviposition were checked daily. The pre-oviposition period was defined as the time between removal from the sand and the production of the first batch of eggs. Eggs were counted daily for 3 to 4 weeks. The rate of oviposition was defined as the average number of eggs laid per ovipositing female.

To examine whether and when the sensitivity to photoperiod is restored, post-diapausing beetles were exposed to either LD or SD conditions. Groups of 10 beetles were transferred to the opposite photoregime after 2, 3 or 4 weeks. Two control groups remained under either constant LD or constant SD conditions for the entire period of 5 weeks. The rate of oviposition was recorded daily before and after the change in photoregime.

To check the effect of photoperiod on ovarian development after diapause, the thorax-abdomens from beetles sacrificed for measuring CA activity (see Chapter 3) were kept in a solution of ethanol (70%). The abdomens were dissected and the maturity of the ovaries was assessed, using the following measurements: percentage of ovarioles containing vitellogenic oocytes, length of the terminal oocyte and number of growing oocytes in the vitellaria. These variables were recorded for 10 out of 34 ovarioles on each side of the abdomen. These results were correlated with CA activity in the same individuals.

Allatectomy of females (2 months in diapause, 25°C) was performed as described earlier (De Wilde and Stegwee, 1958) after anaesthesia using carbon dioxide (5 mins). At the end of the experiment (6 to 8 weeks after the operation), heads were dissected to check that both of the CA had been removed. Unsuccessfully operated females were discarded. As controls, sham-operations were performed by removing some fat body and haemolymph through

the neck-membrane.

RESULTS

Ovarian development during and after diapause

Ovarian development is relatively synchronous in all ovarioles of one ovary. The different stages of ovarian maturation in a reproductive female are represented schematically in figure 19. Differentiation of oocyte and follicle cells occurs in the basal region of the germarium. One oocyte surrounded by follicle cells (or follicle) descends into the vitellarium and undergoes a phase of growth (Stage I). By the time, the first pre-vitellogenic oocyte is fully grown (Stage II), a second follicle has been emitted. Vitellogenesis starts at Stage III. When yolk deposition is completed, chorionization occurs (Stage IV). The mature egg is then expelled into the lateral oviduct. As the production of follicles is continuous, the four stages of maturation coexist in the ovariole of

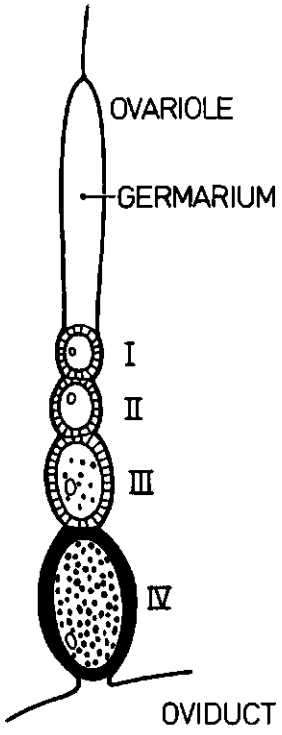

Fig. 19: Schematic representation of ovarian maturation in active reproducing L. decemlineata females.
Each ovary contains 34 ovarioles, in which the vitellarium develops fully under long day conditions. Stage I: growing oocyte surrounded by follicle cells; stage II: fully grown pre-vitellogenic oocyte; stage III: vitellogenic oocyte; stage IV: mature oocyte with chorionic envelope.

reproducing females.

During diapause follicles which are not developed further than Stage II can be observed. We found that the majority of diapausing females also contain vitellogenic oocytes usually in one ovary, but these oocytes showed signs of oosorption. Vitellogenesis most probably started during pre-diapause, since vitellogenins were not detected in haemolymph during diapause (Chapter 2). Results shown in figure 20 indicate that after diapause vitellogenesis is a continuous process followed by oviposition as soon as the first mature eggs reach the lateral oviduct.

After diapause, the stage of ovarian maturation is positively correlated with CA activity (fig. 21). Individual variations in rates of JH synthesis were, however, great.
Neither the length of terminal oocytes nor the percentage of ovarioles with vitellogenic oocytes seemed to bear any relation with the rate of JH-synthesis in the same individual.

Effect of photoperiod on ovarian development after diapause

The effect of photoperiod after diapause completion was examined on the length of the terminal oocytes. Figure 22 shows that photoregime does not significantly affect oocyte length (Wilcoxon-matched pairs signed-ranks

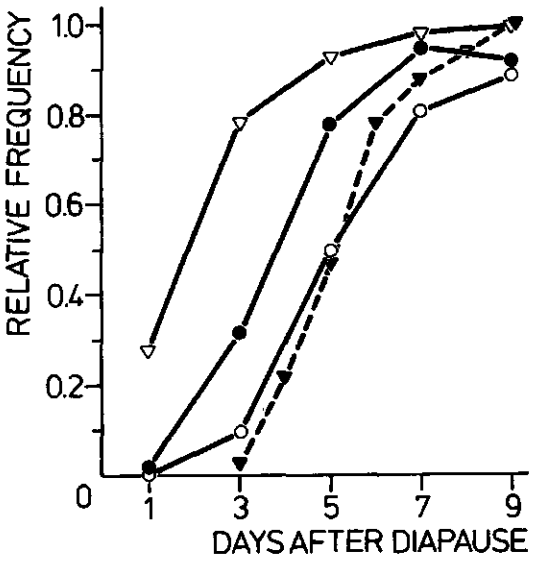

Fig. 20: Frequency distribution of stages of maturation of the ovary after diapause in L. decemlineata. Results of dissection (10 ovarioles examined/ovary) from females exposed to long and short day conditions were pooled. N = 20 to 60 individuals for each point. Stages of maturation were defined as in figure 19. Open triangles represent stage II; closed circles: stage III; open circles: stage IV. Solid triangles and dashed line represent frequency distribution of pre-oviposition period.

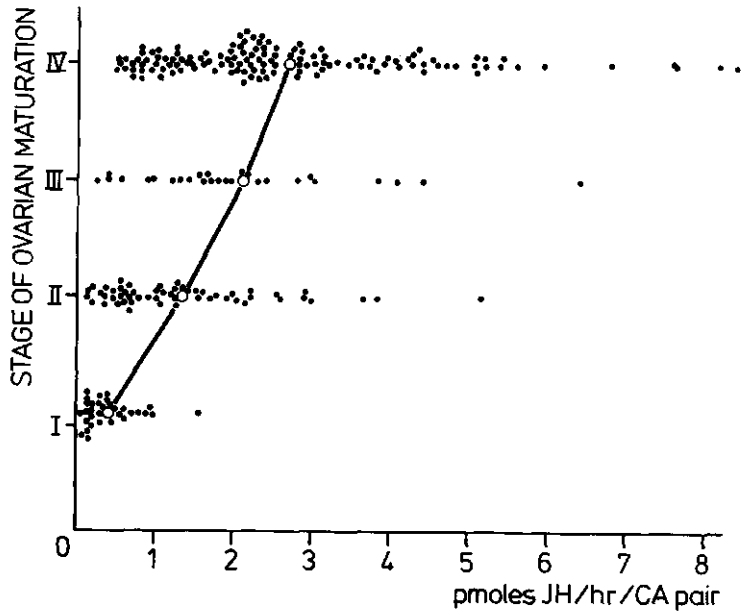

Fig. 21: Relation between the rate of JH synthesis (in pmoles/pair CA/hr) and the stage of ovarian maturation in post-diapause females of L. decemlineata.
Each point represents an individual female; results of ovary dissection and CA activity are pooled from days 1 to 11 after diapause termination (2 months 25°C) under long day conditions. Stages of ovarian maturation were defined as in figure 19. Open circles represent mean rate of JH synthesis for each stages.

test). In contrast, the proportion ovarioles with vitellogenic oocytes increases faster under LD than under SD conditions (fig. 23A). Similarly, the frequency distributions in figure 23B show that females under LD-photoregime begin ovipositing sooner than SD-females.
In conclusion, photoperiod does not seem to affect the length of oocytes, but does influence the rate of vitellogenesis, resulting in a delay in oviposition under SD conditions.

Effect of photoperiod on reproductive behaviour after diapause

Table XI shows that after diapause almost all females oviposit, irrespective of photoperiod. The slightly delayed egg maturation under SD conditions (fig. 23) is not reflected in a difference in the mean pre-oviposition period or in the rate of oviposition (Mann-Whitney U test). On

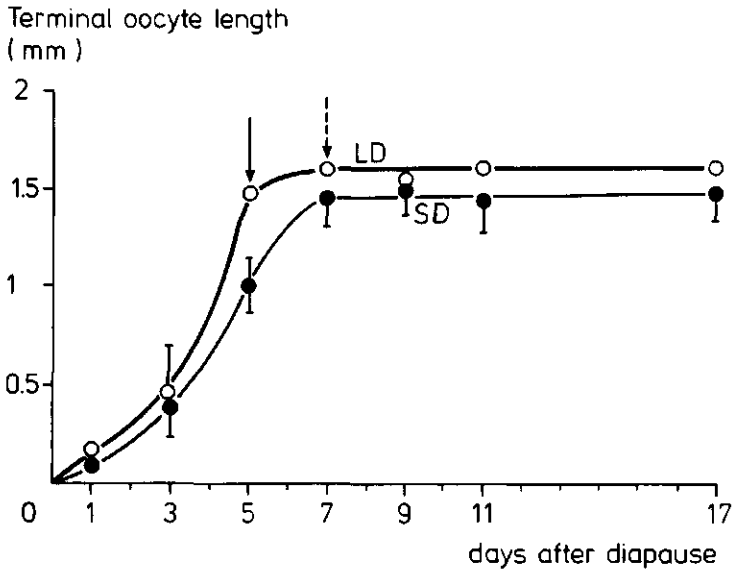

Fig. 22: Post-diapause growth of terminal oocytes in Leptinotarsa decemlineata (2 months diapause at 25°C), exposed to long or short photoperiods.
Each point represent the mean for 10 females (± S.E.M., except when smaller than data points).
Ten terminal oocytes were measured in each ovary. A Wilcoxon-matched pairs signed - ranks test showed no significant difference between the long and short day treatments.
Arrow represents mean pre-oviposition period under LD conditions; dashed arrow, under SD conditions.

Table XI: Reproduction and diapause induction in post-diapause Leptinotarsa females exposed to various photoperiods.

Photoperiod	% females ovipositing	Mean pre-oviposition time (days)	Mean no. eggs/day per female	% females entering 2nd diapause
LD (18L: 6D)	100	5.2 ± 1.2	54.9 ± 16.1	2
SD (10L:14D)	95	6.8 ± 2.3	47.8 ± 16.7	42

N = 50 for the LD group; N = 79 for the SD group. Observations were made daily for 4 weeks. Means are given ± standard deviation. The Mann-Whitney U test revealed no significant differences in oviposition between the two groups.

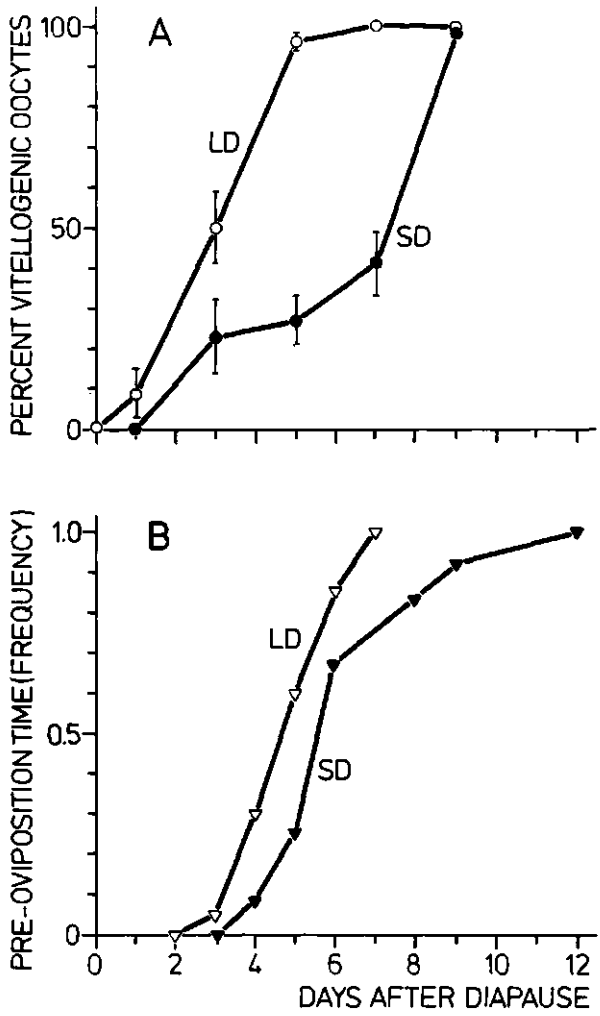

Fig. 23A: Rate of development of vitellogenic oocytes after diapause in L. decemlineata exposed to long or short photoperiods. Vitellogenic development was determined as the percentage of ovarioles (34 in each ovary) containing vitellogenic oocytes. Circles represent means of 20 to 30 females (± S.E.M., except when smaller than data points).
Fig. 23B: Proportions of LD and SD-females ovipositing, in relation to the time since termination of diapause.

the other hand, a certain responsiveness to photoperiod is restored after diapause, as can be concluded from the finding that a number of females enter a second diapause under SD conditions (Table XI). Within 3 weeks after diapause, no digging behaviour was recorded. Only after 4 weeks another period of diapause might begin under SD conditions. As burrowing behaviour normally occurs within 12 days in pre-diapause adults, we can conclude that the sensitivity to photoperiod is initially absent after diapause termination, but is restored later in 42% of the beetles.

The weekly rate of oviposition declined during post-diapause, under both photoregimes (fig. 24). No significant change in rate of oviposition

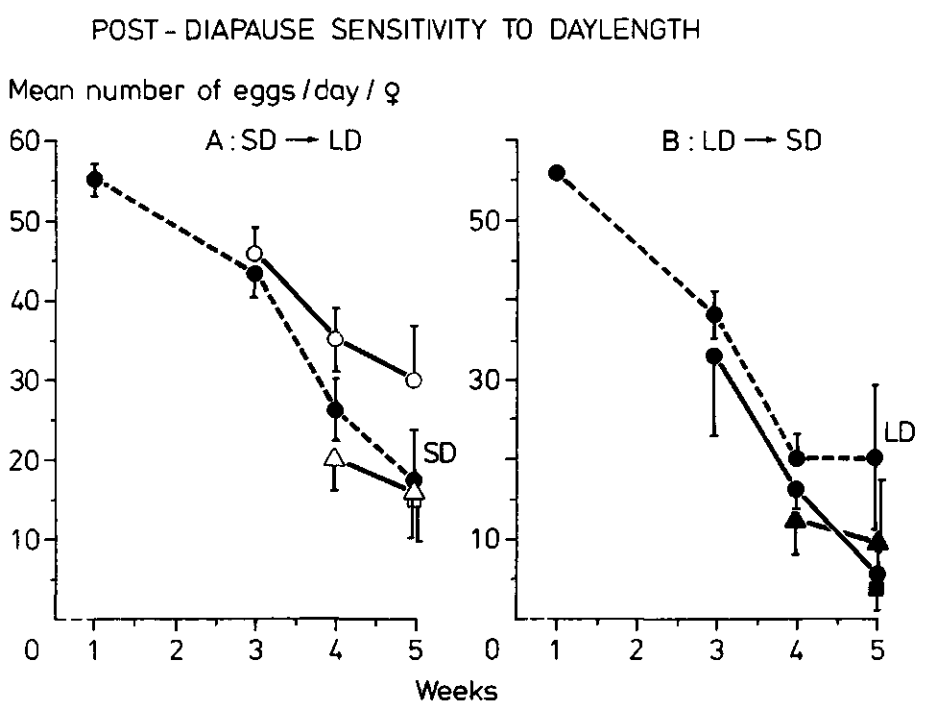

Fig. 24: Weekly rates of oviposition after diapause (2 months 25°C) of beetles exposed to short day (SD) (A) or long day (LD) conditions (B). Transfer from SD to LD (A) and from LD to SD (B) occurred after 2 weeks of constant photoregime (circles), 3 weeks (triangles), or 4 weeks (squares) (mean ± S.E.M.; N=10).
Dashed lines represent results for females that were exposed to constant SD (A) or LD (B) photoperiods for 5 weeks.

was observed when beetles were transferred from SD to LD conditions at various periods after diapause (fig. 24A). However, the rate of oviposition was significantly reduced in females transferred after 3 weeks from LD to SD conditions (fig. 24B: Mann-Whitney U test, $p < 0.05$).
In conclusion, sensitivity to photoperiod is resumed approx. 3 weeks after termination of diapause, resulting in a significant decline in reproduction and in the induction of a second diapause in a number of insects under short photoperiod.

Effects of allatectomy on post-diapause reproduction

Allatectomy was performed at different times after beetles were removed from the soil. The effect of the operation was checked by keeping the insects under LD or SD conditions. Table XII shows that the percentage

When the CA were removed within 24 hrs of diapause break, no beetles entered a second diapause. In contrast, the removal of CA after 3 days or more induced burrowing behaviour. A time-lag thus seems necessary before allatectomy becomes effective in inducing diapause.

The incidence of diapause after allatectomy under LD was, however, lower than under SD conditions (Table XIII). Sham-operated females are sensitive to photoperiod from the time of diapause break (Sign test between LD and SD conditions, $p < 0.01$; Table XIII). One in three of the sham-operated females re-entered diapause under SD, whereas virtually none did so under LD conditions. These results confirm the suggestion made in the previous section i.e.: wounding restores the sensitivity to photoperiod immediately after diapause termination. This responsiveness to photoperiod after the operation also occurs in allatectomized beetles. Under SD conditions, some beetles entered a second diapause within 12 days, while others did not diapause before 37 days. Effects of allatectomy and photoperiod are thus cumulative under SD conditions. Under LD conditions, however, the response to allatectomy is very clear; in a number of insects diapause intervenes between 20 and 35 days after the operation. It should be noted that of the few females which entered diapause after a sham-operation under LD conditions, all burrowed into the sand within 3 days of the operation. These individuals most probably had not completed 'diapause development' at the time of operation.

Females allatectomized at day 3 or later sometimes laid eggs for 2 or 3 weeks before entering diapause.

Finally, beetles in which a second diapause was induced by allatectomy never terminated diapause spontaneously (within 9 months after the operation, at 25°C). Most of them died within 4 to 6 months after reburial in the sand.

DISCUSSION

During diapause fully grown pre-vitellogenic oocytes (Stage II) can be observed in the ovaries and vitellogenic oocytes (Stage III) that may have developed during pre-diapause are resorbed. Ovarian development during adult diapause has been reported in Drosophila obscura (Begon, 1976) and in Aleyrodes proletella (Adams, 1985). Reproductive diapause thus does not imply a total arrest of ovarian development in all species.

Our results also indicate that photoperiod has no effect on the oocyte

length after diapause, but does influence the rate of vitellogenesis and subsequent egg-laying. Dortland (1978) has shown that photoperiod has a drastic effect on vitellogenins synthesis by the fat body in young non-diapausing females. Therefore, it is possible that photoperiod also affects the synthesis and/or the incorporation of vitellogenins in oocytes after diapause. The incorporation of vitellogenins by the oocytes depends on the presence of JH (De Loof and De Wilde, 1970b; De Loof and Lagasse, 1970). We have shown that ovarian maturation after diapause is positively correlated with the rate of JH synthesis.

Our results show that neither the pre-oviposition period nor the eventual rate of oviposition is influenced by photoperiod after the termination of diapause (see also De Wilde et al., 1959; Chapter 1). This apparent loss of sensitivity to photoperiod after adult diapause has been reported in several other insect species as well (e.g. Hodek, 1971; Solbreck, 1974; Hodek and Ruzicka, 1977; Adams, 1986).

On the other hand, our results, which show that photoperiod affects the rate of vitellogenesis and ovarian maturation after diapause, indicate that the sensitivity to photoperiod is never completely lost. The JH titre and the rate of JH synthesis also depend on photoperiod after diapause (Chapter 3). Thus, photoperiod retains a quantitative (modulatory) effect after 'diapause development' is completed. The role of photoperiod as a cue factor for induction of (a second) diapause is temporarily suppressed. The responsiveness, however, is restored 3 weeks after diapause in part of the population. Such restoration of responsiveness had been supposed by De Wilde (1969), but never shown. A similar restoration of sensitivity to photoperiod after 4 weeks has been reported in post-diapausing Coccinella septempunctata. One in three post-diapausing females reproduced under SD conditions, whereas more than 50% did so only for a week. Though most insects showed normal oviposition under LD conditions, 25% of post-diapausing females also showed discontinuous oviposition (Hodek et al., 1977; Hodek and Ruzicka, 1979). Hodek (1986) pointed out recently that the response to photoperiod after diapause is much more complicated than it first seemed. We have shown that in Leptinotarsa, 90% of the post-diapause females oviposit under SD-photoregime and they continue for at least 5 weeks. The other extreme is the clearcut cessation of oviposition within 3 weeks of short photoperiod found in Aelia acuminata (Hodek, 1979). The sensitivity to photoperiod after diapause thus varies greatly between insect species.

Sensitivity to photoperiod seems to be restored in part of the population by the operation trauma in early post-diapause. This is indicated by the relatively low percentage of ovipositing females (approx. 50%) and the induction of a second diapause under SD conditions after a (sham-)operation. A similar 'wounding effect' has been reported to terminate diapause in Leptinotarsa (Rohdendorf and De Wilde, 1972). It seems that injury causes sufficient changes in the endocrine system to restore sensitivity to photoperiod. An effect of carbon dioxide anaesthesia cannot, however, be ruled out. Since the photoperiodic induction of diapause is thought to be the result of a low JH titre in combination with a high peak of ecdysteroids, occurring between 2 and 4 days after adult emergence (Briers and De Loof, 1981), it is likely that an ecdysteroid peak occurs after allatectomy or sham-operation leading to diapause induction under SD conditions.

The allatectomy experiments after diapause have shown that the lack of JH prolongs the pre-oviposition period and reduces the rate of oviposition. The absence of CA does not completely prevent oviposition (in agreement with Dortland's finding, 1979), but oviposition is very irregular and occurs only during a limited period of approximately 12 days after allatectomy, in 90% of the ovipositing females. The few eggs laid had probably matured due to remnants of JH III (possibly in haemolymph and tissues). The period of 12 days corresponds almost exactly to that recorded after allatectomy in young LD beetles (De Wilde and De Boer, 1969; De Loof and De Wilde, 1970b). This corresponds also to the time at which mature oocytes start to be resorbed (De Loof and Lagasse, 1970), but the synthesis of vitellogenins by the fat body already declines significantly 4 days after the operation (Dortland, 1979).
In conclusion, our results confirm the finding of De Loof and De Wilde (1979b) that JH is essential for vitellogenesis and reproduction in L. decemlineata.

Allatectomy of post-diapause females also induces a second diapause. The incidence of diapause depends, however, on the photoperiod. Almost all SD-females entered a second diapause after allatectomy, whereas only 40% did so under LD conditions. This is in contrast to effects of allatectomy in non-diapausing LD-females, in which diapause was induced for 100% after 34 days (De Wilde and De Boer, 1961). This discrepancy may be due either to a partial loss of sensitivity to photoperiod after diapause, or to changes due to artificial selection after 20 years of rearing in the laboratory

(see De Kort et al., 1980).

The finding that allatectomy performed immediately after diapause (within less than 48 hrs) does not induce a second diapause, whereas beetles allatectomized after 3 days or later in post-diapause do enter a second diapause, indicates that the induction of diapause is not primarily caused by the inactivity of CA or lack of JH. This is in contrast with the concept of diapause induction of De Wilde and De Boer (1961). Apparently a period of 3 days has to elapse before diapause induction can take place. Interestingly, Grison (1943) noted that the circadian rhythm of activity is also lost for a short period in post-diapause beetles. He found that the intake of food was responsible for the resumption of rhythmic activity after diapause. We observed that feeding starts at day 2 after diapause. The fact that a second diapause cannot be induced, even when JH is lacking, before feeding, may suggest that the photoperiodic clock and counter (i.e. the responsiveness to photoperiod) are reset at the time of feeding. This corroborates the theory that the primary organ controlling diapause induction is the brain.

GENERAL DISCUSSION

Extrinsic mechanism of diapause regulation

It is clear that environmental factors regulate the physiology of diapause and post-diapause, in such a way that the insect life cycle is accurately synchronized with seasonal changes. Diapause, in our present view, is a complex and dynamic process in which several phases can be distinguished (see scheme). Although Colorado potato beetles remain responsive to photoperiod, humidity and temperature throughout diapause (Chapters 1 to 3) and post-diapause (Chapter 5), their responses change during the different phases of the diapause syndrome (see scheme A). Each phase is characterized by a specific physiological state, as can be seen from our results (scheme B). Quiescence is by definition a facultative phase, which agrees with the finding that virtually no physiological changes (apart from a slight decrease in haemolymph proteins), seem to occur during this period (scheme B).

As diapausing beetles stay in the soil up to a depth of 60 cm (Chapter 1), photoperiod does not seem to be important in the control of diapause development and termination. Nevertheless, beetles artificially exposed to photoperiod, appear to remain sensitive to photoperiod during diapause development (Chapter 1). This photosensitivity is probably a double safety mechanism to ensure diapause maintenance under winter conditions with relatively high temperatures. During quiescence, the sensitivity to photoperiod is lost, since similar rates of oviposition have been obtained after transfer to higher temperatures under long-day and short-day photoregimes (Chapters 1 and 5). However, this loss of photosensitivity is only temporary, because 4 weeks after diapause termination, short photoperiod can induce a second diapause in a certain percentage of the population (Chapter 5). Daylength can still affect, however, the physiology of the insect during quiescence as shown by the dependence on daylength of the rates of juvenile hormone (JH) synthesis by the corpora allata (Chapter 3).

Beetles did not respond to water during diapause development, whereas during quiescence an increase in humidity enhances emergence from the soil (Chapter 1).

The effect of temperature on diapause duration and termination is more

complicated. The rate of diapause development is almost independent of temperature (i.e. temperature compensated: Q_{10}= 1.1), whereas the transient phase of post-diapause development is highly temperature dependent (Q_{10}= 3.2; see Table III, Chapter 1). Therefore, during diapause development, beetles do not respond to temperature changes, whereas quiescent beetles do respond and emerge soon after transfer to high temperature (Chapter 1). Moreover, during diapause development, the corpora allata activity is independent of temperature, whereas the rate of JH-synthesis slightly increases with soil temperature in the transient phase leading to emergence (Chapters 1 and 3). It is indeed important that the neuro-endocrine system, which controls diapause, functions in a relatively independent way from ambient temperature variations during diapause development. On the other hand, during quiescence an increase in soil temperature and/or humidity affects the neuro-endocrine system, which results in the termination of diapause.

Food is not a prerequisite for emergence from the soil (Chapter 1). Starvation did not inhibit the corpora allata activity in post-diapause adults (Chapter 3), in contrast to its effect in young non-diapausing adults (Khan et al., 1982b). This indicates that the development of flight muscles, which depends on the JH level, may occur without food intake. This enables post-diapause beetles to migrate towards feeding sites.
In Pyrrhocoris apterus adults, for example, food is also not required for diapause termination (Hodková, 1982). Starvation apparently accelerates the transient phase (or activation phase of diapause termination) in P. apterus (Hodek and Hodková, 1986).

The following question arises: is the duration of diapause development affected by factors other than temperature? The duration of diapause in pupae of Manduca sexta is determined by the number of short photoperiods received by the larva (Denlinger and Bradfield, 1981). This suggests that diapause duration is governed by a photoperiodic clock. Whether quantitative variations in the photoperiodic treatment of pre-diapause beetles affect the length of diapause development remains an open question.

Intrinsic mechanism controlling adult diapause

The discovery by De Wilde (1954) of the major role of the corpora allata (CA) in controlling reproduction and diapause in Colorado potato beetles, has led to the concept of juvenile hormone (JH) deficiency as the

main cause of adult diapause (De Wilde and De Boer, 1961). De Wilde and De Boer (1969) showed the involvement of the brain in the regulation of the corpora allata (synthesis of JH). Because ecdysone was thought to be absent from the haemolymph in adult insects, the concept that diapause is controlled by the brain, mainly by governing the activity of a master gland, i.e. the corpora allata (CA), has been put forward in De Wilde's work (see De Wilde, 1983, 1984). Since these pioneering studies, major progress has been made in neuroendocrinology, and the complexity of regulatory mechanisms from the brain has been uncovered. Neurosecretory cells in the central nervous system include aminergic and peptidergic neurons, whose numbers had been underestimated. Neurosecretions can act as hormones, neurotransmitters or neuromodulators. The lack of knowledge concerning the numerous neuropeptides in the brain and their respective functions, remains a major obstacle to elucidating the neuro-endocrine control of diapause termination. The brain integrates the information perceived from the environment and transduces it, amongst others, into chemical signals, which regulate the endocrine glands and/or act directly on target organs. The first experiments on the control of diapause termination in Leptinotarsa decemlineata (Grison, 1949) revealed the primary role of the brain. The CA activity is controlled by humoral factors (De Wilde and De Boer, 1969) and neural pathways (Khan, 1983). A neural inhibition of the CA, originating from the lateral neurosecretory cells of the brain, has been shown in pre-diapausing Colorado potato beetles (Khan et al., 1983, 1986). The neuro-endocrine control of the low CA activity in diapausing beetles remains, however, to be investigated. In other species diapausing as adults, diapause could be broken by electrical stimulation of the pars intercerebralis (in Anacridium aegyptium: Girardie et al., 1974) or by removal of the neural inhibition of the CA activity (in Pyrrhocoris apterus: Hodková, 1976, 1977 and in Tetrix undulata: Poras, 1977a, b).

Another major finding since De Wilde's pioneering work has been the presence of ecdysteroids in adult insects. Ecdysteroids have also been detected in Colorado potato beetle adults (Briers and De Loof, 1981), and it has been suggested that the induction of diapause resulted from the lack of juvenile hormone and a peak of ecdysteroids in pre-diapause adults (Briers et al., 1982). Thus the intrinsic control of diapause could be more complex than initially thought.

The use of exogenous hormones to determine the role of endogenous hormones in the control of diapause is unnatural. This approach can,

however, indicate which hormones play a decisive role and reveal some characteristics of the mechanism involved. Based on the assumption that adult diapause is a consequence of the inactivation of CA, several attempts have been made to terminate diapause by implanting active glands in diapausing adults. This operation was successful in many insect species (see Denlinger, 1985), but not in Colorado potato beetles (De Wilde and De Boer, 1961). Moreover, injection of high doses of JH or JH analogues terminated diapause only temporarily (De Wilde and Lutke-Schipholt, 1974; Schooneveld et al., 1977). We confirmed these results by injecting lower doses of JH III during diapause (Chapter 4).

The finding that the ecdysteroid level progressively increases during diapause in Colorado potato beetles (Briers and De Loof, 1981), has led us to consider the role of 20-hydroxyecdysone (20-HE) in diapause termination. Injection of 20-HE alone did not terminate diapause. Only the combined injection of JH and 20-HE resulted in permanent termination of diapause. JH injected during diapause exerted a negative feed-back on CA activity (Chapter 4), which occurs via the brain (Khan et al., 1982c). Exogenous 20-HE preceding an injection of JH, suppressed the effect of exogenous JH on the induction of specific JH-esterase in diapausing beetles (Chapter 4). This induction of esterases is also mediated by the brain (Kramer, 1978b). This indicates that both hormones act on the brain neurosecretory cells which trigger termination of diapause. Schooneveld (1972) also suggested that diapause termination in Colorado potato beetles was mediated by brain neurosecretory cells which become activated after treatment with JH. A stimulatory effect of JH and 20-HE on the neuro-endocrine system has also been reported in diapausing pupae of Manduca sexta (Bradfield and Denlinger, 1980). The data in Chapter 4 suggest that both hormones are involved in termination of adult diapause. Although there are indications that the sequence 20-HE and JH is more effective in breaking diapause than the reverse sequence, it is premature to say that a pulse of 20-HE precedes the activation of the CA at the end of diapause.

We found a synergism of 20-HE and JH in terminating diapause in Colorado potato beetles (Chapter 4). This synergistic effect may be due to the action of exogenous 20-HE on the control of JH metabolism during diapause. Exogenous 20-HE prevents the negative feed-back of exogenous JH III on CA activity and the induction of JH-esterases in haemolymph after JH injection (Chapter 4). Degradation of exogenous JH may be suppressed by the previous injection of 20-HE. The JH titre may then remain high for a longer

time, resulting in diapause termination. This suggests a possible function of ecdysteroids, whose level was reported to increase throughout diapause in L. decemlineata (Briers et al., 1982).

The role of ecdysteroids in adults is still unclear. In view of our results, it is tempting to speculate that 20-HE switches off the 'diapause programming' by the brain, thereby causing the activation of the CA. We were, however, unable to detect any effect of 20-HE on CA activity (Chapter 4). Khan et al. (1982c) also reported no effect of in vivo injection of 20-HE on CA activity in non-diapausing beetles. Doses injected were high and 20-HE has been reported to be effective at very low doses in other species (Lafont and Koolman, 1984, p. 220; e.g. Whisenton et al., 1985). There is growing evidence in the literature that CA activity is regulated by a complex mechanism also involving 20-HE acting via the brain in other species (Stay et al., 1980; Friedel et al., 1980; Whisenton et al., 1985; Watson et al., 1986; Paulson and Stay, 1987). The role of 20-HE in the control of CA activity in L. decemlineata therefore deserves more investigation.

The synergistic effect of 20-HE and JH III on termination of diapause is most conspicuous in the first month of 'diapause development'. A change in response to exogenous hormones occurs after one month of diapause: hormone injections were more effective in breaking diapause before one month than later (Chapter 4). This change in response suggests a switch in the neuro-endocrine system after one month. The proportion of CA with undetectable activity was highest after a diapause period of one month (Chapter 3). Beetles terminating diapause by hormonal treatment before one month, were unable to mature ovaries and oviposit (Chapter 4). This suggests that the intensity of diapause is greater after one month. This agrees with the observation that beetles not only do not terminate diapause spontaneously (Chapter 1), but also are insensitive to exogenous hormones after one month (Chapter 4).

This indicates that another neuro-endocrine process has to be initiated for diapause termination. Which process is involved in diapause termination remains to be assessed, but a possible mechanism was suggested in the previous paragraph.

Diapause syndrome in the adult stage versus reproductive diapause

The behavioural manifestations of diapause in adult insects vary greatly among different species. Most adults remain inactive during diapause, however some species (e.g. some mosquitoes or butterflies) show some behavioural activities (e.g. flight, feeding) and dissections of sexual glands are required to determine their state of diapause. In the Colorado potato beetle the diapause syndrome includes burrowing behaviour which makes it an 'all-or-none' response. The Colorado potato beetle is therefore an ideal model to study adult diapause.

Emergence of beetles at the end of diapause is the result of diapause termination processes which are initiated in the soil. Specific environmental factors i.e. temperature and moisture, control these processes. Once beetles have emerged, photoperiod has to be taken into account. However, under natural conditions, the responsiveness to short photoperiod is irrelevant since daylength is long in spring. The presence of food becomes an important factor: it is a prerequisite for vitellogenesis and oviposition (Chapter 1). Food is an essential requirement for reproduction in most insect species (De Wilde and De Loof, 1973; e.g. Hodek and Hodková, 1986).

Since different environmental factors and requirements exist for diapause termination (emergence and flight muscles development) and post-diapause reproduction, neuro-endocrine processes controlling diapause termination are distinct from those controlling ovarian maturation and oviposition. Diapause in adult Colorado potato beetles is characterized by JH titres below 10 ng/ml haemolymph (Chapter 3). Diapause termination involves both 20-HE and JH, which are produced under control from the brain (Chapter 4). After emergence, the JH titre increases and values above 20 ng JH III equivalent/ml haemolymph are required for oviposition (Chapter 3). Post-diapause reproduction requires a high JH titre sustained by CA activity under cerebral control (Chapter 5). De Loof and De Wilde (1970b) found that in non-diapausing beetles vitellogenesis also requires JH and a brain factor.

The use of the term 'reproductive diapause' as a synonym of adult diapause has led to investigations of the cause of the arrest of reproduction or ovarian maturation, thereby neglecting all other symptoms of the syndrome. Our results have shown the need to regard diapause termination and the resumption of reproduction as two separate processes,

which are regulated by different mechanisms. High doses of JH or JH analogues (JHA) are needed to terminate diapause in most species diapausing as adults (see numerous references in Denlinger, 1985, p. 402). This treatment failed to initiate reproduction in some species or resulted in only temporary reproduction in others. This is probably due to the fact that actual termination processes regulated by other neuro-endocrine factors are bypassed by this drastic treatment. On the other hand, in Riptortus clavatus, for example, adult diapause could be broken with low doses of JHA alone without intervention of protocerebral neurosecretion (Numata and Hidaka, 1984). Vitellogenesis started in treated females, while sexual behaviour was initiated in males. This denotes a great diversity in response among insect species, and draws attention to the fact that in different insect species the termination of diapause may be caused by different regulatory mechanisms.

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Externe en interne regelmechanismen bij de verbreking van diapauze van de Coloradokever.

SAMENVATTING

De effecten van verschillende milieu-factoren (zoals temperatuur, vocht, daglengte en voedsel) op het gedrag en de stofwisseling van enkele bloedbestanddelen, zoals het juveniel hormoon, zijn onderzocht in vrouwelijke Coloradokevers tijdens en na de diapauze. De endocriene regeling van de verbreking van de diapauze is geanalyseerd met behulp van hormooninjecties. Daarnaast is door middel van allatectomie na afloop van de diapauze de rol van het juveniel hormoon in de reproductie bevestigd en werd de endocriene controle van de inductie van een tweede diapauze aan het licht gebracht.

Een analyse van het gedrag in relatie tot temperatuur maakt het mogelijk in de diapauze drie achteréénvolgende fasen te onderscheiden:

i) 'diapause development', of echte diapauze, die ongeveer 3 maanden duurt, maar die langer is bij lage temperaturen;

ii) 'post-diapause quiescence', of rust, die wordt gekarakterizeerd door een beperkte afbraak van eiwitten. Deze tweede fase wordt door lage temperaturen, onvoldoende vocht of gebrek aan voedsel in stand gehouden;

iii) 'transient phase' van post-diapauze ontwikkeling, die een temperatuur boven een drempel van 8 tot 10°C vereist en die gekarakteriseerd wordt door een geringe reactivatie van de corpora allata. Deze fase eindigt met het boven de grond komen van het insekt.

Na verbreking van de diapauze, gaat de post-diapauze ontwikkeling door, hetgeen tot voortplanting leidt.

De gevoeligheid voor daglengte varieert gedurende de verschillende fasen van de diapauze. Tijdens 'diapause development' kan korte dag in opgegraven dieren nog als signaal voor inductie van diapauze fungeren. Daarna wordt daglengte niet meer als een signaal factor, maar wél als directe factor, waargenomen. Na de diapauze zijn de synthese-activiteit van de corpora allata en de titer van het juveniel hormoon in het bloed lager bij korte dag dan bij lange dag. Als gevolg daarvan wordt de vitellogenese vertraagd. In tegenstelling hiermee is de waarneming dat het aantal eieren na de diapauze onafhankelijk van daglengte is. De gevoeligheid voor fotoperiode blijkt slechts tijdelijk verloren te zijn en wordt na drie of vier weken in een aantal kevers hersteld.

Voedsel lijkt geen belangrijke rol te spelen bij de verbreking van de diapauze, maar is wel essentieel voor de vitellogenese en dus voor het herstel van de reproductie. Voedsel is ook van belang voor het herstel van de fotoperiodische klok, noodzakelijk voor een eventuele inductie van een tweede diapauze.

De bloedsamenstelling, specifiek voor de diapauze, is voornamelijk gekarakteriseerd door verhoogde concentraties van lipiden, eiwitten en vrije aminozuren. Daardoor lijkt bij diapauze kevers de weerstand tegen vrieskou te worden verbeterd. Proline, dat als energetisch substraat in actieve adulten verbruikt wordt, blijkt tijdens diapauze bij temperaturen onder nul een beschermende rol te hebben.

De stofwisseling, inclusief die van het juveniel hormoon, bevindt zich op een laag basisniveau gedurende de gehele diapauze-periode. Het verbruik van reservestoffen in het bloed gedurende de diapauze is betrekkelijk laag. Op het moment dat de kevers boven de grond komen (aan het einde van de diapauze) heeft er een sterke stijging van de stofwisseling plaats. De diapauze-eiwitten worden afgebroken en de synthese van juveniel hormoon bereikt een maximum na 5 à 7 dagen. Korte dag induceert echter niet langer specifieke esterasen die de afbraak van juveniel hormoon verzorgen.

Het inspuiten van 20-hydroxyecdyson alléén heeft geen effect op de verbreking van diapauze; juveniel hormoon alléén verbreekt de diapauze tijdelijk, zolang de hormoontiter hoog blijft. Diapauze wordt echter permanent verbroken door een gecombineerde behandeling met beide hormonen. Gedurende de eerste maand van de diapauze treedt er een synergetisch effect tussen het 20-hydroxyecdyson en het juveniel hormoon op. In die periode heeft er een voortgaande inactivering van de corpora allata plaats. Uit deze waarnemingen wordt geconcludeerd dat er een verandering in het neuro-endocrien systeem optreedt na één maand diapauze.

De endogene stofwisseling van juveniel hormoon tijdens diapauze wordt niet beïnvloed door 20-hydroxyecdyson alleen of geïnjiceerd na een injectie van juveniel hormoon. Juveniel hormoon alléén veroorzaakt, door terugkoppeling, een inhibitie van de corpora allata activiteit en induceert bovendien een toename van specifieke esterasen in het bloed. Deze effecten van juveniel hormoon worden onderdrukt door vooraf 20-hydroxyecdyson te injiceren. 20-Hydroxyecdyson lijkt dus een sleutelrol te spelen in de regulatie van de stofwisseling van juveniel hormoon gedurende de diapauze. Deze waarnemingen leiden tot de opvatting dat 20-hydroxyecdyson verantwoordelijk is voor het in de hersenen in gang zetten van het programma voor de

verbreking van diapauze.

Extirpatie van de corpora allata na de diapauze veroorzaakt in een aantal gevallen een tweede (permanente) diapauze, maar alléén als de kevers zich eerst hebben kunnen voeden. Dit suggereert dat een tweede diapauze moeilijker induceerbaar is. De fotoperiodische klok en dus de hersenen zijn essentieel voor de inductie van diapauze. De beschreven resultaten staven de theorie dat de hersenen de voornaamste rol spelen in de regulatie van de diapauze. Onder invloed van korte dag veroorzaken de hersenen een verandering in het endocriene evenwicht tussen ecdysteroiden en het juveniel hormoon, waardoor het diapauze syndroom wordt opgeroepen.

Contrôle extrinsèque et intrinsèque de la levée de diapause chez le doryphore.

RÉSUMÉ

L'influence des différents facteurs environnementaux (tels que température, humidité, photopériode et nourriture) sur le comportement et le métabolisme de quelques constituants de l'hémolymphe, tels que l'hormone juvénile, a été étudiée chez les femelles diapausantes et post-diapausantes du doryphore. Le contrôle endocrine de la rupture de diapause a été analysé par des expériences d'injections hormonales. D'autre part, des expériences d'allatectomie après la rupture de diapause ont confirmé le rôle de l'hormone juvénile dans la reproduction et révélé le contrôle endocrine de la réinduction en diapause.

L'analyse du comportement en fonction de la température a permis de distinguer trois phases successives au cours de la diapause:

i) la diapause vraie ('diapause development') dont la durée est d'environ 3 mois mais celle-ci est prolongée par des températures basses;

ii) une quiescence ('post-diapause quiescence') qui est marquée par une faible utilisation des protéïnes de réserves. Cette deuxième phase est maintenue par des températures basses, une humidité insuffisante ou l'absence de nourriture;

iii) une phase transitoire de développement de post-diapause dont le seuil de température se situe entre 8 et 10°C, et au cours de laquelle une légère réactivation des corps allates s'amorce. Cette phase aboutit à l'émergence du sol.

Après la levée de diapause, le développement de post-diapause se poursuit pour aboutir à la reproduction.

La sensibilité à la photopériode varie selon les différentes phases de la diapause. Chez les doryphores en vraie diapause exposés artificiellement au jour court, la photopériode peut encore fonctionner comme signal d'induction de diapause. Ensuite, ce facteur n'est plus perçu en tant que signal mais bien comme facteur direct. Après la diapause, l'activité de synthèse des corps allates et le titre de l'hormone juvénile dans l'hémolymphe sont inférieurs en jour court à ceux de jour long. La vitellogénèse en jour court s'en trouve par conséquent ralentie. Le taux d'oviposition, par contre, n'est pas affecté par le photorégime après la diapause. Cette perte de sensibilité n'est toutefois que temporaire,

puisqu'elle est réinstaurée après 3 ou 4 semaines chez un certain nombre d'individus.

La nourriture ne semble pas jouer de rôle important dans la levée de diapause, mais elle est nécessaire pour la vitellogénèse, et donc la reprise de la reproduction. En réinstaurant l'horloge photopériodique, la nourriture joue aussi un rôle important pour l'induction d'une seconde diapause.

La composition de l'hémolymphe, spécifique de la diapause, se caractérise principalement par l'accumulation de lipides, protéînes et acides aminés libres. Cette accumulation de substances semble augmenter la résistance au gel des insectes diapausants. En outre, la proline, qui sert de substrat énergétique chez l'adulte actif, paraît avoir une rôle cryoprotecteur pendant la diapause.

Le métabolisme, y compris celui de l'hormone juvénile, est réduit à un niveau basal pendant toute la durée de diapause. Au cours de la diapause, les réserves accumulées dans l'hémolymphe sont relativement peu utilisées. A l'émergence du sol (à la fin de la diapause), le taux de métabolisme augmente rapidement; les protéînes dites de diapause sont dégradées et la synthèse d'hormone juvénile culmine entre le cinquième et le septième jour de post-diapause. Toutefois, le jour court n'induit plus les estérases spécifiques responsables de la dégradation de l'hormone juvénile.

L'injection de 20-hydroxyecdysone seule n'a aucun effet sur la rupture de diapause, tandis que l'hormone juvénile seule rompt la diapause temporairement, pour le temps que le titre de l'hormone reste élevé. La rupture permanente de la diapause n'est obtenue que par les injections combinées des deux hormones. Pendant le premier mois de la diapause, une action synergétique de la 20-hydroxyecdysone et de l'hormone juvénile semble même avoir lieu. Pendant cette période, une inactivation progressive des corps allates semble se faire. Il est conclu de ces observations qu'un changement semble intervenir au niveau du système neuro-endocrine après un mois de diapause.

Le métabolisme endogène de l'hormone juvénile pendant la diapause n'est pas affecté par la 20-hydroxyecdysone seule ou administrée après une injection d'hormone juvénile. Par contre, l'hormone juvénile exerce un feed-back négatif sur l'activité des corps allates et induit en outre une augmentation de l'activité des estérases spécifiques dans l'hémolymphe. Ces effets de l'hormone juvénile sont supprimés si l'injection est précédée d'une injection de 20-hydroxyecdysone. Cette dernière semble donc jouer un

rôle-clé dans la régulation du métabolisme de l'hormone juvénile pendant la diapause. Ces observations conduisent à l'interprétation que la 20-hydroxyecdysone interviendrait au niveau du cerveau dans le déclenchement du programme de rupture de diapause.

Une seconde diapause (permanente) peut être induite chez un certain nombre d'individus par l'ablation des corps allates en post-diapause, mais seulement après que les insectes aient commencé de se nourrir. Une seconde diapause semble donc être plus difficilement induite. L'horloge photopériodique et donc le cerveau sont nécessaires à l'induction en diapause. Les résultats présentés corroborent la théorie que le cerveau est l'organe primordial contrôlant la diapause. Sous l'effet du jour court, le cerveau cause un changement d'équilibre hormonal entre les hormones ecdystéroïdes et juvénile, provoquant le syndrome de diapause.

CURRICULUM VITAE

Kathelijne Sylvie Lefevere is geboren op 1 maart 1956 te Brussel, België. Na het behalen van het diploma secundair onderwijs in de humaniora aan de "Ecole Normale Emile André" te Brussel in 1974, begon zij haar studie aan de "Université Libre de Bruxelles". Het kandidaatsexamen in de biologie werd in juli 1977 afgelegd. Het diploma licentiaat in de wetenschappen (richting zoölogie) werd "met grote onderscheiding" behaald in oktober 1979, met als hoofdvak systematiek-zoölogie en als bijvakken: dierfysiologie, vergelijkende histologie der evertebraten en insekten endocrinologie. In december 1979 werd het diploma "Agrégation de l'Enseignement supérieur (sciences biologiques)" daar behaald. In het jaar 1980 heeft zij onderzoek bij het "Département de Biologie animale et Histologie comparée" verricht aan dezelfde universiteit, onder begeleiding van Dr. J. Naisse. Van september 1980 tot juni 1982 genoot zij een beurs ter specialisatie van het Nederlands Ministerie van Onderwijs en Wetenschap, aan haar toegekend in het kader van de bilaterale culturele betrekkingen. In die periode werkte ze als wetenschappelijk gastmedewerkster bij de vakgroep entomologie aan de Landbouwuniversiteit te Wageningen. Dat onderzoek, onder leiding van wijlen Professor Dr. J. de Wilde, vormt een gedeelte van dit proefschrift. Na een jaar in België, werd promovendus als wetenschappelijk assistent tijdelijk aangesteld bij de Landbouwuniversiteit te Wageningen (van september 1983 tot juli 1985). Verslag van het onderzoek dat in die periode werd verricht aan de vakgroep entomologie, vormt het grootste gedeelte van dit proefschrift.

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