ENDOCRINE CONTROL OF FATTY ACID BINDING PROTEIN EXPRESSION IN FLIGHT MUSCLE OF SCHISTOCERCA GREGARIA

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Endocrine Control of Fatty Acid Binding Protein Expression in Flight Muscle of Schistocerca gregaria

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ABSTRACT

Fatty acid binding proteins (FABPs) are abundant intracellular proteins believed to be involved in fatty acid transport and metabolism. The FABP present in flight muscle of the locust, *Schistocerca gregaria*, is similar to mammalian muscle FABP in its primary structure and biochemical characteristics. The changes of locust muscle FABP concentration and distribution during development were studied using enzyme linked immunosorbent assays and electron microscopy of immunogold labeled tissue sections. Locust muscle FABP is the most abundant soluble muscle protein in fully developed adult locusts, comprising 18% of the total cytosolic protein. At the beginning of the adult stage, however, no FABP is detectable. Its concentration rises during the following 10 days, after which it reaches its maximal value. The protein is abundant in cytosol and nuclei, but virtually absent in mitochondria.

The levels of FABP were examined in insects that had been treated with metamorphosis controlling hormones and anti-hormones. The FABP content was found to be similar in normal and precocious adults, but no FABP was found in last instar nymphs, methoprene induced supernumerary nymphs and azadirachtin induced over-aged nymphs. These results indicate that FABP synthesis does not occur prior to metamorphosis, but is always initiated in differentiated adult flight muscle. FABP synthesis in adult muscle, however, is not directly stimulated by any hormone from the brain or adjacent endocrine glands.

FABP is expressed at high rates only at the beginning of the adult stage. In mature locusts, the protein is a major cytosolic component, but its mRNA transcription is reduced to a minimum level. In these animals, FABP gene expression can be induced by flight activity. Extended flight leads to a 15-fold increase of FABP mRNA within 16 hours; the additional synthesis of FABP may assure a more

efficient fatty acid transport towards mitochondria ß-oxidation. Injection of adipokinetic hormone II (AKH II) into resting locusts also stimulates transcription in a dose dependent manner. FABP mRNA reaches its maximum 16 hours after treatment and returns to its base level within 48 hours. Purified low density lipophorin, when injected into resting locusts, causes a similar increase as AKH II or flight, suggesting that the increased supply of fatty acid is the ultimate cause for the observed induction of FABP gene expression. Additional synthesis of FABP in response to an increase of free fatty acid in the cytosol allows a higher fatty acid flux through the cytosol. Moreover, FABP may be necessary to protect cellular structures from damaging effects of an excess of fatty acids.

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CHAPTER 1. GENERAL INTRODUCTION

The desert locust, *Schistocerca gregaria* is arguably the most devastating insect pest. The threat of a locust swarm arises both from the actual quantity of insects and from its mobility. A swarm of locusts may cover areas of 30 km^2 and contain more than 10^9 locusts with a total weight between two to three tons. Laboratory studies have shown that the energy demands of active flight require locusts to consume plant material equivalent to their own body weight each day. During a heavy locust invasion, several hundred square kilometers of swarms are present, resulting in the consumption of hundreds of thousands tons of plant material. Swarms have been found to migrate over extreme distances, such as 11,000 km from Sahel to West India, and to cause massive crop losses (Rainey, 1989).

The desert locust is a large and powerful insect that is able to fly at a maximal airspeed of around 20 km/h. A cruising airspeed of up to 15 km/h may be maintained for periods of 40 h or longer (Rainey, 1989). The energy expended during flight can be expressed as the metabolic rate, *i.e.* the amount of oxygen consumption of the insect in a given time interval. During rest, a metabolic rate of 0.63 ml O_2/g body weight/h has been observed. In contrast, flying locusts achieve very high metabolic rates of up to 30 ml O_2/g body weight/h (Weis-Fogh, 1952). This almost 50-fold increase during the transition from rest to flight can be achieved in a fraction of a second (Goldsworthy, 1983; Kammer and Heinrich, 1978).

Most metabolic energy in flight is needed by the flight muscles. These muscles which accelerate the wings and control wing movement can be divided into three categories, based on function and anatomy: indirect, direct and accessory flight muscles. The wing-beating is accomplished by the operation of indirect flight muscles, which are located in meso- and metathoracic segments. Contraction of the

dorsal longitudinal muscles, extending from prephragma to postphragma, causes depression of the wings, whereas elevation is brought about by contraction of the dorsoventral flight muscles, inserted on scutum and sternum or coxa. It is the distortion of the shape of the thorax, produced by these contractions, that introduces the movement of the wings, made possible by a particular articulation between wings and scutum (Beenakkers *et al.*, 1985). Direct muscles act on the wing via their insertion on a sclerite in the wing base or via ligamentous attachments between their insertion and wing-base (axillary) sclerite. They may provide power or control wing movements or both. The accessory muscles originate and insert on the thoracic skeleton and influence the mechanical properties of the thorax or the relative positions of components of the flight machinery. These muscles may influence both the form of the wing beat and power production by the indirect muscles (Kammer, 1985).

Gas exchange is facilitated in insects by a vast network of air-filled tubes, the tracheae, that penetrate the body and tissues. The tracheae open to the outside of the body through segmentally arranged spiracles. The tracheal supply to wing muscles is especially adapted to meet the very high requirements for O_2 by working wing muscles: fine branches of the tracheal system penetrate deep into the muscular tissues, and interfibrillar tracheoles are in close contact with the mitochondria, providing them with oxygen (Van den Hondel-Franken and Flight, 1981). Because of the efficient tracheal system, the increase in metabolic rate during flight is not accompanied by an oxygen debt, thus making fuel supply the limiting factor for sustaining flight activity.

Locust flight muscles utilize both carbohydrates and lipids as energy sources. Initially the muscles utilize carbohydrates already present in the muscle cells which are rapidly replenished from the hemolymph. The disaccharide trehalose, the main carbohydrate in the hemolymph, is thus the predominant source of energy on the

initiation of flight. However, there is a subsequent switchover towards oxidation of lipid for sustained flight (Mayer and Candy, 1969; Goldsworthy, 1983), lipids stored in the fat body thus must be mobilized and transported to the muscles.

The energy metabolism and the switch of fuel utilization are mostly controlled by adipokinetic hormones (AKHs). Flight is a primary stimulus for the release of AKHs. Although it has not been possible to detect elevated titers of hormones until about 20 min into flight, there is a rapid increase in hormone titer between 20 and 30 min, peaking at 30 min and decreasing thereafter (Orchard and Lange, 1983; Cheeseman and Goldsworthy, 1979). It has been suggested that the release of the hormones is initiated by the decrease in hemolymph trehalose which occurs soon after the commencement of flight (Houben and Beenakkers, 1973). The ultimate control of release of adipokinetic hormones resides within the brain/retrocerebral axis.

In locusts two adipokinetic hormones, AKH I and AKH II, have been identified. Although both have similar biological activities, they are released in different amounts during flight. AKH I represents at least 80% of the recoverable activity at 30 min (Orchard and Lange, 1983); AKH II also peaks after 30 min of flight, but its titer decreases much more slowly than AKH I (Orchard and Lange, 1983). Thus, it appears that AKH I plays an important role in the early stage of flight, while AKH II may be more important for sustained flight.

Both hormones stimulate lipid release from fat body where lipids are typically stored as triacylglycerol (TAG). Upon the stimulation of AKHs, TAG is degraded by a non-stereospecific lipase to 2-monoacylglycerol (Tietz and Weintraub, 1978) which is subsequently acylated by a stereospecific microsomal monoacylglycerol-acyltransferase (Tietz *et al.*, 1975) which synthesizes preferentially *sn*-1,2-diacylglycerol (DAG) (Tietz and Weintraub, 1980). Due to the low water-solubility of lipids, DAG requires a transport system in the hemolymph (Wheeler

and Goldsworthy, 1985a). In locusts, DAGs are transported as part of the major hemolymph lipoprotein. Once released into hemolymph, DAGs are taken up by high density lipophorin (HDLp). The DAG enriched particle is then stabilized by several molecules of apolipophorin III (apoLp-III), a small amphiphilic apoprotein that has high affinity for the lipid-water interface. Thus apoLp-III binds to the newly created lipid-water interface of DAG-HDLp particle and stabilizes the expanding particle. Because of the uptake of DAG, the lipid content of lipophorin increases to about 50%, leading to the formation of a low density lipophorin (LDLp) which then passes through the hemolymph and delivers DAG to the flight muscle (Mwangi and Goldsworthy, 1977; Chino *et al.*, 1986). At the flight muscle, lipids are hydrolyzed at the cell surface by a membrane-bound lipase, leaving behind the original HDLp particle and free apoLp-III which can return to the hemolymph (Wheeler and Goldsworthy, 1985b). At the flight muscle, the hydrolyzed fatty acids penetrate the muscle cell membranes, are released into the cytosol, and are ultimately oxidized in the mitochondria to produce the ATP needed to support flight (Goldsworthy, 1983).

Long-chain fatty acids are poorly soluble in aqueous media. In the cytosol, diffusion of fatty acids from plasma membrane to mitochondria is extremely slow and can never provide enough fatty acids for mitochondria to meet the high energy requirement of flight. In order to assure efficient transport a water soluble carrier protein is needed. In vertebrate animals, several fatty acid binding proteins (FABPs) have been found in different tissues that are thought to facilitate the transcytosolic movement of fatty acids (Veerkamp *et al.*, 1991).

Fatty acid binding proteins (FABPs) were first detected 20 years ago in mammalian liver (Ockner *et al.*, 1972; Mishkin *et al.*, 1972). Subsequently, these proteins have been detected in several tissues of numerous vertebrate species (Veerkamp *et al.*, 1991). There are at least three distinct classes of fatty acid binding proteins, namely hepatic (L-FABP), intestinal (I-FABP) and cardiac FABP (H-

FABP), that belong to the same superfamily of lipid binding proteins (Matarese *et al.*, 1989). H-FABP was initially purified from heart muscle (Fournier *et al.*, 1978; Unterberg *et al.*, 1986; Claffey *et al.*, 1987), but has since been found to have a wide-spread tissue distribution. It has been identified in skeletal muscle (Miller *et al.*, 1988), brain, ovary, testis, kidney (Bass and Manning, 1986), aorta (Sarzani *et al.*, 1988) and mammary gland (Jones *et al.*, 1988). It is an acidic protein (pI - pH 5.0) with a molecular mass of approximately 15,000 Da that binds fatty acids stoichiometrically in a 1:1 ratio. While metabolic functions of FABP have not been conclusively demonstrated, it appears that cardiac FABP acts primarily in aiding cytosolic fatty acid transport to mitochondria for subsequent ß-oxidation (Kaikaus *et al.*, 1990) and therefore modulates cardiac energy production by controlling the transfer of acyl-carnitine to the mitochondrial ß-oxidative system (Glatz and Van der Vusse, 1989). It has also been proposed that, by binding unesterified fatty acids, FABP might function as a protective mechanism against deleterious effects of high concentrations of free fatty acids in an actively working muscle (Bass, 1988; Veerkamp *et al.*, 1991). Generally, FABP appears to be more abundant in muscles with a high red fiber content, which have a higher oxidative capacity. In mammalian heart tissue which uses primarily fatty acids as energy source FABP concentrations of up to 5% of the total cytosolic proteins have been reported (Kaikaus *et al.,* 1990) and the concentration of FABP in rat heart could be increased 29% by extended muscular activity, which may reflect the increased rate of lipid utilization.

As in mammalian heart, a fatty acid carrier is also needed in locust flight muscle. Locust flight muscles are among the most active muscles known, and their rate of lipid utilization during flight is much higher than that in mammalian heart. Recently, a fatty acid binding protein has been isolated from the flight muscle of the desert locust. Locust M-FABP has a molecular weight of 15,000 and isoelectric point of 5.2. It binds fatty acids stoichiometrically in a 1:1 ratio. Its molecular

characteristics, tissue specificity and electrophoretic behavior are reminiscent of mammalian H-FABP (Haunerland and Chisholm, 1990). Besides, its primary structure conclusively establishes the evolutionary relationship to its vertebrate analogs. Although the vertebrate lineage diverged from the invertebrates 650 million years ago, locust M-FABP shows a 41% sequence identity with mammalian H-FABP, but a far lower degree of homology to hepatic and intestinal FABP. Moreover, all the amino acids believed to be involved in fatty acid binding are either identical or conservatively substituted. The structural similarity between vertebrate H-FABP and invertebrate M-FABP thus suggests that the protein serves identical functions in vertebrates and invertebrates (Price *et al.*, 1992).

Since fatty acid flux in locust flight muscle is much higher than in mammalian heart, the FABP concentration should be higher in flight muscle as well. Indeed, preliminary data indicated that in mature adult locusts FABP levels approach 10% or more of total cytosolic proteins (Haunerland and Chisholm, 1990). Locust nymphs, however, which lack wings and thus cannot fly, do not possess any detectable amount of FABP in their mesothorax muscles. It appears that massive FABP synthesis is occurring at the beginning of the adult stage.

The present project was undertaken to study the expression and synthesis of FABP in flight muscle of the desert locust, *Schistocerca gregaria*. Aim of this research was to gain insights into the endocrine control of FABP gene expression. Towards this goal, FABP synthesis was analyzed quantitatively during locust development as well as in response to the treatment of metamorphosis controlling hormones and anti-hormones, furthermore, changes in FABP mRNA transcription in response to flight and the treatment of flight related hormone and metabolic product were revealed.

CHAPTER 2. MATERIALS AND METHODS

2.1. Materials

All chemicals and buffer components were obtained from Sigma (St. Louis, MO), unless otherwise indicated. Scintillation cocktail and the reagents for the immuno-assays were purchased from Amersham (Oakville, ON), with the exception of Pro-bind microtiter plates (Fisher Scientific, Vancouver, BC). Magic miniprep columns came from Promega (Madison, WI), restriction endonucleases and RNA size standards from Pharmacia (Piscataway, NJ), and Genescreen Nylon membrane from NEN-Dupont (Lachine, PQ). Electron microscopy supplies came from J.B. EM services (Pointe Claire Dorval, PQ) except for gold labeled antibodies (Sigma, St. Louis, MO). Methoprene (mixture of both isomers) was a gift from the Zoecon Corporation (Palo Alto, CA). Anti-locust FABP-antiserum was prepared as described before (Haunerland and Chisholm, 1990); the antiserum was specific for FABP from locust flight muscle and did not cross-react with mammalian H-FABP or any proteins from other locust tissues.

2.2. Buffers and reagents

Ringer's solution: 0.12 M NaCl, 15 mM KCl, 4 mM $CaCl_2$, 8 mM $MgCl_2$, 5 mM Na_2HPO_4, 20 mM N-[tris(hydroxymethyl)methyl-2-amino]ethanesulfonic acid buffer and 60 mM sucrose, pH 7.2.

PBS: 10 mM phosphate-buffered saline, 10 mM sodium phosphate, 0.143 M sodium chloride, pH 7.0.

25 mM PBS: 25 mM sodium phosphate, 0.143 M sodium chloride, pH 6.5.

Sample buffer: 0.125 M Tris, 20% v/v glycerol, 4% w/v SDS, 10% w/v ß-mercaptoethanol, 0.002% w/v bromophenol blue, pH 6.75.

Electrode buffer: 25 mM Tris, 0.192 M glycine and 0.1% w/v SDS, pH 8.3.

Transfer buffer for West blotting: 39 mM glycine, 48 mM Tris, 0.0375% w/v SDS, 20% v/v methanol, pH 8.3.

TBS-T buffer: 20 mM Tris base, 0.137 M sodium chloride, 0.1% v/v Triton X-100, pH 7.6.

Blocking buffer for blotting: 3% w/v BSA, 0.25% w/v gelatin in TBS-T buffer, pH 7.6.

Diethanolamine buffer: 0.1 M diethanolamine, 5 mM magnesium chloride hexahydrate, pH 9.5.

Coating buffer: 0.1 M sodium carbonate buffer, pH 9.6.

PBS-T buffer: 0.2 M sodium phosphate, 0.143 M sodium chloride, 0.1% v/v Triton X-100, pH 7.5.

Blocking buffer for ELISA: 3% w/v BSA, 0.25% w/v gelatin in PBS-T buffer, pH 7.5.

Substrate solution: 4 mM o-phenylenediamine, 25 mM citric acid, 50 mM Na_2HPO_4, 0.016% v/v hydrogen peroxide, pH 5.0.

2.5% glutaraldehyde: 2.5% v/v in 0.2 M sodium phosphate, pH 7.4.

1.0% Osmium tetroxide: 1.0% w/v OsO_4 in 0.2 M sodium phosphate, pH 7.4.

Homogenization buffer: 4 M Guanidinium thiocyanate; 25 mM sodium Citrate, pH 7.0; 0.5% w/v Sarkosyl, 293 ml water.

0.1% DEPC-treated water: 0.1% diethyl pyrocarbonate in water.

10x MOPS buffer: 200 mM Mops, pH 7.0; 50 mM sodium citrate, pH 7.0; 10 mM EDTA.

LB medium or Luria-Bertani Medium: 1% bacto-tryptone, 0.5% bacto-yeast extract, 1% sodium chloride, pH 7.0.

X-gal solution: 0.2 g X-gal dissolved in 10 ml dimethylformamide.

IPTG solution: 2 g of isopropylthio-ß-D-galactoside (IPTG) in 8 ml water.

LB-amp/X-gal/IPTG plate: 30-35 ml of hardened LB agar medium, 40 μl of X-gal solution, 4 μl of IPTG solution.

20x SSPE: salt sodium phosphate EDTA. 3 M NaCl, 0.2 M $NaH_2PO_4 \cdot H_2O$, 20 mM EDTA, pH 7.4.

Solution O: 1.25 M Tris-HCl, 0.125 M $MgCl_2$, pH 8.0. Stored at 4 °C.

Solution A: 1 ml solution O + 18 μl ß-mercaptoethanol + 5 μl dATP, 5 μl dTTP, 5 μl dGTP (each triphosphate previously dissolved in TE (3 mM Tris-HCl, 0.2 mM EDTA, pH 7.0) at a concentration of 0.1 M). Stored at -20 °C.

Solution B (2 M Hepes, titrated to pH 6.6 with 4 M NaOH. Stored at 4 °C.

Solution C (Hexadeoxyribonucleotides, evenly suspended in TE at 90 units/ml. Stored at -20 °C.

Oligo-labeling buffer (OLB buffer): mix solution A:B:C in a ratio of 100:250:150. Stored at -20 °C.

TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 7.4.

50x Denhardt's solution: 1% w/v polyvinylpyrrolidone (PVP-40), 1% w/v Ficoll, 1% w/v bovine serum albumin.

Hybridization buffer: 5x SSPE, 5x Denhardt's solution and 0.3% SDS.

2.3. Insect rearing and treatment

2.3.1. Insects

Schistocerca gregaria were obtained from a colony maintained at the insectary of Simon Fraser University. The insects were reared at 32°C under crowded conditions with a 16:8 light-dark cycle. Within 12 h after molting, freshly emerged nymphs or adults were removed from the colony and reared separately until sacrificed at the specified age for protein or RNA analysis. Individuals between 0 and 12 h after adult molting are referred to as day 0 adults, with each subsequent day representing an additional 24 h period. Individuals between 0 and 12 h after ecdysis

to the third-, forth- and fifth-instar nymphs are referred to as fresh 3rd, 4th and 5th-instar nymphs, with each subsequent day representing an additional 24 h period.

2.3.2. Precocene treatment

Ethoxy-precocene (100 μg in 5 μl acetone) was applied topically to the abdomen during the first day after ecdysis to the third-instar nymph. Control insects received 5 μl acetone in the same manner.

2.3.3. Methoprene treatment

Between 50 and 500 μg of methoprene, dissolved in 5 μl ethanol, were applied topically to the abdomen of 5th-instar nymphs either within 24 h or 3 to 4 days after ecdysis. Control insects received 5 μl ethanol in the same manner.

2.3.4. Azadirachtin treatment

Azadirachtin was dissolved in 70% aqueous ethanol to a concentration of 2 μg/μl and injected within 24 h after ecdysis between the 5th and 6th abdominal terga of 5th-instar nymphs (4 μg/insect). Control insects received 2 μl 70% ethanol in the same manner.

2.3.5. Ligation experiments

For studying the influence of substances from the head and abdomen on FABP synthesis in the adult stage, freshly emerged adults were ligated between head and prothorax as well as between metathorax and the first abdominal segment. These insects were injected daily with 100 μl of locust Ringer's solution (Chen *et al.*, 1978), but otherwise raised under the same condition as described above. Starved adults, fed only with water, were used as control.

2.3.6. Sustained flight

Sustained flight in a laboratory setting was achieved by mounting locusts in the front of a small household fan. The prothorax of the locusts was fixed with

contact cement to the tip of a wooden stick, which was attached to a stand in such a way that the mounted locust faced the fan in an angle of approx. 30 degrees. Mature locusts (20 days after adult ecdysis) were forced to fly continuously for 1 h, 2 h, 4 h, 8 h and 12 h; individuals that stopped to fly were gently stimulated to continue; if a locust was not able to fulfil the required flight duration, it was discarded. Following the experiments, the insects were sacrificed at specified time intervals of rest for RNA isolation, as described in 2.6.1.

2.3.7. Adipokinetic hormone II (AKH II) treatment

Adipokinetic hormone (AKH II) was dissolved in PBS and diluted to a concentration of 25 pmol/μl. All locusts used were 20-day old and mRNA was isolated 16 h later after treatment except when otherwise indicated. Doses between 10 and 500 pmol of AKH II (4-20 μl) were injected between the 5th and 6th abdominal terga. The equivalent volume of PBS was injected into control animals.

2.3.8. Low density lipophorin (LDLp) treatment

Low density lipophorin (LDLp) (1.4 mg) was injected into 20-day old adult locusts between the 5th and 6th abdominal segment and RNA was isolated 16 h later to examine the influence of LDLp on FABP gene expression.

2.4. Protein isolation and analysis

2.4.1. FABP isolation

Pure FABP was prepared from mature adult locusts as described by Haunerland and Chisholm (1990). FABP was desalted and lyophilized in aliquots. The freeze dried material was reconstituted in PBS immediately before use.

2.4.2. LDLp isolation

Adipokinetic hormone (250 pmol in 10 μl of PBS) was injected into fifteen 10-day old locusts between the 5th and 6th abdominal segment. After 45 min,

hemolymph was collected and diluted with PBS to 15 ml. Solid KBr (8.9 g) was added and stirred until dissolved. The solution was then brought with PBS to a final volume of 20 ml (44% KBr, density 1.31g/ml). After transfer to a 40 ml Quick-Seal tube (Beckman, Palo Alto, CA) the sample was overlaid with 20 ml PBS. The sealed tubes were centrifuged at 10 °C for 4 h at 242,000 g (Beckman L8-80 ultracentrifuge, VTi 50 rotor, 50,000 rpm). Following this step, the yellow band which is LDLp was collected and dialyzed against 10 mM ammonium carbonate buffer to remove KBr, and subsequently concentrated by evaporation in a Speed Vac (Savant).

2.4.3. Preparation of cytosol

Flight muscle was dissected out and minced in liquid nitrogen with mortar and pestle, then transferred to a Potter type homogenizer and homogenized in PBS in presence of 1 μl phenylmethyl sulfonylfluoride. The cytosolic fraction obtained by ultracentrifugation at 4 °C for 1 h at 200,000 g (Beckman L100 ultracentrifuge, TLA-45 rotor, 45,000 rpm) was kept frozen in aliquots at -80 °C after protein determination.

2.4.4. Protein determination

Total cytosolic protein was determined spectrophotometrically at 595 nm by the colorimetric assay according to Bradford (1976) with bovine immunoglobulin G as standard protein.

2.4.5. Protein Electrophoresis and Western Blotting

Denaturing SDS-polyacrylamide gel electrophoresis (15% T, 2.6% C; stacking gel: 2.5% T, 20% C) was performed according to Laemmli (1970) in a mighty small Mini gel unit (8 cm x 15 cm x 1.0 mm, Hoefer Scientific, San Francisco, CA). Sample aliquots were mixed with the same volume of sample buffer, and then boiled at 100 °C for 2 minutes. By using an electrophoresis power supply (Bio-Rad, Model 3000xi), electrophoresis was carried out at 25 mA, limits 300 V, 30 mA, 10 W

in electrode buffer until the marker dye had reached the bottom of the gel (1.5 h). The gels were stained with Coomassie brilliant blue R 250 (0.2% in solution with methanol:acetic acid:water = 4:1:5) for 3 h and destained by repeated washing in methanl:acetic acid:water (4:1:5) solution.

For electrophoretic transfer of proteins, unstained gels were blotted onto nitrocellulose on a semi-dry blotting apparatus (LKB Nova Blot), 1 h at 0.8 mA/cm^2. The semi-dry transfer technique uses filter papers soaked in transfer buffer as only buffer reservoir.

Immunological analysis was carried out at room temperature. After electrophoretic transfer, the nitrocellulose sheet was blocked with 3% BSA (w/v) and 0.25% gelatin (w/v) in TBS-T buffer for 1 h on a shaker, then the membrane was washed three times with TBS-T buffer for at least five minutes each wash. Anti-FABP antiserum was diluted 1:500 in TBS-T buffer. The membrane was incubated with the primary antibody solution for 1 h. Following three washes (5 min each) with TBS-T, the biotinylated goat anti-rabbit IgG, diluted 1:500 in TBS-T buffer, and 1:3000 diluted streptavidin-alkaline phosphatase conjugate were added. After 20 min incubation, washing (3 x 5 min), incubation with 1 drop each of NBT (Nitro-blue tetrazolium) and BCIP (5-Bromo-4-chloro-3-indolyl phosphate) solution for every 10 ml of diethanolamine buffer, the reaction was stopped by washing in distilled water. The membrane was dried between sheets of filterpaper and stored at -4 °C.

2.4.6. Enzyme-linked immunosorbent assay

Assays were carried out in microtiter plates. For each data point, equal amounts of cytosolic extracts from each of three or six individuals were combined into a single sample. Cytosolic extracts were diluted in PBS to a final concentration of 25 μg/ml, except for extracts form normal and precocious adults older than 5 days. Due to their high FABP, these samples were diluted to a concentration of 1 μg/ml.

Purified, lyophilized FABP was dissolved in PBS to a concentration of 0.1 μg/ml. Microtiter plate wells were loaded with 40, 20, 10, 5 and 2.5 μl of these solutions and filled with coating buffer to a final volume of 100 μl. Plates were gently shaken at room temperature for 1 h and then incubated overnight at 4 °C. After withdrawal of the samples, wells were washed 4 times with PBS-T buffer; unoccupied binding sites were blocked by 1 h incubation with 5% milk powder in the same buffer. Anti-FABP antiserum was diluted 1:5000 in PBS-T buffer. Each well was incubated with 100 μl of the primary antibody solution for 1 h at 37°C. Following four washes with 200 μl of PBS-T buffer, biotinylated goat anti-rabbit IgG diluted 1:500 with the same buffer was added. After 1 h incubation at 37 °C, washing, incubation with streptavidin conjugated horseradish peroxidase diluted to 1:500 in the same buffer (30 min , 37 °C) and an additional wash step, substrate solution was added. After 20 min incubation in the dark, the reaction was stopped by adding 25 μl of 2.5 M HCl to each well. Absorbance was read in a plate reader (BioTek, Burlington, VT) at 490 nm and 630 nm to correct for variations between individual wells.

2.5. Electron microscopic methods

2.5.1. Electron microscopy

Locust dorsolongitudinal flight muscle was dissected under 2.5% glutaraldehyde at 4 °C. Following 2 h fixation at room temperature, the tissue was rinsed in 0.2 M sodium phosphate buffer, pH 7.4 and post fixed in 1.0% OsO_4. The tissue was rinsed again in the buffer and dehydrated in graded ethanol, transferred to propylene oxide and infiltrated and embedded in Epon/Araldite. Thin sections (80-100 nm) were cut with a diamond knife and stained with 5% uranyl acetate and 2.6% lead citrate (w/v, in water).

2.5.2. Immunocytochemistry

For immunocytochemical processing, flight muscle was embedded in Lowicryl K4M, as described by Wang and Haunerland (1991). The muscle tissue was dissected under 2.5% glutaraldehyde at 4 °C and fixed in the same fixative for 1 h at room temperature. Tissue was rinsed in the buffer (3x10 min), followed by graded dehydration in 50, 70, 90, and twice 100% ethanol (10 min each). For infiltration, increasing concentrations of Lowicryl K4M in ethanol were used, starting with 50% K4M in ethanol (v/v) for 25 min, followed by 66% K4M in ethanol for 30 min, and finally twice 100% K4M for 45 min each. The infiltrated tissue was transferred to pre-cooled K4M in gelatin capsules. Polymerization of the resin was achieved by irradiation with ultraviolet light (360 nm, 20 W, distance 10 cm) at 4 °C for 1 h.

Sections were first treated with 0.2% polyclonal rabbit anti-FABP antiserum and then incubated in 1.0% goat anti-rabbit immunoglobulin G labeled with 10 nm gold particles. Afterwards the sections were counterstained lightly with uranyl acetate and lead citrate. Control experiments were performed under identical conditions except that pre-immune serum was used instead of polyclonal primary antiserum.

The number of gold particles located in mitochondria and associated with the myofibrils were counted in longitudinal sections that represented 30 μm^2 of muscle section (5 replicates each). With the previously determined mitochondria and myofibril volume of the muscle at each time point (Wang *et al.*, 1993.), the data were then transformed to particles per μm^2 of mitochondria or cytoplasm section, respectively. Similarly, gold particles per μm^2 of nucleus section were determined.

2.6. FABP mRNA assay

2.6.1. RNA isolation

Total RNA was isolated by a one step guanidinium thiocyanate/phenol/chloroform extraction method adapted from Chomczynski and

Sacchi (1987). Freshly excised flight muscle tissue, rinsed in ice cold PBS, was immediately homogenized in 2 ml homogenization buffer in presence of 14.4 μl ß-mercaptoethanol with a Potter type homogenizer. After the homogenate was transferred to Falcon 2063 (5 ml) snap-cap tube, 0.2 ml of 2 M NaOAc (pH 4.0), 2 ml H_2O saturated phenol and 0.4 ml of chloroform-isoamyl alcohol mixture (49:1) were added to the homogenate in sequence, mixed gently and thoroughly by inversion after each addition of a reagent. The tubes were shaken vigorously for 10 seconds and cooled on ice for 15 min. Samples were centrifuged at 10,000 g (9,500 rpm in a Sorvall SS-34 rotor) at 4 °C for 20 min. After centrifugation RNA was present in the aqueous phase whereas DNA and proteins were present in the interphase and phenol phase. The aqueous phase was removed to a fresh Falcon tube mixed with 1 volume of isopropanol, and subsequently placed at -20 °C for at least 1 h to precipitate RNA. The samples were spun for 20 min as before, then the supernatant was poured off and the resulting RNA pellet was resuspended in 0.5 ml of homogenization buffer with 3.6 μl ß-mercaptoethanol. Sequentially, the samples were transferred to a 1.5 ml microfuge tube, topped up with pre-cooled 95% ethanol, mixed well and placed at -20 °C, overnight. The samples were centrifuged in a microfuge at 15,000 g (13,000 rpm in Biofuge 13, Baxter) for 15 min, the supernatant was poured off and the pellet was washed with 70% ethanol (ethanol : 0.1% DEPC treated water, 7:3) by spinning down for 5 min in the same microfuge. Following this step, the pellet was dissolved in 500 μl DEPC-treated water, mixed with 31.3 μl of 8.0 M $LiCl_2$, topped up with pre-cooled 95% ethanol and placed at -80 °C for 15 min. Sedimentation in the microfuge for 15 min was again performed, and the pellet was washed with 70% ethanol by spinning in the same microfuge for 5 min, resuspended in 35 μl of DEPC-treated water and stored at -80 °C.

For the spectrophotometric quantification of the amount of RNA, readings were taken at 260 nm and 280 nm (Ultrospec III, Pharmacia LKB). The ratio

between the readings at 260 nm and 280 nm (OD_{260} /OD_{280}) provides an estimate of the purity of RNA and pure preparations of RNA have OD_{260} /OD_{280} values of 2.0. Typically, RNA yields were about 100 μg per muscle.

For each data point, equal amounts of total RNA from each of three individuals were combined into a single sample and diluted to a final concentration of 4.5-6.5 μg RNA/μl. The integrity of each sample was confirmed by gel electrophoresis.

2.6.2. RNA electrophoresis and Northern blotting

With the electrophoresis power supply (Bio-Rad, Model 3000xi) set at 40 V, limits 300 V, 45 mA, 10 W, total RNA (5 μg) was separated by electrophoresis for 3 h in 1x MOPS buffer on a small (10 cm x 10 cm), 1.1% agarose gel containing 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, 2.2 M formaldehyde, pH 7.0. The RNA was visualized by adding ethidium bromide to the sample prior to electrophoresis. Gels were washed with water (2x30 min) and 25 mM sodium phosphate, pH 6.5 (1x30 min) prior to unidirectional capillary blotting to Genescreen Nylon membrane.

2.6.3. Probe preparation and labelling

An M-FABP DNA probe was obtained from a 523-bp cDNA cloned in a Bluescript vector (Price *et al.*, 1992). Following transformation of *E coli* (strain DH5α), recombinants were selected on LB-amp/X-gal/IPTG plates. White-coloured colonies were grown in LB medium containing 60 μg/ml ampicillin overnight 37 °C. Plasmid DNA was isolated using Magic Miniprep columns. For probe preparation, approximately 20 μg of plasmid DNA was double digested with *DraI* and *NarI* restriction endonucleases. The resulting 412 bp fragment contained only FABP cDNA with no flanking sequence. The probe fragment was isolated by electrophoresis in a low melting point agarose gel and eluted through a home made

mini column. A 650 bp *Pisaster ochraceus* ß-actin probe (Kovesdi *et al.*, 1984) was a gift from Dr. M.J. Smith (Simon Fraser University).

The DNA probe was labeled using a random priming method (Feinberg and Vogelstein, 1983, 1984). A 25-μl reaction mixture was prepared by mixing 8 μl H_2O, 10 μl oligo-labeling buffer (OLB buffer), 2 μl of (10 mg/ml) bovine serum albumin and 4 μl of [α-^{32}P]dCTP (40 μCi, specific activity 800 mCi/mmol). Approximately 50 ng of DNA probe in 5 μl agrose and 20 μl H_2O was heated to 100 °C for 10 min, immediately cooled to 0 °C in an ice bath for 5 min, and added to the reaction mixture. Subsequently, the large fragment of *E. coli* DNA polymerase I (BRL No. 8012) was added. Polymerization reaches a plateau in 3 h. The reaction was stopped by addition of 50 μl TE buffer and purified on a home made small gel filtration column of Sephadex G25. Specific activities of the probe ranged from 1 to 5 x 10^8 cpm/μg.

2.6.4. Hybridization and quantification

Prior to hybridization, membranes were equilibrated at 62 °C for 4 h in hybridization buffer. The labeled probe was denatured at 100 °C for 10 min, placed on ice for 5 min and added to 10 ml of the hybridization buffer. Hybridization cocktails typically contained 1.0 - 5.0 x 10^6 cpm/ml. Hybridizations were carried out overnight at 62 °C. After hybridization, the membrane was washed twice with 1x SSPE containing 0.3% SDS, once in 0.1x SSPE containing 0.3% SDS at 62 °C and rinse with 0.1x SSPE. Membranes were exposed to preflashed Kodak XK-1 autoradiographic film or X-OMAT AR film with intensifying screens at -80 °C for 12 h. Quantification was achieved by scintillation counting the radioactivity of actin- and FABP-bands. FABP mRNA was normalized against the slight variations in the actin signal.

CHAPTER 3. DEVELOPMENTAL CHANGES OF FABP CONCENTRATION AND INTRACELLULAR DISTRIBUTION

3.1. Introduction

One of the strongest argument made for the involvement of muscle FABP in fatty acid transport is that the intracellular concentration of FABP generally reflects the extent of lipid utilization. Reflecting the lipid dependency of a particular mammalian muscle, FABP concentrations between 0 and 5% of the cytosolic protein have been reported (Kaikaus *et al.*, 1990). Results from locusts flight muscle gave additional support to this hypothesis. Locusts maintain very high metabolic rates during sustained flight which is fueled exclusively by the oxidation of lipids; preliminary data revealed that the concentration of FABP in mature locusts reached at least 10% of the total cytosolic proteins. Moreover, locust flight activity is limited to mature adults, and thus FABP should not be needed in nymphs. Indeed, FABP was not detected in 5th-instar nymphs (Haunerland and Chisholm, 1990).

While immature locusts do not have wings, the flight muscles develop already during the fifth larval instar and the early part of the adult stage. Four phases of development were distinguished by Brosemer and Coworkers (1963) in *Locusta migratoria*, based on weight, morphology and biochemical parameters: muscle growth in the last nymphal stage, the molting interval, a phase of differentiation during the first 4 days of the adult stage, and a final phase of growth. Locust FABP is absent before metamorphosis, but present at extremely high concentrations in mature muscle, thus, its synthesis must be initiated and maintained during the differentiation and final growth phase. Since the titer changes of different hormones such as ecdysteroids and juvenile hormone have been well studied in both male and female locusts during these stages, the corresponding studies of FABP concentration changes will certainly give us some clues about the possible hormonal control of

FABP synthesis. This chapter describes the quantitative analysis of FABP synthesis and its intracelluar distribution through the maturation period of the locust.

3.2. Results

3.2.1. Developmental changes of FABP content

In order to investigate the developmental changes of FABP content, the cytosolic proteins of flight muscle were analyzed during first 15 days of adult by SDS-PAGE and FABP was detected by Western blotting with anti-FABP antibodies (Fig. 3-1). In flight muscle of newly emerged adults, FABP was not detectable. It became first visible three days after metamorphosis and increased rapidly in subsequent days to become a major flight muscle protein.

Quantification of these changes was achieved by an enzyme linked immunosorbent assay (ELISA). The results are shown in Figure 3-2. When expressed relative to the amount of total cytosolic proteins, the FABP concentration of both male and female insects increased dramatically during muscle growth. No significant differences in FABP concentration was detected between both sexes. Immediately after metamorphosis, the FABP concentration was below the detection limit of the assay. Two days later, however, FABP already comprised 0.65% of the total cytosolic proteins, and its concentration nearly tripled during the next day. Between day 4 and 11, its concentration rose rapidly. After two weeks, however, little change in the FABP concentration could be seen; its value appeared to be stable near 18% of the total cytosolic proteins for several weeks. In 4-week old adults, the FABP concentration was still 17.7 ± 2.5% (mean of 6 determination ± standard deviation).

3.2.2. Intracellular distribution of FABP in flight muscle

The intracellular distribution of FABP during the first 15 days of adult life was determined by immunocytochemical method with anti-FABP antibodies and gold labeled secondary antibodies in glutaraldehyde fixed tissues. At the beginning of

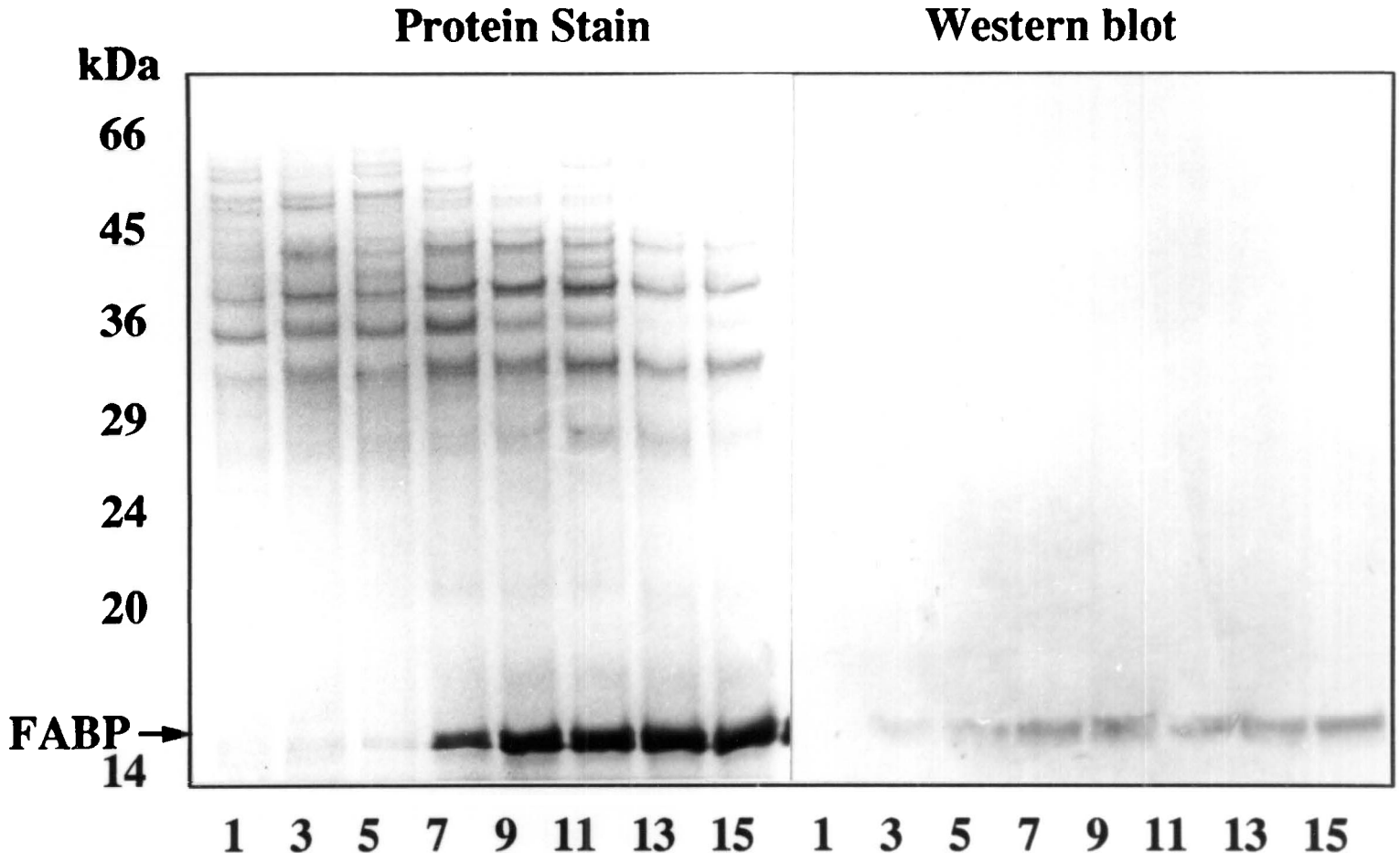

Fig. 3-1. FABP concentration changes during adult maturation
Cytosolic proteins were separated by SDS-PAGE as described in Materials and Methods (2.4.5.). Left: coomassie stain. Right: Western blot processed with anti-FABP antibodies

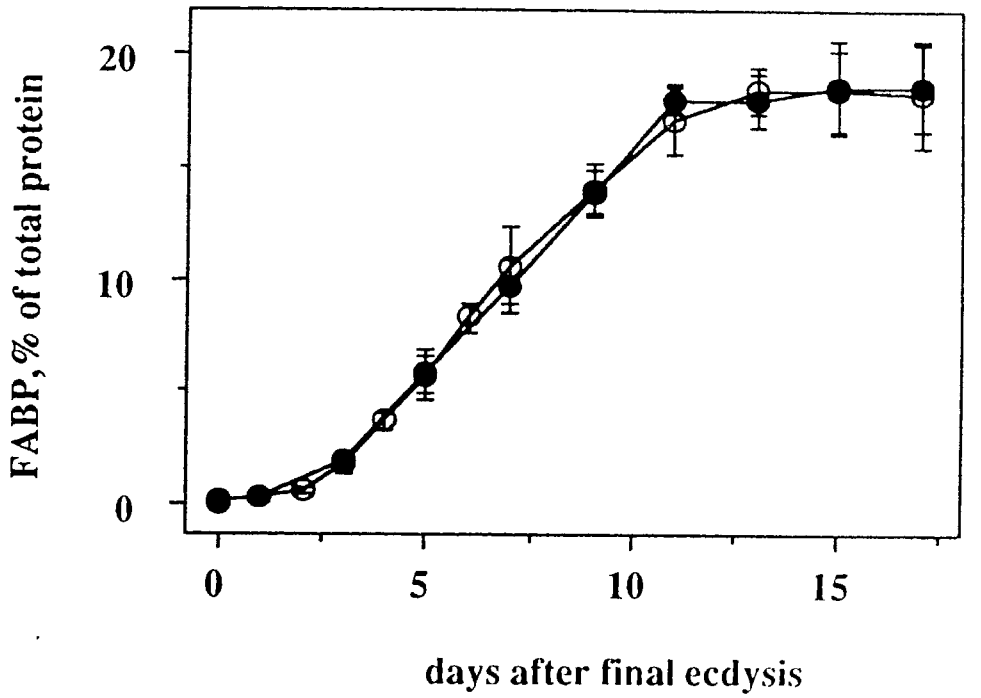

Fig. 3-2. Muscle FABP concentration in male and female locusts.
Intracellular FABP was determined by an ELISA as described in Materials and Methods (2.4.6.). FABP concentration is expressed as percentage of total soluble cytosol proteins. Values are the mean of 3 ± standard deviation. –●– male insects; –○– female insects.

the adult stage, no specific binding of antibodies was observed (Fig. 3-3); only slightly more gold particles were bound to muscle sections after incubation with anti-FABP antiserum than after incubation with pre-immune serum. During the next 10 days of adult life, the number of specifically bound gold particles gradually increased until it reached a maximum at about day 15. The sections also revealed the intracellular distribution of FABP. The protein seemed to be associated mostly with the myofibrils, with few gold particles binding to mitochondria. However, while the cytosolic concentration greatly increased with muscle growth and development, little change could be seen in the mitochondria. A similar increase as in the cytosol, however, was visible in muscle nuclei (Fig. 3-4). Numerous gold particles were present in nuclei of fully grown insects, located primarily in the less intensely stained euchromatin area.

Quantitative evaluation of the FABP distribution was achieved through counting of gold particles on electron micrographs (Fig. 3-5). After background subtraction, the number of gold particles found per μm^2 of mitochondria cross-section is slightly higher after incubation with anti-FABP antiserum than after treatment with pre-immune serum; this number however did not change significantly during development. In contrast, between day 1 and 15 the number of gold particles per μm^2 increased more than 40-fold for myofibrils, and 25-fold for nuclei.

3.3. Discussion

As revealed by ELISA, FABP is the most abundant soluble muscle protein in fully developed adult locusts, comprising 18% of the total cytosolic protein. However, FABP is detectable only at very low level after ecdysis and its concentration increases drastically during next two weeks. The patterns of FABP synthesis during adulthood are very similar in male and female locusts.

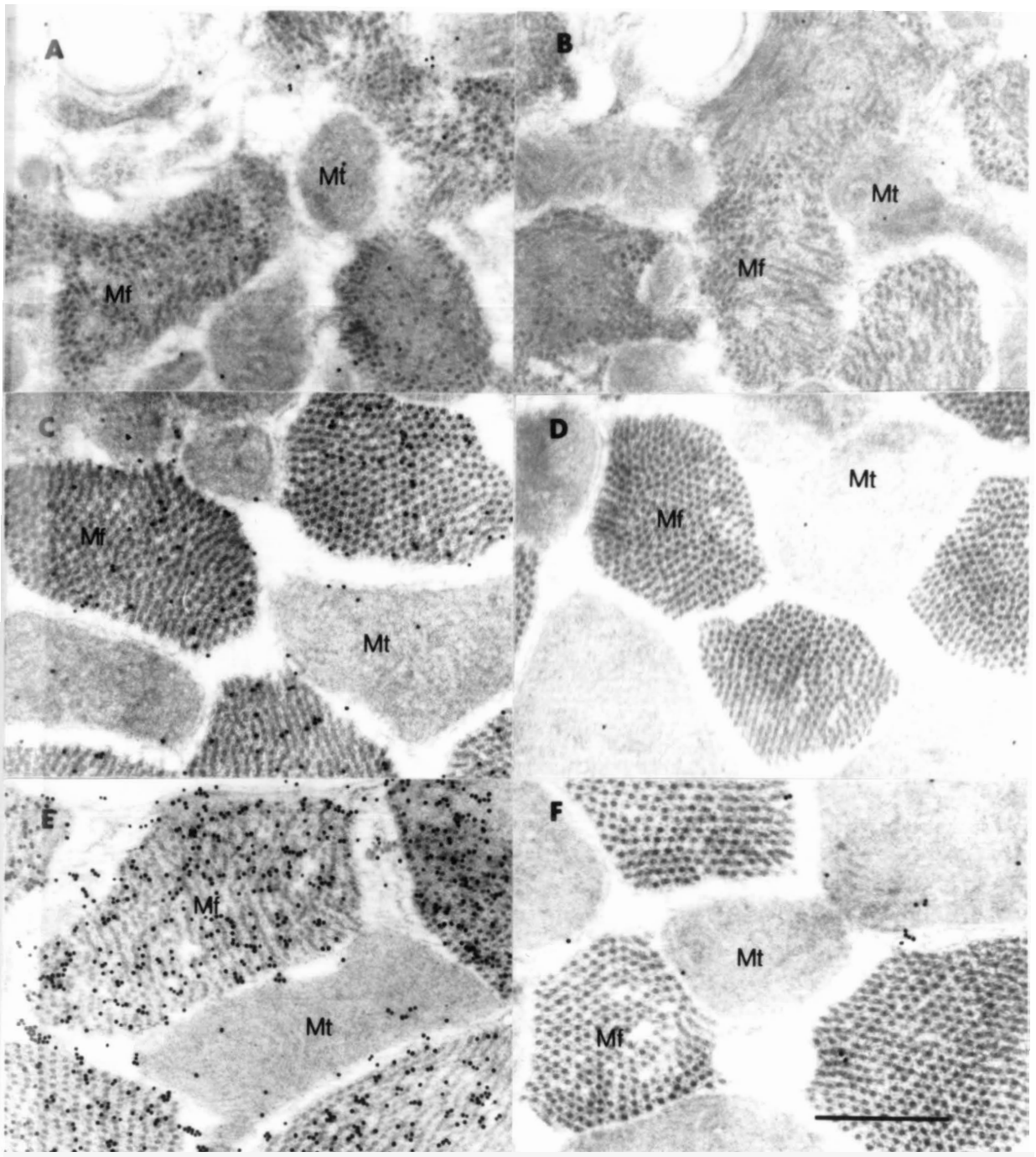

Fig. 3-3. FABP detection in muscle cross-sections of locust adults.
Freshly excised muscles were fixed in glutaraldehyde, embedded in Lowicryl K4M, and stained with primary antiserum and gold labeled secondary antibodies as described in Materials and Methods (2.5.2.). A, B: 1 day after ecdysis; C, D: 7 days after ecdysis; E, F: 15 days after ecdysis. A, C, E: primary antiserum, anti-FABP antiserum. B, D, F: control; primary antiserum, pre-immune serum. Mt = mitochondrion; Mf = myofibril. Space bar = 0.5 μm.

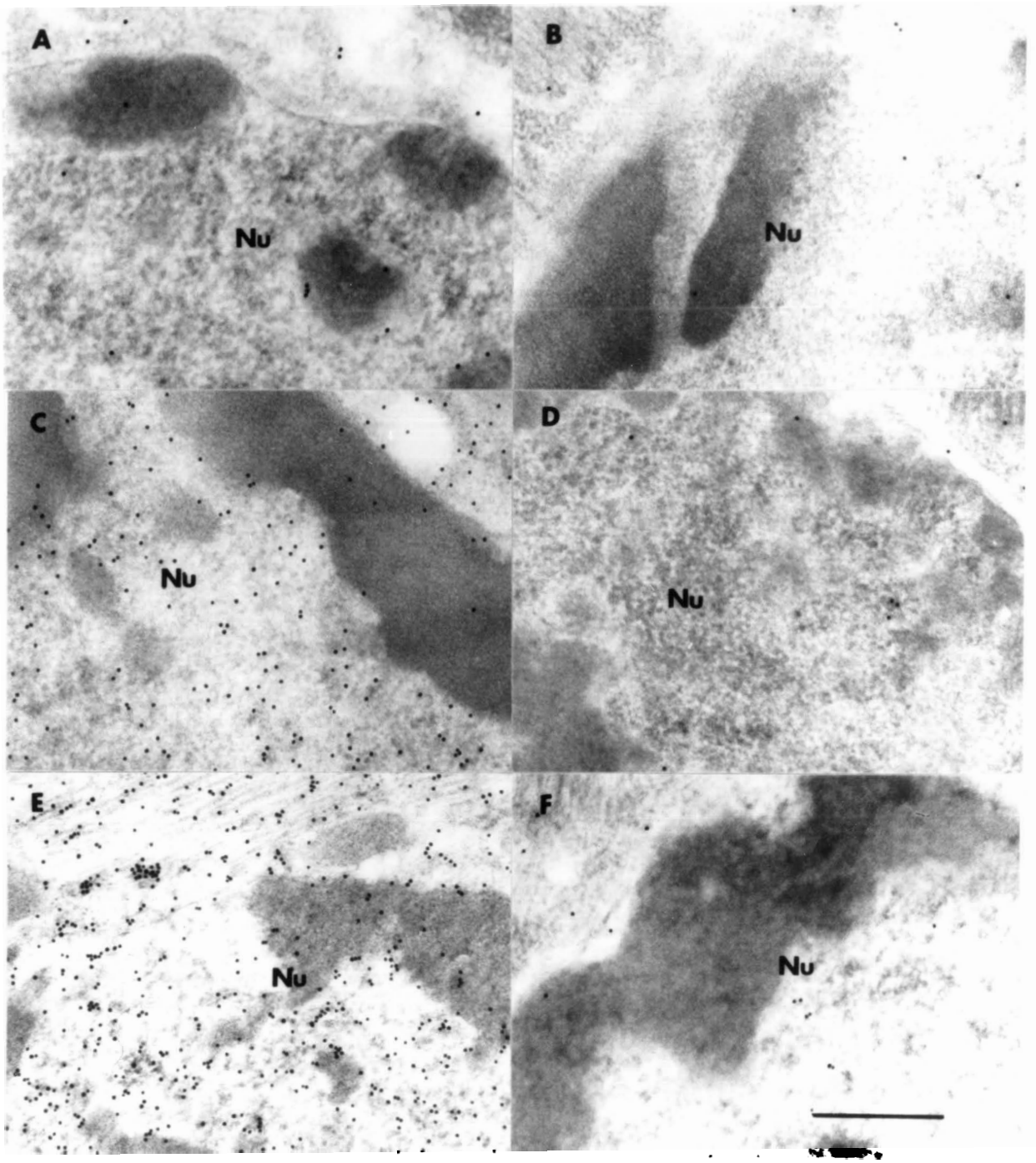

Fig. 3-4. FABP detection in nucleus cross-sections of locust adults.
Freshly excised muscles were fixed in glutaraldehyde, embedded in Lowicryl K4M, and stained with primary antiserum and gold labeled secondary antibodies as described in Materials and Methods (2.5.2.). A, B: 1 day after ecdysis; C, D: 7 days after ecdysis; E, F: 15 days after ecdysis. A, C, E: primary antiserum, anti-FABP antiserum. B, D, F: control; primary antiserum, pre-immune serum. Nu = nucleus. Space bar = 0.5 μm.

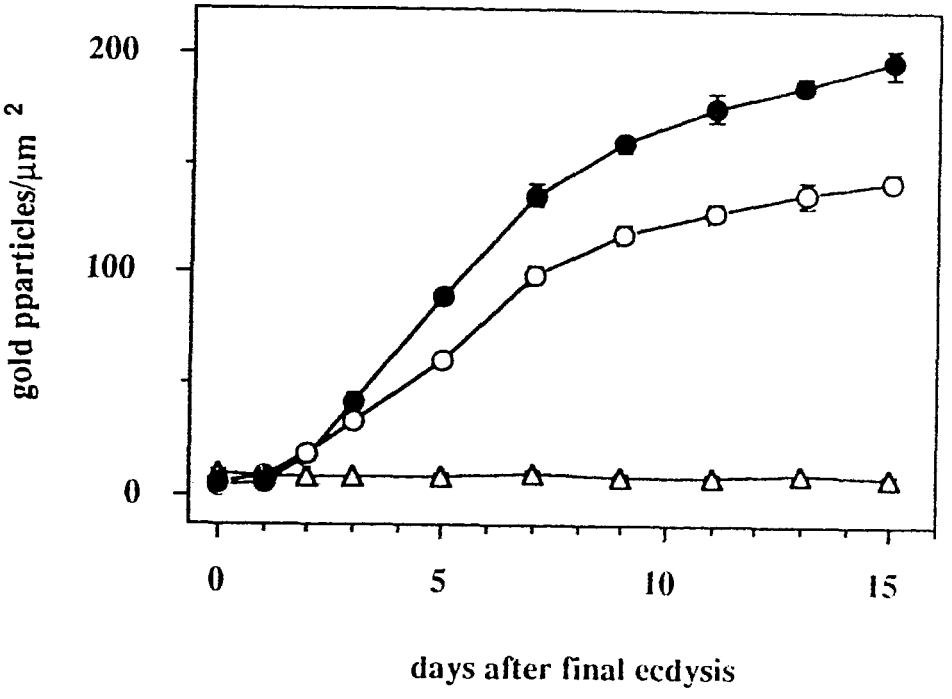

Fig. 3-5. Subcellular distribution of FABP in normal adults.
Gold label on electron micrographs was counted and expressed as gold particles per area of subcellular structure, as described in Materials and Methods (2.5.3.). Values present the mean of 10 ± standard deviation.-O-cytosol; -●-nucleus;-Δ- mitochondria.

The high value of FABP content in flight muscle of fully developed adult locusts seems to reflect the high metabolic rate that can be achieved by these muscles. Locust flight muscle uses fatty acids exclusively to fuel sustained flight. During flight, fatty acids are oxidized at a rate of 0.9 μmol min^{-1} g^{-1} of muscle (Crabtree and Newsholme, 1975) which is three times as high as that of mammalian heart muscle. It is also found that the FABP concentration in locust flight muscle is more than three times as high as in mammalian heart.

Parallel to the increase in the FABP concentration the insect develops its ability to engage in flight activity. After the final molt, the young adult is initially only capable of weak, descending flight from bushes. A week later, however, the flight performance improves rapidly. Strong flight becomes possible, prior to sexual maturation at three weeks, in which much of the long-range migration occurs (Rainy, 1989). It is possible that the low amount of FABP during the first week of adulthood poses limitations on the flight ability. The changes in FABP concentration observed during development are consistent with its proposed role in fatty acid metabolism.

A similar relationship between FABP content and the capacity of tissues to utilize fatty acids as energy substrate has also been observed in vertebrate muscle (Heuckeroth *et al.*, 1987; Kaikaus *et al.*, 1990). It has been proposed that FABP is a determinant of the flux through the fatty acid oxidation pathway; on the other hand, it may be possible that the capacity to oxidize fatty acids controls the cytosolic content of FABP (Glatz and Van der Vusse, 1989). The results of the present study suggest that FABP may be a limiting factor for ß-oxidation, since all other enzymes involved fatty acid metabolism are already present at substantial concentrations before metamorphosis (Beenakkers, 1964). In contrast, FABP is virtually absent in nymphs and young adults and its concentration reaches a substantial level at the same time when sustained flight becomes possible.

The total FABP concentration was also quantified by immunocytochemical gold labeling techniques in electron microscopic sections. The result obtained here indicates that during the first two weeks of adulthood, the number of gold particles associated with myofibrils increases 40-fold between day 1 and day 15, which resembles closely the data from the ELISA which shows an increase of 60-fold during the same period.

In addition to the developmental changes of total cytosolic FABP concentration, EM also reveals the intracellular distribution of FABP. FABP was found primarily in the cytosol, associated with the myofibrils. A similar number of gold particles was also present in nuclei, mostly located in the lighter staining euchromatin area. While similar results have been reported for mammalian muscle FABP (Fournier and Rahmin, 1985; Börchers *et al.*, 1989), it is not generally accepted that FABP is indeed present in the nuclei. It was suggested that possible crossreactivity between FABP antibodies and an unrelated nuclear protein could have led to the results. In the locust, however, the amount of FABP found in nuclei increases in a similar way as the concentration of cytosolic FABP during development. This strongly suggests that the nuclear FABP is indeed the same protein, which has entered the nucleus from the cytosol. Free exchange between nucleus and cytosol is not unlikely for a 15,000 Da protein that should easily pass through nuclear pores (Dingwall and Laskey, 1986).

Whether or not FABP is present in mitochondria is even more controversial. Some reports described a distribution of FABP throughout the cytosol, but it was not associated with mitochondria of rat heart (Crisman *et al.*, 1987; Paulussen *et al.*, 1989). Others found, however, small amounts of heart FABP within mitochondria of bovine and rat heart (Fournier and Rahmin, 1985; Börchers *et al.*, 1989). Recently, Unterberg *et al.*, (1990) reported the isolation of mitochondrial FABP from bovine heart. This mitochondria FABP is identical to the pI 4.9-cytosolic FABP and differs

from pI 5.1-cytosolic FABP in only one amino acid: mitochondrial FABP contain Asp^{98}, whereas in pI 5.1-cytosolic FABP this position is occupied by Asn^{98}. However, it remains unclear how the protein was imported into mitochondria since the FABP transcripts does not contain a mitochondrial targeting sequence. Therefore, others have questioned these results. From the data presented here, it is highly unlikely that FABP is present in locust mitochondria. Although mitochondria bind slightly more gold particles when incubated with FABP antiserum than after preimmune serum treatment, the number of particles did not increase during development, while cytosolic FABP increased more than 40-fold. Thus, the gold particles in mitochondria may be caused by nonspecific interactions between the antiserum and other mitochondrial antigens.

Since the synthesis of FABP appears to be limited to a certain developmental stage (adult), it must be under the control of the two major hormones, ecdysteroids and juvenile hormones (JH), which control insect development (Wigglesworth, 1985). However, ecdysteroids were only found in female adult locusts (Lagueux *et al.*, 1977; Gande *et al.*, 1979), while the pattern of FABP synthesis are similar in both male and female adult locusts, therefore, it is unlikely that FABP synthesis is influenced by the ecdysteroid titers in adult stages. On the other hand, the parallelism of FABP concentration and JH titer during the adult stage suggests a possible role of adult JH in the control of FABP synthesis. Furthermore, in *Locusta migratoria*, a related locust species, the development of the flight muscle and the synthesis of enzymes involved in energy metabolism is strongly influenced by the ecdysteroid and JH titers in nymphal stages (Beenakkers and Van der Broek, 1976; Van den Hondel-Franken and Flight, 1981; Cotten and Anstee, 1990). It is therefore possible that FABP itself, as a major flight muscle proteins, is also under similar endocrine control mechanisms. More experiments designed to elucidate endocrine control of FABP synthesis are described in the next chapter.

CHAPTER 4. ENDOCRINE CONTROL OF THE INITIATION OF FATTY ACID BINDING PROTEIN BIOSYNTHESIS

4.1. Introduction

The developmental profile of FABP synthesis presented in the previous chapter indicates that FABP is normally only found in adult locusts. Its synthesis commences shortly after metamorphosis and continues for about two weeks; at this time, a high FABP concentration has been reached which remains constant for at least a month. Although adult ecdysteroids do not influence FABP synthesis, it is possible that adult JH is exerting direct regulatory action on the synthesis of FABP, since the cytosolic concentration of FABP changes in a similar manner as the hemolymph titer of juvenile hormone (Injeyan and Tobe, 1981). Moreover, it is probable that FABP synthesis is influenced in some way by juvenile hormone and ecdysteroids in the nymphal stages because the interplay of these two hormones in nymphal stages control metamorphosis and locust FABP is only present after metamorphosis.

To investigate physiological actions of these hormones, insects can be treated with both hormone analogs and hormone antagonists. Since the presence of juvenile hormone prevents metamorphosis, treatment of last instar larva of many insect species with methoprene, a potent juvenile hormone analog, has resulted in an additional, supernumerary larval stage (Van der Horst *et al.*, 1989; Cotton and Anstee, 1990, 1991). In contrast, when insects in earlier larval stages had been treated with the anti-juvenile hormone precocene, which prevents juvenile hormone biosynthesis by degenerating the corpora allata, metamorphosis usually took place at the next molt, leading to the formation of precocious adults (Bowers, *et al.*, 1976; Bowers, 1985; Pener *et al.*, 1978; Unnithan *et al.*, 1980). The molting process itself is initiated by ecdysone. Thus, application of an ecdysone antagonist can be used to

prevent molting. Such an antagonist is azadirachtin, and treatment of last instar larvae can prolong the larval stage by many days, producing so called over-aged larva (Rembold, 1984; Koul and Isman, 1991). Since each of these three groups of insects has specific pattern of hormone expression, the hormone effects can be easily examined.

These experimental approaches should also be useful for studying the influence of metamorphosis controlling hormones and anti-hormones on the synthesis of FABP. This chapter describes the generation of precocious adults, supernumerary nymphs, and over-aged nymphs of *S. gregaria* and correlates FABP synthesis with muscle development.

4.2. Results

4.2.1. FABP biosynthesis and intracellular distribution in precocene-induced precocious adults

Precocious metamorphosis of *S. gregaria* was induced by treatment of both third or fourth instar locusts with ethoxy-precocene. While 98.3% of all precocene treated third instar nymphs emerged as precocious adults, only 20.0% of the treated 4th-instar nymphs underwent precocious metamorphosis (Table 4-1). Therefore, precocious adults obtained from third instar nymphs were chosen for FABP analysis. Analysis of the FABP concentration during the two weeks following ecdysis revealed that the synthesis of FABP is very similar in normal and precocious adults (Fig. 4-1). The first occurrence of FABP was delayed by one or two days, and the maximal FABP concentration (21.0%) was slightly higher than in normal adults (18.5%).

The intracellular distribution of FABP in flight muscles of these insects was analyzed in electron micrographs of immunogold labeled sections. As in normal adults, FABP in precocious insects was also confined to cytosol and nucleus; no FABP was found in mitochondria (Fig. 4-2). Quantitative evaluation of the gold

Table 4-1: Effects of ethoxy-precocene on metamorphosis of 3rd and 4th-instar nymphs of S. gregaria. Fresh 3rd and 4th-instar nymphs were topically applied with ethoxy-precocene.

Nymphal instar	Number of treated insect	Mortality [Number]	[%]	Duration of the instar	Precocious adults [Number]	[%]	Normal adults [Number]	[%]
3rd	120	2	1.7	8.1 ±0.8	118	98.3	0	0
4th	50	2	4.0	5.9 ±0.6	10	20.0	38	76.0

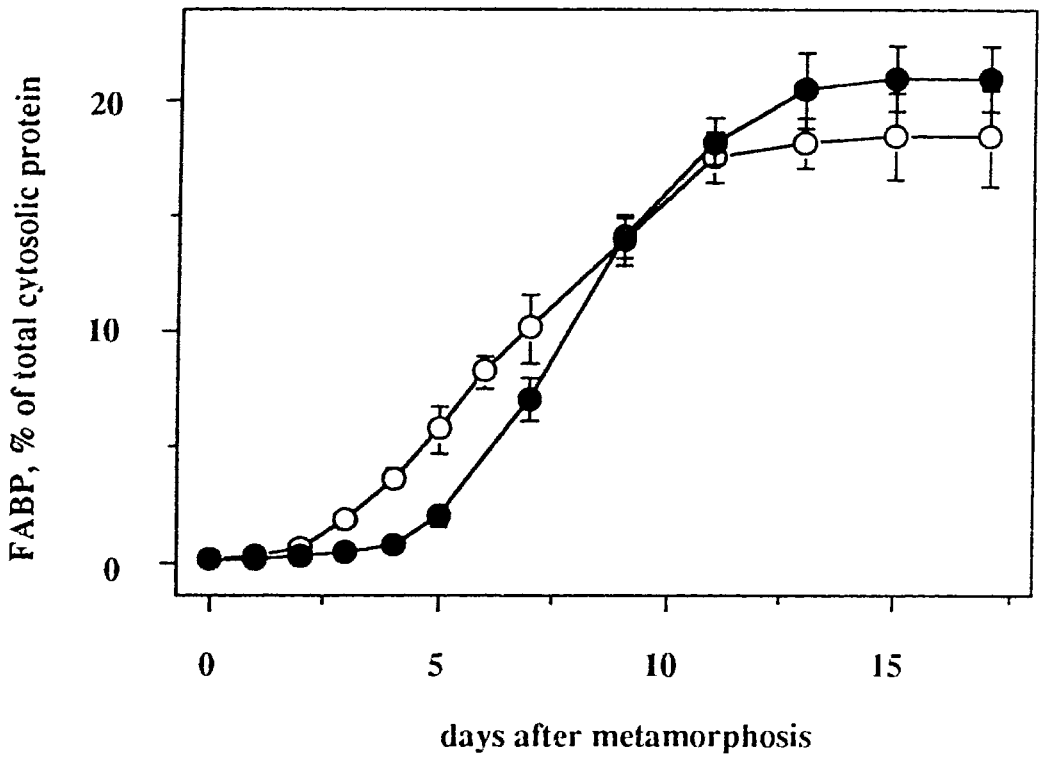

Fig. 4-1. Muscle FABP concentration in precocious and normal adults.
Intracellular FABP was determined by an ELISA as described in Materials and Methods (2.4.6.). FABP concentration is expressed as percentage of total soluble cytosol proteins. Values are the mean of 6 ± standard deviation.–●– precocious adults;–○– normal adults.

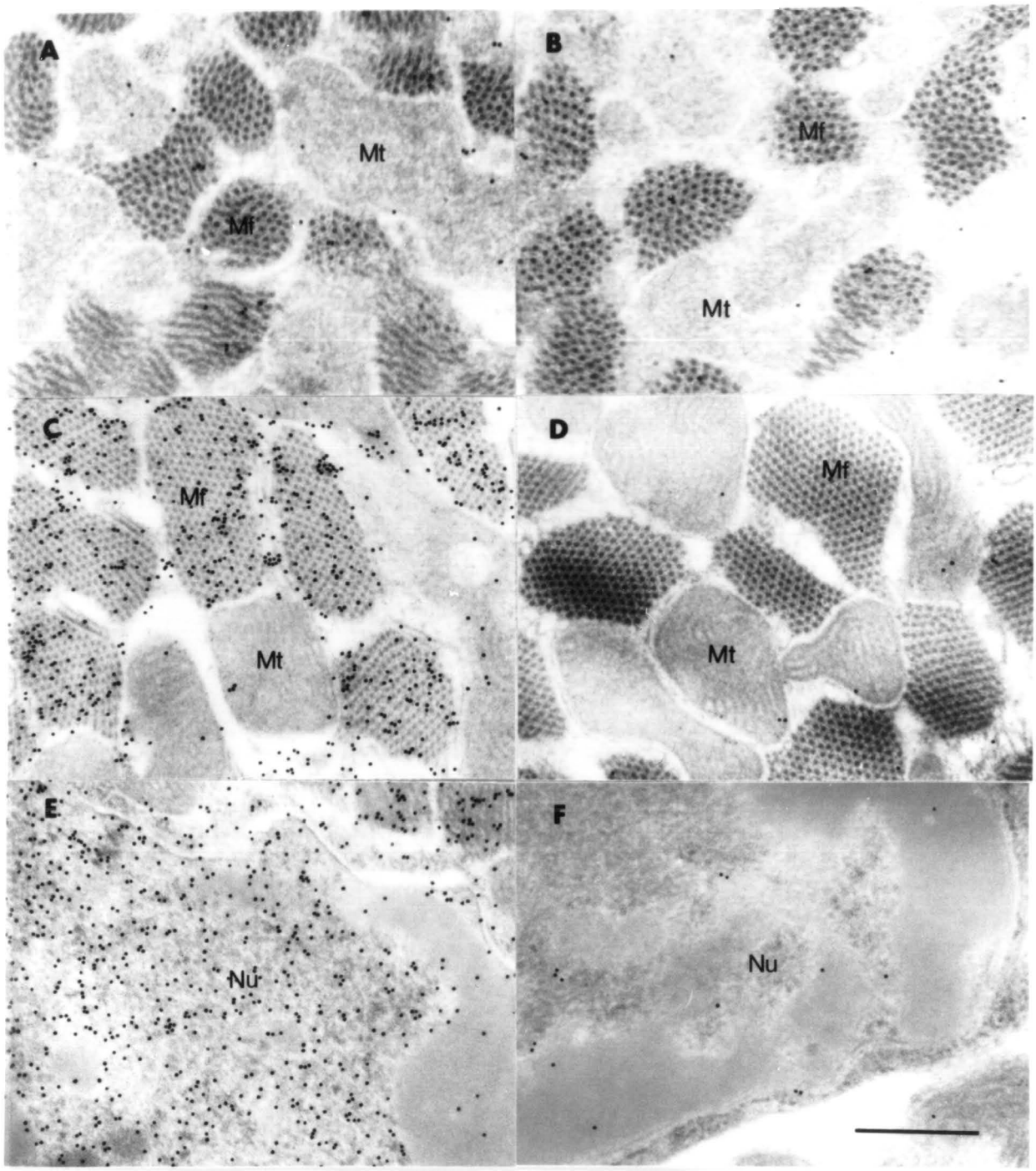

Fig. 4-2. FABP detection in muscle cross-sections of precocious adults.
Freshly excised muscles were fixed in glutaraldehyde, embedded in Lowicryl K4M, and stained with primary antiserum and gold labeled secondary antibodies as described in Materials and Methods (2.5.2.). A, B: myofibrils, 1 day after ecdysis; C, D: myofibrils, 15 days after ecdysis; E, F: nucleus, 15 days after ecdysis. A, C, E: primary antiserum, anti-FABP antiserum. B, D, F: control; primary antiserum, pre-immune serum. Mt = mitochondrion; Mf = myofibril; Nu = nucleus. Space bar = 0.5 μm.

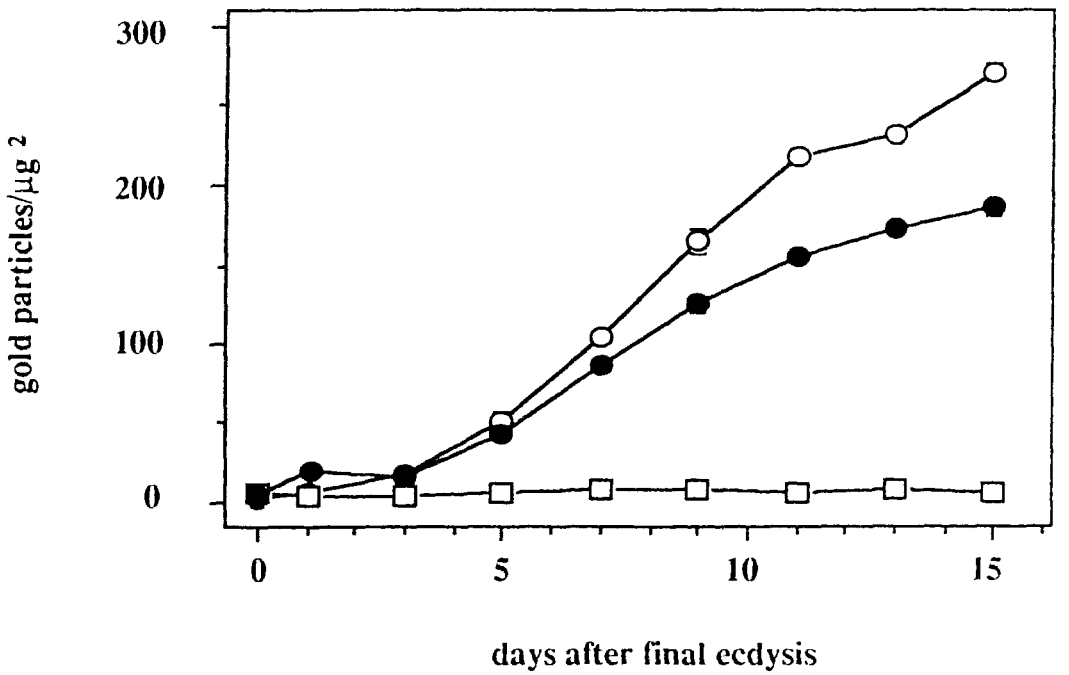

Fig. 4-3. Subcellular distribution of FABP in precocious adults.
Gold label on electron micrographs was counted and expressed as gold particles per area of subcellular structure, as described in Materials and Methods (2.5.3.). Values present the mean of 10 ± standard deviation.-●-cytosol;-○-nucleus; -□- mitochondria.

distribution through the first two weeks of adulthood revealed a rapid increase of FABP in both nuclei and cytosol (Fig. 4-3), also reaching a slightly higher value than that found for normal adults (Fig. 4-3, 3-5).

4.2.2. FABP biosynthesis in methoprene-induced supernumerary nymphs

Metamorphosis could be prevented through the application of juvenile hormone analog at the beginning of the fifth nymphal stage. When locusts were treated with 500 μg methoprene within 24 h after molting to the fifth instar, the subsequent molt again was a nymphal molt, leading to an additional nymphal instar. These supernumerary nymphs exhibited some nymphal pigmentation such as black pigment in dorsal parts of head, pronotum, thorax and abdomen, but the whole body was more green than a typical nymph. Thorax shape was similar to normal nymphs, but the wings were intermediate between nymphs and adults. The flight muscle itself appeared colorless instead of pink or brown. The insects normally died after 12 to 15 days in an unsuccessful attempt to molt again. In supernumerary nymphs, no FABP could be detected at any time by ELISA (Fig. 4-4) and by immunogold labeling methods (Fig. 4-5a,b). However, nymphs treated 3 or 4 days after molting into the fifth instar developed to adults instead of supernumerary nymphs. These insects were distinguished from normal adults only by different cuticle coloration (green instead of pink). Furthermore, FABP was synthesized in these adults in a similar way to control animals (Table 4-2). To induce a supernumerary molt in locusts, high doses of methoprene are normally required. Although the treatment was identical to that used by other investigators (Cotton and Anstee, 1990, 1991), it was necessary to eliminate the possibility of nonphysiological responses of the locust due to a large excess of juvenoid. We therefore analyzed the effects of lower doses of methoprene on metamorphosis and the synthesis of FABP (Table 4-3). Identical results were obtained after application of 500 or 200 μg of methoprene. All surviving animals

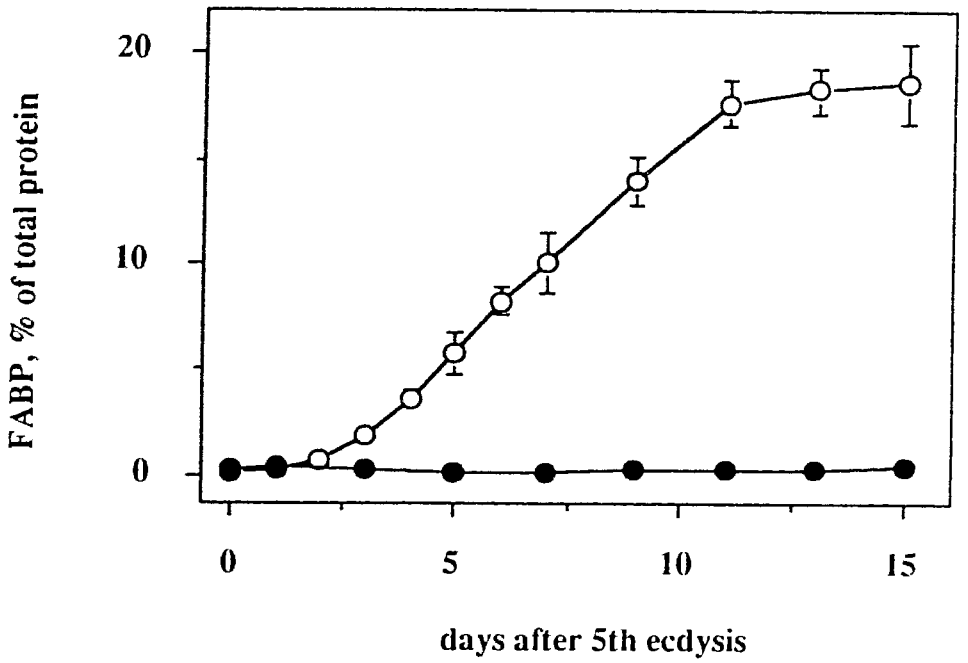

Fig. 4-4. Muscle FABP concentration in supernumerary nymphs and normal adults. Intracellular FABP was determined by an ELISA as described in Materials and Methods (2.4.6.). FABP concentration is expressed as percentage of total soluble cytosol proteins. Values are the mean of 6 ± standard deviation. -●- supernumerary nymphs; -○- normal adults.

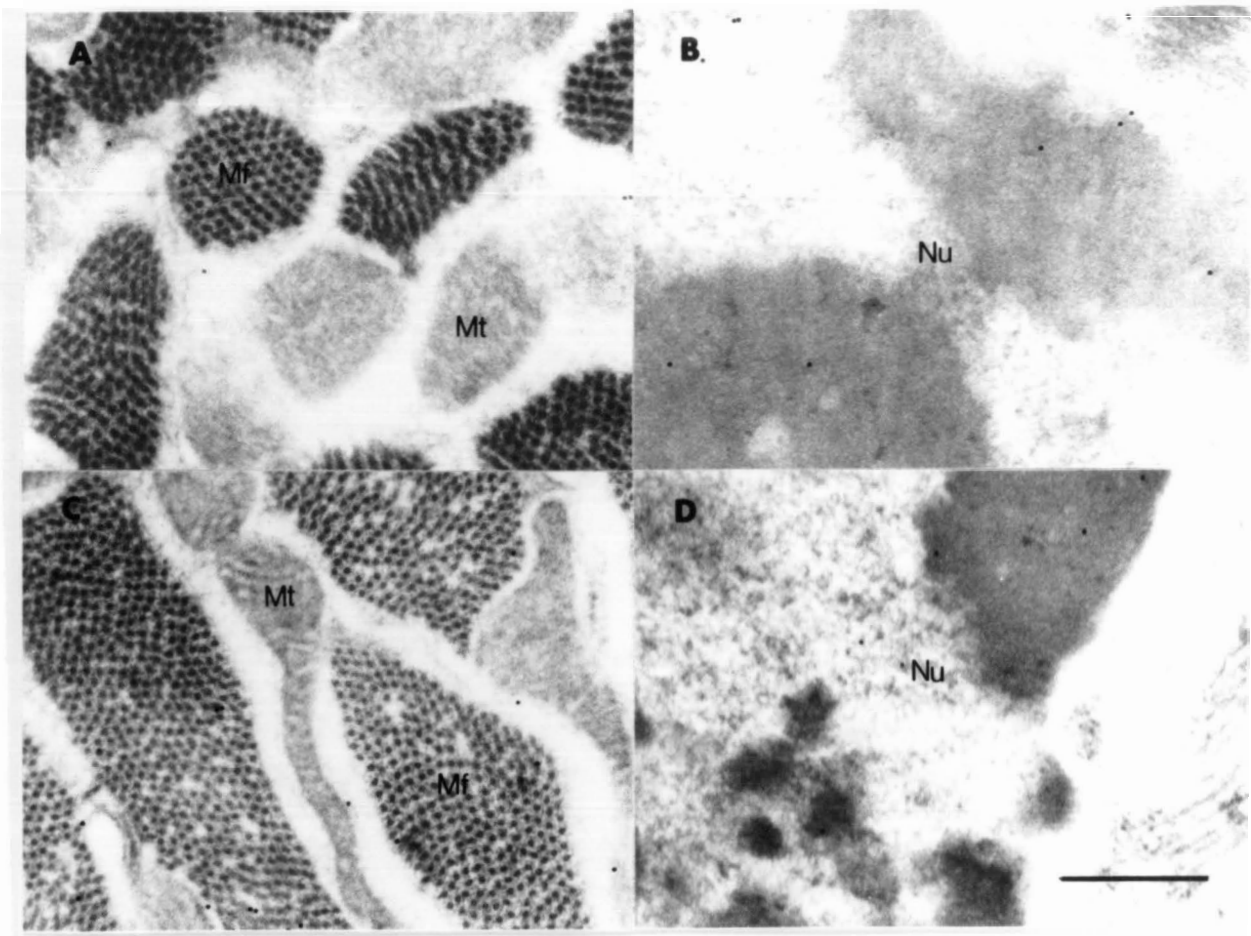

Fig. 4-5. FABP detection in muscle cross-sections.
Freshly excised muscles were fixed in glutaraldehyde, embedded in Lowicryl K4M, and stained with primary antiserum and gold labeled secondary antibodies as described in Materials and Methods (2.5.2.). A, B: supernumerary nymphs, 15 days after ecdysis; C, D: overaged 5th-instar nymphs, 60 days after ecdysis. Mt = mitochondrion; Mf = myofibril; Nu = nucleus. Space bar = 0.5 μm.

Table 4-2: Time response to methoprene treatment. Fifth instar locusts were topically applied with 500 μg methoprene at different time. Thirty insects were treated at each time. FABP concentration was determined at the specified days by ELISA. Values are mean of 6 determinations ± standard deviation.

Time of treatment [hour]	Duration of 5th instar [day]	Mortality [%]	Adults [%]	Supernumerary nymphs [%]	Cuticle color	FABP concentration day 3 [%]	day 9 [%]	day 15 [%]
0-24	7.0 ±0.5	9	0	91	green	0.3 ±0.1	0.3 ±0.1	0.4 ±0.1
60-72	9.0 ±0.5	5	95	0	green	1.8 ±0.3	14.1 ±0.9	18.2 ±1.2
Control	9.1 ±0.4	4	96	0	pink	1.8 ±0.2	14.0 ±2.0	18.5 ±2.6

Table 4-3: Dose response to methoprene treatment. Fresh emerged fifth instar locusts were topically applied with different dosage of methoprene within 24 hours after ecdysis. Between 20 and 30 insects were treated with each dose. FABP concentration was determined at the specified days by ELISA. Values are mean of 6 determinations ± standard deviation.

Dose [µg]	Duration of 5th instar [days]	Adults [%]	Supernumerary nymphs [%]	Mortality [%]	Cuticle color	FABP concentration day 3 [%]	Day 9 [%]	Day15 [%]
0	9.1 ±0.4	96	0	4	pink	1.8 ±0.2	14.0 ±2.0	18.5 ±2.6
50	9.1 ±0.5	90	0	10	pink	1.9 ±0.1	14.0 ±1.1	18.6 ±1.5
100	8.8 ±0.5	95	0	5	pink	1.9 ±0.4	14.1 ±0.8	18.5 ±1.1
150	8.0 ±0.6	91	0	9	pink	1.7 ±0.2	14.6 ±1.6	18.5 ±0.9
200	7.0 ±0.5	0	89	11	green	0.3 ±0.1	0.3 ±0.1	0.3 ±0.1
500	7.0 ±0.5	0	91	9	green	0.3 ±0.1	0.3 ±0.1	0.4 ±0.1

molted to supernumerary nymphs, and in all animals FABP synthesis was absent. Lower doses of methoprene, however, had no obvious influence on metamorphosis. Although this treatment frequently resulted in deformed wings, the rate of FABP synthesis was identical in all cases to that in untreated control animals. Thus, it appears that low doses of methoprene had no influence on FABP synthesis. However, as soon as metamorphosis was prevented with higher doses of methoprene, FABP was completely absent.

4.2.3. FABP biosynthesis in azadirachtin induced over-aged 5th-instar nymphs

Azadirachtin was used to prevent molting in the last nymphal instar, as described by Rembold (1984). In spite of the high mortality some of the azadirachtin treated 5th-instar nymphs survived for up to 60 days without molting (Table 4-4). The optimum amount of azadirachtin was 4 μg in 2 μl. The flight muscle of these over-aged nymphs were very small. In all cases, the FABP concentration in the flight muscles was below detection limit of the ELISA (Fig. 4-6) and of the immunocytochemical gold labeling methods (Fig. 4-5c,d).

4.2.4. FABP biosynthesis in ligated locusts

The influence of other endocrine signals on FABP biosynthesis in adult locusts were studied with ligation experiments. Through ligation between head and prothorax the whole head region was sealed off the body cavity, so that no hormones released from brain or adjacent endocrine glands could reach the flight muscle. Ligation between metathorax and the first abdominal segment further separated large parts of the fat body from the flight muscle. Despite the high mortality of adult locusts that were ligated immediately after the final molt between head and prothorax as well as between metathorax and the first abdominal segment, several animals survived for up to seven days (Table 4-5). As shown in Fig. 4-7, FABP is apparently synthesized in a similar way in ligated, starved and normal adult locusts

Table 4-4: Effects of azadirachtin on development of 5th-instar locusts. Fifth instar locusts were injected with different doses of azadirachtin within 24 hours or between 48-72 hours after ecdysis. Between 20-50 insects were treated with each dose at each time.

Dose [µg]	Time of treatment [hour]	Treated insects [Number]	Mortality [Number]	[%]	Normal adults [Number]	[%]	Overaged nymphs [Number]	[%]
2	0-24	20	3	15.0	17	85.0	0	0
4	0-24	50	26	52.0	0	0	24	48.0
4	48-72	20	2	10.0	18	90.0	0	0
6	0-24	30	25	83.3	0	0	5	16.7
6	48-72	20	4	20.0	16	80.0	0	0
8	48-72	20	6	30.0	14	70.0	0	0

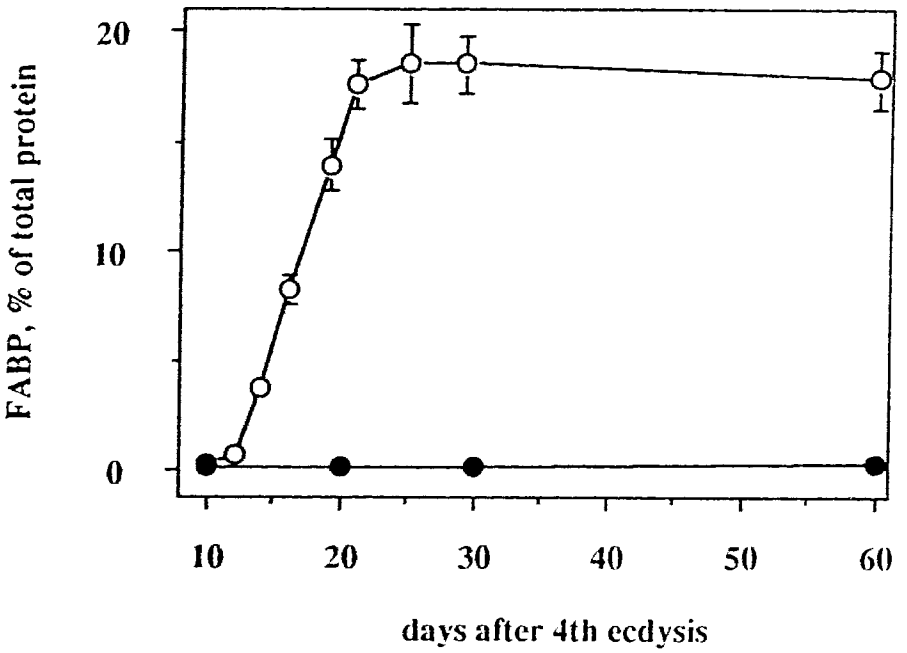

Fig. 4-6. Muscle FABP concentration in over-aged 5th instar nymphs and normal adults. Intracellular FABP was determined by an ELISA as described in Materials and Methods (2.4.6.). FABP concentration is expressed as percentage of total soluble cytosol proteins. Values are the mean of 6 ± standard deviation. –●–over-aged 5th instar nymphs;–○– normal adults.

Table 4-5: Effects of ligation on life-span of adult locusts. Freshly emerged adults were ligated within 24 hours. Three insects were removed for assay each day.

Total adults ligated	Mortality											
	day 1		day 2		day 3		day 4		day 5		day 6	
[Number]	[Number]	[%]	[Number]	[%]	[Number]	[%]	[Number]	[%]	[Number]	[%]	[Number]	[%]
44	2	4.4	4	10.0	6	16.7	7	25.7	6	30.0	4	36.4

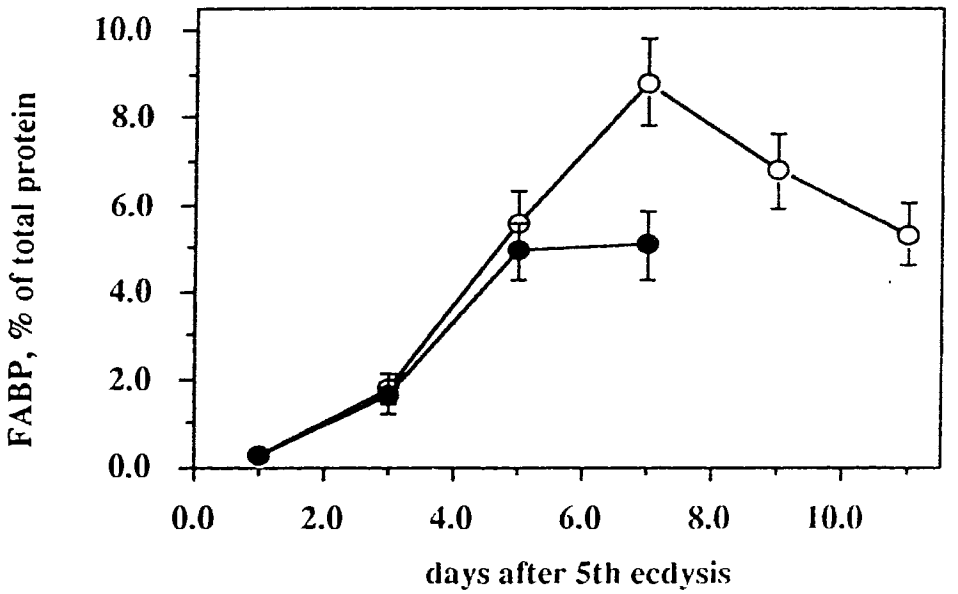

Fig. 4-7. Muscle FABP concentration in ligated and starved adults.
Intracellular FABP was determined by an ELISA as described in Materials and Methods (2.4.6.). FABP concentration is expressed as percentage of total soluble cytosol proteins. Values are the mean of 6 ± standard deviation. -●- ligated adults;-○- starved adults.

during the first 4 days of adulthood; the decline in FABP afterwards is probably caused by the lack of nutrients.

4.2.5. Hormonal influence on locust flight muscle development

In order to link the data on FABP synthesis with muscle development in these insects, it is useful to know what ultrastructural changes occur in muscle after treatment with these hormones or anti-hormones. However, no comprehensive studies of flight muscle development in precocious adults, supernumerary nymphs and over-aged nymphs of the locust have been reported. Due to insufficient fixation the muscle fine structure is not visible in immunogold labeled tissues (Fig. 4-2, 4-5). Therefore, flight muscles from all stages investigated were fixed in glutaraldehyde and osmium tetroxide and embedded in Epon/Araldite before sectioning. The resulting electron micrographs (Fig. 4-8 - 4-11) reveal much more detail. In the later 5th instar of normal locust, flight muscle contained myofibrils in two easily distinguishable shapes: small round myofibrils that were located in the center, and large oblong myofibrils found in the peripheral area. Numerous small mitochondria were visible between myofibrils; the Z discs were well defined (Fig. 4-8a, b). During metamorphosis the oblong myofibrils divided to form round myofibrils; the mitochondria increased sharply in size, and interfibrillar tracheoles became visible. In the early adult stage the flight muscle contained only round shaped myofibrils surrounded by many mitochondria and numerous interfibrillar tracheoles located closely to the mitochondria (Fig. 4-9a,b). Muscle differentiation and development continued for several days, during which myofibrils and mitochondria further increased in size. The number of myofilament present in each myofibril also increased (Fig. 4-9c, d). Growth of myofibrils and mitochondria continued with further development (Fig. 4-9e, f).

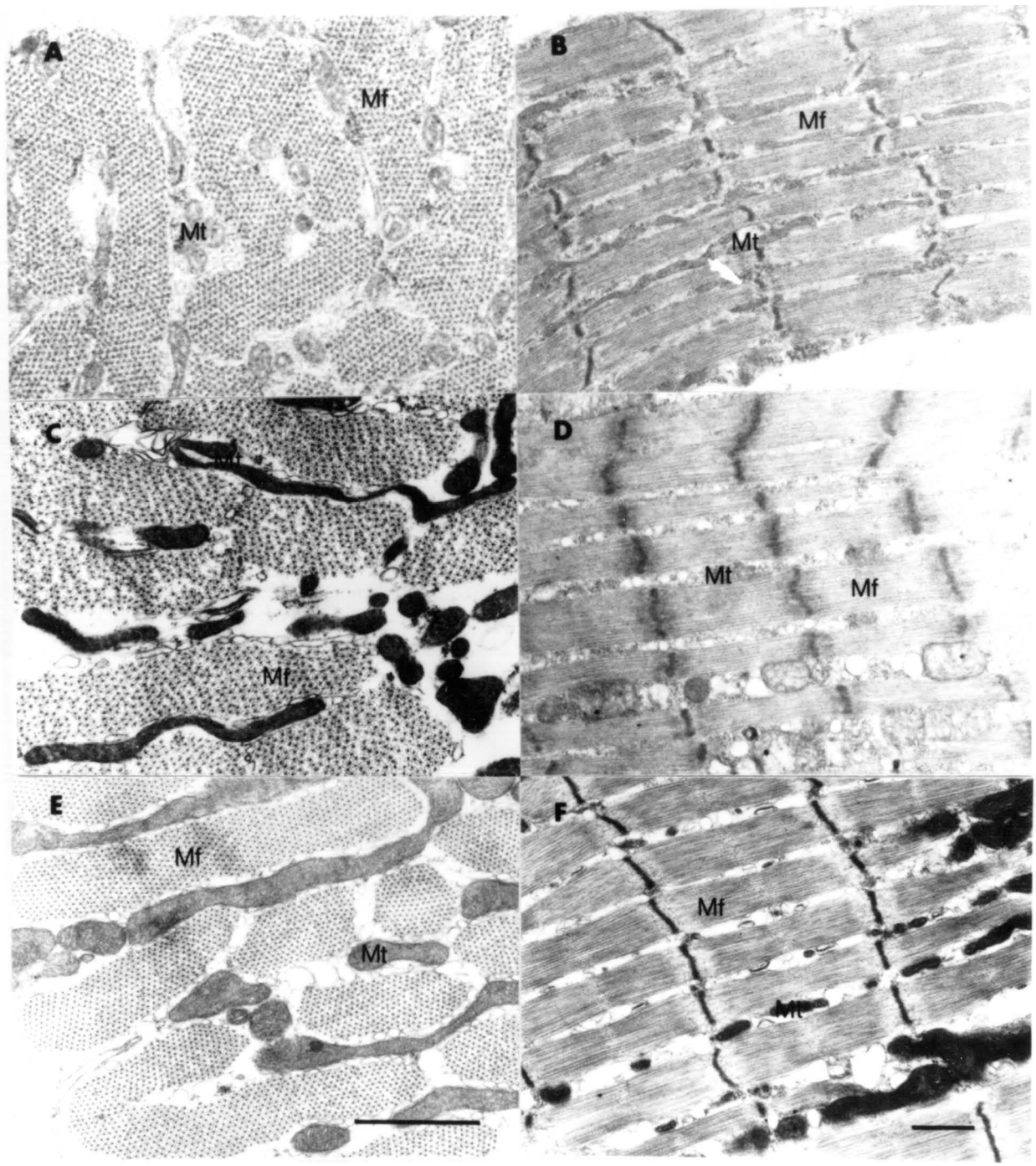

Fig. 4-8. Dorsolongitudinal mesothorax muscle of nymphs.
Flight muscle of locust nymphs were dissected, fixed, sectioned and stained as described in Materials and Methods (2.5.1.). A, B: 9 day old normal 5th-instar nymphs; C, D: 30 day old azadirachtin treated overaged 5th-instar nymphs; E, F: 60 day old azadirachtin treated overaged 5th-instar nymphs. Mf = myofibril, Mt = mitochondrion. Space bar = 1 μm.

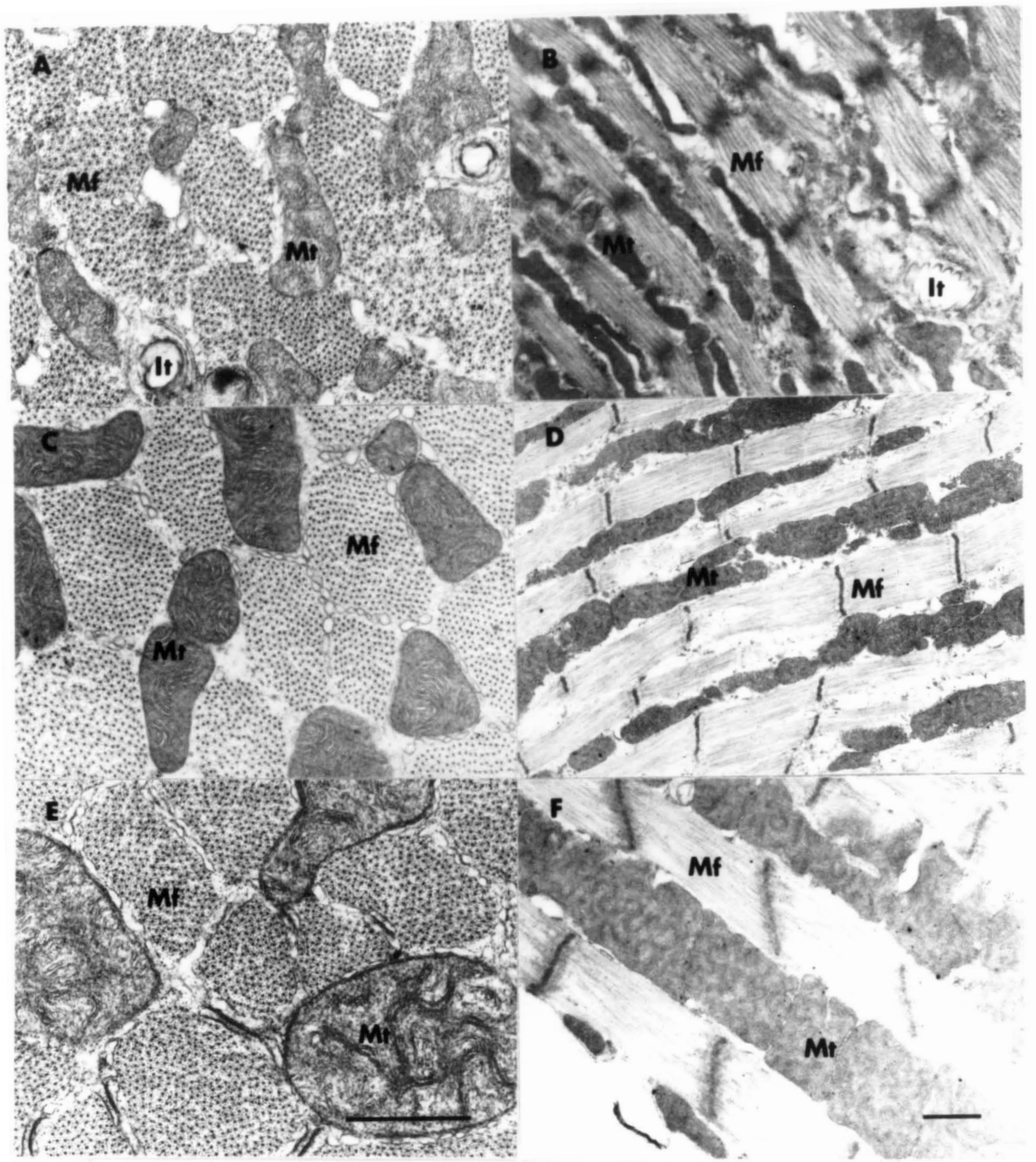

Fig. 4-9. Dorsolongitudinal mesothorax muscle of normal adults.
Flight muscle of locust adults were dissected, fixed, sectioned and stained as described in Materials and Methods (2.5.1.). A, B: day 1; C, D: day 7; E, F: day 15 after imaginal ecdysis. It = interfibrillar tracheol, Mf = myofibril, Mt = mitochondrion. Space bar = 1 μm.

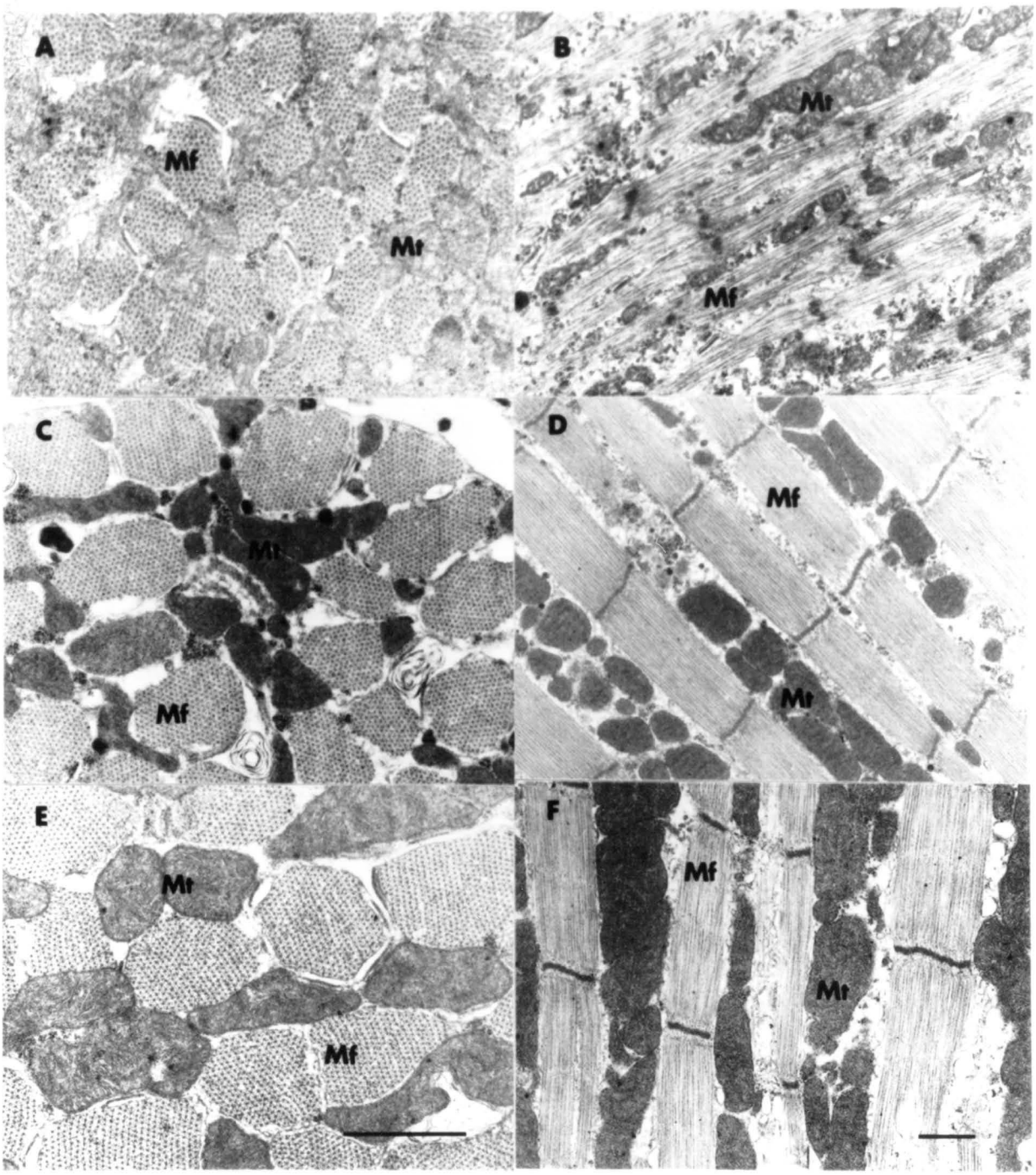

Fig. 4-10. Dorsolongitudinal mesothorax muscle of precocious adults.
Insects were treated with ethoxy-precocene and tissue was processed as described in Materials and Methods (2.5.1.). A, B: 1 day; C, D: 7 days; E, F: 15 days after imaginal ecdysis. Mf = myofibril, Mt = mitochondrion. Space bar = 1 μm.

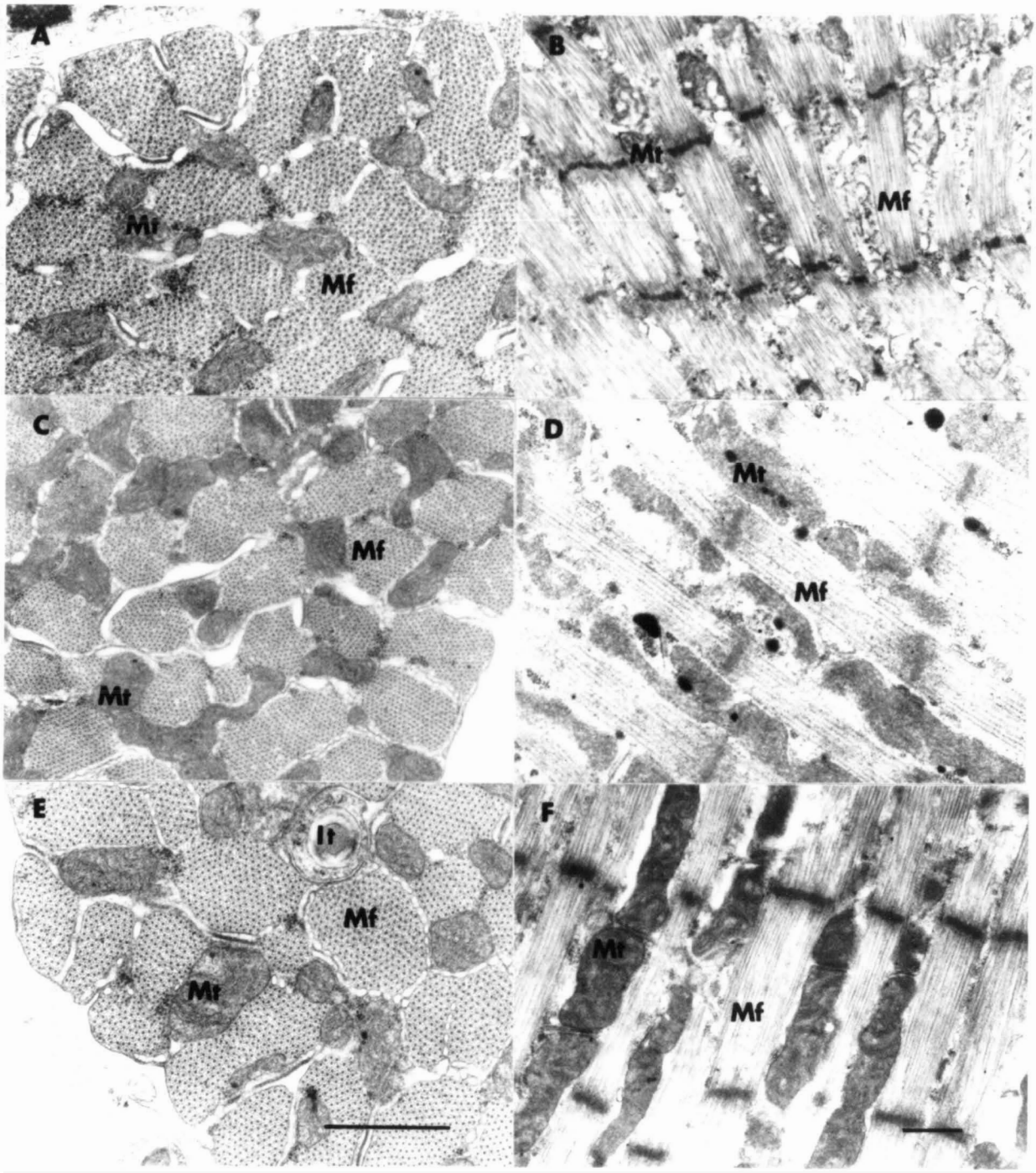

Fig. 4-11. Dorsolongitudinal mesothorax muscle of supernumerary nymphs.
Insects were treated with methoprene and tissue was processed as described in Materials and Methods (2.5.1.). A, B: 1 day; C, D: 7 days; E, F: 15 days after ecdysis to 6th-instar nymphs. It = interfibrillar tracheol, Mf = myofibril, Mt = mitochondrion. Space bar = 1 μm.

The ultrastructure of flight muscle of azadirachtin induced over-aged 5th-instar nymphs remained similar to that of normal late 5th-instar locusts except that mitochondria continued to grow. Both shapes of myofibrils remained present and no interfibrillar tracheoles were formed (Fig. 4-8c-f).

In ethoxy-precocene induced precocious adultiform although the myofibrils and mitochondria were a little smaller, flight muscle development resembled to that of normal adults except for the larger growth of the myofibrils and mitochondria during the first 15 days of precocious adultiform stage (Fig. 4-10), the clustering of mitochondria and the presence of central nucleus (data not shown).

In the early stage of methoprene induced supernumerary nymphs, the flight muscle was characterized by two forms of myofibrils, small mitochondria and absence of interfibrillar tracheoles (Fig. 4-11), similar to the muscle of late 5th-instar nymphs. However, with further development the peripheral oblong myofibrils changed to the round shape, mitochondria grew bigger and finally the interfibrillar tracheoles formed. At this time the ultrastructure was similar to that of the early stage of normal adults.

4.3. Discussion

In order to discuss the effects of hormone treatment on FABP synthesis in the context of insect development, a short review of their physiological functions is needed. The development of insects is regulated primarily by two classes of hormones, ecdysteroids and juvenoids. While ecdysteroids trigger the molting process, it is juvenile hormone that control the nature of the molt. Ecdysone leads to metamorphosis in absence of juvenile hormone, while in presence of JH the insect molts to a new instar similar to the previous one. Thus, juvenile hormone is maintaining the "status quo" (Willis, 1990).

Under normal conditions of development, *S. gregaria* has five nymphal instars. Juvenile hormone is present in the first four nymphal instars and the hemolymph JH activity in 4th-instar nymphs appears to be at its maximum value on the first days of the instar, whereas this activity is virtually absent in the fifth larval instar and so at the subsequent molt the adult form is realized. In the adult stage an increasing activity of JH during the gonotrophic cycle was observed (Johnson and Hill, 1973; Tobe and Pratt, 1975; Tobe, 1977). The JH synthesis at the beginning of adult stage is very low and there is an initial increase between 2 to 4 days. After that, JH synthesis increases continuously and reached maximum levels on day 12 when the first mature oocytes were found (Injeyan and Tobe, 1981).

In all nymphal stages, ecdysteroid levels are low at the start of the instar, rise to a maximal value 1-3 days before ecdysis and fall rapidly again as ecdysis approaches (Gande *et al.*, 1979). It is noteworthy that in the 5th instar of *L. migratoria*, two peaks of ecdysteroids in the hemolymph have been observed, a small commitment peak in the early days of the stadium and a large peak before the 5th molt (Bouthier *et al.*, 1975; Baehr *et al.*, 1979). In *L. migratoria* no ecdysteroids were found in males during the whole adult stadium (Lagueux *et al.*, 1977). In female adults ecdysone and 20-hydroxyecdysone were found, however only after sexual maturity and exclusively as polar conjugates, with the exception of a small amount of free ecdysone and conjugated 20-hydroxyecdysone detected shortly after ecdysis (Gande *et al.*, 1979).

In the adult stage, although the pattern of FABP synthesis is similar to the hemolymph titer of juvenile hormone, this hormone has no direct influence on FABP synthesis. With respect to FABP synthesis, there was no significant difference between normal adults and precocious adults which are not able to produce juvenile hormone (Bowers, 1985). In fact, the results from ligation experiments indicate that no endocrine factor that acts in the adult animal is responsible for the initiation of

FABP synthesis. Insects that were ligated around the neck and abdomen just after metamorphosis made FABP like normal adults, although no hormone from the brain or adjacent endocrine glands could reach the flight muscle. The amount of any fat body product involved in FABP control should also be reduced, since a large part of the fat body is located in the abdominal segment. Thus, it is unlikely that a hemolymph factor released during the first few days of adulthood stimulates flight muscle to synthesize FABP.

It appears that flight muscle is programmed already in the nymphal stadium to synthesize FABP. Juvenile hormone appears to be a crucial factor. If this hormone was absent in the third nymphal stage due to treatment with ethoxy-precocene, FABP was made after molting to a precocious adult in similar amounts and distribution as in normal adults. In contrast, treatment of early 5th-instar nymphs with the juvenile hormone analog methoprene caused molting to an extra instar nymph stage. In the resulting supernumerary nymphs, FABP was never detectable, indicating that the presence of JH in the preceding stage had prevented its expression. The time of treatment is critical with regard to FABP synthesis: treating within 24 h after ecdysis prevented metamorphosis and completely suppressed FABP gene expression, while treating between 60-72 h after ecdysis did neither prevent normal development nor the synthesis of FABP in the resulting adult. The same critical time was also found in *L. migratoria* by other researchers with regard to the influence of exogenous JH on tracheoblast invagination in the flight muscle (Van den Hondel-Franken *et al.*, 1981), these authors concluded that exogenous JH completely prevents the invagination of tracheoblasts into the muscle fibers at the fifth ecdysis only when administered before the commitment peak of ecdysone. In *Lepidoptera*, it has also been demonstrated that during larval-pupal transformation exogenous JH is able to affect the type of cuticle to be made only when applied before the first release of ecdysone (Truman *et al.*, 1974). Therefore, it is very likely

that the JH or JH analog can only inhibit FABP synthesis when applied before the commitment peak of ecdysteroid titer in hemolymph.

My data suggest that a high juvenile hormone titer in immature locusts prevents FABP synthesis in the next instar. Although right now it is difficult to predict how JH acts to prevent FABP synthesis, many mechanisms are conceivable, as recently summarized by Willis (1990). One possibility could be that the hormone is limiting access of transcription factors to the FABP gene before metamorphosis by preventing conformational changes in the chromatin. Juvenile hormone could also, by binding to a nuclear receptor, block the synthesis of other adult specific regulatory proteins that in turn may be required for FABP gene expression. Yet another possibility is that juvenile hormone prevents the translocation of trans-acting factors into the nucleus, for example by stimulating the phosphorylation or dephosphorylation of these proteins.

The absence of juvenile hormone alone, however, in a nymphal instar is not sufficient to initiate FABP synthesis at a later time point, as demonstrated by azadirachtin treatment. Azadirachtin exerts antifeedant, growth-disruptive and toxic effects on a wide range of insects. It also markedly delays, or even completely inhibits the molt in many species. The exact mode of molt inhibitory action of azadirachtin has not yet been elucidated, but it is known that the substance reduces hemolymph ecdysteroid titer and/or delays the appearance of ecdysteroid peak(s), though it does not affect ecdysone production by the prothoracic glands *in vitro* (Warthen, 1979; Redfern *et al.*, 1979; Garcia and Rembold, 1984; Dorn *et al.*, 1986; Pener *et al.*, 1988, 1989; Koolman *et al.*, 1988). In this experiment azadirachtin treated locusts stayed in their 5th instar for up to 60 days; FABP was never detectable. Therefore, it appears that in addition to juvenile hormone absence before the commitment peak of ecdysone the second, major ecdysone peak that precedes metamorphosis is needed for the initiation of FABP gene expression.

In normal insects the onset of FABP synthesis falls just into the phase of flight muscle differentiation. During this phase new growth occurs as in the preceding phase, but now with complete differentiation of the morphological and enzymological pattern (Brosemer, 1963). The ultrastructural data on flight muscle development and differentiation indicate that precocious adults had completely developed and differentiated flight muscle, while in the over-aged nymphs and the supernumerary nymphs the muscle development only reached the level as that of last day of 5th instar and 1st day of adult in normal locusts, respectively. Earlier research data from *Locusta migaratoria* also indicated that the presence of JH or JH analogs in the early 5th-instar nymphs blocked muscle development and differentiation (Van den Hondel-Franken *et al.*, 1980; Van den Hondel-Franken and Flight, 1981; Van den Hondel-Franken, 1982; Cotton and Anstee, 1990). The correlation of flight muscle development and FABP gene expression suggests that the muscle protein FABP could be under the same hormonal regulation as flight muscle itself and any substance, such as ecdysone or juvenile hormone, which stimulates or inhibits muscle differentiation also stimulates or inhibits FABP synthesis.

As outlined above, FABP is a truly adult specific muscle protein that is only synthesized after metamorphosis. While adult specific muscle proteins have never been described, several fat body derived hemolymph proteins are largely specific for the adult stadium (Wyatt, 1990). Vitellogenin and apolipophorin III (apoLp-III) belong to these proteins. Vitellogenin accumulates in female hemolymph from where it is taken up into the ovaries as the major yolk protein vitellin. In adult females, the hemolymph concentration of vitellogenin increases in a similar way, although delayed by a few days, as the hemolymph titer of juvenile hormone rises during the first 2 weeks of adulthood (Injeyan and Tobe, 1981). Therefore, it appears that vitellogenin expression is under direct control of juvenile hormone. Indeed, although normally only expressed in female adults, vitellogenin can be induced before

metamorphosis through treatment with high amounts of juvenile hormone analogs (Dhadialla and Wyatt, 1983). In contrast, FABP gene expression cannot be induced in nymphs, and occurs at similar levels in both male and female adults. Clearly, vitellogenin expression is controlled by different mechanisms from FABP. Another major predominantly adult specific protein is apoLp-III, which, like FABP, plays also a crucial role in lipid transport during extended flight activity. It contributes to the formation of low density lipophorin (LDLp), the main lipid transport protein during flight. The amount of apoLp-III, and consequently the formation of LDLp, increases markedly in adult insects, and apoLp-III has also been found to be elevated in precocious adults. Its synthesis appeared to be suppressed in supernumerary nymphs. While these properties are similar to the findings for FABP, over-aged nymphs seem to possess somewhat more of this apoprotein (Van der Horst *et al.*, 1989). In any case, apoLp-III is present before metamorphosis, albeit in smaller amounts. Of all known major locust proteins, only FABP is a truly adult specific protein which never synthesized before metamorphosis. Studies directed at elucidating the molecular mechanisms that control metamorphosis have frequently been done in fat body, using the expression of vitellogenin as indicator for metamorphic events. Since FABP gene expression is even more specific for insects that underwent metamorphosis, locust flight muscle may be an advantageous tissue to study the induction of metamorphosis.

CHAPTER 5. REGULATION OF FATTY ACID BINDING PROTEIN EXPRESSION IN FULLY DEVELOPED LOCUST FLIGHT MUSCLE

5.1. Introduction

From the research described in the previous chapters it is clear that FABP is a major intracellular protein of locust flight muscle. In support of its function as an intracellular fatty acid transport protein, the amount of FABP is linked to the ability of flight muscle to use fatty acids as energy source. Thus, FABP is absent in nymphs and freshly emerged adults, but its concentration increases rapidly during the first 15 days of the adult stage; it reaches its maximum at the time when insects acquire full competence of long distance flight.

From a series of experiments employing metamorphosis-controlling hormones and antihormones, my earlier research revealed that the initiation of FABP synthesis is possible only following metamorphosis; thus, it is, at least indirectly, controlled by interplay of JH and ecdysteroids prior to metamorphosis. These hormones however do not influence FABP gene expression in the adult.

In mature adult insects, FABP reaches as steady concentration of approx. 18% of the total cytosolic proteins. FABP is a stable protein; thus, the expression of FABP is reduced to minimal rate once this concentration has been reached. The insects then have large amounts of FABP present in their muscle so that during flight fatty acids can be rapidly translocated from the flight muscle membrane to its mitochondria. It is noteworthy that the high concentration of FABP is achieved in insects which never have actually engaged in flight activity.

It has been shown in mammalian skeletal muscle that continuous electrical stimulation (Kaufman *et al.* 1989) or extended exercise (Van Breda *et al.*, 1992) can induce the intracellular FABP gene expression; this may lead to a more efficient transport of fatty acids during long term muscle activity. Nothing, however, is known

about the mechanism by which FABP gene expression is stimulated during exercise. Although locust flight muscle possesses much more FABP than any mammalian muscle, it could be possible that continuous exercise equally stimulates its expression. The present chapter investigates this possibility.

The physiological events that occur during flight are well understood. At the beginning of flight, locusts utilize carbohydrates as fuel; continuous flight leads to the release of the adipokinetic hormones (AKHs) from the corpora cardiaca, which induce the switch from carbohydrates to lipids as the major energy source. AKHs promote the hydrolysis of triacylglycerol in the fat body and the release of diacylglycerol into the hemolymph. The released lipids combine with lipophorin, which changes from its high density form (HDLp) to the lipid rich low density form (LDLp). AKHs may also stimulate diglyceride oxidation and suppress carbohydrate utilization in the flight muscles (Goldsworthy, 1983). The increased use of lipid during flight may necessitate larger amounts of FABP. Thus, it would be logical from a physiological point of view that AKHs and LDLp also influence FABP gene expression. In this chapter I report the influences of sustained flight, AKH II and LDLp on FABP gene expression in fully developed adult locusts.

5.2. Results

5.2.1. Influence of sustained flight on FABP mRNA

In order to test whether sustained flight stimulates FABP gene expression 20-day-old adult locusts were forced to fly for different time intervals; immediately afterwards, mRNA was isolated, electrophoretically separated and analyzed by Northern blotting. The membrane was hybridized with an FABP probe as well as an actin probe, which was used to normalize the FABP signal. Although there was no change in FABP mRNA following 4 h of flight, the increase of FABP mRNA was clearly visible after 8 h of continued flight (Fig. 5-1). However, when mRNA was

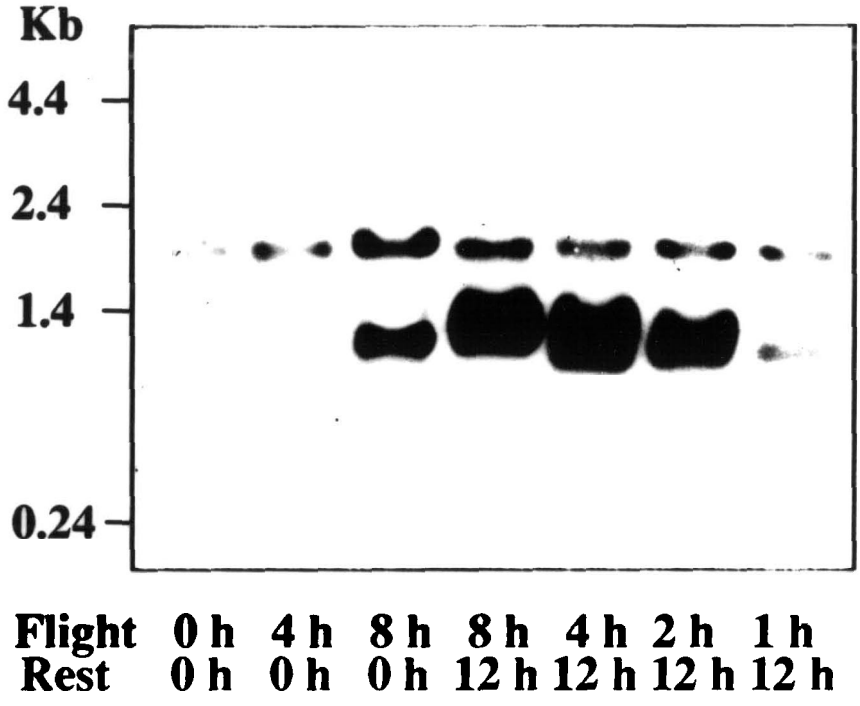

Fig . 5-1. Northern blot of accumulated FABP mRNA after flight.
Total RNA was isolated from flight muscle immediately or 12 hours after flight, separeted by gel electrophoresis and probed simultaneously with FABP cDNA and actin cDNA, as described in Materials and Methods (2.6).

extracted after a waiting period of 12 h following flight, 8 h of flight caused an enormous increase in FABP mRNA content, and even after only 1 h of flight an increase of FABP mRNA was notable (Fig. 5-1). In order to quantify the increase in FABP mRNA, the radioactivity found in the FABP and actin mRNA bands was counted. The relative increase in FABP mRNA was expressed as the ratio between the FABP and actin mRNA bands. As shown in Fig. 5-2, FABP mRNA concentration almost doubled after 8 h of flight and was more than 4 times as high after 12 h of continuous flight. If the insects were allowed to rest for 12 h before being sacrificed, 4 h of flight introduced a similar amount of FABP mRNA as 8 h of flight. Moreover, when the rest time was extended to 14 h, even 2 h of flight resulted in a similar increase. The ratios between FABP and actin mRNA were 4.15 ± 0.15 for 2 h of flight with 14 h rest and 4.28 ± 0.17 for 4 h of flight with 12 h rest. However, 1 h of flight resulted in a much lower amount FABP mRNA, even after 12 h of rest.

5.2.2. Influence of AKH II on FABP mRNA

It is well established that extended flight activity is sustained under the influence of AKHs. Within 30 minutes after initiation of flight, the corpora cardiaca release AKHs which regulate the supply of fuel molecules to the flight muscle. It is therefore possible that these hormones are also involved in the initiation of FABP transcription observed during extended flight. To test this hypothesis, 250 pmol of AKH II were injected into resting locusts, and the relative amount of FABP mRNA was determined. An increase in FABP mRNA could be first seen 8 h after injection; the maximal amount of mRNA was found 16 h after injection. Within 48 h after treatment, the level of FABP mRNA had returned to its base level (Fig. 5-3). Since a lag time of 16 h appears to be needed between stimulus and maximal FABP mRNA, all subsequent RNA extractions were carried out 16 h after treatment.

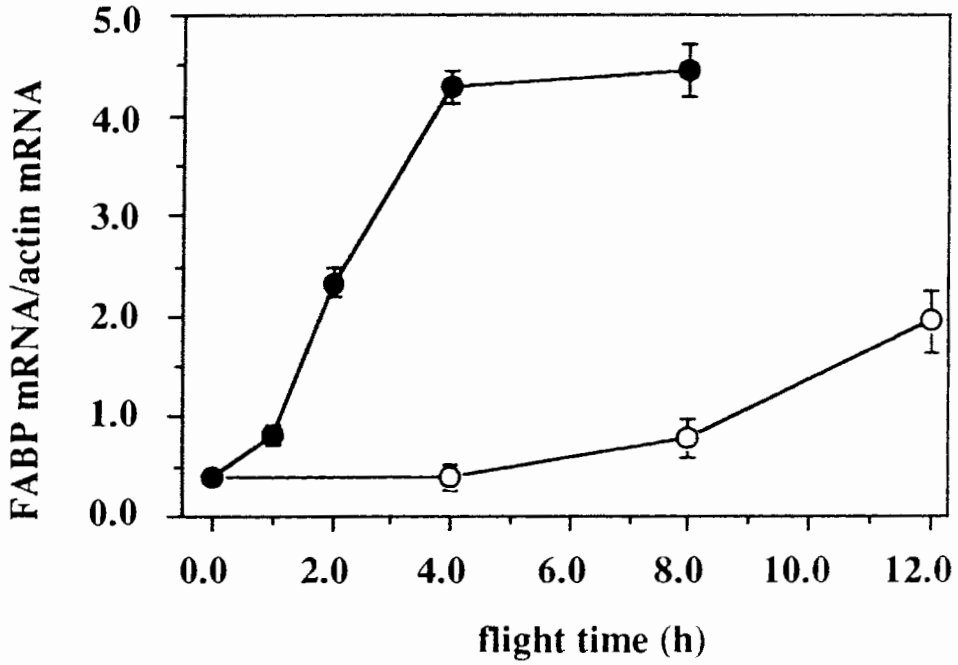

Fig. 5-2. Flight induced FABP mRNA increase. Insects were forced to fly for different time intervals and RNA was isolated immediately (-O-) or 12 hours (-●-) after flight. The relative amount of FABP mRNA was determined as described in Materials and Methods (2.6.4.).

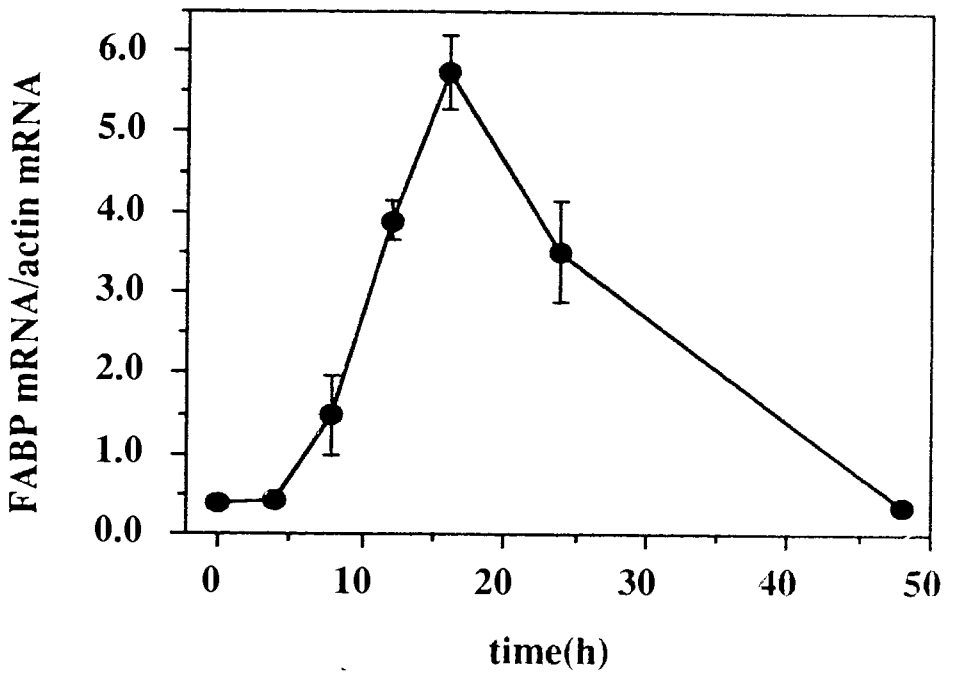

Fig. 5-3. Time dependency of AKH induced FABP mRNA accumulation. Insects were sacrificed and RNA was extracted at different time points following injection of 250 pmol AKH. The relative amount of FABP mRNA was determined as described in Materials and Methods (2.6.4.).

As the amount of AKH II used in the above experiments is probably much higher than encountered by flying locusts, the influence of smaller doses of AKH II on FABP gene expression was analyzed. The dose response curve (Fig. 5-4) showed that 25 pmol of AKH II already stimulated FABP gene expression. Higher doses lead to higher levels of mRNA, up to 250 pmol, which led to maximal stimulation.

5.2.3. Influence of LDLp on FABP mRNA

Adipokinetic hormone is believed to act primarily at the fat body. During flight, AKHs stimulate the hydrolysis of lipid in fat body and its subsequent transport to the flight muscle. Diacylglycerol is released into the hemolymph where it, together with other lipoprotein components, forms LDLp, which subsequently delivers lipid to the flight muscle. This mechanism could be the link between AKH actions at the fat body and FABP gene expression in flight muscle. To investigate this possibility, LDLp was prepared by injecting AKH II into locusts. The protein was purified by density gradient ultracentrifugation, dialyzed and injected into different animals. Sixteen hours later , RNA was extracted and analyzed by Northern blotting. As shown in Fig. 5-5, LDLp, like AKH II and extended flight, can stimulate FABP gene expression as well. An amount of LDLp equivalent to the LDLp content from three insects led to an increase (ratio 5.47 ± 0.47) in FABP gene expression similar to the increase caused by 250 pmol AKH II (ratio 5.74 ± 0.47).

5.3. Discussion

In spite of the extremely high concentrations of FABP present in flight muscles of mature locusts, extended physical activity stimulates FABP gene expression. The amount of FABP mRNA did not change after short flights, but it increased more than 10 fold after continuous flight of 2 or more hours. The increase in expression is apparently independent of the length of flight, as long the flight lasted longer than 2 h. However, FABP mRNA does not appear immediately after

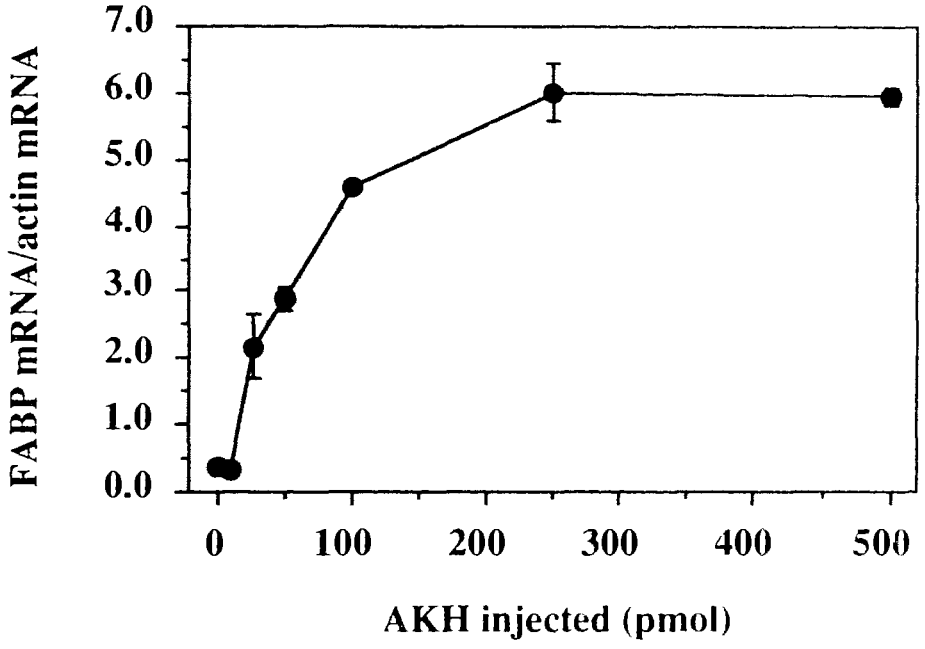

Fig. 5-4. Dose dependency of AKH induced FABP mRNA accumulation. Insects were injected with varying doses of AKH. Sixteen hours later, RNA was extracted and FABP mRNA was determined as described in Materials and Methods (2.6.4.).

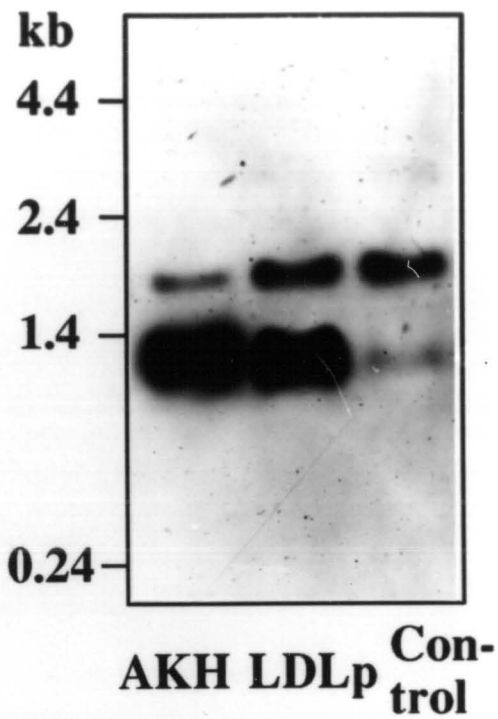

Fig. 5-5. Northern blot of accumulated FABP mRNA.
Total RNA was isolated 16 hours after injection of 250 pmol AKH II, injection of 3 mg LDLp, or initiation of flight that lasted 4 hours. Electrophoresis and Northern blotting were carried out as described in Materials and Methods (2.6.).

termination of flight; instead, a lag time of more than 12 h is required between initiation of flight and the maximal accumulation of mRNA. Indeed, the response of 2 h of flight was similar to 4 h if the total time that elapsed between initiation of flight and analysis was 16 h; the process of FABP transcription is probably initiated within the first two hours of flight but only completed after additional 14 h. Insects that flew 8 h responded similarly after 12 additional hours; this indicates that flight times longer than 2 h and longer lag times between the first two hours of flight and analysis do not increase FABP mRNA levels further.

Although not conclusively demonstrated, fatty acid transport is generally thought to be a major physiological function of FABP (Veerkamp, *et al.*, 1991). Thus, an increase of FABP in response to flight could allow more efficient fatty acid transport towards mitochondrial ß-oxidation. While short term flight is primarily fueled by trehalose, the main hemolymph disaccharide, fatty acid becomes the predominant energy source as flight continues (Beenakkers *et al.*, 1984). This switch between carbohydrates and lipids during flight is controlled by the interplay of endocrine factors (Orchard *et al.*, 1993). Immediately after flight begins, the neuropeptide octopamine is released which serves several functions in flight metabolism. Octopamine acts as a neurohormone on fat body tissue, where it stimulates lipid mobilization; in addition, it is a neurotransmitter which controls the release of peptidergic adipokinetic hormones from the corpora cardiaca. the two hormones, AKH I and AKH II, are secreted approximately 20 min after the initiation of flight. While the concentration of AKH I peaks 10 minutes later and declines rapidly thereafter, AKH II reaches its maximum also 30 minutes after flight initiation, but its titer remains high for at least one hour. During this time, AKH II is believed to constantly stimulate the fat body to hydrolyze stored triacylglycerol and to release the resulting diacylglycerol into the hemolymph, where they associate with high density lipophorin (HDLp) and several molecules of apolipophorin III to form a

lipid rich low density lipophorin particle (LDLp) (Beenakkers *et al.*, 1985). After diacylglycerol has been hydrolyzed and fatty acids have entered the flight muscle cell, HDLp returns again to the fat body, thus serving as a re-usable lipid shuttle.

Adipokinetic hormone alone, without flight and the concomitant increase in the metabolic rate, is capable of stimulating the expression of FABP. Elevated levels of FABP mRNA are detectable 8 h after AKH II injection, but maximal levels are reached 16 h after AKH treatment. Within 48 h, the level of mRNA return to the low base level. The increase of FABP mRNA is dose dependent; while 25 pmoles of AKH II already induced FABP gene expression, higher doses had larger effects, up to 250 pmoles, where FABP mRNA was 20 times higher than in control animals. It should be noted that the AKH II concentration in hemolymph immediately after injection is probably much higher than the hormone concentration that occurs naturally. However, AKHs are normally rapidly degraded in the hemolymph. While the actual AKH concentration and the rate of its degradation is not known, results by Orchard (Orchard and Lange, 1983) indicate a half life for AKH I less than 20 minutes. During flight, the hemolymph titer of AKH II remains constant, an observation that has been attributed to continued release of new hormone. In order to assure that AKH II remains present in the hemolymph for 90 minutes or more, injection of a high initial dose may be required to counteract breakdown.

While the exact mechanisms by which AKHs act are not known, their main function is certainly the mobilization of lipid. Possibly, the increased supply of lipid to the flight muscle initiated by AKH II is the ultimate cause of the effect of the hormone on FABP gene expression. Indeed, injection of LDLp alone leads to the same stimulation of FABP gene expression as high doses of AKH II, a finding that strongly suggests this hormone is not acting directly on the flight muscle. Moreover, the accumulation of FABP mRNA after flight may be entirely due to increased lipid delivery. In fact, the maximal value reached after flight is significantly lower than

after hormone or LDLp administration. It is possible that the actual amount of lipid accumulation in flight muscle is lower during flight since fatty acid is actually metabolized. In contrast, the flight muscles of AKH II or LDLp treated animals were not active and thus did not metabolize significant amounts of fatty acids. Thus, the induction of FABP gene expression after sustained flight may be ultimately caused by an increased concentration of intracellular fatty acids.

Such a feed-back response would offer two major physiological advantages which reflect the postulated roles of FABP in muscle tissue. A higher concentration of FABP in muscle cytosol would assure more efficient fatty acid uptake and transport towards mitochondrial ß-oxidation. In addition, FABP may serve as a buffer to prevent the accumulation of free fatty acids in the cytosol. Due to their amphiphilic nature, an excess of free fatty acids may have deleterious effects on cellular integrity. To avoid a temporary build-up of free fatty acids during or after strenuous muscle activity increased levels of FABP may be required.

CHAPTER 6. GENERAL DISCUSSION

Long chain fatty acids play a vital role in the organism. Apart from providing a major source of energy, fatty acids can also covalently modify the structure of certain proteins, they are the precursors of eicosanoids and are essential constituents of the membrane lipids that maintain cellular and organelle integrity (Kaikaus *et al.*, 1990). Since fatty acids are only poorly soluble in water, a transport mechanism is required for their translocation through hydrophilic media. This transport is mediated in blood and capillary endothelium by serum albumin (Kuhl and Spector, 1975; Ghitescu *et al.*, 1986), while FABP is considered to serve as a carrier inside cells (Glatz and Van der Vusse, 1989). FABPs are relatively small proteins (13-15 kDa) capable of binding long chain fatty acids and possibly their coenzyme A and L-carnitine esters, and found in appreciable amounts in cells of mammalian tissues that are actively involved in uptake or utilization of fatty acids (Bass, 1988; Glatz and Van der Vusse, 1989). Although the functions of FABP are not clearly established, they appear to enhance the transfer of long-chain fatty acids across cytosol. FABP from heart muscle was also reported to modulate cardiac energy production by controlling transfer of acyl-L-carnitine to the mitochondrial ß-oxidative system; these results, however, are controversial. Better established is a cytoprotective function of FABP: the protein is believed to protect the heart against the toxic effects of high intracellular levels of free fatty acid that accumulate during ischemia (Glatz and Van der Vusse, 1989). While mammalian heart FABP is a ubiquitous protein present throughout all developmental stages, its intracellular concentration increases 8-fold through fetal and post-partum development (Crisman *et al.*, 1987). These changes in FABP synthesis generally seem to parallel the increased dependency of the heart on mitochondrial ß-oxidation, which is the major energy producing pathway in cardiac

muscle. Fatty acid guarantee a relatively constant supply of energy and are thus advantageous for the continuous activity of heart muscle.

Similar metabolic conditions are present during migratory flight of locusts. The high oxidative capacity, large number of mitochondria, and the ability to sustain prolonged muscular activity during flight manifest the similarity to mammalian heart muscle (Crabtree and Newsholme, 1975). Locust flight muscle also possesses an FABP that is structurally similar to mammalian heart FABP (Haunerland and Chisholm, 1990; Price *et al.*, 1992). While flight also is fueled primarily by fatty acid oxidation, much more energy is required to sustain flight, and consequently one could expect higher concentrations of FABP. Thus, locust flight muscle is an advantageous system to study quantitatively the control of FABP gene expression and accumulation during development and in periods of prolonged muscle activity.

This thesis revealed that FABP is the most abundant soluble muscle protein in fully developed adult locusts. In fact, the concentration of FABP in locust muscle is more than three times as high as in mammalian heart. The high value seems to reflect the high metabolic rate that can be achieved by flight muscles, which exceeds the maximal metabolic rate of heart by a similar factor. No FABP, however, was detectable at the beginning of the adult stage. The concentration rose dramatically during the following two weeks, after which it reached its maximal value. These changes seem to reflect the ability of locusts to engage in flight activity. While nymphs do not have developed wings and consequently cannot fly, the flight muscle itself is already largely developed at adult ecdysis, as demonstrated in this thesis. Nevertheless, freshly emerged adult locusts are not capable to engage in extended flight for several days (Uvarov, 1977; Rainey, 1989); only short flights are possible (Uvarov, 1977), which use carbohydrates as fuel. These findings may point towards an inability to fully utilize lipid as energy source. Since the intracellular concentrations of enzymes involved in lipid oxidation are already significant in young

adult flight muscle (Beenakkers, 1963), even the lower amounts of these enzymes are not likely to severely restrict ß-oxidation. Thus, it is likely that the lack of FABP in young adults restricts the use of lipid as energy source. My results thus lend more credibility to the proposed physiological function of FABP in intracellular lipid transport.

As expected for an intracellular lipid carrier, locust FABP is mainly a cytosolic protein. However, similar concentrations of FABP are also present in nuclei, but not in mitochondria. During adult maturation, the concentration of FABP in nuclei increases in a similar way as the concentration of cytosolic FABP. This strongly suggests that the nuclear FABP is indeed the same protein, which has entered the nucleus from the cytosol through the nuclear pores. Similar experiments in mammalian heart have failed to conclusively prove FABP in nuclei or exclude FABP in mitochondria. While two reports indicate the presence of FABP in mitochondria (Fournier and Rahmin, 1985; Brchers *et al.*, 1989), others discount these results (Crisman *et al.*, 1987; Paulussen *et al.*, 1989). Because of the dramatic changes in FABP concentration in flight muscle, my results clearly indicate that FABP is absent in the mitochondria, but present in nuclei in similar concentrations as in the cytosol.

In contrast to mammalian heart FABP, locust FABP is completely absent in early development. The initiation of FABP gene expression is under the control of different endocrine factors, as summarized in Fig. 6-1. FABP synthesis in adults is apparently initiated prior to metamorphosis. My research has clearly established that neither ecdysteroids nor juvenile hormones nor peptidergic hormones released from the brain or adjacent endocrine glands stimulate FABP synthesis in the adult. However, the initiation of FABP synthesis is strongly affected by manipulating the insect hormone pattern in the nymph stadium. Precocious 4th-instar adultiforms accumulate FABP in the similar manner as normal locusts, which indicate that FABP

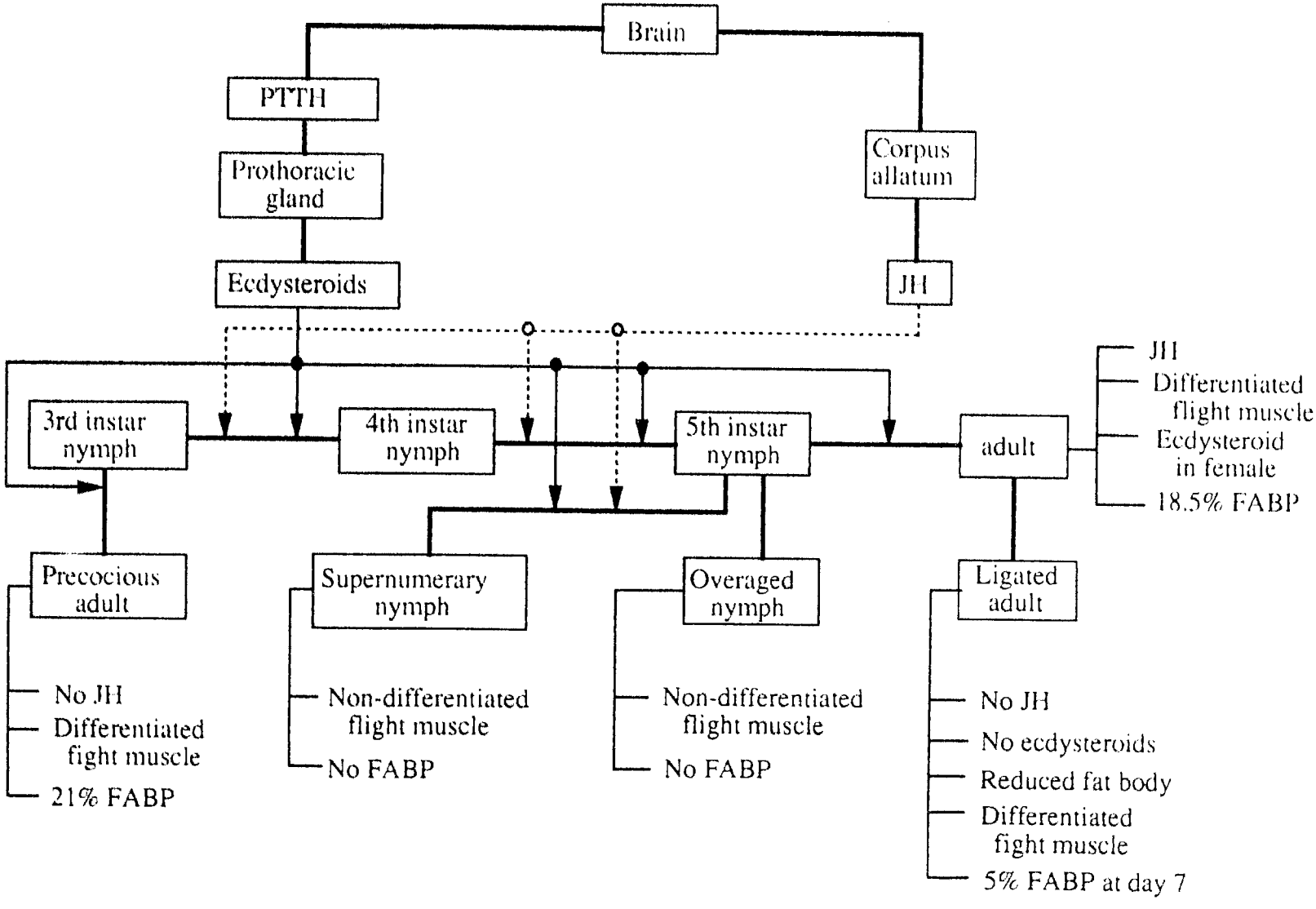

Fig. 6-1. Endocrine control of desert locust development and FABP synthesis.

synthesis takes place following metamorphosis, independent of the time the insect has spent in the nymphal stadium. In contrast, no FABP is made when insects fail to engage in metamorphosis. When metamorphosis of 5th instar nymphs was prevented by treatment with a juvenile hormone analog, no FABP is synthesized in the supernumerary nymphs that emerge after an additional larval molt. Moreover, when normally developed last instar nymphs are prevented from molting into adults by azadirachtin treatment, FABP is not synthesized, even after additional 2 months of larval life. All these results indicate that FABP synthesis dose not occur prior to metamorphosis, but is always initiated in differentiated flight muscle. Flight muscle of supernumerary and over-aged nymphs that do not produce FABP have not undergone final differentiation, while muscles of precocious adults and normal adults are fully differentiated.

The detailed molecular events that control metamorphosis are not yet fully understood, and consequently one can not conclusively explain the observed negative influence of juvenile hormone on FABP synthesis. Willis (1990) proposed that juvenile hormone may suppress the expression of adult specific genes on the chromatin level. Chromatin, condensed DNA complexed with proteins exists in several forms. It may be found in a tightly coiled configuration where regulatory proteins have no access to genes, or regions of chromatin may be "open", forming open-looped domains where regulatory proteins can interact with regulatory elements. Thus, by preventing the opening of chromatin domains, juvenile hormone could block the access of regulatory factors to adult specific genes, including the FABP gene, and inhibit their expression.

Once metamorphosis has occurred, flight muscle differentiation and initiation of FABP synthesis proceeds without further stimulation. Its cytosolic concentration raises until day 15, when a constant level of FABP is reached. FABP mRNA can be detected from the fi[illegible]ay of adult life; it reaches its maximum

several days before the maximal FABP concentration is achieved. After day 15, when the FABP concentration remains constant, its mRNA is reduced to a low level, probably needed only to maintain the established FABP level. This suggest some form of feed back control of FABP gene expression; once the needed FABP amount has been produced, its transcription ceases.

Extended muscular activity however again stimulates FABP gene expression, probably in response to increased lipid utilization. Continuous flight activity of two or more hours strongly stimulates FABP gene expression. FABP mRNA, however, accumulates after a lag time of several hours. It is well established that during sustained flight locust utilize almost exclusively lipid as energy source. Diacylglycerol is released from the fat body and transported through the hemolymph as part of LDLp. At the flight muscle, fatty acid is released by the action of a lipase, taken up into the muscle cell, and carried through the cytosol by FABP. This process of lipid mobilization is controlled by adipokinetic hormones (Goldsworthy, 1983; Orchard, 1987) (Fig. 6-2). Injection of AKH alone, without flight activity, also stimulates FABP gene expression. The amount of mRNA reaches a maximum 16 h after AKH injection. This time is probably needed to initiate transcription of the FABP gene, processing of the mRNA and export into the cytosol. Maximal accumulation of FABP mRNA is reached following 2 h of flight or injection of 250 pmoles AKH. Longer flight times or higher doses of the hormone do not increase further FABP mRNA. The same maximal amount of FABP mRNA is also reached after injection of LDLp. These results suggest that the induction of FABP gene expression in response to flight or AKH treatment is directly mediated by the increased availability of lipid. Although flight and AKH have numerous metabolic effects, the only role of LDLp is to transport lipid to the flight muscle. With increased LDLp content in the hemolymph more substrate is available for the lipase in the plasma membrane of flight muscle, and thus more fatty acid will enter the muscle cytosol. Therefore, it is

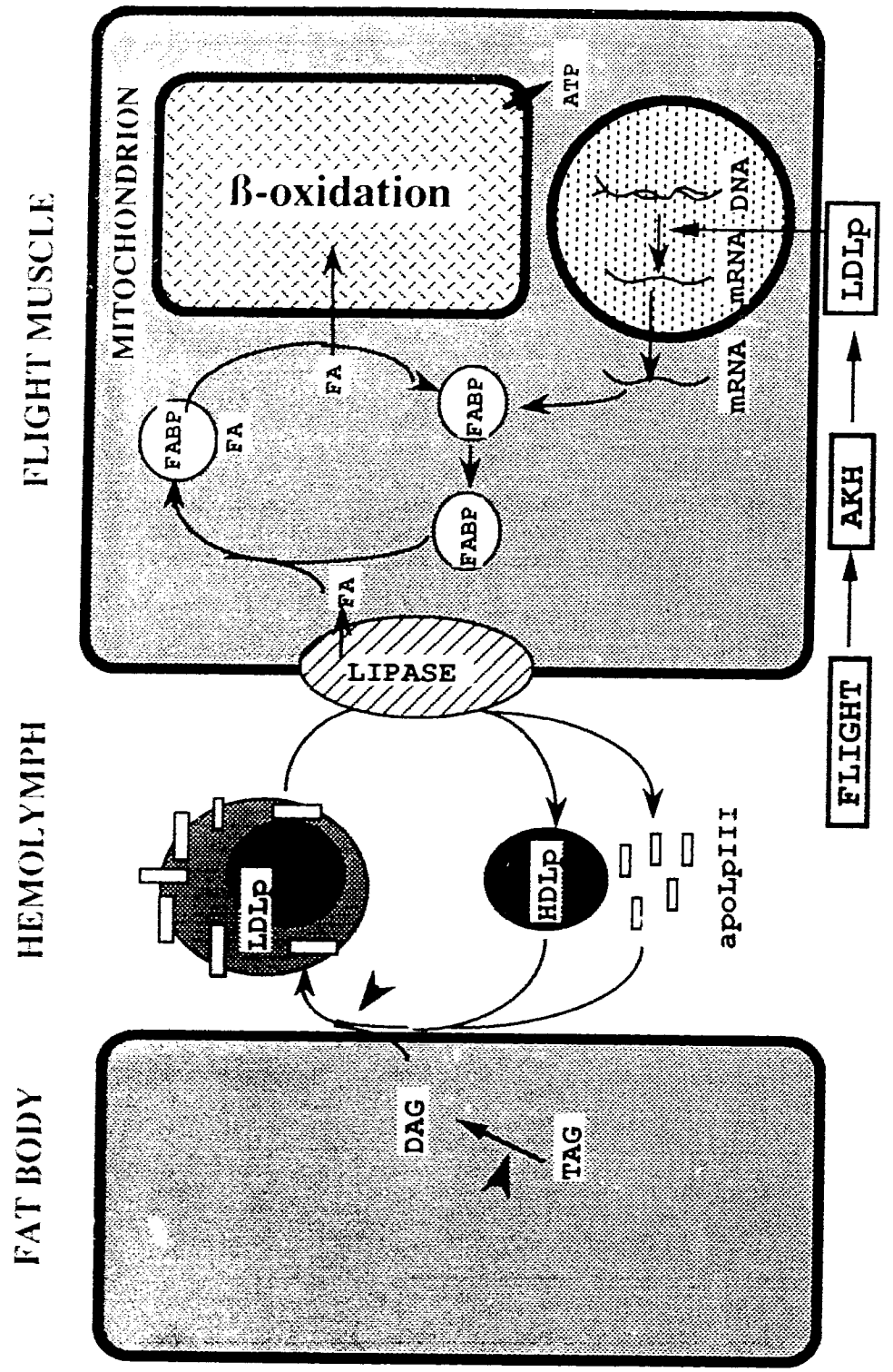

Fig. 6-2. Locust lipid mobilization, transportation and utilization during sustained flight. The arrow indicate the site of AKH action.

conceivable that the ultimate cause of the induction of FABP gene expression is the increased amount of intracellular fatty acids. Such a feed back response to higher levels of intracellular fatty acid could be an important protective mechanisms against potential damage by unbound fatty acids.

From this thesis, it is not clear how free fatty acids may stimulate the transcription of FABP, but an intracellular fatty acid receptor must be present to sense fatty acid. An interesting hypothesis points to FABP itself to represent the link between the cytosolic fatty acid concentration and the FABP gene. The observed similarity between the developmental changes of FABP concentration in both cytosol and nucleus suggests that FABP is small enough to exchange through nuclear pores freely between nuclei and cytosol. The tertiary structure of FABP may change upon binding of fatty acid, and thus FABP may be a sensor for the accumulation of free fatty acids. Consistent with my observations, but nevertheless highly speculative, is a mechanism in which fatty acid free FABP acts as a silencer for the FABP gene. Free fatty acids, when accumulating in the cell, would bind to FABP molecules that suppress gene expression. Upon fatty acid binding, conformational changes in the FABP molecule would weaken the FABP-DNA interactions and lead to gene transcription of the FABP gene. This mechanism could explain the spontaneous synthesis of FABP in adult locusts, the feed-back inhibition after day 12, and the increased synthesis upon increased lipid delivery and utilization.

For mammalian muscle FABP, similar control mechanisms may exist. Prolonged muscle activity leads to increased FABP synthesis, and increased lipid delivery, as experienced in hypertensive animals, also results in elevated FABP levels. To verify the above outlined mechanism molecular studies with the FABP gene will be required. With the detailed knowledge of the physiological regulation of FABP established in this thesis locust flight muscle appears to be an ideal system to study the molecular mechanisms that control the expression the FABP gene.

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ABBREVIATION

AKH I: Adipokinetic hormone I

AKH II: Adipokinetic hormone II

apoLp-III: apolipophorin III

DAG: Diacylglycerol

ELISA: Enzyme-linked immunosorbent assay

FABP: Fatty acid binding protein

HDLp: High density lipophorin

H-FABP: Cardiac FABP

I-FABP: Intestinal FABP

JH: Juvenile hormone

LDLp: Low density lipophorin

L-FABP: Hepatic FABP

M-FABP: Muscle FABP

SDS-PAGE: SDS-polyacrylamide gel electrophoresis

TAG: Triacylglycerol