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HORMONES AND THE STRUCTURAL AND BIOCHEMICAL PROPERTIES OF THE FLIGHT MUSCLES IN THE COLORADO BEETLE

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ABBREVIATIONS

GAPDH	glyceraldehyde-3-phosphate dehydrogenase (EC.1.2.1.12)
GDH	glycerol-1-phosphate dehydrogenase (EC.1.1.1.8)
LDH	lactate dehydrogenase (EC.1.1.1.27)
c-MDH	cytoplasmic malate dehydrogenase (EC.1.1.1.37)
m-MDH	mitochondrial malate dehydrogenase
CE	condensing enzyme (EC.4.1.3.7)
HOAD	ß-hydroxyacyl-CoA dehydrogenase (EC.1.1.1.35)
SDH	succinate dehydrogenase (EC.1.3.99.1)
αGPox	glycerol-l-phosphate oxydase (EC.1.1.2.1)
ChE	acetylcholinesterase (EC.3.1.1.7)
$NADH_2$	nicotinamide adenine dinucleotide, reduced form.
NAD^+	nicotinamide adenine dinucleotide
CoA	coenzyme A
ATP	adenosine-5-triphosphoric acid
TRA	triethanolamine hydrochloride
Tris	tris (hydroxymethyl) aminomethane
EDTA	ethylene diaminotetraacetic acid
RNA	ribonucleic acid

1. GENERAL INTRODUCTION

In 1963 KARLSON introduced his theory on the mode of action of hormones at the molecular level, which has certainly stimulated much work during the last few years. This theory is based on the model proposed by JACOB and MONOD (1961) concerning the regulation of protein synthesis in micro-organisms. As yet there is no definite proof of Karlson's theory, and recent studies suggest that at least in higher organisms regulation of the translation of messenger RNA may be a more important control mechanism for protein synthesis than the rate of genetic transcription (TATA, 1966a). Furthermore it may be questioned whether Karlson's theory can explain the mechanism of action of all hormones.

The study of the mode of action of hormones at the molecular level is complicated by the diversity of the hormonal effects on one hand and the specificity of action on the other. The effects of the juvenile hormone of insects, secreted by the corpora allata, can be used to illustrate this problem. This hormone appears to play an important role in the maintenance of larval characters and to have a gonadotropic effect in many adult insects studied thus far. Moreover, the juvenile hormone appears to be involved in certain types of polymorphism in termites and locusts. It has also often been qualified as a metabolic hormone (WIGGLESWORTH, 1964). Since 1958 studies on the mode of action of juvenile hormone in the Colorado beetle have been carried out in the Wageningen Entomological Institute. In this insect removal of the corpora allata results in the syndrome of diapause, a state of inactivity, low metabolic rate and arrest of reproduction. During the onset of diapause many changes occur, but an outstanding feature is the atrophy of the flight muscles (see chapter 2).

As allatectomy in other insects does not always result in atrophy of flight muscles, a very important question from the endocrinological point of view is, whether the juvenile hormone is indeed directly involved in the maintenance of flight muscle structure and function in the Colorado beetle. In other words, which processes related to this element of diapause are directly controlled by the juvenile hormone? Are other hormones also involved and which processes are influenced indirectly? These questions will be an important topic of the present study, especially in relation to the flight muscles.

As energy production and consumption is the most obvious biochemical characteristic of flight muscles, the activity of some enzymes essential for carbohydrate and fatty acid metabolism was followed. The activity patterns of the enzymes are used as parameters for the functional integrity of the flight muscles. Parallel with the changes in activity of these enzymes, the ultra-structural changes were followed by means of the electronmicroscope. After a discussion of the literature in chapter 2, the material and methods are given in chapter 3. A description of the biochemical and morphological condition of flight muscles in active (non-diapause) beetles is given in chapter 4, the results of changes in hormone level are described in chapter 5 and discussed on the basis of our

knowledge of the hormone titer. In order to investigate whether this hormonal effect is indirect, we studied the influence of the nervous system on the structure and function of the flight muscles, which is reported in chapter 6.

2. LITERATURE

2.1. THE INSECT ENDOCRINE SYSTEM

The centres of hormone production in insects are closely associated with the central nervous system. Specialized neurosecretory cells are present in the pars intercerebralis. The neurosecretory substances are transported through the axons to the corpora cardiaca, which are responsible for the release into the haemolymph (B. SCHARRER, 1962 and 1965). Neurosecretory cells are also found in the ganglia of the ventral nerve cord (RAABE, 1965). Closely associated and contiguous with the cerebral neurosecretory system are the corpora allata, which produce the juvenile hormone. Lastly, the prothoracic glands secrete the moulting hormone, ecdysone (reviews: KARLSON, 1956; GILBERT and SCHNEIDERMAN, 1961; WIGGLESWORTH, 1964; GILBERT, 1964; KARLSON, 1966; HIGHNAM, 1967).

In discussing the functions of these hormones, we may distinguish between their effect on:

a. Larval growth and metamorphosis.
b. Functions in the adult insect, e.g. oogenesis, adult maturation.

The fact that the post-embryonic development of insects is under the control of hormones is well established. Many excellent reviews are dealing with this subject (WILLIAMS, 1952; GILBERT and SCHNEIDERMAN, 1961; GILBERT, 1964; WIGGLESWORTH, 1964). In the literature relating to it there is a certain degree of agreement about the role of these hormones in growth and metamorphosis.

The cyclical growth and moulting of an insect is brought about by two hormones. The brain hormone, produced by the neurosecretory cells of the brain and released by the corpora cardiaca, activates the prothoracic glands, which respond with the release of ecdysone. Ecdysone initiates the moulting process. The juvenile hormone, secreted by the corpora allata, appears to promote the the development of larval characters, and to prevent metamorphosis. Consequently, the juvenile hormone determines whether the result of a moult is a larva, pupa, or adult. When an insect moults in the absence of juvenile hormone, it tends to differentiate towards the adult stage.

There is, however, much less agreement about the relative role of the brain hormone and the juvenile hormone in the adult insect. After the adult moult the prothoracic glands, as a rule, degenerate (SHAAYA and KARLSON, 1965; HERMAN and GILBERT, 1966).

There are, however, many reports concerning the role of the juvenile hormone and the neurosecretory cells in the adult insect. It was WIGGLESWORTH (1936) who first demonstrated the role of the juvenile hormone in the adult blood-sucking bug, *Rhodnius prolixus*. The corpora allata are here necessary for the normal maintenance of sexual functions. Since then this gonadotropic effect has been demonstrated in many other insects (for references see: DE WILDE, 1964; EL-IBRASHY, 1965; ENGELMANN, 1968).

There is at present a good deal of evidence that the morphogenetic and gona-

dotropic effects are due to the same hormone. Larval corpora allata can induce egg maturation in allatectomized adult females and adult corpora allata can inhibit adult differentiation when implanted into allatectomized larvae. Furthermore, cecropia oil (WILLIAMS, 1956), and several juvenile hormone analogues, such as farnesyl-methyl-ether, have both the juvenilizing and the gonadotropic effects. Recently RÖLLER and BJERKE (1966) demonstrated these two functions with their pure juvenile hormone preparations.

That oocyte growth is also dependent upon factors from the neurosecretory cells has been demonstrated by THOMSEN (1952) in *Calliphora erythrocephala*, by HIGHNAM (1962) in *Schistocerca gregaria*, and by MORDUE (1965 a, b, c) in *Tenebrio molitor*. THOMSEN and MØLLER (1959, 1963) have found that the protocerebral neurosecretory cells influence the synthesis of protease in the gut, and they suggested an effect upon protein synthesis in general. The neurosecretory cells also play an important role in the activation of the corpora allata (THOMSEN, 1952; HIGHNAM, 1962). In this connection especially the lateral neurosecretory complexes seem to be important (STRONG, 1965). Moreover, it has been suggested that the corpora allata control to some degree the activity of the neurosecretory cells (HIGHNAM, LUSIS and HILL, 1963; LEA and THOMSEN, 1962) and that the ovaries also affect the activities of the corpora allata and the neurosecretory cells (NAYAR, 1958). It is clear that we are faced with a very complex system, comprising the brain, the corpora cardiaca, the corpora allata, and the ovaries. These organs influence each other by nervous stimuli, by neurosecretion along nervous pathways and by humoral factors in the haemolymph circulating through the body. The relative parts played by these different elements in this system vary from one insect to another (WIGGLESWORTH, 1964; DE WILDE, 1964; ENGELMANN, 1968).

2.2. ENVIRONMENTAL FACTORS AND THE NEURO-ENDOCRINE SYSTEM

Together with intrinsic signals, information from a variety of other sources is passed on to the neuro-endocrine system. Important factors in this respect are photoperiod and food.

It has been observed by GRISON (1958) and confirmed in our laboratory that the fecundity of the Colorado beetle is usually reduced with the ageing of the host plant. In fact, feeding on physiologically old leaves can induce a diapause reaction in *Leptinotarsa* (DE WILDE and FERKET, 1967). The effect of nutrition on the reproductive processes, however, is not necessarily a direct one. There are many indications that the neuro-endocrine system is involved, and that nutrition has an indirect effect (JOHANSSON, 1964; DE WILDE and FERKET, 1967).

The same applies to the photoperiod (DE WILDE, 1965). It has never been observed that the photoperiod has a direct influence on an effector organ. Photoperiodic control is generally effected through the superordinated neuro-endocrine centres. The importance of this arrangement is twofold. The central nervous system receives stimuli other than photic ones, and thus is in a position to integrate the over-all input. Furthermore, more than one function is, as a

rule, light-dependent and this demands for the integration of these functions (E.SCHARRER, 1964).

WILLIAMS (1963) demonstrated in the silkworm, *Antherea pernyi*, that light enters the pupa through an area of transparent cuticle overlying the brain. By surgical methods he was able to show that light acts directly on the pupal brain, inducing the termination of pupal diapause. The photoperiod acts within the brain to control the neurosecretory cells (WILLIAMS, 1963). A similar mechanism seems to exist in the adult Colorado beetle. In this insect the photoperiod remains effective as a controlling factor of adult diapause even after blinding (DE WILDE, DUINTJER and MOOK, 1959).

2.3. THE PHOTOPERIODIC RESPONSE IN THE COLORADO BEETLE

A widely spread phenomenon under the control of the photoperiod is diapause, a condition of very low activity combined with a standstill of growth and reproduction.

DE WILDE (1953) studied different aspects of adult diapause in the Colorado beetle. One of the most obvious characteristics was the change in behaviour. He observed a positive geotactic response instead of an active feeding and reproductive behaviour. The animals ceased reproduction, left the host plant and burrowed themselves. Basal metabolic rate was much reduced.

The photoperiod is the governing environmental factor inducing diapause. The Colorado beetle appeared to be a long-day insect, with a critical photoperiod of 15 hours. Photoperiods applied to the adult insect were decisive although treatment of the larvae had some effect (DE WILDE, 1955; DE WILDE et al, 1959)

That the corpora allata are involved in the induction of diapause was also shown by the experiments of DE WILDE (1955). In later studies DE WILDE and STEGWEE (1958), DE WILDE (1959) and DE WILDE and DE BOER (1961) could prove more definitely that diapause in the Colorado beetle is the result of inactivity of the corpora allata. Allatectomy can produce the change in behaviour, arrest of oogenesis and the low rate of respiration characteristic for diapause. Therefore DE WILDE and DE BOER (1961) described the adult diapause of the Colorado beetle as a case of pseudo-allatectomy.

That the corpora allata were directly involved in the regulation of diapause was concluded from the experiments with tissue homogenates (DE WILDE and STEGWEE, 1958; DE WILDE, 1959; STEGWEE, 1960). They could obtain a significant increase in the oxygen consumption of crude homogenates of diapausing beetles with succinate as a substrate after addition of 4–6 corpora allata of active ovipositing females. The same result could be obtained with the so-called cecropia extract, which is believed to contain the juvenile hormone (WILLIAMS, 1956). But the effects of the cecropia extract upon the succinate oxidation of isolated sarcosomes of diapausing beetles were rather inconsistent (STEGWEE, 1960).

STEGWEE, however, found, within a narrow concentration range of the cecropia oil, a pronounced effect on the rate of oxidative phosphorylation of sarco-

somes from diapausing beetles. Recently MINKS (1967) found the same with mitochondria from *Locusta*.

STEGWEE (1964) started a study on the respiratory metabolism of the flight muscle, because in active animals the thoracic tissues account for some 80 per cent of the total respiration. After he had demonstrated that isolated sarcosomes from normal active beetles have the same characteristics as other insect and vertebrate mitochondria (STEGWEE and VAN KAMMEN-WERTHEIM, 1962), he showed that diapausing beetles yielded sarcosome preparations which were both biochemically and morphologically distinctly different from normal sarcosomes (STEGWEE, 1964). The respiratory activity was only 5% of those from 'active' sarcosomes.

Although the presence of several components of the respiratory chain could be demonstrated in these 'diapausing sarcosomes', their absolute and relative concentrations seemed to have undergone drastic changes. The sarcosomes seemed to be degenerated. Further electronmicroscopic examination substantiated this view (STEGWEE, 1964; STEGWEE, KIMMEL, DE BOER and HENSTRA, 1963). In diapause the flight muscles show pronounced degeneration. The muscle fibrils are greatly reduced in diameter and the sarcosomes are virtually absent. They found the same degeneration after extirpation of the postcerebral complex of endocrine glands. Reimplantation of active postcerebral complexes or of isolated corpora allata resulted in a very rapid regeneration of the muscle fibres and a new formation of sarcosomes. It is worth while pointing out here that these experiments of STEGWEE do not prove that this effect of the corpora allata is due to a direct influence on the flight muscles.

Recently, EL-IBRASHY (1965) made a comparative study on metabolic effects of the corpora allata in two adult Coleoptera, *Tenebrio molitor* and *Leptinotarsa*. He has found that after allatectomy the respiratory rate in both sexes of *Leptinotarsa* decreased, while in *Tenebrio* this operation had no effect on the rate of O_2-consumption. Furthermore, he showed that castration did not lead to a reduction of the oxygen uptake in either of the two species. An electronmicroscopic study of the flight muscles of *Leptinotarsa* showed that castration did not result in degeneration of the flight muscles (DE KORT, unpublished observations). It can be concluded that in *Leptinotarsa* females, the effect of the corpora allata on respiration cannot be explained by an effect on the ovaries, as was described by SLAMA (1964) in *Pyrrhocoris*.

From the data mentioned above concerning the photoperiodic response in the Colorado beetle, the following conclusions may be drawn:

1. The photoperiodic induction of diapause is mediated by hormones; especially the corpora allata appear to be important.
2. Diapause is accompanied by a decrease in the rate of respiration.
3. Flight muscle degeneration is the most important cause of this decrease in respiratory rate.
4. The state of the gonads, although they are also under the control of the neuro-endocrine system, appears to have no direct relations to flight muscle structure.

2.4. Hormones and metabolism

The action of hormones is very often accompanied by an increase in metabolic rate. The juvenile hormone has even been qualified as a 'metabolic hormone'.

Thomsen (1949) demonstrated a decrease of oxygen consumption in adult *Calliphora* after allatectomy. This could not be explained by a lack of ovarian development, because castration did not produce this effect. Comparable results were obtained by Weed-Pfeiffer (1945) in *Melanoplus*, Sägesser (1960) in *Leucophaea* and de Wilde and Stegwee (1958) in *Leptinotarsa*.

Different results, however, were reported by Pflugfelder (1952) with *Dixippus* and by Slama with *Pyrrhocoris* (Slama, 1964a, b, 1965).

The concept of a general metabolic hormone from the corpora allata is questionable in view of the discovery that the neurosecretory cells play an important role in protein synthesis as described above and because of the suggestion that the corpora allata may exert an activating effect on the neurosecretory cells (Highnam et al., 1963; Lea and Thomsen, 1962). But this latter effect may be indirect. In this connection, the experiments of Lüscher and Leuthold (1965) are interesting. They measured the O_2-consumption of the isolated fat body of *Leucophaea* after addition of hormone glands. They found a stimulation of the O_2-consumption after addition of corpora cardiaca. On the other hand, the corpora cardiaca had no effect *in vivo*. Implantation of corpora allata, however, resulted in a stimulation of O_2-consumption after 5–10 days.

Hormone action is often accompanied by an increase in protein synthesis. Because protein synthesis requires an increase in energy production, it is not surprising that hormone action involves changes in rate of O_2-consumption. This effect on O_2-consumption may then be only indirect: the result of a higher demand for energy. Therefore it can be explained in terms of homeostatic regulation.

It is difficult to decide whether a given metabolic effect is a direct or an indirect result of hormone action. The question can be asked if this problem can be solved by 'classical' endocrinological experiments such as: injection, extirpation, implantation and transplantation of hormone glands. Very recently Minks (1967) and Gilbert (1967) used more direct approaches in their studies of the influence of insect hormones on metabolism.

Minks very extensively studied in vitro effects of the corpus allatum hormone on mitochondrial preparations from flight muscles and from the fat body of *Locusta*. He could never obtain any increase in the oxygen consumption of these preparations after addition of corpora allata or cecropia oil, as was reported by Clark and Baldwin (1960). However, he clearly demonstrated the effect on oxidative phosphorylation with both fat body and flight muscle mitochondria. His results with α-glycerophosphate as a substrate are very convincing, but he could not obtain the same result with pyruvate/malate, except with totally uncoupled mitochondria. However, many questions remain to be answered. Isolated sarcosomes, especially from insects, very often show P/O ratios far below their theoretical value. It could always be demonstrated that this was due

to the isolation procedure (VAN DEN BERGH, 1962). Many corrective factors are known to enhance the phosphorylating capacity of isolated mitochondria, in other words, they only may reduce the disruptive effects of the isolation procedure. In view of this the question may be asked, whether the effect of the hormone is different from that of unspecific factors. Is this 'correcting influence' necessary *in vivo?* MINKS could not find a difference in phosphorylating efficiency of mitochondria isolated from normal and allatectomized locusts. Can this correcting effect explain the whole diapause reaction in the Colorado beetle? Is this effect a general principle, and can it be demonstrated in other insects?

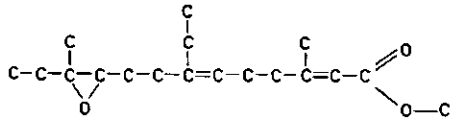
GILBERT (1967) demonstrated the transfer of labelled lipid from the fat body to the ovaries *in vitro*, and suggested that the juvenile hormone is involved in this process. WIENS and GILBERT (1965 and 1967) demonstrated an *in vitro* effect of a corpus cardiacum extract on glycogen metabolism in the fat body of *Leucophaea maderae*. Through these approaches they could confirm the occurence of a hyperglycaemic factor in the corpora cardiaca, described before by STEELE (1961, 1963), BOWERS and FRIEDMAN (1963) and RALPH and MCCARTHY (1964). STEELE (1963) and WIENS and GILBERT (1967) have shown the involvement of phosphorylase in the mobilization of polysaccharides. These results clearly revealed a mechanism comparable to the effect of glucagon in vertebrates. The corpora cardiaca also bring about an acceleration of the oxygen consumption of the fat body *in vitro*, which is in accord with the results of LÜSCHER and LEUTHOLD (1965). The interpretation of the measurements of total body O_2-consumption is very difficult considering the experiments discussed here with isolated tissues.

From all this it is evident that the term 'metabolic hormone' is rather inadequate. Every hormone brings about metabolic changes. Moreover, as shown above, it is difficult to decide whether a given metabolic effect is a direct or an indirect result of hormone action.

The isolation and identification (RÖLLER et al, 1967) and synthesis (DAHM et al, 1967) of juvenile hormone will probably open new possibilities for the study of its mode of action.

2.5. HORMONES AND THE STRUCTURE AND FUNCTION OF MUSCLES

The data about the influence of hormones on the structure and function of muscles are scarce. LINDBERG (1965) and TATA et al (1963) studied the effects of thyroxine on the structure and metabolic rate of rat muscles. But their results could be explained by indirect effects as well.



juvenile hormone = methyl 10-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate.

In insects, comparable results are not available. MINKS (1967) has some indications from electronmicrographs that the amount of mitochondria increases in the flight muscles of *Locusta* after allatectomy, but he gives no correlated enzymatic data. LOCKSHIN and WILLIAMS (1964, 1965a, b) have studied the programmed cell death of the intersegmental abdominal muscles in the silkmoth *Antheraea pernyi*. Indeed this histolysis is directly related to ecdysis, but LOCKSHIN and WILLIAMS could prove that the ultimate cause of the degeneration is the standstill in the conduction of nerve stimuli to the muscle.

Although the diapause reaction involves a whole syndrome of characteristics, we focused our attention on the flight muscles. Furthermore, the degeneration of the flight muscles is the most important cause of the decrease in respiration. Therefore the flight muscles can be considered as the 'target-organs' for the hormonal effect on oxidative metabolism. This paper will give a contribution to the discussion about the relation between hormones and metabolism. BÜCHER and coworkers (BROSEMER et al, 1963; VOGELL, 1965; BÜCHER, 1965; BEENAKKERS, 1964) described the development of the flight muscles in *Locusta*; they used both electronmicroscopic and biochemical methods. This combined enzymological and electronmicroscopic approach seems to us a useful way to describe the condition of the flight muscles.

3. MATERIALS AND METHODS

3.1. Experimental animals

In order to obtain beetles of a constant physiological state, rearing conditions were standarized as much as possible. Especially the quality of the food can greatly influence the effect of the photoperiod (de Wilde and Ferket, 1967). Larvae and adults of the Colorado beetle *(Leptinotarsa decemlineata* Say*)* were reared on fresh potato leaves. Prepupae were placed on sand of appropriate moisture content and soon entered the soil for metamorphosis. The adults emerged after 10–14 days, with a maximum at the 11th day when about 60% emerged. This group was taken together and directly provided with fresh food. The breeding stock was kept in a climate room at 25 °C and treated under two different photoperiods.

1. Long photoperiod (18h photophase).
2. Short photoperiod (10h photophase).

The animals were kept ab ovo under these conditions. In some experiments short-day treated larvae were transferred to long-day conditions and long-day treated larvae to short-day. The transfer always took place during metamorphosis.

For comparison some experiments were carried out on *Musca domestica, Tenebrio molitor* and *Apis mellifica.*

3.2. Operations and injections

For some experiments allatectomized animals were used. Allatectomy was carried out by the method described by de Wilde and de Boer (1961). Denervation was carried out after CO_2 narcosis under Ringer solution. The nerves of the third thoracic ganglion were carefully cut and the wound in the cuticle was closed with paraffin wax.

Injections were always given laterally in a soft part of the abdomen with the aid of a Hamilton microsyringe.

3.3. Electronmicroscopy[1]

The dorso-longitudinal flight muscles were fixed *in situ* with one drop of cold 6.25% glutaraldehyde according to Sabatini et al (1963) and directly dissected and fixed for another 2 hours in the same glutaraldehyde solution in the cold. After removal of the aldehyde the specimen was rinsed with several changes of 0.2 M sucrose in 0.1 M Na-cacodylate buffer pH 7.4 during 24 hours. Post-fixation in 1% osmium tetroxide at 4 °C according to Palade (1952) was carried out during 2 hours and the specimen was embedded in the resin Epon 812 according to Luft (1961). Ultrathin sections were cut with a

[1] Electronmicroscopy was carried out in collaboration with the section Electronmicroscopy of the Service Institute for Technical Physics in Agriculture, Wageningen.

glass-knife on an L.K.B. ultramicrotome type 'Ultratome III'. The sections were examined with a Siemens Elmiskop I or Philips E.M. 300 electronmicroscope.

3.4. Extraction of the enzymes from muscles

The dorso-longitudinal flight muscles were too small for collection in sufficient amounts, and therefore total thoraces were used for the extraction of the enzymes. The fractionated extraction procedure of Delbrück et al (1959) was applied for the determination of the activities of c-MDH and m-MDH.

For determination of the other enzymes this procedure could not be used because it inactivated about 75% of the mitochondrial SDH and αGPox activities. The use of an ultrasonic desintegrator for disruption of the mitochondria was mainly responsible for the drastic inactivation of the SDH and αGPox activities. This is illustrated in fig. 1.

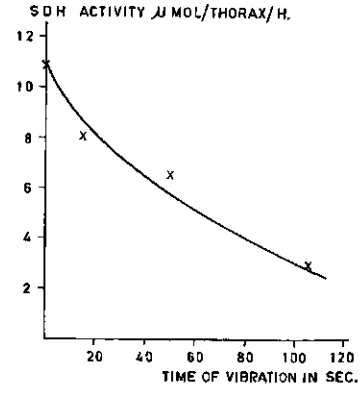


Fig. 1. Effect of ultrasonic vibration on the SDH activity of flight muscle mitochondria of the Colorado beetle.

Also van den Bergh (1962) and Lennie and Birt (1967) found an inactivation of αGPox by ultrasonic treatment, but they found no effect on SDH. However, Bellamy (1958) and Clements (1959) observed an inactivation of SDH during homogenization of the fat body of locusts. We found no effect of ultrasonic treatment on SDH from thoraces of *Tenebrio*, housefly, honey-bee or locust. We avoided this treatment for the extraction of the enzymes from thoraces of the Colorado beetle. In our routine experiments the following extraction procedure has been followed: Four thoraces were carefully isolated, minced and homogenized in 4.0 ml 0.32 M sucrose + 5 mM EDTA pH 7.4 solutions in a handdriven Potter-Elvehjem (teflon and glass) homogenizer. For removal of the cuticle fragments the homogenate was filtered through fine nylon tissue by suction into a small tube. The filtered homogenate was transferred to a centrifuge tube and centrifuged during 20 min. at 60,000 *g* in a Christ-Omega ultracentrifuge. The soluble cytoplasmic enzymes GAPDH, GDH and LDH were measured in the supernatant fraction. The pellet, suspended in 4.0 ml sucrose, contained the mitochondrial SDH and αGPox activities. For determina-

tion of the CE and HOAD activities part of the total homogenate was treated with an ultrasonic desintegrator and measured separately.

3.5. Determination of the enzyme activities in the muscle extracts

Determination of the enzyme activities was carried out by the so-called optical test, under optimal conditions in standardized testmedia as described by Beizenherz et al (1953), Bücher et al (1964) and Beenakkers (1964).

The composition of the testmedia was:

GAPDH: 0.05 M TRA-buffer pH 7.6; 5 mM EDTA; 3.3 mM $MgSO_4$; 0.15 mM $NADH_2$; 2.4 mM reduced glutathione; 1.5 mM ATP; 7 mM phosphoglycerate; 4.5 units/ml 3-phosphoglyceratekinase (EC 2.7.2.3).

GDH: 0.05 M TRA-buffer pH 7.6; 5 mM EDTA; 0.15 mM $NADH_2$; 0.4 mM dihydroxyacetonephosphate.

HOAD: 0.10 M TRA-buffer pH 7.0; 5 mM EDTA; 0.45 mM $NADH_2$; 0.10 mM acetoacetyl-CoA.

LDH: 0.05 M TRA-buffer pH 7.6; 5 mM EDTA; 0.15 mM $NADH_2$; 2.4 mM pyruvate.

MDH: 0.05 M Phosphate buffer pH 7.4; 20 mM aspartate; 13 mM α-oxoglutarate; 0.20 mM $NADH_2$ and 0.36 units/ml glutamate-oxaloacetate transaminase (EC 2.6.1.1).

CE: 0.20 M TRIS-buffer pH 8.0; 5 mM EDTA; 5 mM NAD^+; 50 mM malate; 36 units/ml malate dehydrogenase; 0.15 mM acetyl CoA.

SDH: 0.10 M phosphate buffer pH 7.4; 5 mM EDTA; 1 mM KCN; 0.1% cytochrome c; 20 mM succinate.

αGPox; 0.10 M phosphate buffer pH 7.4; 5 mM EDTA; 8 mM $MgSO_4$; 1.0 mM KCN; 0.1% cytochrome c; 20 mM α-glycerophosphate.

All the solutions were made in redistilled water and adjusted to the appropriate pH. The oxidation of $NADH_2$ and the reduction of cytochrome c were followed in an Optica CF4R spectrophotometer at 340 mμ (for HOAD 366 mμ was used) and 550 mμ respectively. From the change in optical density the activities were calculated with the aid of the difference in extinction coefficients between reduced and oxidized NAD and cytochrome c (Bücher et al, 1964; Margoliash et al, 1959) and expressed as μmol substrate/thorax/h. The concentrations of substrates and co-substrates were carefully estimated by means of the optical test with the aid of pure enzyme preparations.

3.6 Preparation of substrates

To 25 mg dihydroxyacetonephosphate-dimethylketal 1.10 ml water and 0.5 ml Dowex $50H^+$ was added. After thoroughly mixing for 30 sec. the Dowex was centrifuged and the substrate solution was incubated in a water bath at 40 °C for 4 hours. The pH of the substrate solution was adjusted to 4.5 with a con-

centrated $NaHCO_3$ solution. Before use Dowex was activated with 4 N HCl and washed till the elution liquid was neutral.

To 1.84 g 3-phosphoglycerate. 2 H_2O (Ba-salt) 2.5 ml 4 N H_2SO_4 was added and the $BaSO_4$ was centrifugated down. The precipitate was washed with water and the pooled supernatants were adjusted to pH 7.5 with 4 N NaOH and further diluted to a volume of 10 ml.

For preparations of aceto-acetylCoA a modification of the method described by DRUMMOND and STERN (1960) was used:

1. Reduction of CoA: To a solution of 0.5 ml 0.1 M Tris-buffer pH 9.0, 2.0 ml 2 mM KOH and 20 mg KBH_4 an amount of 10 mg CoA was added. After incubation during 15 min. at 37 °C, the solution was adjusted to pH 7.2 with 2 N H_2SO_4 at 0 °C. The solution had to be shaken till the free hydrogen gas had disappeared (10–15 min.).
2. Synthesis of aceto-acetylCoA: To the reduced CoA solution 0.050 ml freshly distilled diketene (Schuchardt, Münich) was added. After 15–20 min the solution was adjusted to pH 4.5. The excess of diketene was removed with diethyl-ether saturated with H_2O.

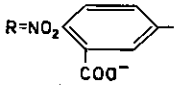
The same procedure could be used for the synthesis of acetylCoA, except that instead of diketene 6 µl distilled acetic anhydride was used.

3.7. ESTIMATION OF CHOLINESTERASE ACTIVITY

Routinely 15 heads were homogenized in 3.0 ml 0.1 M phosphate buffer pH 7.6 + 3% NaCl in a handdriven Potter-Elvehjem homogenizer and afterwards treated 2 × 1 min. in a MSE-ultrasonic desintegrator. For removal of the cuticle fragments the homogenate was filtered through a layer of fine nylon tissue.

The ChE-activity was measured by the method of ELLMAN et al (1961), with acetylthiocholine iodide as a substrate. The amount of thiocholine was determined with the use of 5:5 dithio bis-2-nitrobenzoate (DTNB), according to the reaction:

$$(CH_3)_3N^+CH_2CH_2S^- + R\text{-}S\text{-}S\text{-}R \longrightarrow (CH_3)_3N^+CH_2CH_2\text{-}S\text{-}S\text{-}R + RS^-$$



The yellow coloured anion 5 thio-2-nitrobenzoic acid was formed, which could be measured at 412 mµ. Very recently LEWIS (1967) reported a significant increase in ChE activity of crude homogenates after addition of organic solvents.

n-Butanol appeared to have the strongest effect, it stimulated the activity about 400%. As n-butanol enhanced the ChE activity in crude homogenates of the Colorado beetle with ca 300%, we added butanol to the medium throughout our experiments. The composition of the medium for estimation of the ChE activities was as follows:

0.10 M phosphate buffer pH 7.6 + 3% NaCl; 2% n-butanol; 3.3 mM DTNB; 1 mM acetylthiocholine iodide.

With the aid of the extinction coefficient of the anion the activity could be calculated. It was expressed as μmol/5 heads/h.

3.8. Protein determinations

The protein content of the homogenate was measured as follows. The proteins were precipitated by the addition of perchloric acid (PCA) to a final concentration of 2%. After washing with cold 2% PCA, the lipid material was removed with ethanol/ether (3 : 1 v/v). The proteins were dissolved in 1 N NaOH and the concentration was estimated according to the method of Lowry et al (1951). As a standard, solutions of bovine serum albumin were used.

3.9. Quantitative determinations of some body constituents

The amount of glycogen was determined with the anthrone reagent according to the method described by van Handel (1965). The carbohydrate concentration in the haemolymph was also measured with anthrone, after precipitation of the haemolymph proteins with methanol, final concentration 66%. Trehalose was used as a standard.

The total lipids were extracted with chloroform/methanol (2 : 1 v/v) according to Folch et al (1957) and measured gravimetrically.

The concentration of the glycerides in the haemolymph was determined spectrophotometrically by the method described by Eggstein and Kreutz (1966). Haemolymph was collected with the aid of a glass capillary after clipping a hind-leg. The haemolymph was chilled in ice in the presence of a small amount of reduced glutathione to inhibit tyrosinase activity. A known haemolymph sample (0.10 ml) was saponified in a small centrifuge tube with 0.50 ml alcoholic potassium hydroxide (0.5 M KOH in 96% ethanol) during 30 min. at 70 °C in a water bath. After cooling, 2.5 N PCA was added, to a pH value of about 7 (6–8) and the centrifuge tube was allowed to stand in an ice bath for 30 min. After centrifugation, the amount of glycerol was measured in the clear supernatant according to the following reactions:

$$\text{glycerol} + \text{ATP} \rightleftarrows \text{glycerol-1-phosphate} + \text{ADP}$$
$$\text{phosphoenolpyruvate} + \text{ADP} \rightleftarrows \text{pyruvate} + \text{ATP}$$
$$\text{pyruvate} + \text{NADH}_2 \rightleftarrows \text{lactate} + \text{NAD}^+.$$

The oxidation of $NADH_2$ was followed spectrophotometrically as described in 3.5. The composition of the test medium was: 0.1 M TRA-buffer pH 7.6; 4 mM $MgSO_4$; 6 mM $NADH_2$; 33 mM ATP; 11 mM phosphoenolpyruvate; 7 units LDH; 1.5 units pyruvate kinase (EC. 2.7.1.40) and 1.7 units glycerokinase (EC. 2.7.1.30) in a final volume of 1.0 ml.

The amount of free glycerol was determined in unsaponified haemolymph and subtracted from the total glycerol. The glyceride concentration in the haemolymph was calculated from the change in optical density (see 3.5) and

expressed as glycerideglycerol in mg/100 ml haemolymph. The amount of protein was estimated as described in 3.8.

3.10 Chemicals

All enzymes, coenzymes and substrates were obtained from Boehringer, Mannheim.

The normal chemicals used were from Baker Chemicals, except: trehalose (E. Merck A.G., Darmstadt), osmium-tetroxide (Brocades, Arnhem), glutaraldehyde 25% (Koch-Light Laboratory Ltd., England), Epon 812, dodecenylsuccinic anhydride and methylnadic anhydride (G.T. Gurr Ltd. England), propylene oxide (Hopkin and Williams Ltd., England) and potassium borohydride (Fluka, Switzerland).

All these chemicals were of p.a. quality.

PLATE 1. Cross section of the dorsal longitudinal flight muscle at the moment of emergence (10,500 x).
M = mitochondrion; N = nucleus; T = tracheole; F = myofibril.

4. DEVELOPMENT OF THE STRUCTURAL AND BIOCHEMICAL PROPERTIES OF THE FLIGHT MUSCLES IN LONG-DAY TREATED BEETLES

4.1. Introduction

In chapter 2 we discussed extensively the data showing that both the ultrastructure and the metabolic acitivity of the flight muscles of the Colorado beetle are influenced by the photoperiodic treatment. Therefore both ultrastructure and metabolic activity were studied together here. Already in an early phase of our study it appeared that a significant development of the flight muscles takes place after emergence. We followed this development in order to compare its characteristics with those of short-day beetles.

The development of the flight muscles of *Locusta migratoria* has been described very extensively by Bücher and his associates (Brosemer et al. 1963; Vogell, 1965; Bücher, 1965). They demonstrated a positive correlation between the development of the structural components and the enzymic pattern. They showed, furthermore, that several enzymes from a number of metabolic pathways behave like 'constant-proportion groups'. The activity ratios of the enzymes of a group are constant at all stages of development. The same constant-proportion groups could be found in several tissues from a variety of other organisms; e.g. rat heart, liver, brain, beef heart, a number of muscles from rabbit, pigeon, rat and even in baker's yeast. Consequently, one enzyme is truly representative of a whole group. (Pette, Luh and Bücher, 1962 Pette, Klingenberg and Bücher, 1962; Beenakkers, 1964; Pette, 1965). Therefore the activity of one enzyme at different times of development can be taken as a measure for the capacity of that particular metabolic chain, e.g. HOAD forms a constant proportion group with the other enzymes of the fatty acid oxidation chain, and consequently it is a measure for the capacity for fatty acid oxidation at different times of flight muscle development.

Such systematic investigations as have been done on *Locusta* have never been carried out on holometabolous insects. It would be interesting to compare the flight muscle development of these different kinds of insects. This will be done in this chapter.

4.2. Structure of the flight muscles at different times after emergence

Our electronmicrographs were taken from the dorso-longitudinal indirect flight muscles. At the time of emergence these muscle fibers are very small. We measured an average diameter of the fiber of 34 μ, if we assume that the fibers are completely cylindrical. Plates 1,2,3, show the fiber at this stage. In a transverse section at low magnification (plate 1) an enormous network of tracheoles can be seen between the myofibrils, suggesting a very efficient oxygen

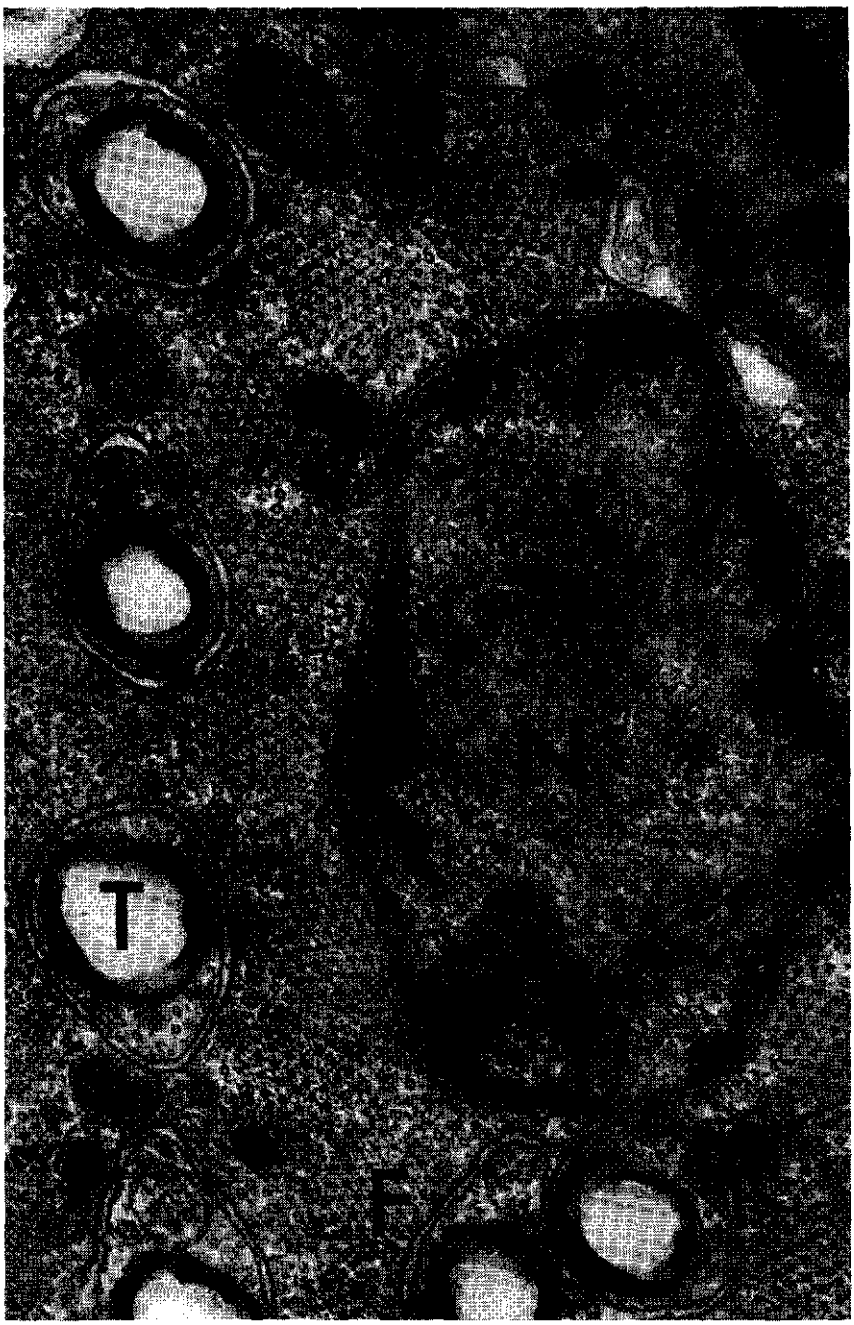


PLATE 2. Cross section of the flight muscle at the moment of emergence (54,000 x).

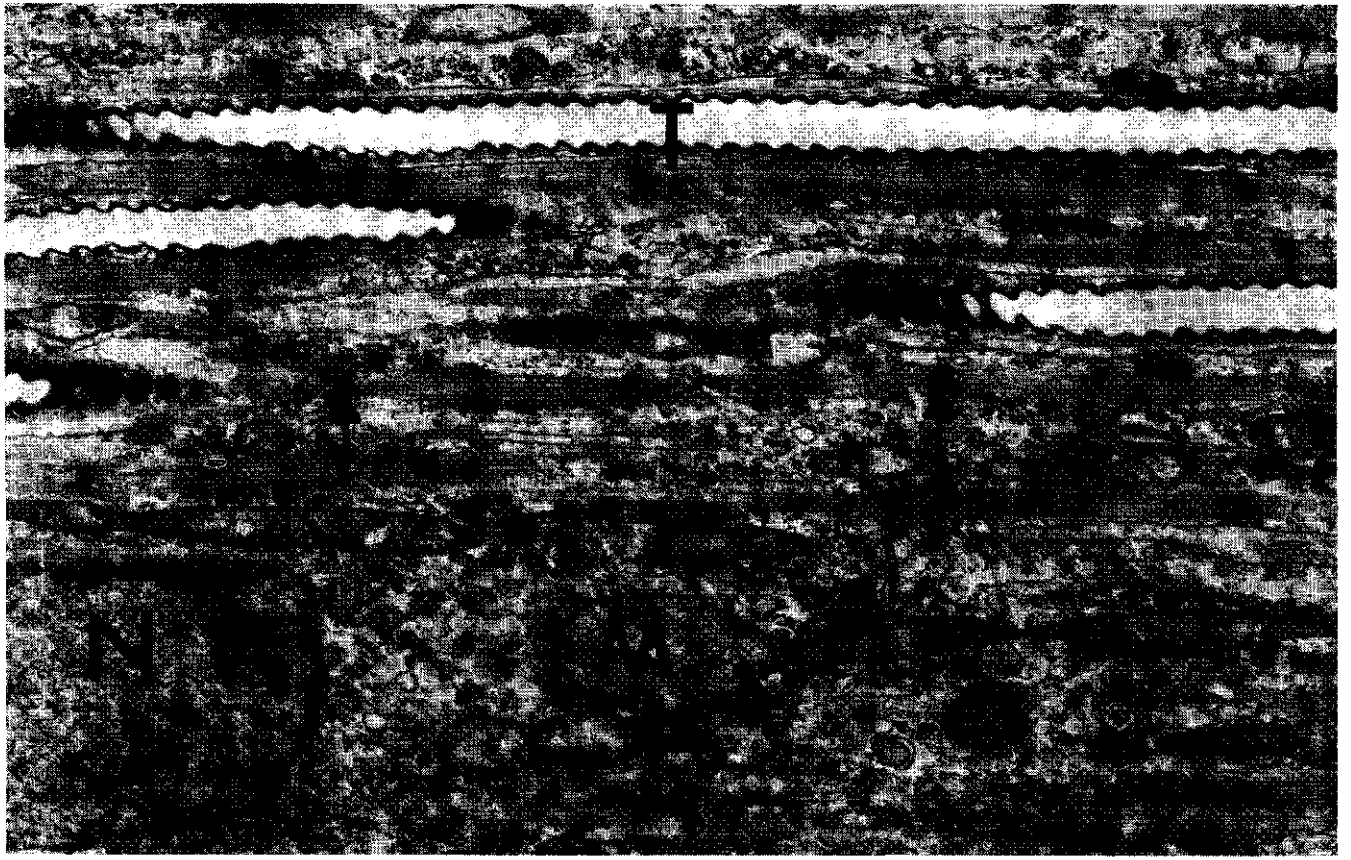


PLATE 3. Longitudinal section of the flight muscle immediately after emergence (21,600 x). The arrow indicates the irregular Z-line

supply. These tracheoles must be considered as external invaginations, as they are surrounded by the sarcolemma and by their own plasma membrane (plate 2).

Numerous oval nuclei, about 5 μ in length, can be seen laying throughout the fiber (plate 1). The characteristic double nuclear envelope is present (plate 2). The accumulation of chromatin at the periphery indicates the beginning of extensive synthetic activity.

From the structure of the myofibrils it can be seen that at this stage the flight muscle has a low level of development. The diameter of the fibrils is small, 0.3–0.4 μ. We can calculate that one fiber contains about 2,000 myofibrils at this stage. Each myofibril contains no more than about 50 myosin filaments. However, the thick and thin filaments can clearly be distinguished and the regular hexagonal arrangement is already present (plate 2). In longitudinal section (plate 3) the cross-striation of the fiber is almost absent. The irregular Z-band is recognizable. The sarcomere length is 3.5 μ at this stage. The A- and I-band can hardly be distinguished. The I-band seems to be much wider in comparison to later stages.

Between the fibrils small mitochondria are visible, 0.2–0.3 μ in diameter. They are recognizable by the characteristic double membrane, and cristae can also be seen (plate 3). They often have a filamentous appearance, and the great number of constrictions strongly suggest fission. Between the mitochondria and the myofibrils numerous small granules of about 150 Å can be seen. These granules become more numerous in later stages and we can see similar granules associated with the outer nuclear envelope. Although we have not identified them by treatment with ribonuclease, we would suggest them to be ribosomes, because BROSEMER et al (1963) described similar granules in the developing flight muscles of *Locusta*.

Our electronmicrographs sometimes showed nerve terminals, which indicate that, at least morphologically, innervation is already present at this moment.

Directly after emergence this 'precursor muscle' increases in size. The myofibrils increase very quickly in diameter through the augmentation of the number of myofilaments at the periphery (plate 4). After 3 days the fibril diameter is about 1.5 μ. The growth of the fibrils is completed after about 5 days. (For more quantitative data see section 4.4.) In longitudinal sections the striations become more regular, and the sarcomere length attains its normal value, i.e. 2.2–2.5 μ (plate 5).

It appeared from our electronmicrographs that the development of the fibrils is completed after 5 days. The myofibrils of the adult muscle are characteristic for fibrillar flight muscles: large circular myofibrils, with a diameter of more than 2 μ (plate 7). The sarcomere has a narrow I-band, (plate 6) which is in agreement with the well-known isometric contraction of these muscles.

Parallel with the development of this contractile system the mitochondria also show a rapid differentiation. First of all there is a significant increase in number, probably by fission of pre-existing mitochondria. Their diameter has hardly changed (0.24 μ) in the first few days. Most of the mitochondria, however, clearly possess more cristae after 3 days (plate 4). After this phase of multi-

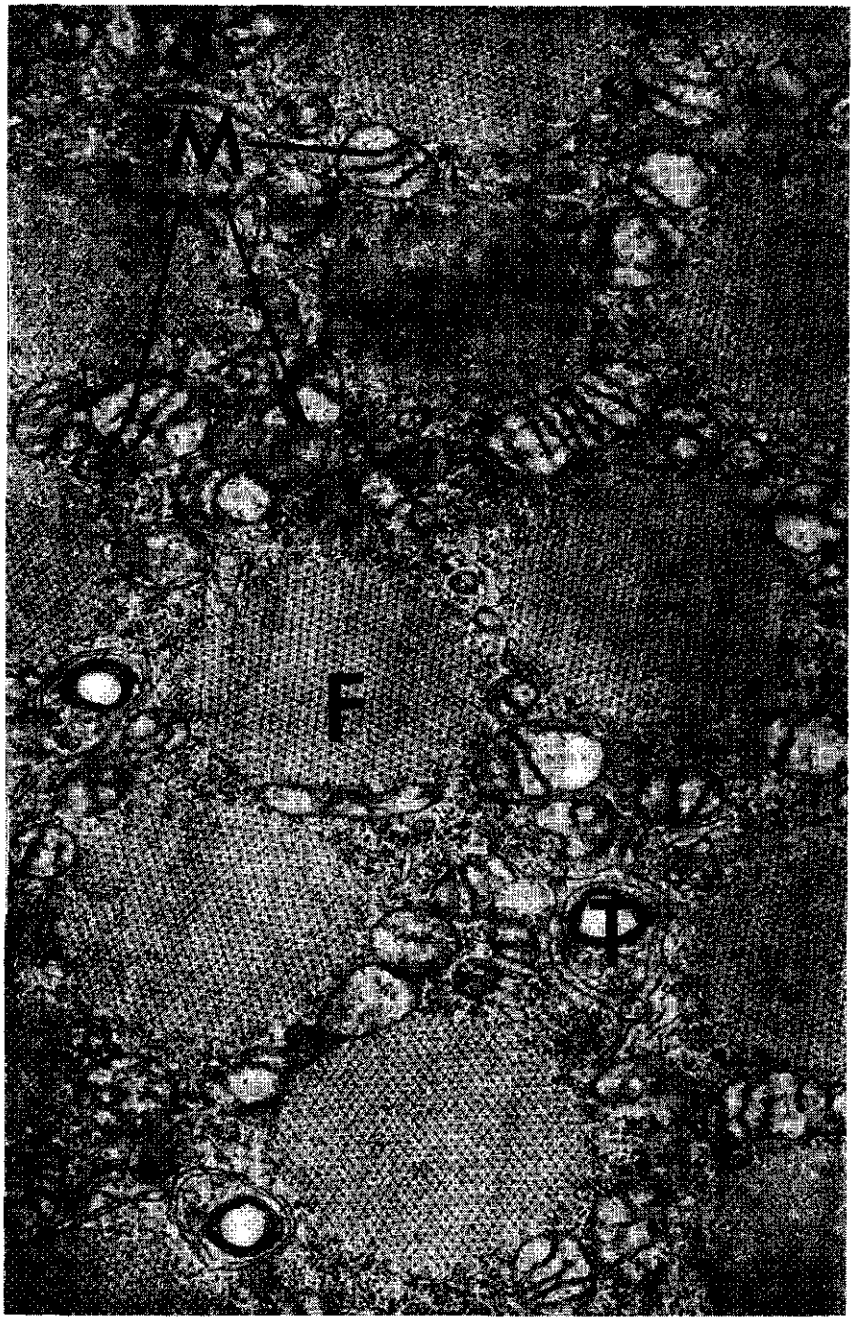


PLATE 4. Cross section of the flight muscle of a three days old long-day beetle (25,000 x).

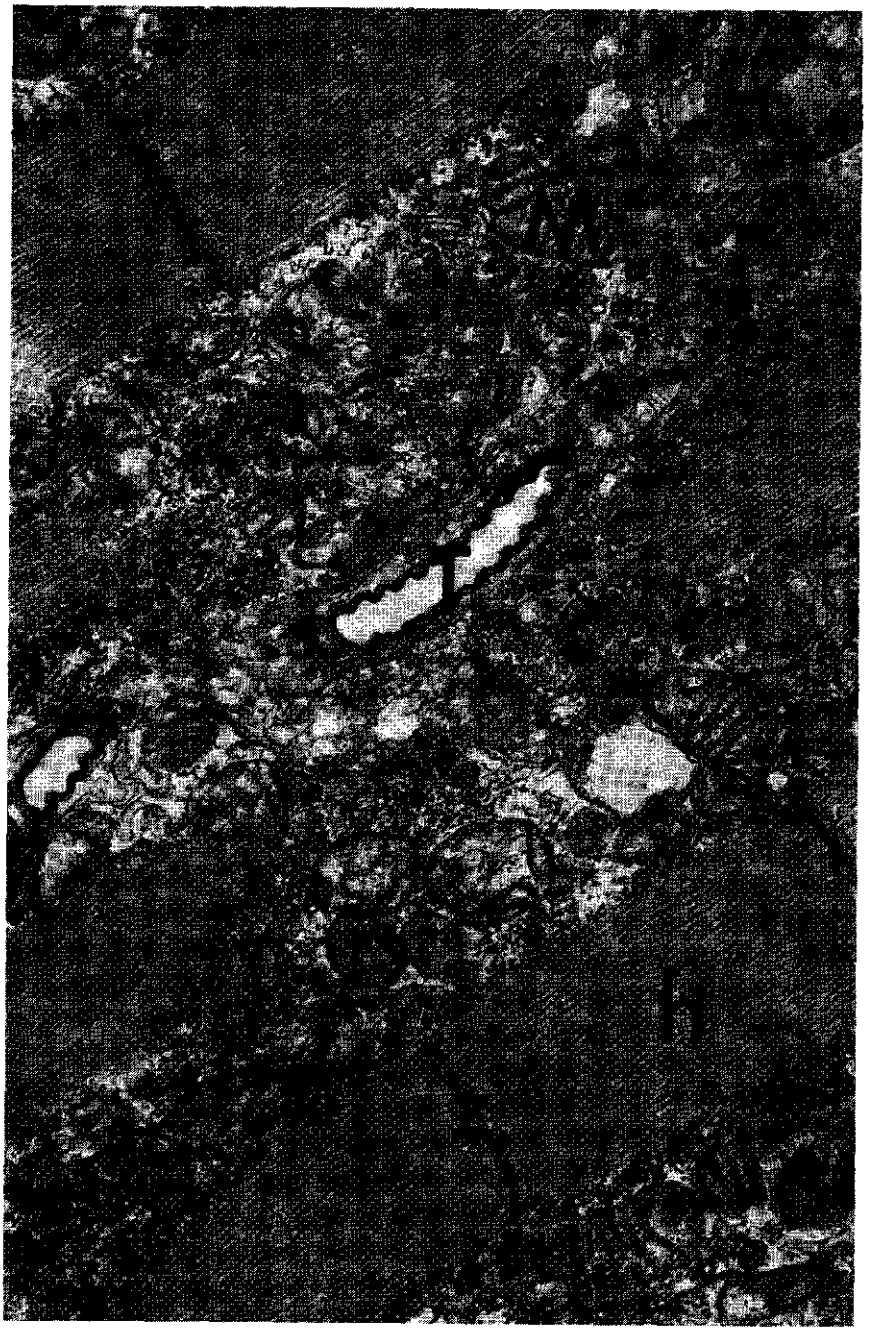


PLATE 5. Longitudinal section of the flight muscle of a three days old long-day beetle (25,000 x). The Z- and L-lines are indicated.

PLATE 6. Longitudinal section of the flight muscle of a twelve days old long-day beetle (25,000 x). The Z and h-lines are indicated.

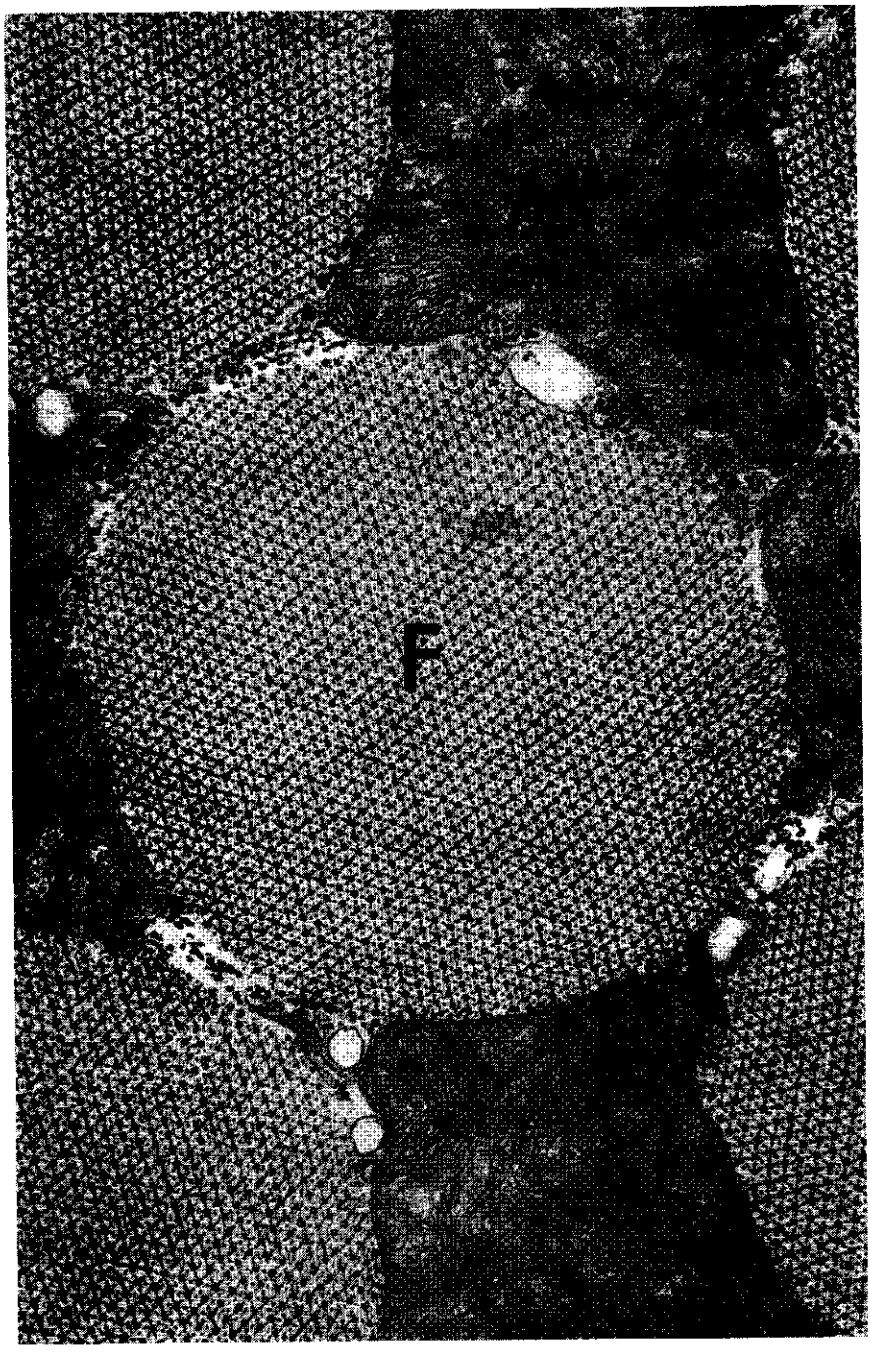


PLATE 7. Cross section of the flight muscle of a twelve days old long-day beetle. (50,000 x).

plication the mitochondria start to grow and at the same time the number of their membrane profiles increases. This phase lasts for about 7–8 days. As a result an extensive sarcosomal system can be seen in the adult muscle, which occupies more than 35% of the total volume of the fiber (for more quantitative data see section 4.4.).

The mitochondria are characterized by a great number of cristae. Long rows of these organelles alternate with the fibrils (plate 6).

The interfibrillar tracheoles run parallel to the fibrils in the immediate vicinity of the mitochondria, which ensures a very efficient oxygen supply.

Another important component of the muscle fiber is the sarcoplasmic reticulum (PORTER and PALADE, 1957). This system is morphologically poorly developed in this muscle.

4.3. ENZYME ACTIVITIES IN THE FLIGHT MUSCLES AT DIFFERENT TIME-INTERVALS AFTER EMERGENCE

Parellel to the changes in structure described in the previous section, we determined the activities of some enzymes in order to study if a correlation exists

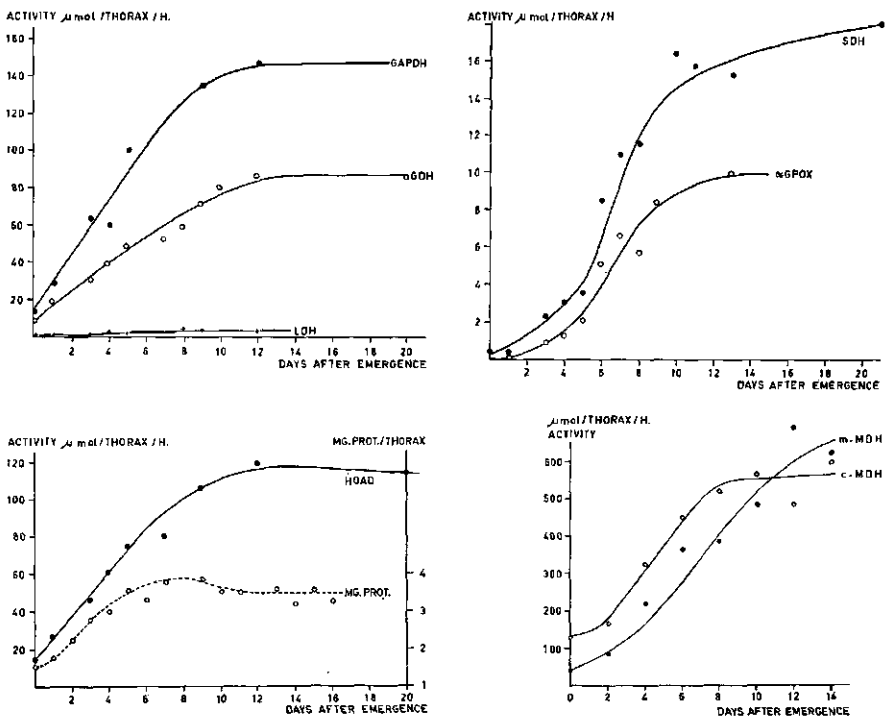


FIG. 2. The activities of different enzymes and the protein content per thorax as a function of time in the adult Colorado beetle reared under long-day conditions. Males were used throughout most of the experiments unless otherwise indicated. Each point reflects an average value derived from at least three determinations.

between the metabolic capacity and the ultrastructure of these muscles. We measured the activities of key enzymes from different important metabolic pathways: GAPDH (Embden-Meyerhof pathway), LDH (glycolysis), GDH and αGPox (α-glycerophosphate cycle), CE, SDH and MDH (Krebs cycle) and HOAD (fatty acid oxidation chain).

At the time of emergence the enzyme activities are very low. The SDH and αGPox can hardly be detected at this moment. A significant development can be deduced from fig. 2, where the activities of some enzymes and the protein content per thorax are illustrated at different times. The specific activities of two of these enzymes are given in fig. 3.

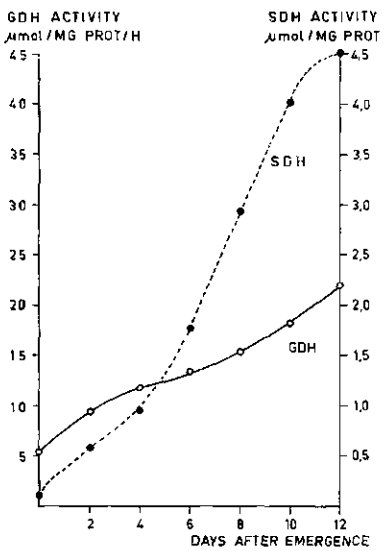


FIG. 3. The specific activities of GDH and SDH as a function of time after emergence in the adult Colorado beetle, reared under long-day conditions

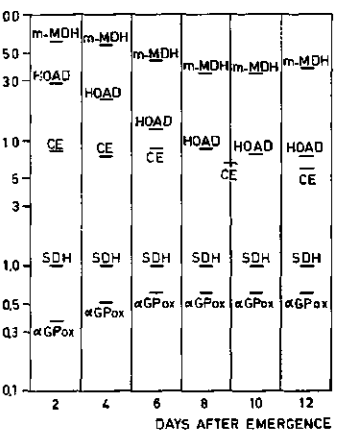


FIG. 4. Pattern of mitochondrial enzyme activities during development of the flight muscle. The mitochondrial enzyme activities are plotted relative to that of SDH.

As was pointed out before, several enzymes are known to behave like constant-proportion groups. The activities at different stages of development of some mitochondrial enzymes relative to SDH are plotted in fig. 4. From this figure it is clear that the activity of several mitochondrial enzymes can be considered as direct proportional to that of SDH during development. However, this proportionality does not exist between HOAD and SDH, in other words, the growth of mitochondria is not synchronous for all the enzymatic components.

According to our enzyme measurements the development of the flight muscles

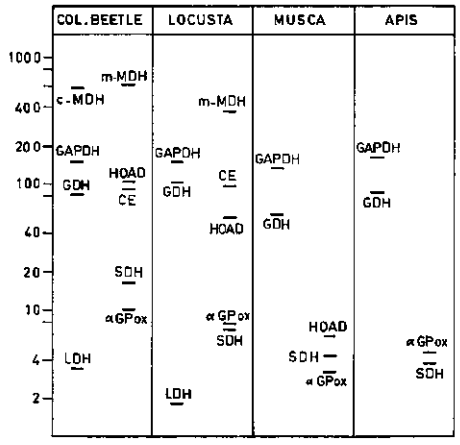


FIG. 5. Enzyme activity patterns of flight muscles of different insect species. The activities are expressed as follows: for the Colorado beetle and housefly in μmol/thorax/h; for Locusta in μmol/100 mg muscle/h; for the honeybee in μmol/0.25 thorax/h.

is completed after about 12 days. At this time the characteristic enzyme pattern of an adult insect flight muscle has been attained. This is illustrated in fig. 5, where the enzyme patterns of 4 different insects are given.

4.4. COMPARISON OF THE STRUCTURE AND ENZYME ACTIVITIES OF THE FLIGHT MUSCLES

In order to compare more precisely the structure and function, we measured the electronmicrographs quantitatively according to the method of LOUD (1962). In doing this we distinguished 3 compartments in the muscle fiber:

a. The volume occupied by the myofibrils.
b. The volume of the mitochondria.
c. The volume of the remaining elements, including nuclei, tracheoles and the sarcoplasm.

Although we realized that the tracheoles must be considered as extracellular, we have included them in this last compartment. The results are summarized in table 1. We could not distinguish different stages in the development. The differentiation of both the contractile system and the enzyme pattern takes place continuously during a single phase of about 2 weeks.

Our results on the development of the mitochondria show an evident parallelism between the mitochondrial structure and the SDH content. We chose SDH as representative for the mitochondrial enzymes, because it is directly bound up with the mitochondrial membranes. However, the correlation between mitochondrial structure and enzyme content only exists for those enzymes which are directly proportional to SDH, viz. the enzymes of the Krebs cycle and perhaps the enzymes of the electron transport chain. This is not the case with HOAD, which is not proportional to SDH.

We could not find a clear correlation between the differentiation of the myofibrils and the enzyme pattern. Neither the activities of the mitochondrial en-

TABLE 1. Results of histiometric analysis of flight muscle development in the Colorado beetle under long-day conditions.

Days after emergence	0	2	4	6	8	10	12
Mg prot./thorax	1.55	2.50	3.15	3.72	3.90	3.65	3.50
Myofibril							
diameter (μ)	0.3	1.0	2.0	2.3	2.4	2.4	2.0
% total volume	21	47	49	52	53	57	56
sarcomere length (μ)	3.5	–.–	2.4	2.5	2.4	–.–	2.2
Mitochondria							
(a) membrane profile μ/μ^2	1.0	2.2	2.8	3.1	3.5	4.0	4.7
(b) % total volume	4	14	16	27	34	38	36
a × b	4	31	45	84	120	152	169
SDH							
μ mol/mg prot/h	0.09	0.58	0.95	1.72	3.06	4.01	4.49
$\frac{\text{SDH}}{\text{a} \times \text{b}} \times 10^2$	2.3	1.9	2.1	2.1	2.5	2.6	2.6

zymes nor those of the extramitochondrial enzymes could be related to the differentiation of the myofibrils. Because we could not find such a parallelism, we also tested the situation in three other holometabolous insects. In table 2 a comparison is given of the activities of some enzymes in these insects directly after emergence and after about 10 days of adult life. It is known from these three insects that the myofibrils are fully developed at emergence. (MARUYAMA and MORIWAKI, 1958; MARUYAMA and SAKAGAMI, 1958; KOSHIHARA and MARUYAMA, 1958; HEROLD and BOREI, 1963; HEROLD, 1965; SMITH, 1961; CLARK and ROCKSTEIN, 1964). These figures clearly show that in some insects *(Apis mellifica* and *Tenebrio molitor)* the differentiation of the enzyme pattern takes place after the formation of the fibrils.

TABLE 2. Enzyme activities in the flight muscles of three different holometabolous insects immediately after emergence and about 7–10 days later.

	Apis mellifica		Tenebrio molitor		Musca domestica	
	at emergence	adult	at emergence	adult	at emergence	adult
GAPDH	36.0	645	59.9	571.0	59.2	133.2
GDH	8.7	344	24.0	260.9	37.9	56.4
HOAD	–	–	–	70.9	–	6.2
SDH	1.86	14.7	3.10	13.1	1.95	4.30
αGPox	2.05	18.6	0.85	7.8	1.90	3.25

Activity in μmol/thorax/h.

4.5. Discussion

Although such investigations on flight muscle development as have been made on *Locusta* (Brosemer et al. 1963; Vogell, 1965; Bücher, 1965), have never been carried out in holometabolous insects, several data exist in literature referring both to enzymic and electronmicroscopic studies. Levenbook and Williams (1956) found that in the housefly the wing-beat frequency was at its maximum after about 7 days of adult life. They correlated the increase in wing-beat frequency with an increase in the dry-weight of the sarcosomes, and with the cytochrome c content of the isolated sarcosomes. They showed that this was not caused by an increase in the number of mitochondria, but by growth of pre-existing sarcosomes. The increase in cytochrome content was also noted by Keilin (1925) in his historic paper on the cytochrome system in animals and plants. Sacktor (1950) and Bodenstein and Sacktor (1952) showed an increase in the cytochrome oxidase activity during the first week, and Lewis and Slater (1954) found lower P/O ratios with sarcosomes isolated from younger blow flies. Although van den Bergh (1962) could not confirm this result in the case of housefly mitochondria, yet he also found an increased yield of sarcosomal proteins in older flies.

These isolated data represent fragments of a pattern of sarcosomal development, which is eventually linked with the post-emergence maturation of the flight muscles. Rockstein has studied this phenomenon in the honeybee and housefly, and used the term 'Metachemogenesis' for the post-emergence biochemical maturation (Rockstein, 1959; Rockstein and Brandt, 1962 and 1963; Bhatnagar and Rockstein, 1964; Clark and Rockstein, 1964).

The results given in this chapter describe the post-emergence maturation of the flight muscles in the Colorado beetle. The rate of this process in the Colorado beetle clearly differs from that in many other insects.

From the literature on flight muscle development in holometabolous insects it can be seen that a great deal of this development takes place in the pupa. Very well studied are the development in the honeybee (Maruyama and Sakagami, 1958; Maruyama and Moriwaki, 1958; Koshihara and Maruyama, 1958; Herold and Borei, 1963; Herold, 1965), in *Drosophila* (Shafiq, 1963), in *Antheraea pernyi* and *A. polyphemus* (Nuesch, 1963, 1965; Eigenman, 1964) and in *Hyalophora cecropia* (Michedja, 1964).

From these studies it appeared that in most holometabolous insects the differentiation of the myofibrils has been completed at the moment of emergence. Maruyama and Sakagami (1958) e.g. measured the same M-(myofibrillar) ATP-ase activities at the moment of emergence as in 10 days old bees. Shafiq (1963) described the differentiation of the fibrils which is completed in the pupal stage in *Drosophila*. The electronmicrographs of the flight muscles of *Tenebrio* made by Smith (1961) clearly showed the same diameter at emergence as in the mature muscle.

On the other hand a significant post-emergence biochemical maturation of the sarcosomes has been found in most of the insects studied thus far. It has

been shown in the honeybee (MARUYAMA and SAKAGAMI, 1958; MARUYAMA and MORIWAKI, 1958; HEROLD and BOREI, 1963), in *Tenebrio* (SMITH, 1961), in the housefly (LEVENBOOK and WILLIAMS, 1956; SACKTOR, 1950; VAN DEN BERGH, 1962), and in the sheep blowfly (LENNIE and BIRT, 1967).

From all these data it appeared that although the development of mitochondria starts during pupal stage, the main mitochondrial characteristics are formed in the early adult life. In the Colorado beetle, however, we found that both the myofibrils and the sarcosomes were poorly developed structurally at the moment of emergence, and that the whole enzyme content originates together with the final differentiation of these structural elements.

BROSEMER et al (1963) pointed out a good correlation between the morphological and enzymological patterns during the development of the flight muscles in *Locusta*. In particular they directed attention to the correlation between the amount of contractile filaments and the activity of the phosphotriose-glycerophosphate group. We could not find such a correlation in the Colorado beetle. As a matter of fact, our results in table 2 clearly show that in other holometabolous insects a correlation between the amount of myofilaments and the activities of these enzymes could not be found during development either. This suggests that the contractile and enzymic systems develop in a more or less independent way. There exists a variation in different insects, as far as the synchronization of the development of these two systems is concerned. The question how, as in the honeybee, a young muscle cell can synthesize such amounts of contractile proteins without sufficient enzymes for the energy supply cannot be answered at the moment. HEROLD and BOREI (1963) discussed the role of cytochrome b5 for the synthesis of these contractile proteins. However, according to our results this seems unlikely, because the most important enzymes of the carbohydrate pathway are lacking.

A very important question is also: which factors control the programming of flight muscle development? Can hormones be considered as inducers for this development? It is not yet possible to answer these questions. However, if the contractile system and the enzyme pattern can be induced more or less independently, this would suggest that more than one programme is needed for the development of flight muscles.

Our results concerning the development of the mitochondria are in agreement with those of the BÜCHER-group. We found a good correlation between the mitochondrial mass and the SDH activity. Furthermore, different mitochondrial enzymes form constant proportion groups with SDH during development. However, we could not find a proportionality between SDH and HOAD, in other words the growth of mitochondria is not accompanied by the synchronous increase of all enzymatic components. BÜCHER (1965) stated that the growth of the mitochondria is synchronous in all elements and that 'this parallelism between increase in morphological mitochondrial structure and the enzyme content does not leave any doubt about the independent growth of mitochondrial structure'. However, the exact meaning of this 'independent growth' is not clear. BROSEMER et al (1963) stated: 'dass die mitochondrialen Strukturen

einschliesslich des koordinierten extra- und intracristalen Bereichs aus kleinen Elementen an Ort und Stelle zusammengesetzt werden'. In other words, the increase in mitochondrial mass is a result of division and growth of pre-existing mitochondria. Independent growth does not mean that growth of mitochondria can take place independently from other cellular activities. On the contrary, the recent data on mitochondrial DNA (BORST et al, 1967) clearly indicate that mitochondrial DNA can maximally code for 5000 amino acids and therefore a large part of the mitochondrial macromolecules must be specified by nuclear genes. In other words, mitochondrial growth depends on cooperation with extra-mitochondrial ribosomes (ROODYN and WILKIE, 1968). If this were true, the disproportionality between HOAD and the other mitochondrial enzymes would be understandable.

Although the development of the flight muscles in the Colorado beetle takes place chiefly in the adult, it compares well with those in other holometabolous insects. The first visible fibrils in *Drosophila* (SHAFIQ, 1963b) have the same diameter as the fibrils in the Colorado beetle directly after emergence, although the sarcomere length is smaller in *Drosophila*. The changes in mitochondrial structure during development are similar to those described by HEROLD (1965) in the honeybee.

We cannot compare our results on the development of the enzyme pattern in holometabolous insects with data in the literature, because such studies have never been carried out, except those on the post-emergence maturation of the sarcosomal enzymes. Our results differ from those of *Locusta* as far as the behaviour of GDH is concerned. BÜCHER (1965) found a twenty-fold increase of this enzyme during the phase of differentiation, while GAPDH increased only by a factor 3. He concluded that this must be considered as an important aspect of the differentiation of flight muscles, because GDH plays a key role in the metabolism of this tissue. In later studies BROSEMER (1965, 1967) discussed the importance of this enzyme for the relation between tissue function and enzymic development. The importance of this enzyme for the functioning of the flight muscles is also suggested by the work of ROCKSTEIN and BRANDT (1963) who found a rapid decline in the activity of this enzyme just before the loss of wings in the male housefly during ageing. However, our results show that the same increase in GAPDH and GDH can be found in the Colorado potato beetle, and *Tenebrio*. In the housefly we found a higher increase in GDH in comparison to GAPDH. We think it is dangerous to state that 'the potentiality for flight may be controlled at the enzymic level by the activity of a single enzyme' (BROSEMER, 1965) if other enzymes have not been measured at the same time.

The structure of the adult muscle is the same as in many other fibrillar flight muscles (HANSON, 1956; SMITH, 1961, 1963, 1965, 1966; SHAFIQ, 1963a). A great number of very large myofibrils (diameter 2 μ or more) alternate with long rows of mitochondria characterized by an extensive system of cristae. We found the same sarcomere length and the typical narrow I-band as these authors. The interfibrillar tracheoles must be considered as external invaginations, which were first described by EDWARDS et al (1955). The poorly developed sarcoplasmic

reticulum clearly indicates that the Colorado beetle possesses asynchronous flight muscles. In the synchronous flight muscles of *Locusta migratoria* and in synchronous insect muscles in general, this sarcoplasmic reticulum is well developed. The enzyme pattern of the adult muscles in the Colorado beetle has the same characteristics as that found in many other insects. SACKTOR (1965) reviewed the metabolism of insect flight muscles extensively. The most important characteristics are:

1. The low activity of LDH.
2. The importance of the α-glycerophosphate cycle (GDH and αGPox).
3. The high activities of the mitochondrial enzymes.

When we compare the GAPDH and HOAD activity, we can conclude that fatty acid oxidation plays an important role in this insect (PETTE, 1965). This is in agreement with the results of EL-IBRASHY (1965) who found values of the respiratory quotient below unity.

As a matter of fact, the enzyme pattern of the Colorado beetle clearly resembles that of *Locusta*, the flight muscles of which are known to use fatty acids for energy supply during flight (KROGH and WEIS-FOGH, 1951; BEENAKKERS, 1964).

Recently LE BERRE (1965) studied the flying ability of the Colorado beetle at different times after emergence. He found that the animals could not fly during the first 4 days of adult life. The results described in this chapter give a structural and biochemical explanation of this inability. After 4 days LE BERRE found an increase in flying ability untill the 9th day after emergence, where the maximal flying ability is reached. According to our results we can say that during this period the flying ability is limited by the enzyme content of the muscles. In contrast to the finding of BROSEMER (1965) our data do not show that one single enzyme can be held responsible for this limited ability for flight. Thus for the maintenance of normal flying ability both normally developed contractile and enzymic systems are required.

5. CORRELATION BETWEEN THE LEVEL OF JUVENILE HORMONE AND THE BIOCHEMICAL AND STRUCTURAL PROPERTIES OF THE FLIGHT MUSCLES

5.1. INTRODUCTION

As described in chapter 4, a normal development of the flight muscles takes place under long-day conditions, i.e. if the corpora allata are active. In this chapter we will study the effect of changes in juvenile hormone level on the structure and biochemistry of the flight muscles.

In chapter 2 we discussed the changes which take place in the Colorado beetles under short-day conditions and after allatectomy. Both treatments resulted in diapause, a state of immobility, lack of reproduction and low metabolic rate. Together with behaviour and reproduction a number of biochemical characteristics have also changed. The response of the Colorado beetle to short-day treatment and allatectomy involves a complex of symptoms.

In an early phase of our study (DE KORT, 1966) we have measured quantitatively the changes in some body constituents. They can be used to illustrate the drastic changes which take place after induction of diapause (table 3).

DE WILDE (1955) showed that, although the adult is both the sensitive and responsive stage for the induction of diapause, photoperiodic treatment of the larvae did have some effect as well. Long-day pretreatment of the larvae sometimes induced ovarian activity in the adult subjected to short-day conditions. This might suggest that the level of activity of the corpora allata can directly or indirectly be changed by different pretreatment of the larvae.

TABLE 3. Body constituents in active long-day beetles and during diapause.

	Active about 14 days old		Diapause 1–2 months	
	♀	♂	♀	♂
Fresh body weight (mg)	183(43)	129(43)	142(38)	117(38)
% Dry weight	30.5 ± 0.6	35.8 ± 1.7	44.5 ± 2.1	44.4 ± 1.9
Total glycogen*	8.8 ± 2.7	14.1 ± 7.7	19.5 ± 7.8	7.8 ± 7.7
Total lipid*	128 ± 12	176 ± 12	272 ± 62	545 ± 61
Total protein*	329 ± 5	290 ± 13	210 ± 11	221 ± 22
Haemolymph:				
† protein (g/100 ml)	3.90	3.40	10.0	10.6
†† glycerides (mg/100 ml)	25.7 ± 1.3		39.0 ± 1.6	
carbohydrate (mg/ml)	0.77 ±0.04		0.34 ±0.02	

* expressed in mg/g dry weight.
† these figures were taken from de Loof (1969).
†† expressed as glyceride glycerol in mg/100 ml haemolymph.
The number of determinations of fresh body weight is given in brackets.

The question whether a different level of flight muscle development is correlated with a difference in juvenile hormone titer will be the subject of this chapter. This will be done by following the flight muscle development under short-day conditions and after allatectomy, after different pretreatment of the larvae and after termination of diapause.

5.2. Structure and enzyme activities of flight muscles in normal short-day beetles and in allatectomized long-day beetles

In the same way as for long-day beetles, we studied the structural and enzymatic development of the flight muscles of animals, kept *ab ovo* under short-day conditions. The structure of the flight muscles at emergence under short-day conditions is comparable to the situation under long-day conditions. The development also starts directly after emergence. The diameter of the fiber is 78 μ after 4 days. The same initial fast differentiation of the myofibrils is found as in long-day beetles. However, they never reach the maximal value of those of long-day beetles. After 6 days an average diameter of 1.68 μ can be measured. This diameter is fairly constant during the next eight days. The development of the mitochondria is initially also comparable to that in long-day beetles. This can be illustrated by the following quantitative data derived from the electronmicrographs. The volume percentages of the 3 components of the muscle fiber are:
after 4 days: Fibrils 46%, Mitoch. 13%, remaining elements 41%.
after 6 days: Fibrils 47%, Mitoch. 26%, remaining elements 27%.
At 9 days after emergence we find the individual variations in the electronmicrographs too large to warrant more quantitative results; this variability is caused by differences in the time of onset of muscle degeneration. This process starts before the muscles attain a level of development comparable to that of long-day beetles. At 14 days a significant decrease in the percentage of mitochondrial volumes can already be seen in all electronmicrographs, whereas the decrease in myofibrillar diameter is insignificant. We measured an average value of about 15% mitochondrial volume at this time (cf. table 1).

As the enzyme measurements were carried out on pooled homogenates, which minimizes the effect of individual variations, we can better use these data to describe the development during the prediapause period. From chapter 4 we may conclude, that enzyme activities can be used very well as a parameter for the condition of the flight muscles. As has been shown, the activity of SDH can be used as a measure for the amount of mitochondrial mass. Therefore only this enzymological aspect of the flight muscles is studied in the following sections. The activities of several enzymes and the protein content per thorax are given in fig. 6. The moment at which 50% of the beetles have disappeared into the soil is indicated by arrows; this is taken from de Wilde et al (1959). For comparison enzyme activities of long-day beetles are indicated by the dotted lines. From these figures it is clear that the degeneration starts with a decrease in the mitochondrial content (= SDH activity) followed by a decrease in the

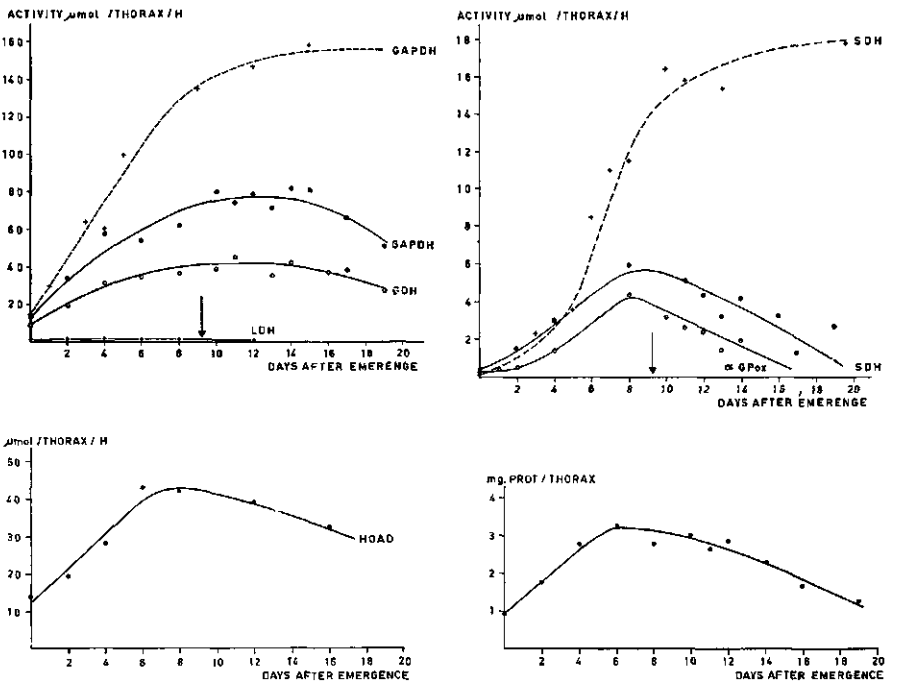


FIG. 6. The activities of different enzymes and the protein content per thorax in the adult Colorado beetle reared under short-day conditions.

activities of the other enzymes. The decrease in diameter of the myofibrils is a secondary phenomenon. We could not establish it in this early phase of degeneration.

We also carried out some measurements on allatectomized long-day animals, to see whether allatectomy is equivalent to short-day treatment with regard to flight muscle development. The results are comparable to the situation in short-day beetles, although we found that the moment of operation is very important. Operation at emergence resulted in a higher rate of development than operation at ecdysis, i.e. about 2 days before adult emergence. This is illustrated in table 4.

TABLE 4. Comparison of the effect of allatectomy at emergence and at ecdysis on flight muscle development in long-day beetles.

Enzymes	at ecdysis	at emergence
GAPDH	42 ± 9	77 ± 8
GDH	17 ± 5	40 ± 10
SDH	1.55 ± 1.7	6.7 ± 3.3
αGPox	0.75 ± 0.99	2.7 ± 0.4
Mg prot/thorax	2.604	2.687

The enzyme activities (µmol/thorax/h) were measured 10 days after emergence.

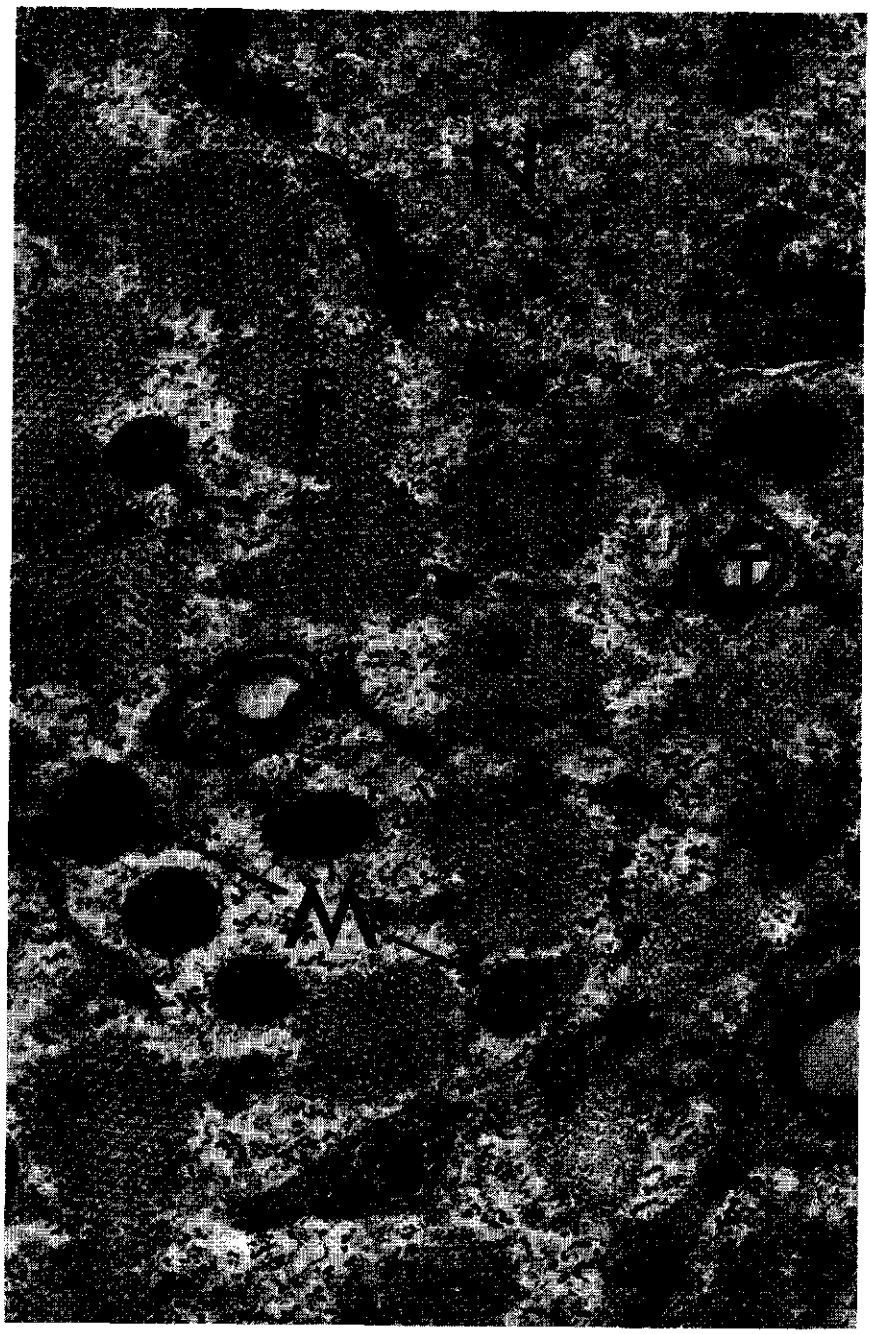


PLATE 8. Cross section of the flight muscle of a diapausing adult Colorado beetle (35,600 x).

TABLE 5. Comparison of the activities of various enzymes in beetles directly after emergence and during deep diapause.

Enzyme	at emergence	diapause
GAPDH	14.5	18.4
GDH	8.2	16.0
HOAD	13.5	10.5
LDH	0.45	0.30
c-MDH	101	130
SDH	0.17	-
α GPox	0.14	-
m-MDH	39	23
Mg prot/thorax	1.550	1.571

Enzyme activities in μmol/thorax/h.

This effect of the moment of operation can probably also explain the variations in the operated animals, especially those found in the activity of the mitochondrial enzymes.

During deep diapause, when the flight muscles have completely degenerated structurally, the activities of the enzymes of special importance for flight muscle metabolism are very low. Mitochondrial enzyme activity could hardly be detected. During diapause the muscle closely resembled the 'precursor muscle' of a newly emerged beetle. This is illustrated in plate 8 and table 5.

5.3. ENZYME ACTIVITIES OF SHORT-DAY BEETLES AFTER LONG-DAY PRETREATMENT OF THE LARVAE

DE WILDE (1955) found that beetles treated under short-day conditions during the entire life cycle, never show vitellinized ova. Long-day treatment of the larvae sometimes induced yolk production in the adult subjected to short-day photoperiods. This suggests that the corpora allata are more or less activated by this pretreatment. As this might also influence the development of the flight muscles, we repeated these experiments and studied the enzyme activities of the flight muscles. Our results were quantitatively different from those of de Wilde. We always found normal oviposition in short-day during the first few weeks after a previous long-day treatment of the larvae. This suggests that the corpora allata have become active in the normal way. We therefore would expect a normal development of the flight muscles as well, and as shown in fig. 7, this appears to be the case.

In a later phase, i.e. after about 40 days, short-day treatment of the adult appeared to have an effect. At that time the animals stopped reproduction and entered into diapause a few days later. However, this diapause induction is very irregular and we have not followed the enzyme activities at this stage. The conclusion from these experiments must probably be that a drastic change has taken place in our laboratory strain of Colorado beetles, during the last ten

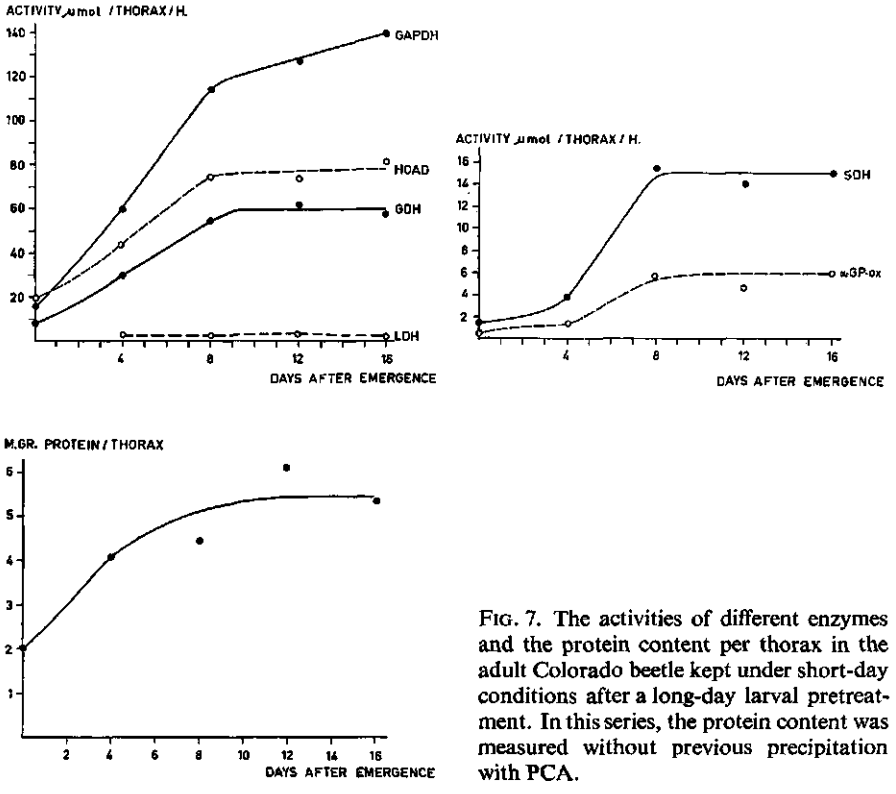


FIG. 7. The activities of different enzymes and the protein content per thorax in the adult Colorado beetle kept under short-day conditions after a long-day larval pretreatment. In this series, the protein content was measured without previous precipitation with PCA.

years. In the present strain short-day responses appear to have weakened. This is in accordance with the recent findings by HODEK and DE WILDE (1969).

5.4. ENZYME ACTIVITIES IN THE FLIGHT MUSCLES DURING LONG-DAY, AFTER SHORT-DAY PRETREATMENT OF THE LARVAE

The preceding sections have revealed that there is a great difference between the effects of long-day and short-day larval pretreatment with regard to the activation of the corpora allata. It is important to investigate whether short-day pretreatment of the larvae has some delayed effect on the flight muscle development in the long-day treated adult.

However, it appeared that a few long-day photocycles – or perhaps even one – applied to the adult, completely reverse the effect of the short-day treatment of the larvae. Oviposition is normal under these conditions, although somewhat delayed. This suggests that the corpora allata are activated very rapidly. The development of the flight muscles appears to be normal, and the same holds for the enzyme values.

The results, given in this and the foregoing section, clearly show that short photoperiods are relatively ineffective in reversing a previous long-day effect (see 5.3) whereas long photoperiods have a strong positive influence (see also LEES, 1968), and can easily overcome retardations evoked by previous short-day treatment.

5.5. ENZYME ACTIVITIES AFTER TERMINATION OF DIAPAUSE

The diapause of animals which have been in the soil for more than 3 months, can easily be terminated by removing them from the soil and providing them with fresh potato leaves, even while exposed to short-day. After a few hours the animals fail to show any burrowing behaviour, which indicates that diapause is definitely broken. After 2 days they start feeding and after 7 days some females start laying eggs. After 8 days oviposition is normal. Under these circumstances the development of the flight muscles starts immediately compared to feeding activity and oviposition. A striking difference in the velocity of development between males and females can be seen. The recovery of enzyme functions, especially those of the mitochondrial enzymes, is very fast in females. The development of the enzymes in males is delayed compared to the situation in females. This is illustrated in table 6. The maximal values reached are those of long-day beetles two weeks after emergence. These data, together with those given in table 5, clearly show the similarity of flight muscle development during early adult life and after break of diapause. It seems likely that the same factors are responsible for the initiation of flight muscle development in these two different stages.

TABLE 6. The activities of various enzymes and the protein content per thorax in the adult Colorado beetle after termination of diapause.

	0 days		3 days		6 days		12 days
	♀	♂	♀	♂	♀	♂	♀
GAPDH	35	18	40	24	90	79	152
GDH	31	16	23	12	52	32	78.2
SDH	0.40	–	2.17	1.23	9.20	4.09	17.70
α GPox	–	–	1.40	0.65	5.46	3.24	10.95
Mg prot/thorax	2.549	1.571	2.857	2.225	3.112	2.379	4.175

Enzyme activities in μmol/thorax/h.

5.6. THE JUVENILE HORMONE TITER IN THE HAEMOLYMPH

Until now, our knowledge about the real juvenile hormone titer circulating in the body was incomplete. Very recently we succeeded in a direct determination of the juvenile hormone level in the haemolymph of the adult Colorado beetle, as a function of different conditions, with the aid of a very sensitive bio-

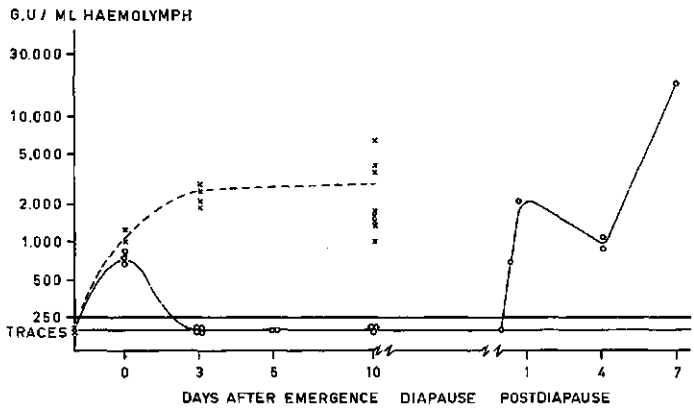


FIG. 8. Juvenile hormone titers (in Galleria units, G.U.) in the haemolymph of the adult Colorado beetle. Titers below 250 G.U. per ml of blood could not be evaluated quantitatively and are indicated as 'traces' After de Wilde et al (1968).
- - - - - long-day animals
——— short-day animals

assay. Although the experimental details are given elsewhere (DE WILDE et al, 1968), the result is illustrated in fig. 8 and will be used for the discussion of the correlation between juvenile hormone titer and flight muscle development.

5.7. DISCUSSION

From the results given in this and the foregoing chapter, we may conclude that for the preservation of normal structure and function of the flight muscles in the Colorado beetle a number of long photoperiods, i.e. normal active corpora allata are indispensable. We may conclude that, if a certain level of juvenile hormone is present, the normal structure and function of the flight muscles can develop and be maintained.

The correlation between the level of hormone titer in the haemolymph and the different stages of flight muscle development described in this and the foregoing chapter is a striking confirmation of this conclusion.

The flight muscle development under long-day conditions can be correlated to the increase of juvenile hormone titer in the haemolymph during the first days of adult life. The development during short-day can be correlated to the rapid initial increase of juvenile hormone titer at emergence. The growth of the muscles is arrested soon after the hormone titer reaches a lower level. Different pretreatment of the larvae does not result in a significant difference of hormone titer at adult emergence. Consequently, we found no difference in flight muscle development during the first few days of adult life. This means that the effect of different larval pretreatment cannot be explained on the basis of a difference in hormone titer of the newly emerged adult. This photoperiodic effect must therefore be localized in higher centres, possibly in the brain, which regulate the secretory activities of the corpora allata.

The correlation between hormone level and flight muscle development is also evident from our experiments with allatectomized beetles. The degree of flight muscle development is directly correlated with the time at which the operation has been performed. As operation soon after ecdysis results in a lower hormone titer as compared to the operation at emergence, the different degrees of flight muscle development correlate quite well to these different hormone titers.

Although the positive correlation between hormone titer and flight muscle development favours the idea of a direct effect of the corpus allatum hormone on flight muscles, the possible role of the neurosecretory cells cannot entirely be excluded. In 1952 THOMSEN described the functional significance of the neurosecretory brain cells and the corpora cardiaca in the female blowfly, *Calliphora erythrocephala*. She wrote: 'It seems logical to assume that the function of the neurosecretory cells of the adult insect should be similar to the function of these cells during development. The fact that these cells in both *Rhodnius* and *Platysamia* produce a growth-promoting factor during the development supports my suggestion that the neurosecretory cells of the adult *Calliphora* stimulate growth. Because the adult insect does not grow any more, one has been accustomed to think of growth in insects in connexion with the developmental stages only, therefore the hypothesis that the neurosecretory cells of the adult *Calliphora* should be concerned with growth may sound like a paradox. However, in many adult insects some organs do grow, in most cases this applies to the fat body, and in the case of *Calliphora* also to the corpus allatum and the accessory gland'. In the Colorado beetle, and as discussed in chapter 4 in many other insects, the maturation of the flight muscles can also be considered as 'growth' of the adult insect. If the neurosecretory cells exert a general growth-promoting effect, the degeneration of the flight muscles by allatectomy could be explained as secondary. As shown by LEA and THOMSEN (1962) and HIGHNAM et al (1963) allatectomy results in an inactivity of the medial neurosecretory cells. THOMSEN (1952) also stated that as a result of the excision of the medial neurosecretory cells the growth is arrested and the organs could be said to enter into diapause.

In the literature the relative roles played by the neurosecretory cells and the corpora allata are disputed. While HIGHNAM (1964) stresses the importance of neurosecretion for the regulation of protein synthesis, DE WILDE (1964) and ENGELMANN (1968) on the other hand do not preclude an indirect role of the neurosecretory cells via the corpora allata. A difficulty in the dispute is that different insects are used in the experiments, while it is very dangerous to generalize. The dispute is hampered further because very little is known about the real secretory activity of the corpora allata under different conditions (HIGHNAM, 1964; ENGELMANN, 1968). The size of the corpora allata has been used as a measure for hormone production, but according to STAAL (1961) growth of the corpora allata and the increase in secretory activity are two different processes. (see also HIGHNAM 1964 and ENGELMANN, 1968). Our recent succesful determinations of the juvenile hormone titer in the haemolymph will perhaps stimulate this same approach in other insects.

However, an other explanation for the degeneration of the flight muscles is

also possible. If the juvenile hormone should stimulate the central nervous system, the absence of this hormone could result in a diminution or even complete cessation of motoric and trophic nerve activity. The cessation of nervous stimuli might have as a secondary effect atrophy of the flight muscles. The observation in fig. 6 that the onset of decrease in enzyme activity coincides with the moment the animals disappear into the soil, and loose their mobility, is compatible with this hypothesis. A low motoric nerve activity during pre-diapause could possibly be the explanation for the standstill of flight muscle development during this period. The change in behaviour is possibly also an indication that the juvenile hormone has an effect on the nervous system.

A very important question also is why allatectomy in the Colorado beetle results in a degeneration of the flight muscles, whereas in other insects this effect is not observed. This difference is difficult to explain on the assumption that in all insects the juvenile hormone directly influences the development and maintenance of the flight muscles. A possible explanation could be sought in supposing that in the Colorado beetle the effect of the juvenile hormone on the muscle is mediated – at least partly – through the nervous system.

A study of the effect of innervation on the structure and function of the flight muscles could therefore be important for a better understanding of the role played by the juvenile hormone.

6. THE ROLE OF THE NERVOUS SYSTEM IN THE DEVELOPMENT AND MAINTENANCE OF THE FLIGHT MUSCLE STRUCTURE.

6.1. INTRODUCTION

Diapause in relation to the activity of the central nervous system in insects has been an extensively studied topic since the work of VAN DER KLOOT (1955). He found that at the onset of pupal diapause, the brain of *Hyalophora cecropia* became electrically silent and cholinesterase (ChE) activity disappeared. However, later work by SCHOONHOVEN (1963), TYSHTCHENKO et al (1965) and SHAPPIRIO et al (1967) could not confirm these results. After an extensive study of different diapausing and non-diapausing insect species MANSINGH and SMALLMAN (1967) concluded that the cholinergic system of the brain is not functionally related to the induction and termination of diapause. They found that changes in the cholinergic activity also occurred in non-diapausing insects and that these changes were associated with the growth and development of the insect nervous system. It is interesting to look whether the induction of diapause in the Colorado beetle is also accompanied by an effect on the development of the nervous system, because the brain of adult insects is indeed involved in diapause, as can be concluded from the work of KUTYNA and TOMBES (1966) with the Alfalfa weevil during aestivation, and from the pronounced change in behaviour of the Colorado beetle after induction of diapause.

A direct influence of insect hormones on the electrical activity of the nervous system was described by OZBAS and HODGSON (1958), by MILBURN et al (1960), MILBURN and ROEDER (1962), HASKELL and MOOREHOUSE (1963) and HASKELL et al (1964), but the juvenile hormone was not included in these studies. Moreover, ODHIAMBO (1965, 1966 a, b) in his work with *Schistocerca gregaria* suggested that the corpora allata regulate the rate of locomotory activity by a direct effect on the central nervous system.

For more than one reason innervation is important for the normal functioning of a muscle. The innervating nerve is not only responsible for activation or inhibition, but the trophic function of the nerve is needed for the maintenance of the integrity of the muscle.

Muscles with their motor nerves must be considered as functional units; in other words, the functioning of a muscle is not only characterized by the properties of this muscle, but also by the properties of the innervating motor fibres. This has been extensively studied on vertebrate muscles, (see the symposium on The Use and Disuse of Neuromuscular Function, GUTMANN et al, 1964). This, and the recent histochemical and biochemical work by ROMANUL et al (1967), KARPATI et al (1967) DUBOWITZ et al (1967) and PREWITT et al (1967) clearly revealed that not only the contractile mechanism, but also the metabolic elements of skeletal muscles are dependent on the supplying motoneuron. Cross-innervation of fast and slow muscles reverses their characteristics of contraction and is accompanied by a corresponding change in the enzyme pattern.

Quantitative biochemical work by PETTE (see TATA, 1966a) suggested that the state of the mitochondrial population is under control of innervation. He studied the effect of cross-innervation of the nervus tibialis and nervus peronaeus in the rabbit and found a change in the activity of the mitochondrial enzymes in the muscles.

Moreover, recent work of KENDRICK-JONES and PERRY (1965, 1967 a, b) and HAJEK and PERRY (1967) revealed an enzymatic adaptation to contractile activity in skeletal muscles of vertebrates. From all this work it can be concluded that the mechanical properties and the preferential metabolic pathways of a muscle are dependent on the appropriate innervation.

Also in insects a trophic effect of the nervous system has been observed by different workers (KOPEC, 1923; WILLIAMS and SCHNEIDERMAN, 1952; NUESCH, 1952; 1968; USHERWOOD et al, 1968). Especially during development innervation appeared to be important as has been shown in *Antheraea pernyi* (NUESCH 1952, 1968; WILLIAMS and SCHNEIDERMAN, 1952). A direct effect of the nervous system on the onset of degeneration of the intersegmental abdominal muscles of the moth *Antheraea pernyi* has been shown by LOCKSHIN and WILLIAMS (1965 a, b, c).

In view of all these data it seems reasonable to investigate the role of the nervous system in the development of the flight muscles in the Colorado beetle. As it could be expected that changes in activity level of the brain would involve changes in the cholinergic system, ChE activity was studied in the brain. Moreover, the effect of denervation on structure and enzyme pattern of the flight muscles was studied in an attempt to a more direct approach. The effect of substances influencing neuromuscular activity will also be reported in this chapter.

6.2. CHOLINESTERASE ACTIVITIES IN THE BRAIN

We studied the cholinesterase activity in the brains directly after emergence and ten days after emergence, and compared the activities under long-day and short-day conditions and in diapause. The results are given in table 7.

These figures clearly illustrate a post-emergence development of the cholinergic system in the Colorado beetle. There is no significant difference between long-day and short-day beetles, although we found a considerable variation in short-day beetles. Moreover, the activity in diapausing beetles is significantly lower, which confirms that the nervous system is involved in the diapause reac-

TABLE 7. ChE activities at different times after emergence.

long day		short day		diapause
at emergence	after 10 days	at emergence	after 10 days	
4.60 ± 0.87	8.75 ± 1.09	4.89 ± 0.71	8.17 ± 1.54	3.99 ± 0.01

Activity μmol/5 heads/h.

tion. However, data about total cholinesterase activity give only an approximate picture of the physiological activity. Moreover, it is not clear whether this change is under hormonal control.

6.3. Effect of denervation on the structure and biochemistry of the flight muscles

The effect of denervation on the structure and function of flight muscles was studied directly by dissection of the peripheral nerves of the third thoracic ganglion. The operation was carried out directly after emergence. Long-day females were used for the experiments, because in females it is easier to check whether the operation interferes with hormone production. As reproduction can be used as an indicator for activity of the corpora allata, we followed oviposition of the operated animals individually. From the animals that survived, those with normal feeding and oviposition were used in the experiments.

As it appeared that a remarkable degree of regeneration might take place, we carefully examined the results of the operation before using the thoraces for electronmicroscopy and enzyme determination. The enzyme activities were measured at different time intervals after the operation. As a control, appropriate sham-operated animals were used. The results are given in table 8.

Table 8. Effect of denervation on the enzyme activities in the flight muscles of long-day beetles.

	Operated						Sham-operated
Days after operation	12	14	15	16	18	19	14
Number of animals	5	3	2	6	2	4	13
GAPDH	70	45	49	51	59	60	117 ± 36
GDH	33	27	37	32	15	32	79 ± 28
SDH	3.94	3.57	4.60	4.73	4.5	4.1	13.7 ± 2.6
α GPox	2.22	1.80	2.80	1.71	1.7	2.6	8.4 ± 0.29
Mg prot/thorax	3.272	1.855	4.950	2.486	4.672	4.359	4.108

6.4. The influence of drugs on the enzyme activities of the flight muscles

If the nervous system plays an important role in the maintenance of normal structure and function of the flight muscles, one can try to influence these by activating or inactivating the nervous system. However, one must be sure not to interfere with the hormone balance in the body. There are two possibilities to do this:

1. To activate the nervous system under short-day conditions without disturbance of the normal diapause reaction.
2. To inactivate the nervous system under long-day conditions without interference with normal oviposition.

We investigated whether activation or inactivation of the nervous system is accompanied by an increase or decrease of the enzyme activities. An activation of the nervous system during short-day could possibly stimulate a further development of the flight muscles or inhibit the onset of normal degeneration. We started experiments in this direction, because LOCKSHIN and WILLIAMS (1965c) were able to demonstrate that activation of the nervous system – electrically or by administration of certain drugs (e.g. pilocarpine and physostigmine) – can prevent the degeneration of certain abdominal intersegmental muscles of the moth *Antheraea pernyi* and because MCCANN and REECE (1967) found repetitive action-potentials in the flight muscles of the fly *Sarcophaga bullata* after injection of physostigmine. Therefore we tried the effect on the enzyme activities of the flight muscles of single and repeated injections of physostigmine and other drugs, which are known to influence the nervous system: atropine, γ-amino-butyric acid, glutamate, phenylhydrazine chloride. We could not find significant effects in these experiments. But it does not mean of course that the nervous system has no effect whatsoever on the flight muscles.

As a change in ion composition of the haemolymph can affect neuromuscular activity, we also studied the effect of injection of potassium ions on the enzyme activities in long-day beetles. Rather high concentrations of potassium chloride (10μl of 1.0 M KCl) were injected at 4 and 8 days after emergence and the enzyme activities were measured at 12 days after emergence. For control, animals after ringer injection were used. The results are given in table 9.

TABLE 9. Effect of injection of potassium chloride on the enzyme activities in the flight muscle of long-day beetles.

	KCl injection	Ringer injection
GAPDH	106 ± 8.5	138 ± 15
GDH	60 ± 11	63 ± 1
SDH	9.2 ± 1.7	13.7 ± 1.7
α GPox	4.6 ± 0.8	11.4 ± 2.8

Enzyme activities in μmol/thorax/h.

We found a significantly lower activity of the mitochondrial enzymes. Although this effect of high potassium concentration might perhaps be explained by a direct repressive effect of high potassium on protein synthesis in the muscle, this seems to be unlikely, because oviposition, which also requires an intensive protein synthesis, is not influenced. So it is reasonable to suppose that potassium affects neuro-muscular activity and as a result the enzyme activity as well.

However, it must be pointed out that this increase in potassium concentration does not occur under physiological conditions in the Colorado beetle. Although a drastic change in percentage of dry weight was shown in table 3 and a drastic

TABLE 10. Ionic composition of the haemolymph of long-day and short-day Colorado beetles.

	Na^+	K^+	Ca^{++}	Mg^{++} in m.equiv./l
Long-day beetles	1.75	35	46	195
Short-day beetles	1.75	35	48	171

Na^+ and K^+ concentrations were measured with a flamephotometer. The Ca^{++} and Mg^{++} concentrations were determined with a mass-spectrograph.

change in protein composition was found by de Loof (personal communication), the ionic composition of the haemolymph is not significantly different in short-day and long-day beetles. See table 10.

6.5. DISCUSSION

It is by no means certain that all symptoms of the diapause syndrome in the Colorado beetle are caused by a single factor. As discussed in chapt. 5, an important role is played by the corpora allata, but the action of the neurosecretory cells cannot entirely be excluded.

Moreover, nonhormonal influences could also effect syndrome elements. For one of the symptoms of this syndrome, the degeneration of the structure and biochemical constitution of the flight muscles, it seems at least questionable whether it is caused by a direct hormonal effect only. In Locusta e.g. allatectomy does not result in flight muscle degeneration (MINKS, 1967) and the same is true for several other insects. In this connection it is important to point out that flight muscle degeneration is always associated with all the other symptoms of the syndrome. It might be possible that one of the elements is primarily affected and that muscle degeneration is a consequence of this primary event. Theoretically a number of different primary effects might cause secondarily the degeneration of the flight muscles:

1. Because HOYLE (1952, 1953, 1954) and ELLIS and HOYLE (1954) found an effect of potassium ions on neuromuscular activity and marching behaviour of the African migratory locust, a drastic change in the composition of the haemolymph might possibly affect the flight muscles. More recently PICHON and BOISTEL (1963) reported comparable results in *Periplaneta americana.* However, the change in ionic composition of the haemolymph will not affect the flight muscles specifically and from data of section 4 of this chapter it appears to be of no significance in the Colorado beetle.
2. An insufficient oxygen supply to the muscles might lead to degeneration. It is possible that the aerobic conditions of diapausing beetles in the soil are not optimal. This might particularly affect the flight muscles, which are preeminently specialized in aerobic metabolism. However, it is difficult to see how the inhibition of flight muscle development in pre-diapause as described in chapt. 5 could be explained by it.
3. If the innervation of the muscles was primarily affected, this could lead to

their degeneration, as nervous influences on structure and function of muscles have been described (see introduction to this chapter).

This last possibility appeared to be the most attractive. Theoretically, both hormonal and nervous effects on the flight muscles could be integrated in different ways:

a. Both factors could be cooperative. In some insects (e.g. *Locusta*) the nervous stimuli could be more important for development and maintenance of flight muscle structure and function, in others (e.g. *Leptinotarsa*) the hormonal influence would have preponderance.
b. Both factors could be consecutive, i.e. work in the order: hormone – nervous system – muscle.

The experiments given in this chapter are not consistent with the idea that both factors, hormones and nervous stimuli, act consecutively. The ChE activity in the brain of long-day and short-day beetles increases in a similar fashion (section 5.2.) but the level of juvenile hormone is much lower in short-day beetles. This would indicate that both parameters are not interdependent, i.e. are not consecutive. It indicates moreover, that the activity of the brain alone – at least as judged by its ChE activity – is not sufficient to warrant a normal development of flight muscles. It must be admitted that total ChE activity gives only an approximate impression of the real physiological activity. An accurate electrophysiological study of the electrical activity of the brain, thoracic ganglia and motoric nerves will give more direct information concerning the physiological activity of the nervous system.

A significant development of the flight muscles still takes place after denervation if the level of juvenile hormone is high enough. This does not fit into a consecutive mechanism of nervous and humoral effects either.

The same holds true for the effect of different pharmaca, which did not interfere with the structural and biochemical properties of flight muscles. However, there could exist various reasons why the injection of pharmaca did not have an effect:

1. The identity of the neuromuscular transmitter in insects is unknown. Evidence has been accumulating from a variety of scattered sources indicating that neuro-muscular transmission in insects differs radically from that of vertebrates in not being cholinergic (FLOREY, 1966; TREHERNE, 1966: KERKUT, 1967).
2. Only limited (sub-lethal) doses of the drugs can be used for these experiments. The success of LOCKSHIN and WILLIAMS (1965c) can possibly be explained by the much higher doses (50 times for physostigmine) they used in their experiments. Much more work is also needed regarding the excretion of the drugs used.
3. It is not certain whether a drug reaches the target enzymes in the central nervous system.

That both humoral and neural stimuli work additively can be deduced from the denervation experiments. The significance of persistent nervous stimuli for the development of the flight muscles can be seen in table 8. The enzyme activities in denervated muscles are significantly lower than those after sham-opera-

tion, especially the mitochondrial enzymes. From the fact that atrophy progresses much more slowly after denervation than after allatectomy it may be concluded that the humoral factor is much more effective.

Moreover, injection of high (= unphysiological) doses of potassium chloride does have an effect, especially on the amount of mitochondrial enzymes. It is tempting to think that this effect can be explained by the inhibition of motor nerve activity. However, BRONSERT and NEUPERT (1966) found a marked decline of the rate of aminoacid incorporation into proteins of isolated locust flight muscle mitochondria at a concentration of 0.15 M potassium chloride.

Considering the above arguments, the conclusion of this chapter must be that both factors, hormones and nervous stimuli, are effective on muscle structure and function. Although the influence of the nervous system on the development of the flight muscles is an important one, a direct effect of the hormone seems te be at least equally important. Very probably both the hormone and the nervous system and perhaps even more factors are influencing the developing muscle fibre at the same time in a very complicated way.

7. GENERAL CONCLUSION AND SUMMARY

In the Colorado beetle, the corpora allata have rather profound effects on metabolic activities (DE WILDE and STEGWEE, 1958; STEGWEE, 1960, STEGWEE et al, 1963; STEGWEE, 1964; DE WILDE, 1964; EL-IBRASHY, 1965). We therefore examined more closely the mode of action of the juvenile hormone with regard to metabolic processes.

Hormones can influence metabolism in two fundamentally different ways:

1. By changing the activity of enzymes, either by a direct action on enzyme conformation or by a change in the permeability barriers resulting in an increased accessibility of substrates.
2. By changing the synthesis of the enzymes, in other words by an effect on protein synthesis.

In most of the literature concerning the action of juvenile hormone these two possibilities are not clearly distinguished. The term 'metabolic hormone' can only be used when a growth-promoting effect is excluded. SLAMA (1964 a) was able to correlate the changes in O_2-consumption in *Pyrrhocoris* after allatectomy to the growth of the ovaries and concluded that the juvenile hormone is not a metabolic hormone. The situation is analogous to the mode of action of thyroxine, which has equally been qualified as a metabolic hormone. However, TATA (1966a) explained the metabolic effects of thyroxine by a selective hormonal control of the synthesis of mitochondrial respiratory and phosphorylative constituents. At this moment, only the hyperglycaemic factor in the corpora cardiaca as found by STEELE (1961, 1963) as well as the recently found so-called adipokinetic factor in the corpora cardiaca (MAYER, 1968) can definetely be qualified as metabolic hormones in insects. In this connection we can also mention the finding by STEGWEE (1960) and MINKS (1967) that juvenile hormone affects oxidative phosphorylation in isolated mitochondria (i.e. acts as a metabolic hormone *in vitro)*, although it is not clear whether this effect is also important *in vivo*.

However, the results given in this paper reveal that the changes in O_2-consumption in the Colorado beetle under short-day conditions or after allatectomy (EL-IBRASHY, 1965) can be explained by an effect on the growth of the flight muscles.

For several reasons we do not believe that the effect of juvenile hormone on oxidative phosphorylation (STEGWEE, 1960 and MINKS, 1967) is of primary importance in muscle development even if it is important *in vivo*:

1. STEGWEE (1964) found the same P/O ratios with mitochondria isolated from active and diapausing beetles, and MINKS (1967) found the same with mitochondria from normal and allatectomized locusts.
2. The effect on oxidative phosphorylation was found with mitochondria from locusts and Colorado beetles, although flight muscle degeneration could only be observed in the Colorado beetle.

3. It is questionable whether energy supply is rate-limiting during protein synthesis in flight muscles.

Thus, the action of juvenile hormone must primarily be sought on the level of general protein synthesis in the muscle fibre. In our opinion it is not justified to call this the action of a metabolic hormone.

Therefore the following scheme can be proposed for the explanation of flight muscle degeneration in the Colorado beetle during the onset of diapause. The normal structure of the flight muscle is controlled by humoral and neural stimuli. In *Leptinotarsa* the humoral stimuli seem to be the more important (chapt. 6). A low hormone level results in a decreased rate of protein synthesis, which will be followed by a degeneration (chapt. 5), because the rate of protein break-down in the muscle is the same under this condition. A decreased trophic activity of the nervous system can probably accelerate flight muscle degeneration. The drastic change in protein synthesis, combined with a normal food intake during pre-diapause will result in an accumulation of reserve materials, especially lipids.

Our results clearly show that the Colorado beetle is rather immature at emergence. The flight muscles are in a primordial state (chapt. 4) and a significant development of the cholinergic system still has to take place (chapt. 6), which is probably linked to the post-emergence differentiation of the central nervous system. Moreover, the increase in juvenile hormone titer after emergence (DE WILDE et al, 1968) and the development of the reproductive system (DE LOOF, 1969), including fat body, ovaries and haemolymph, also underline the immature state of the newly emerged beetle. The Colorado beetle represents a rather extreme example of post-emergence maturation, a feature known to occur also in other insects (ROCKSTEIN, 1950, 1959).

Our detailed study of flight muscle development in the Colorado beetle compared with the post-emergence development in some other holometabolous insects (chapt. 4) indicates a continuity of the pupal and adult stages and, moreover, demonstrates a diversity in the state of adult development at the moment of emergence. The occurrence of the last moult is not directly related to the state of development of the internal organs; it can take place at different moments during the development of the pharate adult. Therefore it is objectionable to take adult emergence as an indication of the state of development.

From the endocrinological point of view it follows that operation at emergence in different insects can result in different effects, with regard to onset of reproduction or flight muscle development. Allatectomy at emergence in the Colorado beetle must be compared to the same operation during the pupal stage in other insects. Some discrepancies in the literature concerning the effects of extirpation or implantation of hormone glands in adult insects can perhaps be explained by the differences in developmental level at the moment of operation.

SUMMARY

By means of electronmicroscopical and biochemical methods the effects of changes in juvenile hormone level on the structural and biochemical properties of the flight muscles were studied in the Colorado beetle, *Leptinotarsa decemlineata* Say.

Under long photoperiods, when a rapid increase in juvenile hormone titer can be measured after adult emergence, a development of the flight muscles was observed during the first twelve days of adult life. This process was studied in detail and compared to that in some other insect species (chapt. 4).

Flight muscle development in holometabolous insects is found to start in the pupal stage and to continue in the adult. In the insect species studied, the moment of adult emergence was not directly related to the state of flight muscle development. It follows that the development is continuous during pupal and adult stage.

In the Colorado beetle interfibrillar tracheoles are present in an early phase of flight muscle development, which is in contrast to the corresponding phase in the flight muscles of *Locusta*. In the beetle, both contractile system and enzyme pattern develop during one single phase.

No direct correlation could be established between the development of the myofibrils and the enzyme pattern in the Colorado beetle or in the other holometabolous insects. We established a positive correlation between the quantity of mitochondria and the activity of some mitochondrial enzymes. The significance of this finding was discussed in relation to theories on mitochondrial growth proposed in the literature.

In order to correlate flight muscle development more closely with changes in juvenile hormone titer, this development was also studied under short-day conditions, after allatectomy, after different pretreatment of the larvae and after termination of diapause (chapt. 5) and compared to the level of juvenile hormone under these different conditions.

Under short-day conditions and after allatectomy muscle development did start, but was not completed. Furthermore, the rate of flight muscle development after allatectomy in long-day treated beetles was correlated to the moment of operation. Allatectomy at ecdysis resulted in a lower rate of flight muscle development than operation at emergence. Our experiments with different pretreatment of the larvae revealed that the corpora allata were rapidly activated by long photophases, whereas short photophases were rather ineffective in reversing a previous long-day effect. The observations on flight muscle development were in agreement with this result. After termination of diapause a rapid regeneration of the flight muscles can be observed, which is paralleled by a rapid increase in juvenile hormone titer.

These findings clearly show the striking correlation between flight muscle development and the juvenile hormone titer under these various conditions, suggesting an important role of juvenile hormone in this process. However,

as discussed in chapt. 5, a role played by the neurosecretory cells could not entirely be excluded.

In order to find out whether the juvenile hormone directly affects the flight muscles, we studied the role of the nervous system as one of the possible other effectors (chapt. 6).

We found that flight muscle development is paralleled by a development of the cholinergic system in the brain, which is probably linked with the post-emergence maturation of the nervous system. During diapause, the activity of the cholinergic system has decreased. Moreover, denervation affected flight muscle development, but the atrophy was not as fast as during short-day. This suggests that flight muscle atrophy during diapause is not exclusively a neural effect.

In a general discussion (chapt. 7) we tried to put our data and data from the literature into one coherent theory. This theory, while allowing for an understanding of many aspects of the relation between corpora allata and diapause metabolism, still leaves a number of problems which need further study.

SAMENVATTING

Door middel van biochemische en electronenmicroscopische methoden zijn de effekten bestudeerd van veranderingen in de titer van het juveniel hormoon op de strukturele en biochemische eigenschappen van de vliegspieren bij de Coloradokever, *Leptinotarsa decemlineata* Say.

Onder langedag-omstandigheden, wanneer na het ontpoppen een snelle toename in juveniel hormoon titer gemeten kan worden, is een ontwikkeling van de vliegspieren waargenomen gedurende de eerste twaalf dagen van het volwassen leven. Dit proces is in detail bestudeerd en vergeleken met dat in enkele andere insektensoorten (hfdst. 4).

Vliegspierontwikkeling in holometabole insekten blijkt te beginnen in de pop, maar gaat door in het volwassen stadium. Een continuiteit van het pupale en volwassen stadium wat betreft de ontwikkeling van de vliegspieren kan ook afgeleid worden uit het feit, dat er bij de verschillende onderzochte insektensoorten geen direkte relatie bestaat tussen het moment van ontpoppen en het stadium van vliegspierontwikkeling.

In de Coloradokever zijn reeds in een vroeg stadium van de vliegspierontwikkeling interfibrillaire tracheolen aanwezig; dit in tegenstelling tot de overeenkomstige fase in de vliegspier van *Locusta*. In de kever ontwikkelen het contractiele systeem en het enzympatroon zich gedurende één enkele fase.

Geen direkte relatie kon worden gevonden tussen de ontwikkeling van de myofibrillen en de differentiatie van het enzympatroon in de Coloradokever, noch in andere holometabole insekten. Wel is een duidelijke korrelatie gevonden tussen de hoeveelheid mitochondriën en de aktiviteit van enkele mitochondriale enzymen. Het belang hiervan werd besproken in verband met de bestaande theorieën omtrent mitochondriale groei.

Om vliegspierontwikkeling meer direkt te kunnen korreleren met veranderingen in de titer van het juveniel hormoon, is deze ontwikkeling ook bestudeerd onder korte-dag kondities, na allatectomie, na verschillende voorbehandelingen van de larven en na het verbreken van diapauze (hfdst. 5).

Onder korte-dag kondities of na allatectomie begint de vliegspierontwikkeling wel, maar wordt niet voltooid. Verder blijkt de mate van vliegspierontwikkeling na allatectomie in lange-dag dieren direkt gekorreleerd te zijn met het tijdstip van operatie. Allatectomie op een vroeg tijdstip resulteert in een geringere vliegspierontwikkeling dan wanneer deze operatie later wordt uitgevoerd. Onze experimenten met dieren met een verschillende larvale voorbehandeling tonen aan, dat de corpora allata snel worden geaktiveerd door lange-dag fotocycli, terwijl korte-dag periodes niet zeer effektief zijn om een voorafgaand lange-dag effekt om te keren. Onze waarnemingen over de vliegspierontwikkeling komen hiermee overeen. Na het verbreken van diapauze kan een snelle regeneratie van de vliegspieren waargenomen worden, die parallel gaat met een snelle toename in juveniel hormoon titer.

Deze resultaten tonen duidelijk aan, dat er een opvallende korrelatie bestaat tussen vliegspierontwikkeling en de juveniel hormoon titer onder verschillende

omstandigheden, wat suggereert dat het juveniel hormoon een belangrijke rol speelt bij dit proces. Maar zoals besproken is in hfdst. 5 kan een rol van de neurosecretoire cellen bij dit proces niet volledig uitgesloten worden geacht.

Om te zien of het juveniel hormoon zijn effekt op de spierontwikkeling direkt uitoefent, dan wel via de trophische werking van het zenuwstelsel, hebben wij het effekt van denervatie vergeleken met dat van korte-dag behandeling en allatectomie (hfdst. 6).

Wij vonden, dat vliegspierontwikkeling parallel loopt met een toename van de cholinesterase aktiviteit in de hersenen, welke waarschijnlijk gekoppeld is aan de ontwikkeling van het zenuwstelsel in het volwassen stadium. Gedurende diapauze is de aktiviteit van het cholinergische systeem afgenomen. Verder leidt denervering van de vliegspier tot degeneratie, maar de atrophie ontstaat niet zo snel als gedurende korte-dag. Dit suggereert, dat vliegspieratrophie gedurende diapauze niet alleen een neuraal effekt is.

In een algemene diskussie is getracht om onze gegevens en die uit de literatuur in een samenhangende hypothese samen te vatten. Deze hypothese stelt ons in staat om verschillende aspekten van de relatie tussen de corpora allata en diapauze te begrijpen. Maar een aantal problemen blijven onopgelost, zodat verder onderzoek noodzakelijk is.

REFERENCES

BEENAKKERS, A. M. TH. Vetzuuroxidatie in de vliegspieren van *Locusta migratoria* L. Thesis. Utrecht (1964).

BEIZENHERZ, G., BOLTZE, H. J., BÜCHER, T., CZOK, R., GARBADE, K.H., MEYER-ARENDT, E. and G. PFLEIDERER. Diphosphofructose-Aldolase, Phosphoglyceraldehyde-Dehydrogenase, Milchsaure-Dehydrogenase, Glycerophosphat-Dehydrogenase und Pyruvatkinase aus Kaninchenmuskulatur in einem Arbeitsgang. Z. Naturforsch. **8B** (1953) 555–577.

BELLAMY, D. The structure and metabolic properties of tissue preparations from *Schistocerca gregaria* (desert locust) Biochem. J. **70** (1958) 580–589.

BERGH, S. G. VAN DEN. Respiration and energy production in the flight muscle of the housefly *Musca domestica L.* Thesis, Amsterdam (1962).

BHATNAGAR, P. L. and M. ROCKSTEIN. Physiological and Morphological changes in the flight muscle of the aging housefly, *Musca domestica* L. Proc. XII Intern. Congr. Entomol. (1964) 184.

BODENSTEIN, D. and B. SACKTOR. Cytochrome c oxidase during the metamorphosis of *Drosophila virilis*. Science, **116** (1952) 299–300.

BORST, P., KROON, A. M. and G. J. C. M. RUTTENBERG. Mitochondrial DNA and other forms of cytoplasmic DNA. in Genetic Elements D. SHUGAR ED. (1967) 81–116. Acad Press. London, N.Y.

BOWERS, W. S., and S. FRIEDMAN. Mobilization of fat-body glycogen by an extract of corpus cardiacum. Nature, **198** (1963) 685.

BRONSERT, U. and W. NEUPERT. Protein synthesis in Locust-flightmuscle sarcosomes. In: Regulation of metabolic processes in mitochondria, TAGER, J. M., PAPA, S., QUAGLIARIELLO, E. and E. C. SLATER eds. B.B.A. Library 7 (1966) Elsevier publishing Co. Amsterdam-London-N.York. 426–437.

BROSEMER, R. W. Changes in glycerophosphate dehydrogenase activity during development of the grasshopper *Schistocerca vaga*. Biochim. biophys. Acta. **96** (1965) 61–65.

BROSEMER, R. W. The levels of extramitochondrial glycerophosphate dehydrogenase in the wing muscle of a flightless grasshopper. J. Insect Physiol. **13** (1967) 685–690.

BROSEMER, R. W. The levels of extramitochondrial glycerophosphate dehydrogenase in the wing muscle of a flightless grasshopper. J. Insect Physiol. **13** (1967) 685–690.

BROSEMER, R. W., VOGELL, W. and TH. BÜCHER. Morphologische und enzymatische Muster bei der Entwicklung indirekter Flugmuskeln von *Locusta migratoria*. Biochem. Z. **338** (1963) 854–910.

BÜCHER, TH. Formation of the specific structural and enzymic pattern of the insect flight muscle. In: Aspects of Insect Biochemistry. T. W. GOODWIN ed. (1965) 15–28 Acad. Press. London-N.York.

BÜCHER, TH., WILFRIED, L. and D. PETTE. Einfache und zusammengesetzte optische Tests mit Pyridinnucleotiden. In: Handbuch der Physiologisch und Pathologisch-Chemischen Analyse- Hoppe-Seyler/Tierfelder. VIA (1964) 292–339.

CLARKE, K. V. and B. W. BALDWIN. The effect of insect hormones and of 2:4 dinitrophenol on the mitochondria of *Locusta migratoria*. J. Insect Physiol. **5** (1960) 37–46.

CLARK, A. M. and M. ROCKSTEIN. Aging in insects. In: The physiology of Insecta. M. Rockstein ed. **1** (1964) 227–281. Acad. Press. N.York-London.

CLEMENTS, A. N. Studies on the metabolism of locust fat body. J. Exp. Biol. **36** (1959) 665–675.

DAHM. K. H., TROST, B. M. and H. RÖLLER. The juvenile hormone. V Synthesis of the racemic juvenile hormone. J. Am. chem. Soc. **89** (1967) 5292–5294.

DELBRÜCK, A., ZEBE, E. and TH. BÜCHER. Über Verteilungsmuster von Enzymen des Energie liefernden Stoffwechels im Flugmuskel, Springmuskel und Fettkörper von *Locusta migratoria* und ihre cytologische Zuordnung. Biochem. Z. **331** (1959)273–296.

DRUMMOND, G. L. and J. R. STERN. Enzymes of ketone body metabolism II Properties of an

acetoacetate- synthesizing enzyme prepared from ox liver. J. biol. Chem. **235** (1960) 318–325.

DUBOWITZ, V. and D. L. NEWMAN. Change in enzyme pattern after cross-innervation of fast and slow skeletal muscles. Nature, **214** (1967) 840–841.

EDWARDS, G. A. and H. RUSKA. The function and metabolism of certain insect muscles in relation to their structure. Quart. J. Micr. Sci. **96** (1955) 151–159.

EGGSTEIN, M. and F. H. KREUTZ. Eine neue Bestimmung der Neutralfette im Blutserum und Gewebe. Klin. Wschr. **44** (1966) 262–267.

EIGENMANN, R. Biochemischer Untersuchungen der Flugmuskelentwicklung von *Antheraea pernyi Guer* (Lep). Rev. Suisse Zool. **71** (1964) 561–568.

EL-IBRASHY, M. T. A comparative study of metabolic effects of the corpus allatum in two adult Coleoptera, in relation to diapause. Meded. Landbouwhogeschool, Wageningen **65**–11 (1965) 1–65.

ELLIS, P. E. and G. HOYLE. A physiological interpretation of the marching of hoppers of the African migratory locust *(Locusta migratoria migratorioides R&F)*. J. exp. Biol. **31** (1954) 271–279.

ELLMAN, G. L., COURTNEY, K. D., ANDREO, V. JR. and R. M. FEATHERSTONE, A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. **7** (1961) 88–95.

ENGELMANN, F. Endocrine control of Reproduction in Insects. Ann. Rev. Entomol. **13** (1968) 1–26.

FLOREY, E. Neurotransmitters and modulators in the animal kingdom. Fed. Proc. **26** (1967) 1164–1178.

FOLCH, J., LEES, M. and G. H. SLOANE STANLEY. A simple method for the isolation and purification of total lipids from animal tissues. J. biol. Chem. **226** (1957) 497–511.

GILBERT, L. I. Physiology of growth and development: endocrine aspects. In: The physiology of Insecta, M. Rockstein, ed. **1** (1964) 149–225. Acad. Press. N.York-London.

GILBERT, L. I. Changes in lipid content during the reproductive cycle of *Leucophaea maderae* and effects of the juvenile hormone on lipid metabolism in vitro. Comp. Biochem. Physiol. **21** (1967) 237–257.

GILBERT, L. I. and H. A. SCHNEIDERMAN. Some biochemical aspects of insect metamorphosis. Am. Zool. **1** (1961) 11–51.

GRISON, P. L'influence de la plante-hôte sur la fécondité de l'insecte phytophage. Ent. exp. & appl. **1** (1958) 73–93.

GUTMANN, E. and P. HNIK. The effect of use and disuse on neuromuscular functions. Elsevier Publishing Co. Amsterdam-London-N.York. (1963).

HAJEK, I. and S. V. PERRY. Enzyme adaptation in isolated muscle. Biochem. J. **105** (1967) 45P–46P.

HANDEL, E. VAN. Estimation of Glycogen in small amounts of tissue. Anal. Biochem. **11** (1965) 256–265.

HANSON, J. Studies on the cross-striation of the indirect flight myofibrils of the blowfly *Calliphora*. J. biophys. biochem. Cytol. **2** (1956) 691–710.

HASKELL, P. T., CARLISLE, D. B., ELLIS. P. E. and J. E. MOOREHOUSE. Hormonal influences in locust marching-behaviour. Proc. XII Intern. Congr. Entomol. (1964) 290–291.

HASKELL, P. T. and J. E. MOOREHOUSE. A blood-borne factor influencing the activity of the central nervous system of the desert locust. Nature, **197** (1963)56–58.

HERMAN, W. S. and L. I. GILBERT. The neuroendocrine system of *Hyalophora cecropia* (L) (Lepidoptera: Saturniidae). I The anatomy and histology of the ecdyseal glands. Gen. comp. Endocr. **7** (1966) 275–291.

HEROLD, R. C. Development and ultrastructural changes of sarcosomes during honeybee flight muscle development. Devl. Biol. **12** (1965) 269–286.

HEROLD, R. C. and H. BOREI.Cytochrome change during honey bee flight muscle development. Devl. Biol. **8** (1963) 67–79.

HIGHNAM, K. C. Neurosecretory control of ovarian development in *Schistocerca gregaria*. Quart. J. micr. Sci. **103** (1962) 57–72.

HIGHNAM, K. C. Endocrine relationships in insect reproduction. In: Insect Reproduction Symposium no. 2 R. ent. Soc. Lond. (1964) 26–42.
HIGHNAM, K. C. Insect hormones. J. Endocr. **39** (1967) 123–150.
HIGHNAM, K. C., LUSIS, O. and L. HILL. The role of the corpora allata during oocyte growth in the desert locust, *Schistocerca gregaria Forskål*. J. Insect Physiol. **9** (1963) 587–596.
HODEK, I. and J. DE WILDE. (1969) in press.
HOLYE, G. High blood potassium in insects in relation to nerve conduction. Nature, **169** (1952) 281–282.
HOYLE, G. Potassium ions and insect nerve muscle. J. exp. Biol. **30** (1953) 121–135.
HOYLE, G. Changes in the blood potassium concentration of the African migratory locust *(Locusta migratoria migratorioides R&F)* during food deprivation and the effect on neuromuscular activity. J. exp. Biol. **31** (1954) 260–270.
JACOB, F. and J. MONOD. Genetic regulatory mechanisms in the synthesis of proteins. J. mol. Biol. **3** (1961) 318–356.
JOHANNSON, S. Feeding and Nutrition in reproductive processes in insects. Insect Reproduction. Symposium no. 2, R. ent. Soc. London. (1964). 43–55.
KARLSON, P. Biochemical studies on insect hormones. Vit. Horm. **14** (1956) 227–266.
KARLSON, P. New concepts on the mode of action of hormones. Perspectives Biol. Med. **6** (1963) 203–215. The University of Chicago Press.
KARLSON, P. Ecdyson, das Häutungshormon der Insekten. Naturwissenschaften **53** (1966) 445–453.
KARPATI, G. and W. K. ENGEL. Transformation of the histochemical profile of skeletal muscle by 'foreign' innervation. Nature, **215** (1967) 1509–1510.
KEILIN, D. On cytochrome, a respiratory pigment, common to animals, yeast and higher plants. Proc. R. Soc. Lond. (B) **98** (1925) 312–339.
KENDRICK-JONES. J. and S. V. PERRY. Enzymatic adaptation to contractile activity in skeletal muscle. Nature, **208** (1965) 1068–1070.
KENDRICK-JONES, J. and S. V. PERRY. The enzymes of adenine nucleotide metabolism in developing skeletal muscle. Biochem. J. **103** (1967) 207–214.
KENDRICK-JONES, J. and S. V. PERRY. Protein synthesis and enzyme response to contractile activity in skeletal muscle. Nature, **213** (1967) 406–408.
KERKUT, G. A. Biochemical aspects of invertebrate nerve cells. In: Invertebrate nervous systems. C.A.G. Wiersma ed. The University of Chicago Press. Chicago-Lond. (1967) 5–37.
KLOOT, W. G., VAN DER. The control of neurosecretion and diapause by physiological changes in the brain of the Cecropia silkworm. Biol. Bull. **109** (1955) 276–294.
KOPEĆ, S. The influence of the nervous system on the development and regeneration of muscles and integuments in insects. J. exp. Zool. **37** (1923) 15–25.
KORT, C. A. D., DE. The influence of the corpus allatum hormone on the flight muscle metabolism in the adult Colorado potato beetle. Symp. on Insect Endocrines, Brno, (1966) in press.
KOSHIHARA, H. and K. MARUYAMA. Changes in the fine structure of honeybee thoracic muscle during pupal development. Sci. Papers Coll. Gen. Education. Univ. Tokyo **8** (1958) 213–216.
KROGH, A. and T. WEIS-FOGH. The respiratory exchange of the desert locust *(Schistocerca gregaria)* before, during and after flight. J. exp. Biol. **28** (1951) 344–357.
KUTYNA, F. A. and A. S. TOMBES. Bio-electric activity of the central nervous system in normal and diapausing Alfalfa weevils. Nature, **212** (1966) 956–957.
LEA, A. O. and E. THOMSEN. Cycles in the synthetic activity of the medial neurosecretory cells of *Calliphora erythrocephala* and their regulation. In: Proc. Third Intern. Symp. Neurosecretion H. Heller and R. B. Clark eds. (1962) 345–347.
LE BERRE, J. R. Variations en fonction de l'age, du pouvoir d'envol d'un insecte, *Leptinotarsa decemlineata Say* (Coleoptera, Chrysomelidae). C. R. Acad. Sci., Paris **263** (1965) 913–916.
LEES, A. D. Photoperiodism in insects. In: Photophysiology. A.C. Giese ed. **IV** (1968) 47–137.

Acad. Press. Inc. New York.
LENNIE, R. W. and L. M. BIRT. Aspects of the development of flight muscle sarcosomes in sheep blow fly, *Lucilia cuprina*, in relation to changes in the distribution of protein and some respiratory enzymes during metamorphosis. Biochem. J. **102** (1967) 338–350.
LEVENBOOK, L. and C. M. WILLIAMS. Miiochondria in the flight muscles of insects. J. gen. Physiol. 39 (1956) 497–512.
LEWIS, D. K. Activation of honey-bee head cholinestesterase by water-miscible organic solvents. Nature, 213 (1967) 205–206.
LEWIS S. E. and E. C. SLATER. Oxidative phosphorylation in insect sarcosomes. Biochem. J. 58 (1954) 207–217.
LINDBERG, O. Morphogenetische und metabolische Wirkungen des Hormons des Schilddrüse. Naturwissenschaften **52** (1965) 379–388.
LOCKSHIN, R. A. and C. M. WILLIAMS. Programmed Cell Death- II Endrocrine potentiation of the breakdown of the intersegmental muscles of silkmoths. J. Insect Physiol. **10** (1964) 643–649.
LOCKSHIN, R. A. and C. M. WILLIAMS. Programmed Cell Death- I Cytology of degeneration in the intersegmental muscles of the Pernyi silkmoth. J. Insect Physiol. **11** (1965a) 123–133.
LOCKSHIN, R. A. and C. M. WILLIAMS. Programmed Cell Death- III Neural control of the breakdown of the intersegmental muscles of silkmoths. J. Insect Physiol. **11** (1965b) 601–610.
LOCKSHIN, R. A. and C. M. WILLIAMS. Programmed Cell Death- IV The influence of drugs on the breakdown of the intersegmental muscles of silkmoths. J. Insect Physiol. **11** (1965c) 803–809.
LOOF, A. DE. Thesis, University of Ghent. (1969).
LOUD, A. V. A method for the quantitative estimation of cytoplasmic structures. J. Cell Biol. **15** (1962) 481–487.
LOWRY, O. H., ROSENBROUGH, N. J., FARR, A. L. and R. J. RANDALL. Protein measurement with the Folin phenol reagent. J. biol. Chem. **193** (1951) 265–275.
LUFT, J. H. Improvements in epoxy resin embedding methods. J. Cell. Biol. **9** (1961) 409–414.
LÜSCHER, M. and R.LEUTHOLD. Uber die hormonale Beeinflussung des respiratorischen Stoffwechsels bei der Schabe *Leucophaea maderae (F)*. Rev. Suisse Zool. **72** (1965) 618–623.
MCCANN, F. V. and R. W. REECE. Neuromuscular transmission in insects: Effect of injected chemical agents. Comp. Biochem. Physiol. **21** (1967) 115–124.
MANSINGH, M. N. and B. N. SMALLMAN. The cholinergic system in insect diapause. J. Insect Physiol. **13** (1967) 447–467.
MARGOLIASH, E. and N. FROHWIRT. Spectrum of horse-heart cytochrome c. Biochem. J. **71** (1959) 570–572.
MARUYAMA, K. and K. MORIWAKI. Respiratory enzyme systems and muscular function in honyebee thoracic muscle. Enzymol. **19** (1958) 211–219.
MARUYAMA, K. and S. F. SAKAGAMI. Aktivität der Myofibrillen- und Sarkosomen Adenosintriphosphatasen im Flugmuskel der Bienerarbeiterinnen. Z.f. vergl. Physiol. **40** (1958) 543–548.
MAYER, R. J. Flight muscle metabolism in the desert locust *Schistocerca gregaria*. Thesis, Birmingham. (1968).
MICHEDJA, J. Physiology and structure of flight muscle sarcosomes in silkworm, *Hyalophora-Cecropia L.* Bull. Soc. Amis Sci. Lett. Poznán, (D) **4** (1964) 61–102.
MILBURN, N. and K. D. ROEDER. Control of efferent activity in the cockroach terminal abdominal ganglion by extracts of corpora cardiaca. Gen. comp. Endocr. **2** (1962) 70–76.
MILBURN, N., WEIANT, E. A. and K. D. ROEDER. The release of efferent nerve activity in the roach *Periplaneta americana* by extracts of corpus cardiacum. Biol. Bull. **118** (1960) 111–119.
MINKS, A. K. Biochemical aspects of juvenile hormone action in the adult *Locusta migratoria*. Archs. Neerl. Zool. **17** (1967) 175–257.
MORDUE, W. Studies on oocyte production and associated histological changes in the neuroendocrine system in *Tenebrio molitor L.* J. Insect Physiol. **11** (1965) 493–503.
MORDUE, W. The neuro-endocrine control of oocyte development in *Tenebrio molitor L.*

J. Insect Physiol. **11** (1965) 505–511.
MORDUE, W. Neuro-endocrine factors in the control of oocyte production in *Tenebrio molitor L.* J. Insect Physiol. **11** (1965) 617–629.
NAYAR, K. K. Probable endocrine mechanism controlling oviposition in the insect *Iphita limbata* Stal. 2nd. Intern. Symp. Neurosecretion Berlin. (1958) 102–104.
NUESCH, H. Uber den Einfluss der Nerven auf die Muskelentwicklung bei *Telea polyphemus*. Rev. Suisse Zool. **59** (1952) 294–301.
NUESCH, H. The development of muscle functions in *Antheraea polyphemus* (Lep). Proc. Intern. Congr. Zool. **16** (1963) 80.
NUESCH, H. Uber die strukturelle und funktionelle Entwicklung der Muskeln bei *Antheraea* (Lepidoptera). Z. Naturfosch. **20B** (1965) 343–347.
NUESCH, H. The role of the nervous system in insect morphogenesis and regeneration. Ann. Rev. Entomol. **13** (1968) 27–44.
ODHIAMBO, TH. R. Metabolic effects of the corpus allatum hormone in the desert locust, *Schistocerca gregaria*. Nature, **207** (1965) 1314–1315.
ODHIAMBO, TH. R. The metabolic effects of the corpus allatum hormone in the male desert locust: I Lipid metabolism. J. exp. Biol. **45** (1966a) 45–50.
ODHIAMBO, TH. R. The metabolic effects of the corpus allatum hormone in the male desert locust: II Spontaneous locomotor activity. J. exp. Biol. **45** (1966b) 51–63.
OZBAS, S. and E. S. HODGSON. Action of insect neurosecretion upon central nervous system in vitro and upon behaviour. Proc. natl. Acad. Sci. **44** (1958) 825–830.
PALADE, G. E. A study of fixation for electronmicroscopy. J. exp. Med. **95** (1952) 285–298.
PETTE, D. Plan und Muster im Zellulären Stoffwechsel. Naturwissenschaften. **52** (1965) 597–616.
PETTE, D. and H. BRANDAU. Intracellular localization of glycolytic enzymes in cross-striated muscles of *Locusta migratoria*. Biochem. biophys. Res. Comm. **9** (1962) 367–370.
PETTE, D., KLINGENBERG, M. and TH. BÜCHER. Comparable and specific proportions in the mitochondrial enzyme activity pattern. Biochem. biophys. Res. Comm. **7** (1962) 425–429.
PETTE, D., LUH, W. and TH. BÜCHER. A constant proportion group in the enzyme activity pattern of the Embden-Meyerhof chain. Biochem. biophys. Res. Comm. **7** (1962) 419–424.
PFLUGFELDER, O. Entwicklungsphysiologie der Insekten. Akad. Verl. Gesell. Geest and Portig K.G. Liepzig. (1952) 332 pp.
PICHON, Y. and J. Boistel. Modifications of the ionic content of the haemolymph and of the activity of *Periplaneta americana* in relation to diet. J. Insect Physiol. **9** (1963) 887–891.
PORTER, K. R. and PALADE, G. E. Studies on the endoplasmic reticulum. III Its form and distribution in striated muscle cells. J. biophys. and biochem. Cytol. **3** (1957) 269–299.
PREWITT, N. A. and B. SALAFSKY. Effect of cross innervation on biochemical characteristics of skeletal muscles. Am. J. Physiol. **213** (1967) 295–300.
RAABE, M. Etude des phénomènes de neurosécrétion au niveau de la chaine nerveuse ventrale des Phasmides. Bull. Soc. Zool. France **90** (1965) 631–654.
RALPH, C. L. and R. MCCARTHY. Effects of brain and corpus cardiacum extracts on haemolymph trehalose of the cockroach, *Periplaneta americana*. Nature, 203 (1964) 1195–1196.
ROCKSTEIN, M. The relation of cholinesterase activity to change in cell number with age in the brain of the adult worker honey-bee. J. cell. comp. Physiol. **35** (1950) 11–23.
ROCKSTEIN, M. Metachemogenesis- postemergence biochemical maturation in insects. Smithsonian Miscell. Coll. **137** (1959) 263–286.
ROCKSTEIN, M. and K. F. BRANDT. The biochemical basis for ageing of flight ability in the male house fly. Am. Zool. **2** (1962) 182.
ROCKSTEIN, M. and K. F. BRANDT. Enzyme changes in flight muscle correlated with ageing and flight ability in the male house fly. Science, **139** (1963) 1049–1050.
RÖLLER, H. and J. S. BJERKE. The juvenile hormone: Its purification and isolation. Symp. on Insect Endocrines, Brno. (1966) in press.
RÖLLER, H., DAHM, K. H., SWEELEY, C. C. and B. M. TROST. The structure of the juvenile hormone. Angew. Chemie Intern. Ed. **6** (1967) 179–180.
ROMANUL, F. C. A. and J. P. VAN DER MEULEN. Reversal of the enzyme profiles of muscle

fibres in fast and slow muscles by cross-innervation. Nature, **212** (1966) 1369–1370.
ROODYN, D. B. and D. WILKIE. The biogenesis of mitochondria. Methuen's Monographs on biological Subjects. Methuen & Co. Ltd. Lond. 1968.
SABATINI, D. D., BENSCH, K. and R. J. BARRNETT. Cytochemistry and electronmicroscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. J. Cell Biol. **17** (1963) 19–58.
SACKTOR, B. A comparison of the cytochrome oxidase activity of two strains of houseflies. J. econ. Ent. **43** (1950) 832–838.
SACKTOR, B. Energetics and respiratory metabolism of muscular contraction. In: The physiology of Insecta II. M. Rockstein ed. (1965) 483–580. Acad. Press. New York – London.
SÄGESSER, H. Über die Wirkung der corpora allata auf den Sauerstoffverbrauch bei der Schabe, *Leucophaea maderae L.* J. Insect Physiol. **5** (1960) 264–285.
SCHARRER, B. The fine structure of the neurosecretory system of the insect *Leucophaea maderae*. In: Proc. Third. Intern. Symp. Neurosecretion. H. Heller and R. B. Clark eds. (1962) 89–97.
SCHARRER, B. Recent progress in the study of neuroendocrine mechanisms in insects. Arch. Anat. microsc. **54** (1965) 331–342.
SCHARRER, E. Photo-neuro-endocrine systems: General Concepts. Ann. N.Y. Acad. Sci. **117** (1964) 13–22.
SCHOONHOVEN, L. M. Spontaneous electrical activity in the brain of diapausing insects. Science, **141** (1963) 173–174.
SHAAYA, E. and P. KARLSON. Der Ecdysontiter während der Insektenentwicklung. IV Die Entwicklung der Lepidopteren *Bombyx mori* L. und *Cerura vinula* L. Devl. Biol. **11** (1965) 424–432.
SHAFIQ, S. A. Electronmicroscopic studies on the indirect flight muscles of *Drosophila melanogaster*. J. Cell Biol. **17** (1963 a) 351–362.
SHAFIQ, S. A. Electron microscopic studies on the indirect flight muscle of *Drosophila melanogaster*. J. Cell Biol. **17** (1963 b) 363–373.
SHAPPIRIO, D., EICHENBAUM, D. M. AND B. R. LOCKE. Cholinesterase in the brain of the Cecropia silkmoth during metamorphosis and pupal diapause. Biol. Bull. **132** (1967) 108–125.
SLAMA, K. Hormonal control of respiratory metabolism during growth, reproduction and diapause in female adult of *Pyrrhocoris apterus* L. (Hemiptera). J. Insect Physiol. **10** (1964a) 283–303.
SLAMA, K. Hormonal control of respiratory metabolism during growth, reproduction and diapause in male adults of *Pyrrhocoris apterus* L. (Hemiptera). Biol. Bull. **127** (1964b) 499–510.
SLAMA, K. Effect of hormones on the respiration of body fragments of adult *Pyrrhocoris apterus L.* (Hemiptera). Nature, **205** (1965) 416–417.
SMITH, D. S. The structure of insect fibrillar flight muscle. J. Cell Biol. **10** (1961) 123–158.
SMITH, D. S. The structure of flight muscle sarcosomes in the blowfly *Calliphora erythrocephala* (Diptera). J. Cell Biol. **19** (1963) 115–138.
SMITH, D. S. The organization of flight muscle in an aphid, *Megoura viciae* (Homoptera). J. Cell Biol. **27** (1965) 379–393.
SMITH, D. S. The organization of flight muscle fibers in the Odonata. J. Cell Biol. **28** (1966) 109–127.
STAAL, G. B. Studies on the physiology of phase induction in *Locusta migratoria migratorioides* R&F. Thesis, Wageningen (1961) 124 pp.
STEELE, J. E. Occurence of a hyperglycaemic factor in the corpus cardiacum of an insect. Nature **192** (1961) 680–681.
STEELE, J. E. The site of action of insect hyperglycaemic hormone. Gen. comp. Endocr. **3** (1963) 46–52.
STEGWEE, D. Metabolic effect of a corpus allatum hormone in diapausing *Leptinotarsa decemlineata* Say. Verh. XI Intern. Kongr. Entom. Wien (B) **3** (1960) 218–221.
STEGWEE, D. Respiratory chain metabolism in the Colorado potato beetle. II Respiration and

oxidative phosphorylation in 'sarcosomes' from diapausing beetles. J. Insect Physiol. **10** (1964) 97–102.

STEGWEE, D. and A. R. VAN KAMMEN-WERTHEIM. Respiratory chain metabolism in the Colorado potato beetle: I Respiration and oxidative phosphorylation in sarcosomes from active beetles. J. Insect. Physiol. **8** (1962). 117–126.

STEGWEE, D., KIMMEL, E. C., BOER, J. A. and S. HENSTRA. Hormonal control of reversible degeneration of flight muscle in the Colorado potato beetle, *Leptinotarsa decemlineata Say* (Coleoptera). J. Cell Biol. **19** (1963) 519–527.

STRONG, L. The relationships between the brain, corpora allata and oocyte growth in the central american locust, *Schistocerca sp.* II The innervation of the corpora allata, the lateral neurosecretory complex and oocyte growth. J. Insect Physiol. **11** (1965) 271–280.

TATA, J. R. The regulation of mitochondrial structure and function by thyroid hormones under physiological conditions. In: Regulations of metabolic processes in mitochondria. Tager, J., M. Papa, S., Quagliariello, E. and E. C. Slater eds. B.B.A. Library **7** (1966) 489–507 Elsevier Publish. Co. A'dam-Lond.-N.York.

TATA, J. R. Hormones and the synthesis and utilization of ribonucleic acids. In: Progress in nucleic acid research and molecular biology. Davidson, J. N. and W. E. Cohn eds. **5** (1966) 191–250.

TATA, J. R., ERNSTER, L., LINDBERG, O., ARRHENIUS, E., PEDERSEN, S. and R. HIDMAN. The action of thyroid hormones at the cell level. Biochem. J. **86** (1963) 408–428.

THOMSEN, E. Influence of the corpus allatum on the oxygen consumption of adult *Calliphora erythrocephala* Meig. J. exp. Biol. **26** (1949) 137–149.

THOMSEN, E. Functional significance of the neurosecretory brain cells and the corpus cardiacum in the female blowfly *Calliphora erythrocephala* Meig. J. exp. Biol. **29** (1952) 137–172.

THOMSEN, E. and I. MØLLER. Neurosecretion and intestinal protease activity in an insect, *Calliphora erythrocephala* Meig. Nature, **183** (1959) 1401–1402.

THOMSEN, E. and I. MØLLER. Influence of neurosecretory cells and of corpus allatum on intestinal protease activity in the adult *Calliphora erythrocephala* Meig. J. exp. Biol. **40** (1963) 301–321.

TREHERNE, J. E. The neurochemistry of Arthropods. Cambridge University Press. Cambridge (1966).

TYSHTCHENKO, V. P. and J. E. MANDELSTAM. A study of spontaneous electrical activity and localization of chlolinesterase in the nerve ganglia of *Antheraea pernyi* Guer at different stages of metamorphosis and in pupal diapause. J. Insect Physiol. **11** (1965) 1233–1239.

USHERWOOD, P. N. R., COCHRANE, D. G. and D. REES. Changes in structural, physiological and pharmacological properties of insect excitatory nerve-muscle synapses after motor nerve section. Nature, **218** (1968) 589–590.

VOGELL, W. Phasen der Bildung morphologischer und enzymatischer Muster der Flugmuskels der Wanderheuschrecke. Naturwissenschaften. **52** (1965) 405–418.

WEED-PFEIFFER, I. G. Effect of the corpora allata on the metabolism of adult female grasshoppers. J. exp. Zool. **99** (1945) 183–233.

WIENS, A. W. and L. I. GILBERT. Regulation of cockroach fat-body metabolism by the corpus cardiacum in vitro. Science, **150** (1965) 614–616.

WIENS, A. W. and L. I. GILBERT. Regulation of carbohydrate mobilization and utilization in *Leucophaea maderae*. J. Insect Physiol. **13** (1967) 779–794.

WIGGLESWORTH, V. B. The function of the corpus allatum in the growth and reproduction of *Rhodnius prolixus* (Hemiptera). Quart. J. micr. Sci. **79** (1936) 91–121.

WIGGLESWORTH, V. B. The hormonal regulation of growth and reproduction in insects. Advanc. Insect Physiol. **2** (1964) 247–336.

WILDE, J. DE, Aspects of diapause in adult insects, with special regard to the Colorado beetle, *Leptinotarsa decemlineata* Say. Arch. néerl. Zool. **11** (1953) 375–385.

WILDE, J. DE, The significance of the photoperiod for the occurence of diapause in the adult *Leptinotarsa decemlineata* Say. Proc. 1st Intern. phtobiol. Congr. (1955).

WILDE, J. DE, Diapause in the Colorado beetle *(Leptinotarsa decemlineata* Say*)* as an endocrine deficiency syndrome of the corpora allata. Proc. Symp. on the Ontogeny of Insects,

Praha (1959) 226–230.
WILDE, J. DE, Photoperiodism in insects and mites. Annal. Rev. of Entom. **7** (1962) 1–26.
WILDE, J. DE. Reproduction-endocrine control. In: Physiology of Insecta. **1** (1964) 59–90. M. Rockstein ed. Acad. Press Inc. N.York.
WILDE, J. DE, Photoperiodic control of endocrines in insects. Archs. Anat. Microsc. **54** (1965) 547–564.
WILDE, J. DE, and J. A. DE BOER. Physiology of diapause in the adult Colorado beetle: II Diapause as a case of pseudo-allatectomy. J. Insect Physiol. **6** (1961) 152–161.
WILDE, J. DE, DUINTJER, C. S. and L. MOOK. Physiology of diapause in the adult Colorado beetle *(Leptinotarsa decemlineata* Say*)*: I. The photoperiod as a controlling factor. J. Insect Physiol. 3 (1959) 75–85.
WILDE, J. DE, and P. FERKET. The host plant as a source of seasonal information. Med. Rijksfac. Landbouwwetensch. Ghent. **32** (1967) 387–392.
WILDE, J. DE, STAAL, G. B., DE KORT, C. A. D., DE LOOF, A. and G. BAARD. Juvenile hormone titer in the haemolymph as a function of photoperiodic treatment in the adult Colorado beetle *(Leptinotarsa decemlineata* Say*)*. Proc. Koninkl. Nederl. Akad. Wetensch. Series C, **71** (1968) 321–326.
WILDE, J. DE, and D. STEGWEE. Two major effects of the corpus allatum in the adult Colorado beetle. Arch. néerl. Zool. 13 (1958) 277–289.
WILLIAMS, C. M. Morphogenesis and the metamorphosis of insects. The Harvey Lect. **47** (1952) 126–155.
WILLIAMS, C. M. The juvenile hormone of insects. Nature, **178** (1956) 212–213.
WILLIAMS, C. M. Control of pupal diapause by the direct action of light on the insect brain. Science, **140** (1963) 386.
WILLIAMS, C. M. and H. A. SCHNEIDERMAN. The necessity of motor innervation for the development of insect muscles. Anat. Rec. **113** (1952) 77.