POLYPLOIDIZATION IN THE FAT BODY CELLS OF

THE DESERT LOCUST, SCHISTOCERCA GREGARIA

by

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Polyploidization in the Fat Body Cells of the Desert Locust,

Schistocerca gregaria

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ABSTRACT

The purpose of this study was to examine the significance of polyploidization as a developmental mechanism in the fat body cells of Schistocerca gregaria during growth of the last larval instar and adult maturation of female insects. Cytochemical determination of DNA content was done by microspectrophotometry of Feulgen- stained nuclei. Results showed that the predominant ploidy class of DNA in fat body tissue of late fourth instar and fifth instar nymphs is tetraploid (4C). Fat body tissue of the maturing adult female was found to contain 4C nuclei as well as an increasing proportion of octaploid (8C) nuclei. DNA synthesis in this tissue was assessed by ^{3}H-thymidine incorporation. Autoradiograms of squash preparations indicated that DNA synthesis occurs cyclically in the fat body of S. gregaria during growth of the fifth instar and maturation of the adult female. In the absence of mitosis this DNA synthesis is presumably involved in endomitotic cycles.

The influence of juvenile hormone (JH) on fat body ploidy was investigated in precocious adult S. gregaria. These insects were obtained by treating second and third instar nymphs with the anti-allatal compound precocene, and are considered to be lacking in endogenous JH which is elaborated by the corpus allatum (CA). After topical application of JHIII or juvenile hormone analogue (JHA) ZR-512 to precocious adults it was found that fat body nuclei doubled their DNA content in response to exogenous JH or JHA. The results of this study suggest that vitellogenin synthesis by the fat body cells is preceded by the replication of the DNA content.

DEDICATION

For Violet

ACKNOWLEDGEMENTS

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GENERAL INTRODUCTION

Increase in cell size rather than cell number is a growth phenomenon well known in the Insecta. This phenomenon includes somatic polyploidization which is achieved by endomitosis. It is usually cells that synthesize tissue-specific proteins which undergo polyploidization, and the function of this process is thought to be a consequence of the simultaneous need for cell growth and tissue function (Brodsky and Uryvaeva, 1977), so that in the competition for cellular resources the mitotic cycle is modified. As a result, the proliferation of equivalent cells is replaced by replication of the genome. Somatic polyploidization or endopolyploidization has recently been extensively reviewed by Nagl (1978).

Endomitosis is a phenomenon originally described by Geitler (1939) in somatic nuclei of _Gerris lateralis_ (Heteroptera) and refers to structural changes of the chromatin which resemble those of mitosis but which occur within the nuclear membrane and without spindle formation. A distinction has also been made between two types of observed endomitoses on the basis of morphological changes of the chromosomes during the endomitotic cycle. The process observed in plants differs from that seen in the insects in that the former has a phase of temporary heterochromatic decondensation similar to early prophase of the mitotic cycle.

Endopolyploidization has been implicated as part of the differentiation process (Kafatos, 1972; Nagl, 1978). Nagl has suggested that the abolition of mitosis opens the way for continuous and increasing RNA synthesis and therefore an increased potentiality for protein synthesis in the cell . By quantitative autoradiography he has shown that the capacity for RNA synthesis is higher in the gap 1 phase of a polyploid endomitotic nucleus than it is in the gap 2 phase of a diploid mitotic nucleus.   Essentially the break in RNA synthesis during mitosis   is responsible for the inverse relationship between cell division and RNA   production (Nagl, 1973).

Kafatos (1972) studied the cocoonase zymogen cells of silk moths and found that these specialised cells undergo polyploidization in order to increase their secretory potential. However, in his model of terminal cell differentiation for specific protein synthesis, polyploidization is only one of several steps in the development of such cells. In contrast to Nagl's (1973) findings, Kafatos reports that template multiplicity is not needed if the mRNA for a specific protein is unusually stable, which seems to be the case for cocoonase zymogen mRNA.

Other advantages of endopolyploidy have been suggested. It may be that endocycles provide more immunity from disturbances that would upset mitotic activity with its more complex events. Polyploidy has been found to protect cells from damage by radiation (Wagenheim, 1976) and to alkylating mutagens (Zutshi and Kaul, 1975; Evans, 1976). It is also

possible that the coordination of cellular activities is more easily controlled in a population of endopolyploid cells than it is in a larger group of diploid cells.

The method of choice for accurate detection of endopolyploidization is the microspectrophotometry of Feulgen-stained nuclei. This technique involves the partial hydrolysis of tissues such that aldehyde groups of DNA are exposed for subsequent staining with leucofuchsin (Schiff's reagent)(Pearse, 1968). The stained nuclei are scanned in a microspectrophotometer at the appropriate wavelength thus obtaining total extinction measurements on an arbitrary scale. A method whereby these measurements are converted to $\log_2$ values facilitates detection of genome doubling when such values are compared to a haploid standard (Mittwoch et al., 1966). By examining several insect tissues in this manner Fox (1969) was able to demonstrate that some insects have polyploid somatic tissues which conform to members of a doubling series (Fox, 1969), whereas the tissues of other species contain interclass values of DNA (Fox, 1970). Such interclass DNA values are suggested to have implications for mechanisms of tissue differentiation in insects.

Insect fat body is a versatile organ which has been functionally compared to vertebrate liver (Kilby, 1963). When an insect is dissected the fat body is generally the most conspicuous tissue. It is arranged in lobes or sheets in contact with the other internal organs. Fat body functions include: (1) glycogen synthesis and storage; (2) trehalose

biosynthesis; (3) amino acid metabolism; (4) lipid synthesis and storage; (5) pigment storage; and (6) uric acid storage. Reactions of the citric acid cycle and cytochrome system also occur in this tissue. Vitellogenic proteins are synthesized in the mature female fat body of some species (Engelmann, 1970,1979; Keeley, 1978; Wyatt et al., 1978). The histochemistry of S. gregaria fat body has been familiar for some time (Coupland, 1957; Odhiambo, 1967).

This study was intended to investigate the role of polyploidization in the growth and development of fat body tissue from the desert locust. In Chapter I we examined the fat body of S. gregaria for polyploidization during development and metamorphosis. The desert locust is a hemimetabolous insect which passes through five nymphal instars prior to the imaginal moult and the average duration of each stadium is five to seven days under our rearing conditions. Beginning with the fourth instar, through the fifth and into the maturing adult stage, we examined fat body nuclei for endopolyploidization.

The present study looked at ^{3}H-thymidine incorporation into fat body cells concurrently with the examination of their DNA content. Thus DNA synthesis was determined autoradiographically and the DNA content assessed microspectrophotometrically. This is important when determining whether DNA synthetic activity is involved in mitosis or in endocycles.

Chapter II of this study examines the factors that are responsible for further polyploidization seen in the fat body of adult females. In most insects the corpus allatum (CA) is activated in the adult to produce juvenile hormone (JH) which assumes a gonadotropic function after metamorphosis (Engelmann, 1970). The juvenile hormone is necessary for the synthesis of vitellogenic proteins in the fat body (Engelmann, 1970,1979; Wyatt, 1978; Keeley, 1978). This system is therefore conducive to studying the action of JH on a target tissue.

A methodological modification introduced in the present study was to use a chemical, precocene, to eliminate the endogenous source of JH. This method of chemical allatectomy has several advantages over surgical removal of the CA and the precocious adult insects produced by application of precocene have every indication of an irreversibly inhibited corpus allatum (Unnithan et al., 1977,1979,1980; Pedersen, 1978).

CHAPTER I

Polyploidization During Development and Metamorphosis

INTRODUCTION

Polyploidization in insect somatic tissues is a phenomenon which results from endomitosis, a modified mitotic process involving the duplication of chromosomes within an intact nuclear membrane (Geitler, 1953). Polyteny, a closely related situation found in dipterous insects, is brought about by endoreduplication, a process similar to endomitosis but for the fact that homologues remain paired (Levan and Hauschka, 1953). Polyteny and endopolyploidization are functionally similar in that both phenomena are probably related to the need for producing a large amount of secretion for export. But whereas polyteny has been rather extensively studied due to its associated phenomenon of chromosome puffing (Ashburner, 1970), endopolyploidy has only recently been the focus of investigations (Nagl, 1978).

The quantitative cytochemical technique of assaying DNA content by Feulgen microspectrophotometry has been found fruitful in studying endopolyploidy in the mammalian liver (Geschwind et al., 1958; 1960; Mahon et al., 1979). Mittwoch et al. (1966) used Feulgen microspectrophotometry of honey bee larvae to demonstrate a contrast in ploidy between dividing and non-dividing cells. Wigglesworth (1967) in

a study of _Rhodnius_ fat body, has suggested that nuclear fusion might play a role in endopolyploidization. He suggested that a basic level of endopolyploidy arises initially by endomitosis but that sporadic polyploidization occurs subsequently during various circumstances such as starvation. His study was largely based on chromosome counts and his cytophotometric results were inconclusive because they were based on the results of measurements on only 20 nuclei. Fox (1969) used densitometric technique to describe the pattern of DNA values in two somatic tissues of Dermestid beetles, a pattern that was found to be discontinuous and consistent with a doubling series of DNA values. Subsequently Fox (1970) determined the DNA content of various tissues in two species of locusts, _Locusta migratoria_ and _Schistocerca gregaria_ of unknown age, and found tissue specific DNA contents with peaks in the frequency distributions that did not conform to a doubling series (Fox, 1970). Fox has interpreted this latter result to indicate a differential replication of those chromosome regions which are active in particular tissues.

The present study was designed to examine the DNA content of fat body nuclei during the development and metamorphosis of _S. gregaria_. Although the cytological aspects of insect development have been extensively studied, not much work has been done on the nuclear aspects of differentiation. Since endopolyploidization has been implicated as a mechanism for insect tissue development (Nagl, 1978), we felt it useful to study the fat body with respect to polyploidization during a part of

nymphal development and during maturation of the adult insect. Moreover, we sought to relate the polyploidization process to DNA synthetic activity by carrying out autoradiographic studies of fat body tissue. Using these techniques we were able to examine intranuclear events with respect to some developmental aspects of this insect tissue.

MATERIALS AND METHODS

The locusts were raised on a diet of whole wheat bran and fresh

grass and reared under a light regime of 16 hr. photoperiod and 8 hr.

scotoperiod at temperatures of 35 and 31°C respectively and 60-70%

relative humidity. Only females of the following age groups were used

in this study: late fourth instar nymphs one day before ecdysis; one-,

three- and five-day-old fifth instar nymphs; one-, three-, five-, and

eight-day-old adults. The ages were determined from the time of

ecdysis. Three to four insects were used from each age group for the

experiments outlined below.

DNA Synthesis

The locusts were injected under CO_2 anaesthesia with $10\mu Ci$ of

methyl ^3H-thymidine (3.8 Ci/mmole, Amersham-Searle Corporation,

Oakville, Ontario) through the arthroidal membrane at the base of the

third pair of legs. After injection the insects were returned to the

incubator for four hours. The perivisceral fat body from the

metathoracic segment was dissected out from the above age groups,

squashed on gelatinized slides and cover slips removed by the dry ice

technique. After air-drying the squashes were fixed in ethanol:acetic

acid (3:1v/v) for two hours at room temperature. Following hydration

through a graded ethanol series the slides were placed in 5%

trichloracetic acid at 4°C for 15 minutes and then in 0.1%

non-radioactive thymidine solution for 20 minutes to remove free

radioactive thymidine. The slides were washed in three successive

changes of distilled water, dehydrated, air dried and coated with NTB-2
nuclear emulsion (Eastman Kodak). One to two sets of slides from each of
the age groups were treated with DNAse (Sigma Chemical) (Dietch, 1966)
prior to coating with the nuclear emulsion. All slides were exposed in
the dark at 4°C for six weeks. After developing they were stained with
methyl-green pyronin (Pearse, 1968). At least 500 nuclei per age group
were examined for tritium labelling. From these the percentage of
labelled nuclei was calculated for each of the age groups.

DNA Content

Squashes of fat body which were fixed as described above were
hydrolysed in 3.5N HCl at 37°C (Fand, 1970). 20 minutes was found to
be the optimum time of hydrolysis for fat body nuclei taken from
different age groups . After hydration the squashes were stained in
Schiff's reagent (Basic Fuchsin, Harleco) prepared according to Deitch
(1966), dehydrated through ethanol series, cleared in xylene and mounted
in oil of matching refractive index (nD=1.56, Cargille, Cedar Grove,
N.Y.). In addition, pieces of testis from one-day-old adult males and
brain from mature females were squashed on slides, fixed and stained as
described for the fat body in order to obtain reference haploid and
diploid DNA standards respectively.

Microspectrophotometry

Extinction measurements on 100 to 150 Feulgen-stained nuclei per age group were taken at 570nm using the Scanning Microscope Photometer (SMP, Carl Zeiss, Oberkochen, Germany)at 1 μ m intervals. The SMP used in this study was on line with a PDP-12 computer (Digital Equipment Corporation, Maynard, Mass.). All measurements were carried out with ultrafluar objective (X100, N.A.1.25) and condenser (N.A.0.8) lenses at 1 μm steps along the X and Y axes. The diameter of the scanning aperture was 1.4 $_{\mu}$m. Fifty early spermatid nuclei from testis squashes and neuronal nuclei from brain squashes were measured and their respective averages (1C and 2C) used to determine the different DNA ploidy classes in the fat body nuclei.

The instrumentation used in this study provides us with several parameters. The SMP reads a pre-selected number of points over a stained nucleus and the sum of these readings is expressed as the total extinction of light absorbed over the nuclear area. A digitized image of such a scanned nucleus is shown in Figure 1. The mean extinction is the average of these values in the scanned image. Nuclear area is the total number of points having a transmittance between 5 and 95%. These parameters are provided by the computer which is interfaced to the SMP and coordination of these components achieved by the APAMOS (automatic photometric analysis of microscopic objects by scanning) programme.

Figure 1. A. Digitized image of a Feulgen-stained fat body nucleus (B) showing extinction values (x100) at 1µm intervals. The sum total of these values represents total extinction (DNA content), the average of these is the mean extinction, and the number of points represents the area in 1µm steps. Scale in Fig. B is 25µm.

A

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                        10 18 15
                6 24 47 61 66 60 48 33 15
            9 49 70 66 65 67 70 69 61 57 45 33  8
         11 48 72 73 67 67 68 73 69 62 67 58 61 55 20
       6 36 63 66 75 69 64 57 69 69 70 72 69 72 70 56 28  6
      35 60 65 61 59 64 57 56 69 70 67 67 73 76 67 69 63 34 11
   20 55 61 69 65 62 61 50 66 78 75 74 74 79 73 68 61 63 59 29  6
  8 46 57 58 60 63 69 58 48 69 75 79 75 69 72 68 70 65 59 60 49 19
 22 60 61 54 49 66 79 65 61 69 60 69 66 74 79 74 62 63 73 66 65 39
 39 61 63 63 61 66 73 73 72 68 53 68 65 78 76 78 70 66 75 66 68 61 13
14 50 63 63 62 73 69 70 82 80 68 67 76 73 74 68 79 78 70 68 61 79 70 38
21 50 61 59 62 75 76 79 85 76 63 67 79 76 62 58 69 72 70 73 78 86 68 52  9
23 52 54 55 69 72 74 78 79 75 70 74 78 76 66 50 42 49 62 68 69 72 65 44  6
24 56 60 55 68 62 62 62 69 64 74 78 74 79 79 53 33 42 56 54 57 61 61 41
27 53 56 58 68 65 72 74 76 70 79 82 76 76 74 52 38 46 65 65 72 70 68 38
27 52 56 63 61 60 61 64 67 67 64 79 74 72 80 72 56 62 75 82 80 78 70 25
27 56 62 54 55 57 61 63 62 61 61 74 76 80 79 72 65 60 76 80 78 86 56 11
21 50 62 58 56 68 76 75 70 61 61 72 79 75 72 70 73 67 79 74 67 61 20
11 43 61 53 60 68 68 74 72 67 72 74 78 82 78 85 79 80 92 80 66 39
 6 33 52 61 68 70 58 63 66 58 72 75 68 72 73 70 73 74 90 86 56 19
   20 45 61 63 68 63 69 73 70 68 70 66 67 76 74 70 69 88 68 33  6
      20 44 55 61 63 64 70 65 57 65 72 82 85 88 85 78 69 40 14
         16 33 49 59 61 68 74 62 61 75 83 79 83 83 74 38 12
            19 37 52 61 67 69 62 64 70 69 63 53 33 11
               8 21 28 35 40 39 36 33 21 13  8
                     6  9  7  6
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B

The DNA content was expressed as $\log_2$ of the total extinction. This facilitated graphic presentation of the data since an increase in one unit on a $\log_2$ scale represents a complete duplication of the genome (Mittwoch et.al., 1966; Fox, 1969).

The correlation between the DNA content and the nuclear area was determined using the statistical package for the social sciences (SPSS) (Klecka et al., 1975).

Mitotic Index

As the Feulgen stain can be used to detect mitotic figures (Fig.2A,B), all squash preparations were examined for mitotic figures. In the case of the fourth instar, fat body squashes were made at 24hr intervals from day 1 till the end of the fourth instar, which took approximately five days under our rearing conditions. Two insects were used for each time interval. The feulgen-stained squashes were scored for the mitotic figures on slides where such figures were found.

RESULTS

DNA Synthesis

Intense labelling is seen in some of the individual nuclei in all of the age groups studied (Fig.2C,D). Since treatment with DNA-ase removes over 90% of the label, it is presumed that these nuclei are active in DNA synthesis. It is evident from the data obtained (Fig.3) that DNA synthesis occurs in a cyclic fashion with peaks of synthetic activity in the late fourth instar, five-day-old fifth instar larvae and five-day-old adults. Since mitosis is not found after the third day of the fourth instar, it can be assumed that this DNA synthesis is involved in endomitotic cycles. In the fourth instar mitotic figures were found on the third day and of the 2,348 nuclei counted for that period there was a mitotic index of 3.5%.

Figure 2. A. Photomicrograph of Feulgen-stained fat body nuclei from a 3-day-old fourth instar $\underline{S}$. $\underline{gregaria}$ showing mitotic figures. Scale bar 30μm. B. Same as (A) at higher magnification. Scale 30μm. C. Autoradiograms of fat body nuclei of fifth instar $\underline{S}$. $\underline{gregaria}$ after ^{3}H-thymidine incorporation. Scale 30μm. D. Same as (C) at higher magnification. Note intense labelling of two nuclei. Scale 30μm.

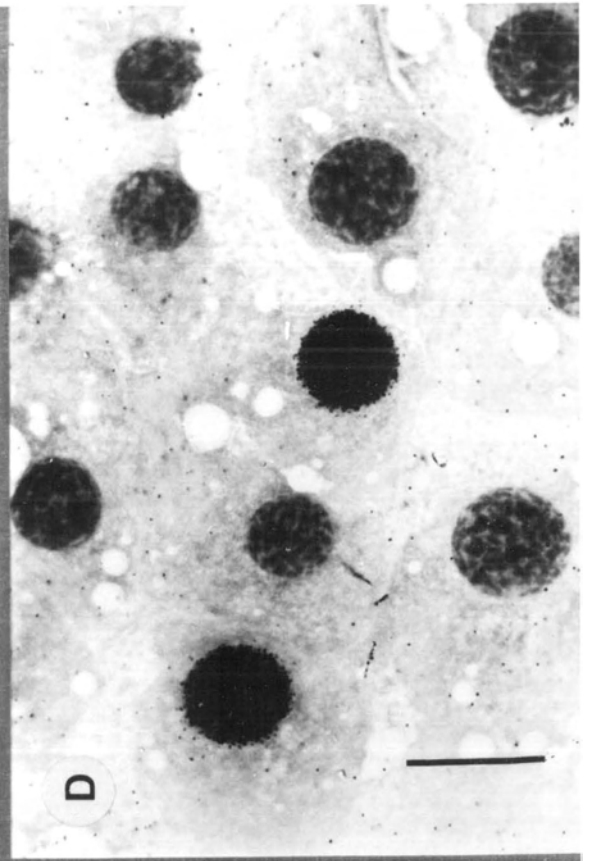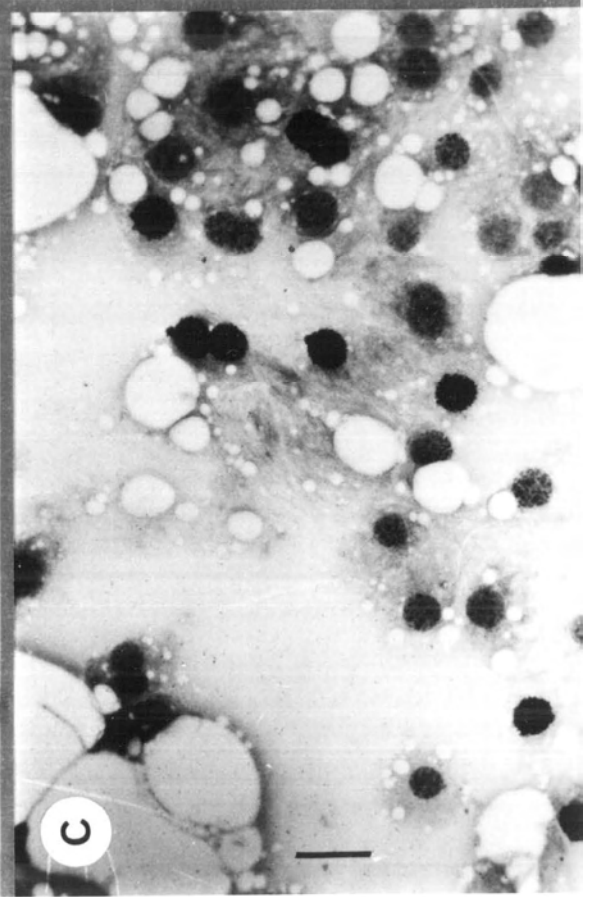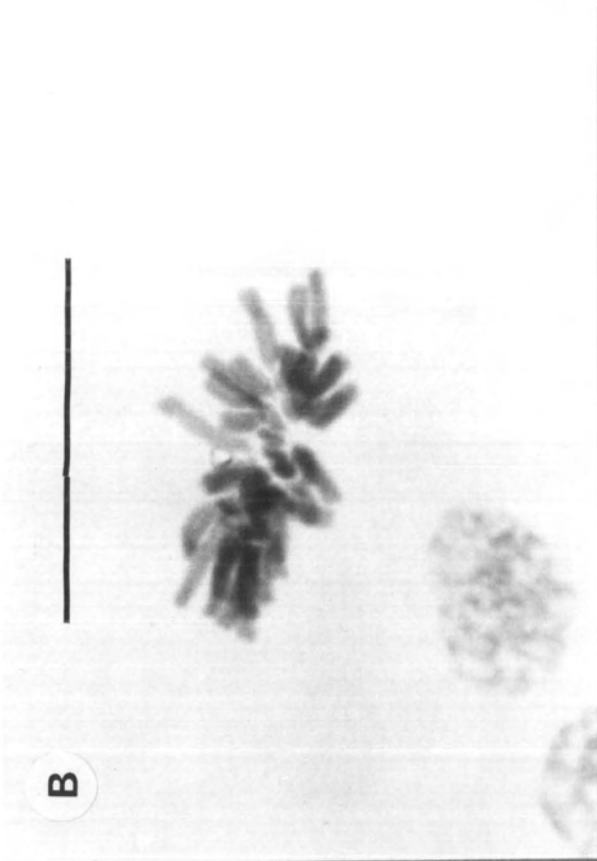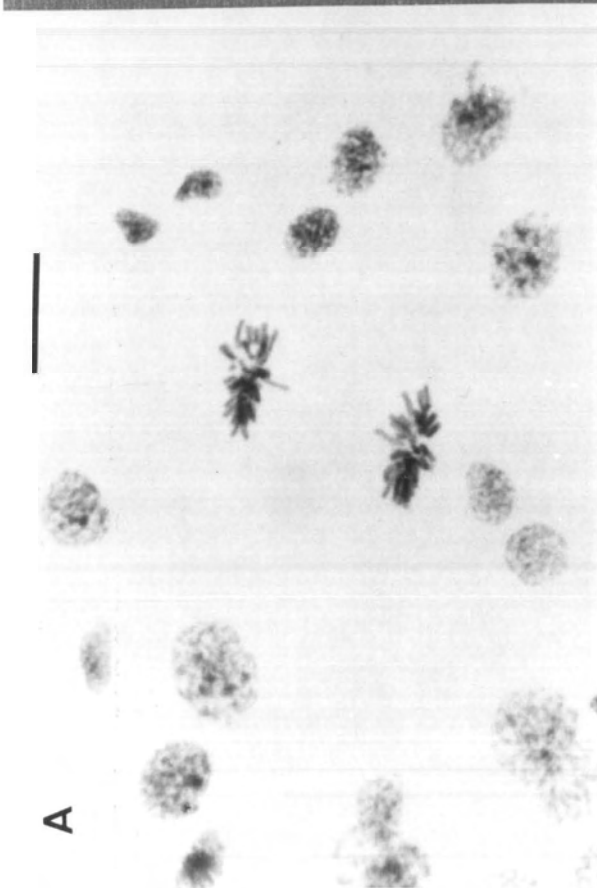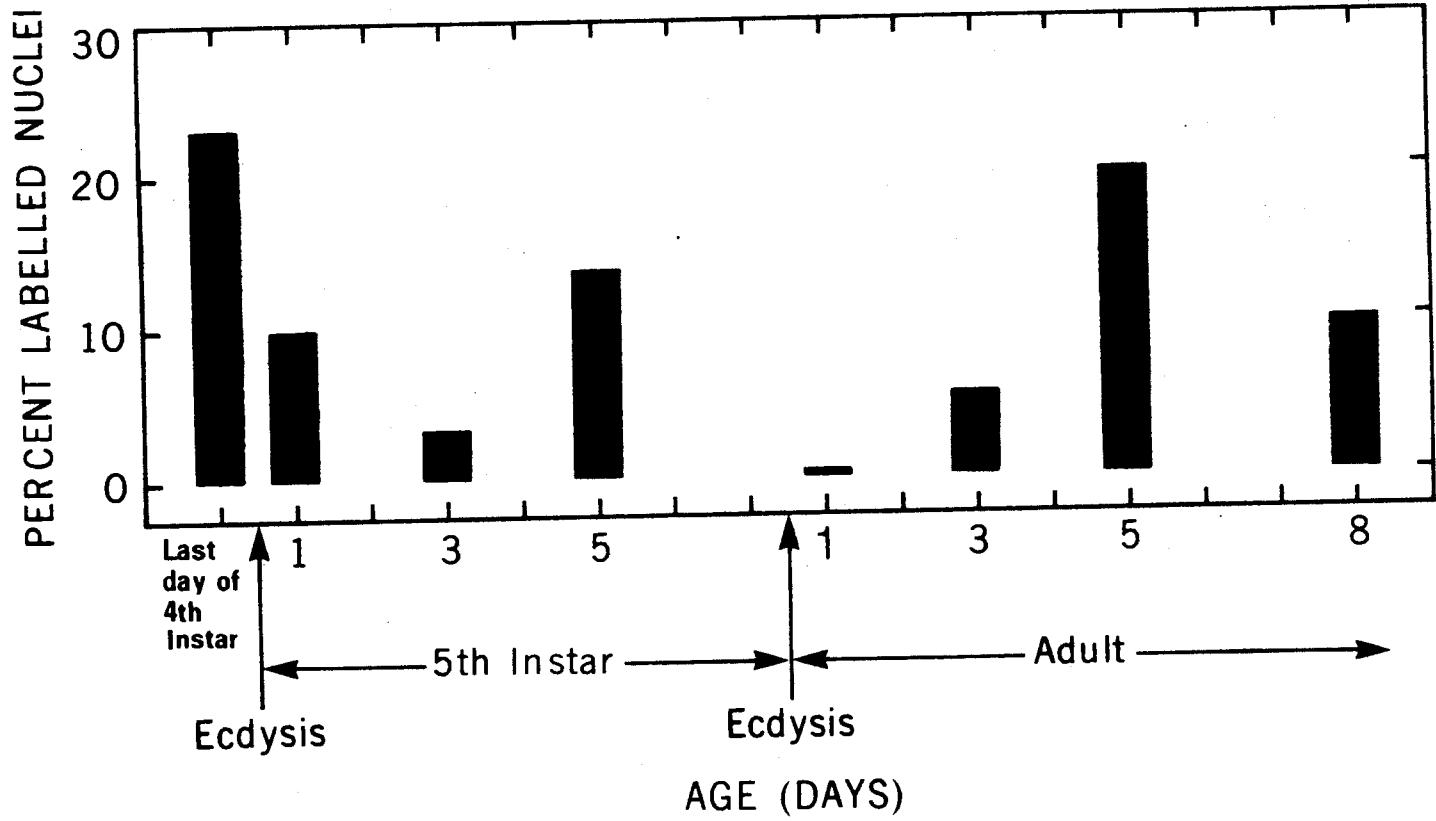Figure 3. Histograms showing the percent frequency of labelled fat body nuclei after a four hour pulse of ^{3}H-thymidine incorporation. The ages of the various groups are estimated in days from the day of previous ecdysis.

DNA Content

The microspectrophotometry results show that endopolyploidy is involved in fat body tissue growth during the development of <u>S.</u> <u>gregaria</u>. By establishing a reference diploid value from brain nuclei measurements the histograms in Figures 4 and 5 were obtained. The distribution of the various DNA classes in the fat body nuclei shows that the fat body cells are polyploid . In the fourth larval instar the major class of DNA on the last day is 4C (Fig.4B). During the growth of the fifth instar the major class of DNA is 4C (Fig.4C-E), and this ploidy class gradually decreases in frequency (90 to 75%) with corresponding increases in the 8C (8 to 21%) (Fig.4). In the adult insect the major classes of DNA are 4C and 8C with a small percentage of 16C nuclei in the eight-day-old adults (Fig.5). This increased DNA content is associated with a concomitant increase in nuclear area since a linear relationship between nuclear area and total extinction is demonstrated (Fig.6). The correlation coefficient in all of these cases was found to be between 0.87 and 0.92 .

Figure 4. Frequency distribution of the DNA content of Feulgen-stained brain nuclei (A), and fat body nuclei from fourth and fifth instar S. gregaria. The numbers parenthesized in the legend represent the total number of nuclei measured for each group. B. Last day fourth instar (n=100). C. One-day-old fifth instar (n=100). D. Three-day-old fifth instar (n=150). E. Five-day-old fifth instar (n=150).

18b

Figure 5. Frequency distribution of the DNA content of Feulgen-stained fat body nuclei in adult female <u>S</u>. <u>gregaria</u>. The numbers parenthesized in the legend represent the total number of nuclei measured. A. One-day-old adult (n=150). B. Three-day-old adult (n=150). C. Five-day-old adult (n=150). D. Eight-day-old adult (n=150).

PERCENT NUCLEI
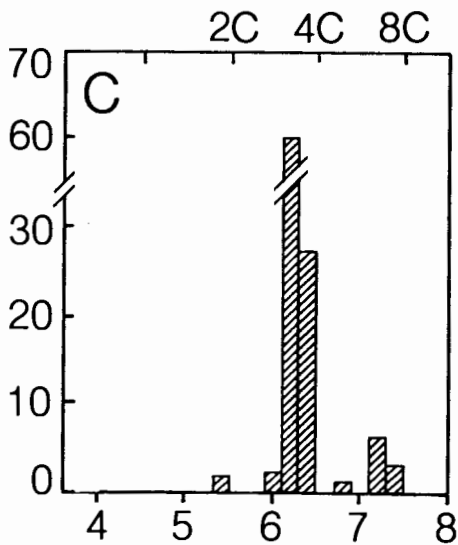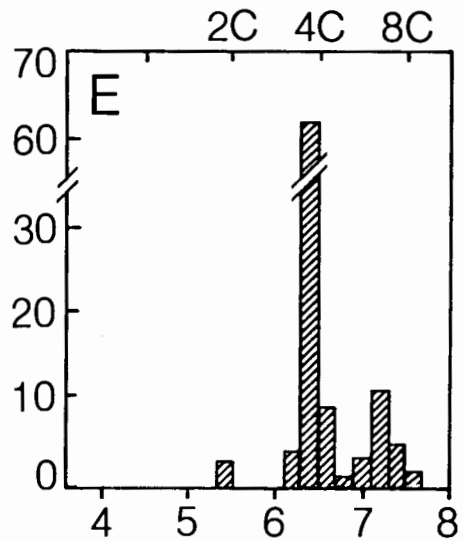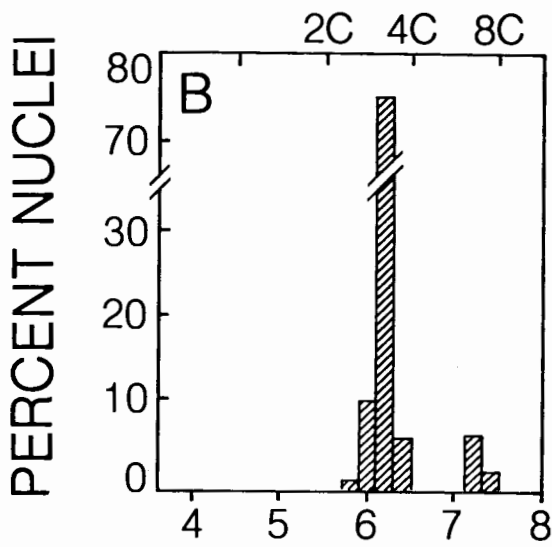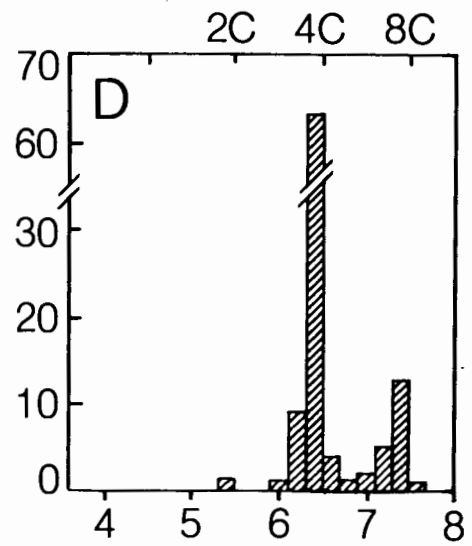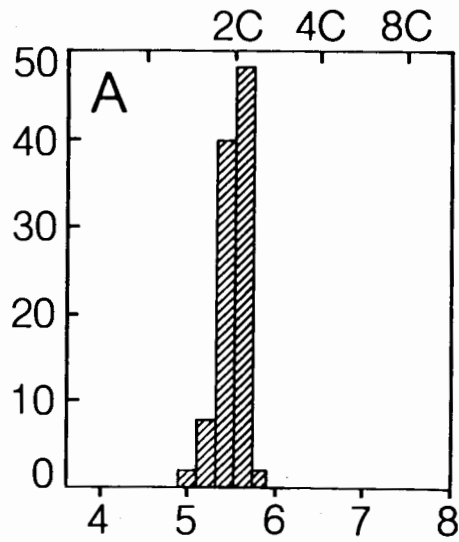
DNA CONTENT (LOG$_2$ TOTAL EXTINCTION)
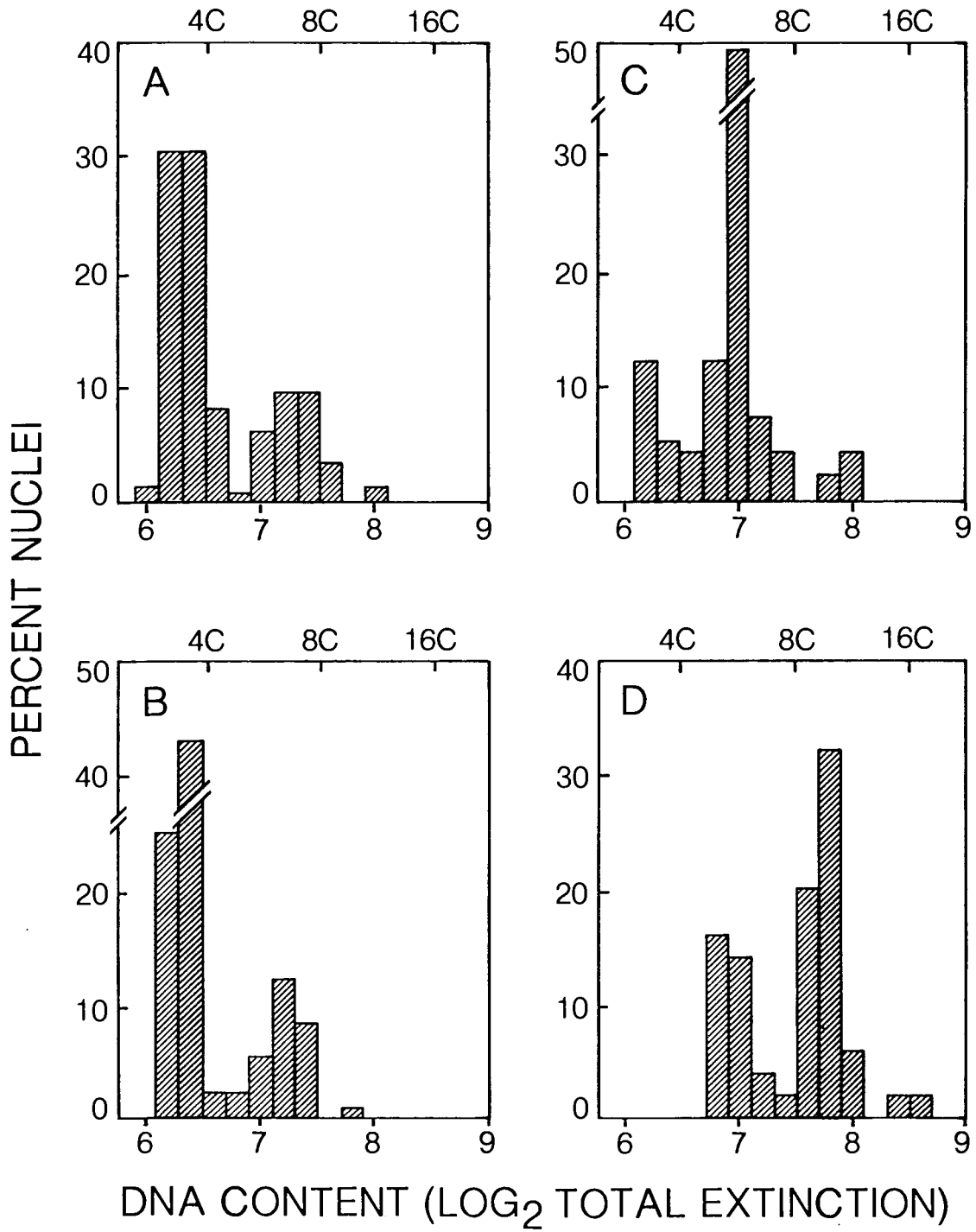
DISCUSSION

Although mitosis is involved in fat body growth during the early fourth instar, it appears that endomitosis is the major mechanism as indicated by the predominance of tetraploid nuclei and a peak of DNA synthesis in the late fourth instar (Figs.3,4B). But during the fifth nymphal instar and the maturation of the adult, it would appear that the DNA synthesis is involved only in endocycles since no further mitoses are seen. Moreover, the gradual appearance of higher ploidy classes is indicative of endomitosis. This result is at variance with the data of Fontana (1974) on an albino strain of S. gregaria, which suggest that the predominant DNA class in the fifth instar is 4C, and that any 8C nuclei are probably due to the presence of oenocytes. However, we find that oenocytes can be distinguished from larger fat body cells in that the nuclei of the latter have areas of condensed chromatin, whereas the chromatin of oenocyte nuclei is more diffuse. Coupland (1957) has shown that oenocytes are numerous in the peripheral fat body of S. gregaria, whereas they are fewer in number in the perivisceral fat body. Moreover, Fontana (1974) used the Barr and Stroud densitometer. This instrument has an an upper limit to the nuclear area which can be scanned since its measuring grid is limited at any one magnification resulting in a measuring size bias. The scanning microphotometer used in this study, on the other hand, can measure over a variable area. This feature is particularly useful where nuclei of higher ploidy values are found. The increase in polyploidy of fat body nuclei is also accompanied by an increase in nuclear area as shown in Figure 6.

Figure 6. Regression plots of area vs. total extinction, 570nm., (DNA content), for nuclei from one-day-old fifth instar (A), three-day-old fifth instar (B), five-day-old fifth instar (C), one-day-old adult (D), three-day-old adult (E), and five-day-old adult (F).
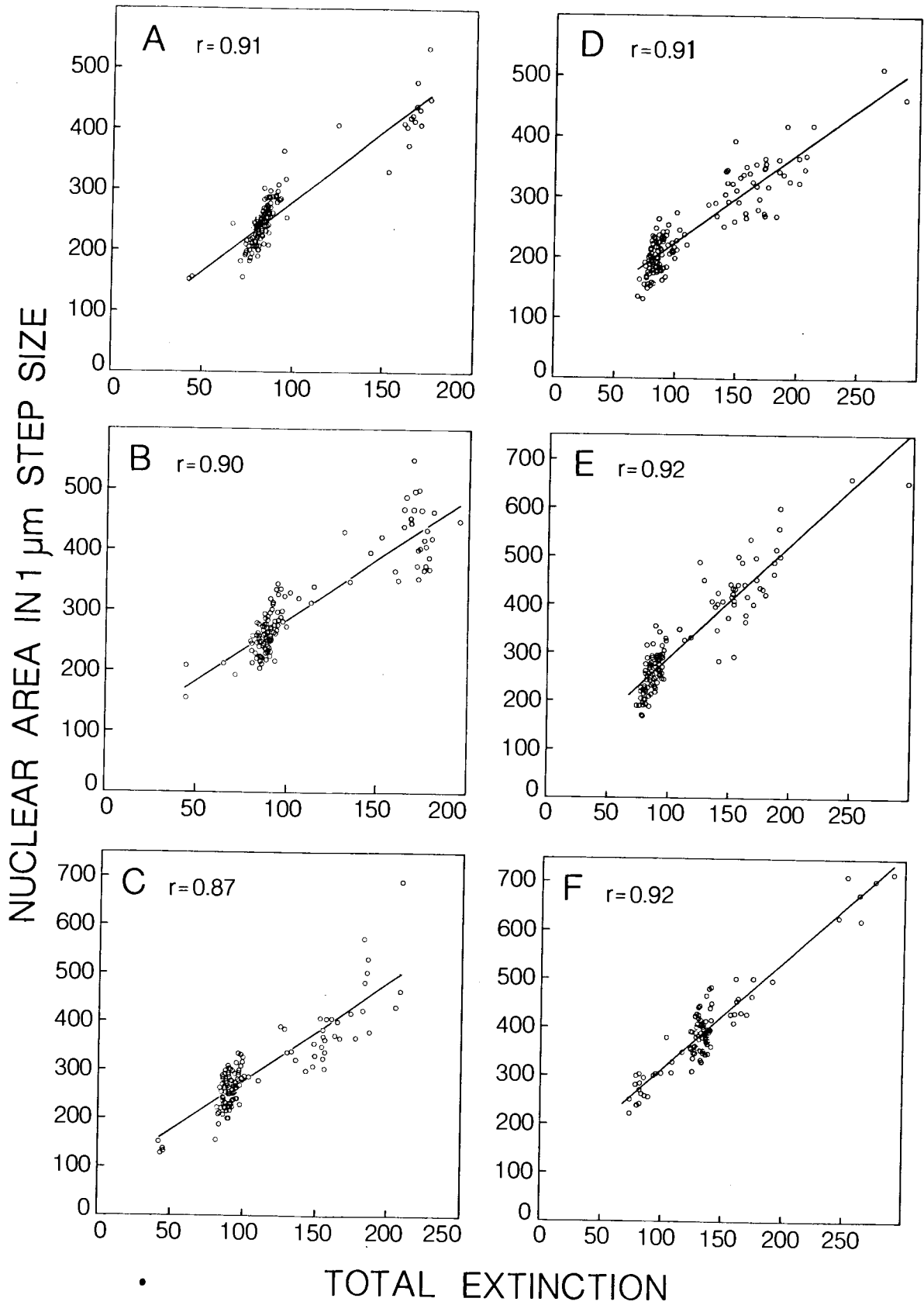
The distribution of the DNA classes shows also that in the adult levels of ploidy are higher than those in the nymphal instars (Fig.5). Our data of the maturing adult fat body show an increase in the number of 8C nuclei. That this increase in ploidy is achieved by endocycles in the fat body nuclei is suggested by the presence of DNA synthesis as demonstrated by autoradiography (Fig.3). In L. migratoria this enhanced DNA synthesis and polyploidization in the adult female fat body has been associated with vitellogenin production and shown to be dependent on the presence of juvenile hormone (Nair et al., in preparation). Chapter II of this thesis examines the relationship between polyploidization and JH in S. gregaria.

The appearance of nuclei in the 4C - 8C interclass range needs to be explained. Fox has suggested a number of factors which may contribute to this phenomenon: (1) DNA synthesis at the time of fixation will result in intermediate values. Similarly, Roberts and Roberts (1972) have suggested that interclass DNA values found in larval tissues of Tricholioproctia impatiens are due to cells that were fixed while in the process of endoreplication. (2) Nuclear fusion will influence the distributions of DNA classes but its effect will depend upon the true frequency of fusion and the classes of nuclei which undergo fusion. From his own data Fox (1970) concludes that nuclear fusion has a minimal influence on the distribution of DNA class values. This would agree with the studies of nuclear fusion by Wigglesworth (1967) who determined the phenomenon to be of sporadic occurrence only. Fox (1970) therefore suggests that the most reasonable explanation for the finding of

interclass DNA values is that ''during the differentiation of most of the tissues studied here, each round of DNA replication consistently excludes a part of the DNA from the replication process''. He adds the evidence from <u>Drosophila</u> somatic tissues where the heterochromatin is more or less consistently under-replicated (Berendes and Keyl, 1967). There are several instances where under-replication of the genome in plant and animal cells has been noted by cytophotometry and these have been adequately reviewed by Nagl (1978). In some cases buoyant density analyses of nuclear DNA have established differential replication of euchromatin and heterochromatin. If replication of euchromatin and heterochromatin depends on the thresholds of molecules that initiate DNA synthesis (Barlow, 1972) one may expect to find intermediate classes of DNA in various tissues of an organism. What is needed is concurrent studies involving cytophotometry and buoyant density analysis of nuclear DNA to substantiate or disprove the phenomenon of under-replication of the genome in locusts.

A study on the moulting hormones of the desert locust (Morgan et al., 1975) has determined the titres of 20-hydroxyecdysone at daily intervals throughout the fifth instar and adult. Their results show a maximum concentration of this substance on the day of ecdysis and a variation in the levels during the instar and adult growth. Although the fifth instar lasted for 12 days under their rearing conditions while ours lasted for 7 days, a cyclic variation in the titre of this hormone during development might be extrapolated. The authors suggest that

moulting hormones derived from prothoracic gland extracts cause a growth of epidermal cells as determined by histological examination of the cuticle. Such an increase in epidermal cell size rather than increased cell division is suggestive of a moulting hormone-dependent DNA synthesis in this tissue.

Two studies on the effects of 20-hydroxyecdysone in Calliphora stygia larvae suggest that ecdysone stimulates the general capacity of the fat body for protein synthesis (Neufeld et al., 1968; Thomson et al., 1971). Moreover, the time course for ecdysone-stimulated protein synthesis was identical in both the fat body and the epidermis of these insects. The cyclic variation in DNA synthesis observed in S. gregaria fat body may be related to the action of ecdysone. The role of ecdysone and JH during morphogenesis, and especially the interaction of these hormones remains unclear (Willis, 1974) and present models of control are necessarily vague (Kumaran, 1976). More studies at the molecular level are needed to resolve this difficulty.

CONCLUSIONS

Our results indicate that DNA synthesis is taking place in the fat body of S. gregaria in a cyclic manner during the nymphal stages examined and in the maturing adult female. When this data is related to the DNA content of fat body cells as determined by microspectrophotometry of Feulgen stained nuclei, it would appear that the DNA synthetic activity is involved in endocyles. The predominant ploidy class is 4C throughout development with a gradual increase in the appearance of 8C nuclei. Interclass DNA values are also found and their possible significance discussed. Since mitoses are not found beyond the fourth nymphal instar it would seem that endomitosis is the major method of growth in the fat body throughout the fifth instar and during adult maturation.

CHAPTER II

Juvenile Hormone-induced Polyploidization in Fat Body Cells

INTRODUCTION

Vitellogenesis is a major component of sexual maturation in female insects and has recently been reviewed by Engelmann (1979). Research on insect vitellogenin has demonstrated the involvement of control mechanisms similar to those found in birds and amphibians, since in both instances yolk proteins are produced outside the ovary (Schwartz et al., 1976). In the vertebrates these proteins are synthesized in the liver under the regulation of estradiol and in insects a corpus allatum (CA) hormone directs vitellogenin synthesis in fat body.

Beginning with the work of Wigglesworth (1936) investigations have demonstrated that the CA is necessary for oocyte development in many insects (reviewed in Highnam and Hill, 1977). Coles (1965), in his work with Rhodnius studied the electrophoretic patterns of hemolymph proteins and compared them to egg proteins thereby demonstrating the presence of adult female specific proteins . The same author showed that radioactively labelled amino acids first appeared in the proteins of the fat body and subsequently in the hemolymph of insects. Since that work a number of other insect species have been shown to synthesize proteins in the fat body which are then taken up by the developing oocytes from the hemolymph, hence the term vitellogenins for this particular group of

proteins. In the desert locust Highnam et al. (1963) showed that JH stimulated ovarian uptake of hemolymph proteins in vivo, and Hill (1965) demonstrated the incorporation of ^{14}C-glycine into the proteins of S. gregaria fat body during ovarian development. Dufour et al. (1970) showed that the ontogenesis of a female specific protein in S. gregaria was closely related to ovarian development. In Locusta migratoria Chen et al. (1976) demonstrated a JH-controlled vitellogenin synthesis in female fat body which was dose responsive. Engelmann (1971) used in vivo studies of allatectomized Leucophaea females to demonstrate a graded response by these insects to exogenous JH. Following this insight into endocrine control over oocyte development, the fat body of female insects has been a system of choice for studying the action of JH at the molecular level (Engelmann et al., 1971; Koeppe et al., 1976).

Since knowledge of the protein product is useful in studying gene regulation, biochemical studies of Chen et al. (1978) focussed on the properties and post-translational modification of vitellogenin in the fat body. Vitellin (yolk protein) and vitellogenin (yolk precursor protein) from Locusta migratoria were identified by SDS gel electrophoresis and pulse labelling of tritiated amino acids. Analysis of immunospecific products suggested that the large initial polypeptides may represent the products of two structural genes. These products are subsequently subjected to proteolytic cleavage as well as dimerization and addition of carbohydrate and lipid before secretion from the fat body cells.

A following study sought to investigate the role of JH in
vitellogenin synthesis (Chen et al., 1979). It was found that
vitellogenin synthesis as well as increases in RNA and DNA were
prevented by allatectomy. Moreover, enhanced DNA synthesis, as
determined biochemically, was found in the fat body within 24 hours of
treatment with the juvenile hormone, JHI. A steep dose-response curve
for the induction of vitellogenin synthesis after topical application of
JHI or the analogue ZR-515 to allatectomized locusts was demonstrated.
Cytological examination of JH-stimulated female fat body revealed
cytoplasmic changes which reflect a conversion of the main role of fat
body from nutrient storage to the synthesis and secretion of protein
(Couble et al., 1979). Such changes included enlargement and increased
basophilia of the nucleus, diminished lipid droplets and enhanced rough
endoplasmic reticulum (RER) in the cytoplasm. Since this work, the
JH-induced DNA synthesis has been investigated cytophotometrically and
related to increased ploidy of the fat body cells in L. migratoria (Nair
et al., in preparation).

The present study was undertaken to determine whether the fat body
of the precocious adults of S. gregaria would respond to JH by
synthesizing DNA. Moreover, we have used a modified form of allatectomy
by treating nymphs with the anti-allatal compound precocene (Unnithan et
al., 1980). Ultrastructural studies of insects treated with precocene
demonstrate an irreversible degeneration of the CA, thus eliminating the
source of endogenous JH. A study on the precocene-induced metamorphosis

of <u>S</u>. <u>gregaria</u> established that premature adult differentiation is apparently due to the degeneration of the CA (Unnithan et al., 1980). Moreover, examination of the ovaries revealed that most of the terminal oocytes from precocious insects treated with a JH analogue had restored yolk deposition whereas terminal oocytes of untreated precocious adults were arrested in the pre-vitellogenic phase. Our study may therefore link these observations to hormonally-influenced synthetic activity in the fat body.

MATERIALS AND METHODS

Chemical Allatectomy

Second and third instar larvae of S. gregaria were treated with precocene by the contact method (Unnithan et al., 1980). Chemically clean petri dishes were coated with $15 \mu g/cm^2$ of precocene II (6,7-dimethoxy-2,2-dimethyl chromene) dissolved in acetone, and an average of 12 insects were confined to these dishes for 24 hours. At the end of the treatment period the insects were returned to their normal rearing jars in the incubator. Within a period of ten days precocious adults emerged from the treated group and were characterized by a diminutive adult morphology. Four cohorts of precocious adults were obtained in this manner and the experiments outlined below were repeated for each cohort, using only the female insects.

DNA Content

JHIII or an analogue (ZR-512) were applied topically to precocious adults which ranged in age according to experimental cohort. The hormone was dissolved in acetone and applied in divided doses over an interval of 24 hours. Control insects received the solvent only. Different doses were used for different experimental groups (Table I). Three days after the application of hormone the locusts were sacrificed and fat body processed as described in Chapter I. The fat bodies of 1-day-old and 10-day-old normal female insects were also processed for microspectrophotometry.

DNA Synthesis

21-day-old female precocious adult insects were treated with 200μg
of ZR-512 divided in two doses over a 24 hour period. One day later two
insects were injected with 10μCi of ^{3}H-thymidine (specific activity 5Ci/
mmol) and sacrificed four hours later. This tritium labelling was
repeated in insects 48 and 72 hours after the last dose of ZR-512.
Fat body was dissected and processed for staining with Schiff's reagent
after which the slides were coated with NTB-2 nuclear emulsion and
stored in the dark at 4°C for three weeks. After development of the
slides, the percent labelled nuclei was calculated for each of the
three time intervals. In a second experiment the female precocious
adults were treated with a single dose of ZR-512 (200μg) followed by
labelling at intervals of 4h., 8h., 24h., 48h., and 72h. with ^{3}H-
thymidine.

TABLE I

Treatment Schedule of Precocious Adult

S. gregaria with JH or JHA

Exp. #	Treatment
1.	10-day-old control precocious adult 10-day-old Rx with ZR-512 (100+40μg) 10-day-old Rx with JHIII (100+40μg)
2.	8-day-old control precocious adult 8-day-old Rx with ZR-512 (100+100μg) 8-day-old Rx with ZR-512 (200+200μg)
3.	30-day-old control precocious adult 30-day-old Rx with JHIII (100+40μg)
4.	45-day-old control precocious adult 45-day-old Rx with ZR-512 (100+100μg)

Precocious adults in experiments #1 were obtained from the treatment of second instar nymphs with precocene. Precocious adults in the experimental groups 2, 3, and 4 were obtained from third instar nymphs treated with precocene.

Rx is an abbreviation for treatment.

RESULTS

In each experiment there is an increase in the DNA content of fat
body nuclei following treatment of the insects with JHIII or with the
analogue ZR-512 (Figs. 7 and 8). This increased DNA content is expressed
in enhanced ploidy levels, mostly in the 8C and 16C range. These results
are contrasted with a normal immature female (one day old) and a normal
mature female (ten day old) in Fig.7 A and B. Fat body from the former
contains mostly 4C nuclei whereas by day ten of adult life the level of
ploidy has increased to include 8C and 16C nuclei. A cursory glance at
Fig. 9 shows that the increase in DNA content is accompanied by an
increase in nuclear size.

The largest increase in fat body ploidy was achieved in treated
insects obtained from the second instar (Fig. 8). In comparing Fig. 8B
with Fig. 7C it will be seen that control precocious adults from the
former group have fat body nuclear ploidy levels in the 8C and 16C range
whereas third instar precocious adults have mostly 4C nuclei. In Fig. 8A
a normal third instar nymphs is shown to have fat body nuclei with a DNA
content of 2C. It will also be noted that ZR-512 has a stronger
polyploidization effect than JHIII (Fig.8C,D) which may be attributed to
the slower rate of degradation of the analogue.

Figure 7. Frequency distribution of total extinction (log base 2) of Feulgen-stained fat body nuclei from $\underline{S}$. $\underline{gregaria}$ females. A. One-day-old adult. B. Ten-day-old adult. C. Precocious adult control. D. Precocious adults treated with two doses of JHIII (100+40μg). E. Precocious adults treated with two doses of JHA, ZR-512 (100+100μg). F. Precocious adults treated with double the dose of JHA (200+200μg).
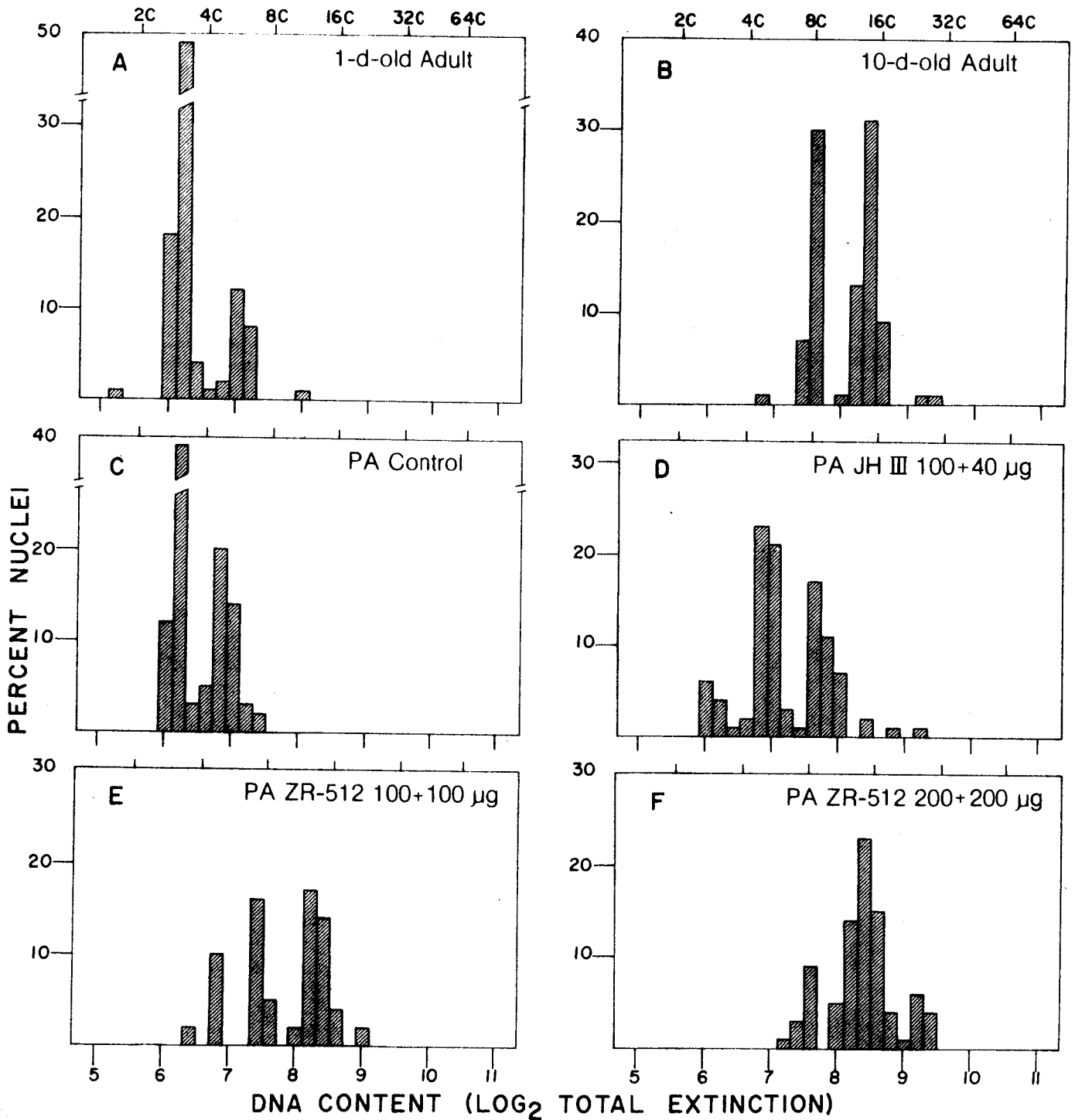
Figure 8. Frequency distributions of total extinction (log base 2) of Feulgen-stained fat body nuclei from S. gregaria third instar (A) and precocious adult females. B. Precocious adult from second instar. C. Precocious adult from second instar treated with two doses of JHIII (100+40μg). D. Precocious adults from second instar treated with two doses of JHA (100+40μg).
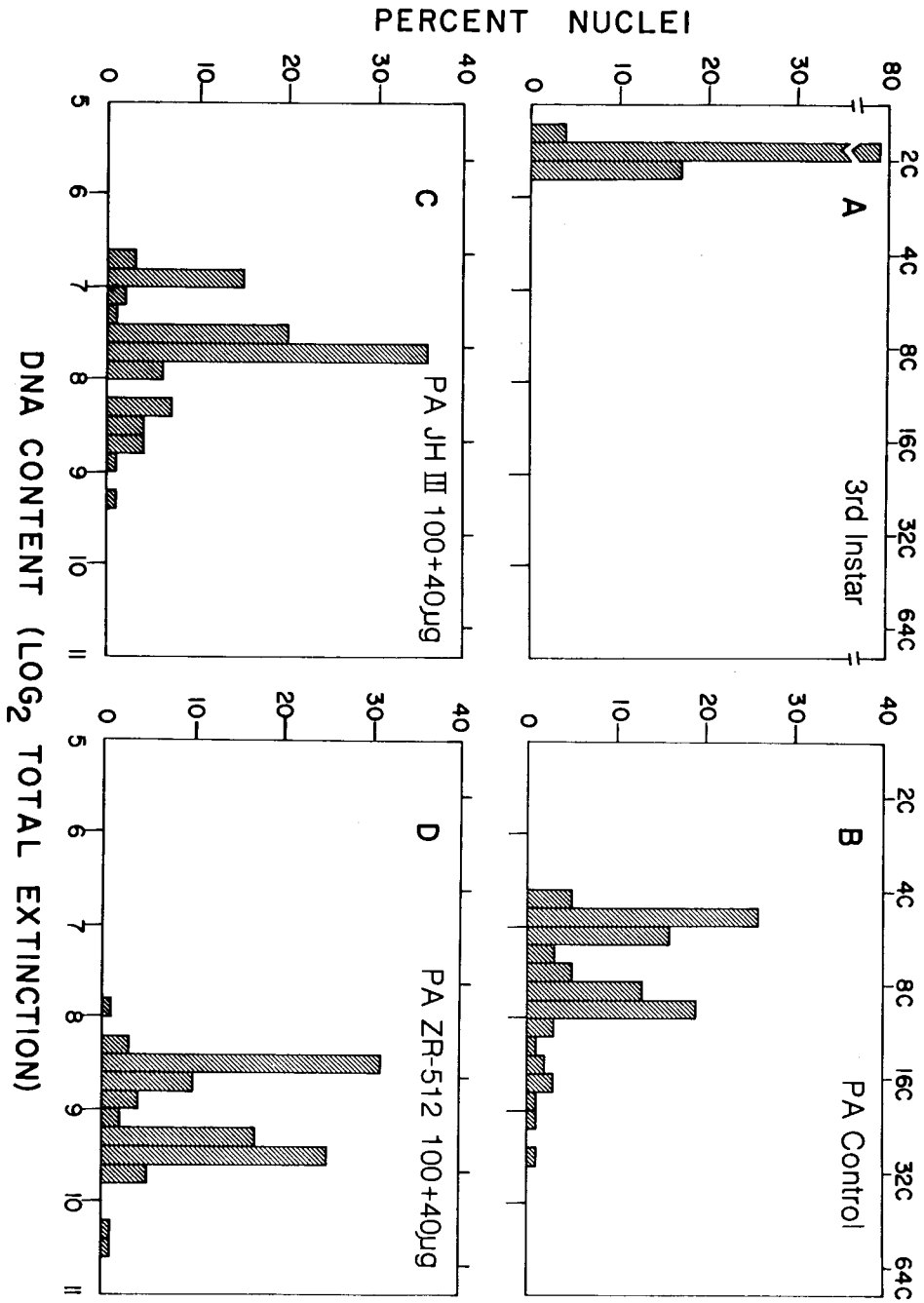
Figure 9. Feulgen-stained fat body nuclei of one-day-old (A) and ten-day-old (B) adult female <u>S</u>. <u>gregaria</u>. Scale bars 30μm.
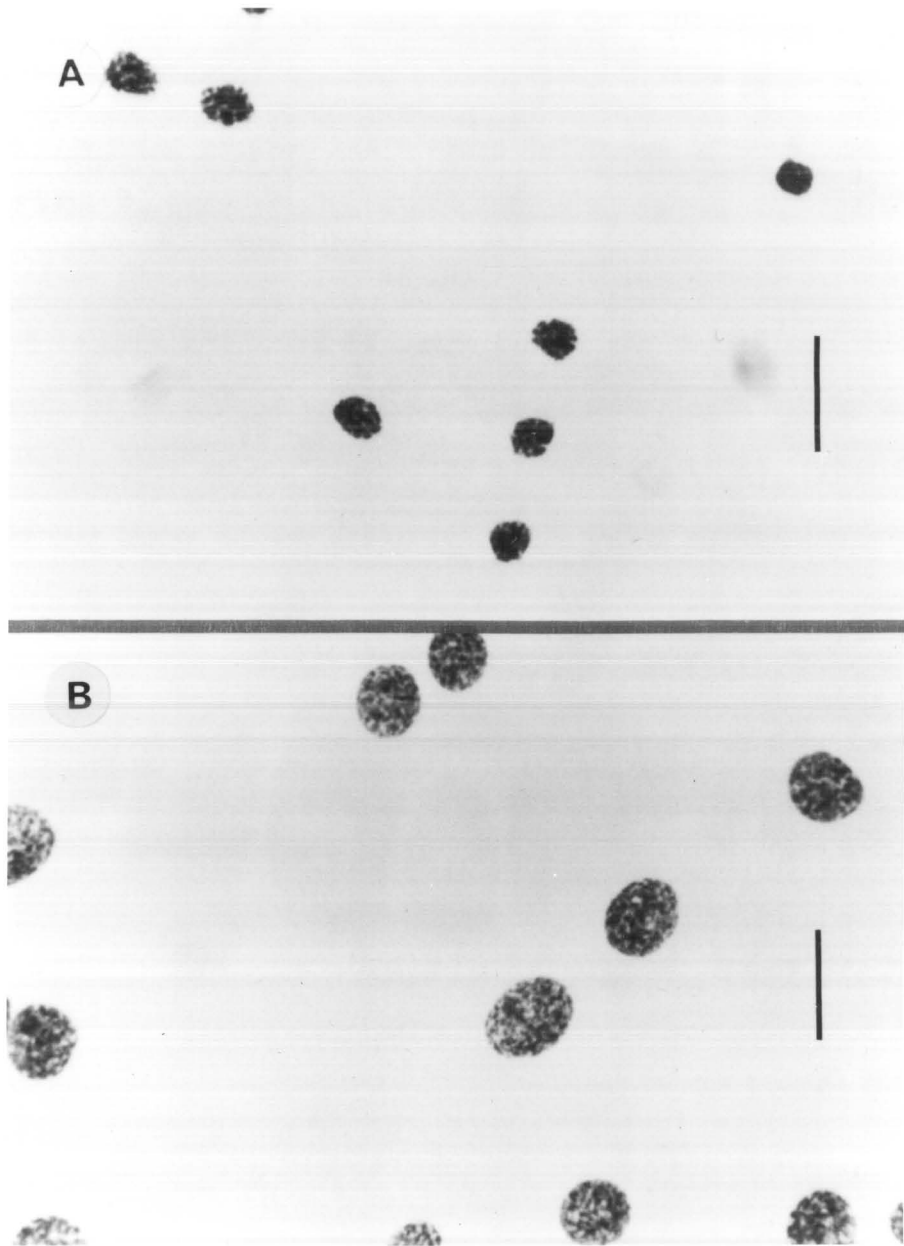
The autoradiography results show that DNA synthesis in the fat body occurs within the first 24 hours following hormone treatment(Table II). The first trial of this experiment showed that 28% of nuclei were labelled 24 hours after hormone treatment. A second experiment which included labelling after 4 and 8 hours following hormone treatment revealed that ^{3}H-thymidine is incorporated by 6% of the fat body nuclei 8 hours after the application of JHA. By 24 hours this percentage has increased to 52% and after 48 hours 19% of the nuclei are labelled. The higher rate of incorporation in the second experiment can be attributed to the single dose of JHA given (200 g), whereas in the first experiment this dose was given in two applications 24 hours apart.

TABLE II

Frequency of Labelled Nuclei in Fat Body of S. gregaria
Precocious Adults Treated with JHA

Exp. #	Time After Hormone Treatment	# Nuclei Counted	% Nuclei Labelled
	24h	2281	28
I.	48h	3157	9
	72h	1224	4
	4h	3635	0.6
	8h	2523	6
II.	24h	2746	52
	48h	2352	19
	72h	1204	11

In experiment I the insects were treated with two doses
of ZR-512 (100+100μg) which were given 24 hours apart.

In experiment II the insects received a single dose of
ZR-512 (200μg).
^{3}H-thymidine was injected after the various time intervals
indicated following hormone treatment.

DISCUSSION

The results clearly show an increase in the polyploidization of fat body cells in response to treatment with JHIII or the analogue ZR-512 (Fig.7,8). This trend is suggested in the normal maturing female wherein the CA is activated to produce JH which acts as a gonadotropic hormone (Wyatt, 1972).

The histograms do not fall precisely on the expected ploidy values (Figs.7,8). Nagl (1978) suggests that the differences between the actual values measured and the theoretical DNA content of an endomitotic nucleus are either not significant or only weakly so. This might be the expression of an almost completed S phase or it may be interpreted as the result of a systematic measurement error due to the altered texture of the chromatin.

These data can be related to a previous study by Unnithan et al.(1980) which demonstrated that precocious adult female S. gregaria would resume yolk deposition after the application of ZR-512. Whereas control precocious adults were shown to have arrested oogenesis in the previtellogenic stage, insects treated with the JH analogue had significantly larger terminal oocytes (1.8±0.2mm;4.7±1.1mm). Since our results show that DNA synthesis occurs within 24 hours of hormone treatment, it can be concluded that DNA synthesis in the fat body precedes vitellogenesis and yolk deposition.

Our data can also be compared to those of Chen et al. (1979) who observed increased DNA synthesis in fat body nuclei of L. migratoria within 24 hours of treatment with juvenile hormone. Moreover, our study demonstrates the absence of mitoses and the presence of polyploidy thereby indicating that DNA synthesis is involved in endocycles.

Since we know that the significant process occurring in mature female fat body of the locust is vitellogenin synthesis (Engelmann, 1979), it remains to be elucidated just what role polyploidization plays in promoting this enhanced synthesis. Nair et al. (in preparation) suggest that the JH-dependent synthesis of DNA and resultant polyploidy in female fat body of L. migratoria permits accelerated production of mRNA for vitellogenin and possibly other proteins. This would agree with the suggestion that polyploidization is a mechanism whereby the synthesis of specialised proteins is enhanced (Brodsky and Uryvaeva, 1977). The study of JH-controlled vitellogenin synthesis in L. migratoria fat body (Chen et al., 1979) showed that hormonal induction in vivo occurred initially after a lag of some 48 hours whereas a second application of hormone after ten days renewed vitellogenin synthesis with very little initial lag. It is tentatively suggested here that the establishment of polyploid fat body cells is responsible for the initial observed lag in vitellogenin production. The second response to hormone treatment may be faster due to the presence of synthetic machinery "primed" by polyploidization.

The levels of ploidy observed in the fat body nuclei of precocious adults that emerged from the second instar are higher than those found in insects obtained from third instar (Fig.8B,7C). This may be due to the following reasons. In early instars, up to the end of the fourth, the fat body cells increase in number by mitosis. Precocene treatment of second instars induces degeneration of their CA and in the absence of JH the fat body cells as well as those of other tissues begin differentiation towards the adult form. These cells compensate for the reduction in their number by increasing their DNA content, thus accounting for higher ploidy levels in fat body from precocious adults that emerged from the second instar.

A model for endocrine controlled vitellogenin synthesis and vitellogenesis has been proposed by Engelmann (1979). The CA elaborates JH which acts on the fat body genome to transcribe mRNA for vitellogenin. It is also postulated that JH acts at a cytoplasmic level to stimulate the proliferation of RER. These two actions are synergistic in causing a net increase in the synthesis of vitellogenin for export to the developing oocytes. The model further suggests that JH may influence the ability of follicular epithelium to incorporate the vitellogenin. The results of this study suggest that an additional step may be inserted into this model. Prior to the transcription of mRNA for the vitellogenic protein it appears that the fat body nuclei first undergo polyploidization, and this process is directly stimulated by JH.

Some broad implications are forthcoming from our results. Studies on gene regulation have heretofore concentrated on prokaryotic systems. Insect tissues provide a good tool for studying the regulation of eukaryotic genomes, particularly the system of vitellogenin synthesis in fat body. In locusts especially, the finding of JH-induced polyploidization of fat body cells suggests the interaction of hormones with genetic material. Moreover, the phenomenon of under-replication in locust fat body DNA is worth investigating with respect to the ontogeny of tissues. Perhaps the C-value paradox, which notes a lack of correspondence between evolutionary complexity and genome size, may eventually be resolved by such studies.

CONCLUSIONS

Endopolyploidization occurs in the fat body cells of adult female S. gregaria during maturation in response to juvenile hormone. This has been demonstrated by the application of JHIII and the analogue ZR-512 to precocious adult insects. It appears that polyploidization precedes vitellogenin synthesis in the fat body of female S. gregaria, and DNA synthesis occurs in this tissue within 24 hours of JH treatment.

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