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Dietary influences over proliferating cell nuclear antigen expression in the locust midgut

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Summary

We have studied the influence of variations in dietary protein (P) and digestible carbohydrate (C), the quantity of food eaten, and insect age during the fifth instar on the expression of the proliferating cell nuclear antigen (PCNA) in the epithelial cells of the midgut (with special reference to the midgut caeca) in the African migratory locust, *Locusta migratoria*. Densitometric analysis of PCNA-immunostained cells was used as an indirect measure of the levels of expression of PCNA, and a PCNA cellular index (PCNA-I) was obtained. Measurements of the DNA content of the cells have also been carried out by means of microdensitometry of Feulgen-stained, thick sections of midgut. A comparison between the PCNA nuclear level and the DNA content was performed. The PCNA levels were significantly different among the cells of the five regions studied: caeca, anterior ventricle, medial ventricle, posterior ventricle and ampullae of the Malpighian tubules. We have studied in more detail the region with highest PCNA-I, i.e. the caeca. The quality and the quantity of food eaten under *ad libitum* conditions were highly correlated with both the PCNA and DNA levels in the caeca cells. Locusts fed a diet with a close to optimal P:C content (P 21%, C 21%) showed the highest PCNA and DNA content. In locusts fed a food that also contained a 1:1 ratio of P to C but was diluted three-fold by addition of indigestible cellulose (P 7%, C 7%), a compensatory increase in consumption was critical to maintaining PCNA levels. Our measurements also showed that the nuclear DNA content of the mature and differentiated epithelial cells was several-fold higher than the levels in the undifferentiated stem cells of the regenerative nests. These results, combined with the low number of mitotic figures found in the regenerative nests of the caeca and the marked variation in PCNA levels among groups, suggest that some type of DNA endoreduplication process may be taking place. Our data also indicate that the DNA synthetic activity in the midgut is related to feeding in locusts. The possible dietary and nutritional regulatory mechanisms and the significance of the differences found are discussed.

Key words: proliferating cell nuclear antigen, PCNA, *Locusta migratoria*, Insecta, BrdU incorporation, DNA synthesis, protein, carbohydrate, midgut, feeding behaviour, diet, nutritional balance.

Introduction

The midgut wall of insects is mainly comprised of a columnar epithelium surrounded by visceral striated muscle fibres. The main cell type of the epithelium is the enterocyte, which has absorptive and secretory functions. The existence of a variety of endocrine cell types spread among the enterocytes of the midgut wall of the desert locust has also been reported (Montuenga et al., 1989, 1993). Some suggestions about the possible role of the endocrine cells in digestive physiology have been proposed (Fuse and Orchard, 1998; Zudaire et al., 1998a,b). A third type of epithelial cells, of a proposed regenerative nature, is also found interspersed between the enterocytes (Uvarov, 1977). In some insect orders, such as Orthoptera, the regenerative cells of the midgut are clustered in regenerative 'nidi' located at the basis of the epithelium.

Little is known about cell proliferation in insects and especially about the turnover of the insect midgut epithelial cell population. There have been studies on isolated aspects of cell proliferation in insect neurogenesis, muscle remodelling in metamorphosis and glandular growth (Hegstrom and Truman, 1996; Lee et al., 1995; Fahrbach et al., 1995). Endo (1984), using incorporation of tritiated thymidine, reported differences in the rate of renewal between enterocytes and endocrine cells in the cockroach midgut. In addition, proliferation of embryonic tissues during *Drosophila* development (including the gut) has been studied by immunocytochemical techniques using antibodies raised against proliferating cell nuclear antigen (PCNA; Yamaguchi et al., 1991).

PCNA is a 36-kDa polypeptide present in the nuclei and cytoplasm (Yamaguchi et al., 1991; Grzanka et al., 2000) of mitotically active (S phase) cells (Miyachi et al., 1978). It is

known to be involved in DNA replication through an association with DNA polymerase δ (Bravo et al., 1987) and in DNA repair (Fairman, 1990), although there is yet no evidence for a role in other processes that require DNA synthesis, such as DNA endoreduplication. PCNA shows a high degree of molecular similarity throughout the animal and plant kingdoms (Sukuza et al., 1989; Mathews et al., 1984; Bauer and Burgers, 1990; Daidoji et al., 1992).

A number of assays have been used to assess cell proliferation levels, including direct counting of mitotic figures, tritiated thymidine autoradiography and bromodeoxyuridine (BrdU) immunocytochemistry (Gratzner, 1982). Each of these methods has disadvantages, however. Counting mitotic figures is cumbersome and represents only one phase of the cycle. Tritiated thymidine autoradiography and BrdU immunocytochemistry are also restricted to one phase of the cell cycle, and these methods require previous administration of a thymidine analogue, one of which is a radioactive substance. The immunohistochemical assay for PCNA avoids some of these disadvantages (Foley et al., 1991). The PCNA antigen is stable and can be detected by immunohistochemistry during the S phase of cells of all tissues (Kurki et al., 1986; Foley et al., 1991). Several monoclonal antibodies have been developed and used in immunocytochemical techniques to detect PCNA in paraffin-embedded tissues (Ogata et al., 1987a,b; Hall et al., 1990). This technology has been applied mostly to mammalian tissues (Foley et al., 1991). PCNA has been immunocytochemically demonstrated in only a few non-mammalian species, including the fruit fly (Yamaguchi et al., 1991). A number of studies have characterised the PCNA gene in *Drosophila* (*mus209*, NT_033778.1) and have described the molecular interactions of PCNA and other proteins and its relevance to the development of this insect (Yamaguchi et al., 1990, 1991, 1995, 1996; Yamamoto et al., 1997). Several cDNAs with high homology to *Drosophila* PCNA have also been sequenced in other insect species such as *Anopheles gambiae* (gi/31236419), *Bombyx mori* (gi/3334291) *Spodoptera frugiperda* (gi/21717394), *Hyphantria cunea* (gi/21717396) and *Sarcophaga crassipalpis* (gi/3334293).

The aim of the present work was to study the differences in the expression of PCNA in the regenerative compartment of the epithelium of the locust midgut in relation to feeding and food nutritional quality. The possible cellular and physiological implications of these differences are discussed. We also report our modification of the immunocytochemical technique to show the expression of PCNA in the epithelial cells of the locust midgut, which is likely to be useful for other insect tissues and a variety of invertebrate epithelial models.

Materials and methods

Insects and artificial diets

Approximately 200 fifth-instar locusts (*Locusta migratoria* L.) of both sexes were used. Experimental insects were reared at the Department of Zoology, Oxford University, using seedling wheat and wheat germ as a food source. Locusts weighing 400–580 mg for males and 480–660 mg for females were collected within 4 h after ecdysing to the fifth instar (termed day 0). Six dry, granular synthetic foods were used across four experiments and contained the following percentages of protein (P) to digestible carbohydrate (C): 7:7, 7:35, 14:28, 21:21, 28:14 and 35:7. Casein, peptone and albumen (3:1:1) comprised the protein source, while sucrose and dextrin (1:1) provided digestible carbohydrate. Changes in the levels of these nutrients were compensated for by altering the amount of indigestible cellulose added, while all other nutrients were present at the same level (4%; for further technical details, see Simpson and Abisgold, 1985).

Experimental procedure

Locusts were housed individually in clear plastic boxes (17×12×6 cm) containing an aluminium perch, a water dish and a Petri dish of food. They were kept at 30°C under a 12 h:12 h light:dark photoregime. Two experiments were performed.

Experiment 1

Thirty newly ecdysed fifth-instar nymphs (15 males, 15 females) were kept until day 1 or 4 on one of the five synthetic foods listed above (P%:C% 7:35, 14:28, 21:21, 28:14 or 35:7). Nymphs were taken during the light phase of day 4 as they commenced a meal during *ad libitum* feeding (defined as having walked from the perch to the food dish and fed for 10 s). After crushing the head capsule, the tip of the abdomen was cut and the head pulled until the cervical membrane tore. Next, the entire gut was gently pulled out of the body by the head and opened along its length with bowspring scissors under Ringer solution (Mordue, 1969). The gut contents were removed and the tissue pinned flat onto a piece of photographic paper (3×1 cm) in a wax dish. The dissected and pinned gut, with its paper support, was then placed into Bouin's fluid for 24 h. Thereafter, the fixed guts were removed from the photographic paper, washed and stored in 70% ethanol prior to embedding in paraffin.

Experiment 2

Eighty-four nymphs (42 males and 42 females) were fed one of two synthetic foods (P%:C% 7:7, 21:21) and their guts were sampled (as described above) on days 0 (before feeding commenced for the stadium), 1, 2, 3, 4, 6 and 8. The mean stadium duration under the prevailing experimental conditions is 10–11 days on both diets, with insects ceasing feeding by day 8–9 (Raubenheimer and Simpson, 1993; Zanotto et al., 1993).

Immunocytochemistry

For the immunocytochemical localisation of PCNA in paraffin sections (4 μm in thickness), a variant of the avidin–biotin complex (ABC) technique of Hsu et al. (1981) was employed. After removal of paraffin with xylol, followed by 10 min in absolute ethanol, endogenous peroxidase was

blocked by treatment with 3% H_2O_2 in absolute methanol. Sections were then hydrated through a graded series of ethanol (96%, 80%, 70%) and rinsed for 5 min in deionized H_2O to remove any remaining alcohol.

PCNA epitopes masked by fixation were revealed by antigen retrieval by heating the sections in a retrieval solution [citric buffer (CB): 0.01 mol l^{-1} citric acid]. To obtain optimal antigen retrieval, several different antigen retrieval heating protocols using different pHs (2.3–11) of CB and different microwave heating times (0–90 min) were tested. For the microwave treatment, a glass slide rack was immersed in a plastic container with 1000 ml CB. The open container was heated in a 700 W microwave oven for 15 min. The solution was allowed to bubble.

After the microwaving protocols, slides were cooled for 15 min, rinsed in deionized H_2O and then placed in Tris-HCl buffered saline (TBS: 0.05 mol l^{-1} Tris buffer, pH 7.4 and 0.5 mol l^{-1} NaCl). Non-specific binding sites were blocked with 5% mouse normal serum in TBS, and the sections were then incubated overnight at 4°C with anti-PCNA serum (mouse anti-PCNA immunoglobulins; clone PC10; Dakopatts, Glostrup, Denmark) diluted in TBS. Several dilutions of the primary antiserum were tested (1:50, 1:100, 1:200, 1:400, 1:800) to obtain optimal immunolabelling. Following treatment with the primary antiserum, sections were rinsed in TBS (5 min) and then incubated for 30 min at room temperature with biotinylated rabbit anti-mouse antiserum (Dako, Cambridge, UK) diluted 1:200 in TBS. After a second rinse in TBS, the sections were treated for 30 min at room temperature with an avidin–biotin peroxidase complex (Dako) diluted 1:100 in TBS prepared 30 min in advance. The sections were then washed in TBS and then in acetate buffer (AB: 0.1 mol 1^{-1} acetic acid, pH 6). Peroxidase activity was demonstrated by the diaminobenzidine (DAB)/H_2O_2 method (Sigma Chemical Co., Madrid, Spain). The reaction was intensified with nickel according to Shu et al. (1988). The solution was made by mixing a solution of 50 mg of DAB in 50 ml of deionized H_2O, with a second solution made up by adding 2.5 g of ammonium nickel sulphate [di-ammonium nickel (II) sulphate 6-hydrate; BDH Laboratory Supplies, Dorset, UK], 200 mg of b-D-glucose [b-D(+) glucose; Sigma Chemical Co., St Louis, MO, USA], 40 mg of ammonium chloride (Sigma Chemical Co.) and 30 mg of glucose oxidase (Sigma Chemical Co.) in 50 ml of AB. Finally, the sections were washed in distilled water, lightly counterstained with haematoxylin, dehydrated and mounted in a mountant for microscopy (DPX).

Negative controls (omission of any of the layers of the immunocytochemical protocol and the use of non-immune mouse serum as the first layer) were performed and gave no immunocytochemical reaction. Also, the lack of antigen retrieval protocol was used as a negative control in the PCNA detection.

Quantification of the immunocytochemical reaction

Two different systems were used to assess the quantity of PCNA nuclear immunoreactivity: (1) by counting PCNA-positive nuclei and (2) by measuring the optical density of PCNA-like immunoreactive nuclei.

Counting PCNA-positive nuclei

Four sections of the gut of each experimental locust were randomly selected among a total of 50 serial sections performed. An arbitrary threshold was set, with the help of the image analysis system Visilog 4 (Noesis, Velicy, France), in order to discriminate the PCNA-positive cells from the background. Four fields of the appropriate midgut region were randomly selected so that ~400 nuclei per locust were counted.

Positive and negative cells were counted, and the index of cells immunostained for PCNA (PCNA-I) was calculated using the following equation:

$$\text{PCNA-I} = (\text{+ nuclei/total cells}) \times 100 ,$$

in which '+ nuclei' is the number of nuclei labelled with PCNA and 'total cells' is the sum of the immunostained and non-immunostained nuclei within the same field.

Measuring optical density of PCNA-like immunoreactive nuclei

The optical density (PCNA-OD) of the nuclei counted as described in the above section was measured using the Photoshop 4.0 image software (Lehr et al., 1997).

Bromodeoxyuridine immunocytochemistry

The BrdU labelling was carried out by abdominal injection of 100 μg BrdU in 10 μl of saline. The midgut was dissected and fixed (following the same fixation protocol described above) within one hour of the BrdU injection. For the immunocytochemical localization (Montuenga et al., 1992; Hyatt and Beebe, 1992), after blocking endogenous peroxidase with H_2O_2, the sections were treated with the nuclease supplied with the primary anti-BrdU-serum (Amersham Pharmacia, Little Chalfont, UK) for one hour at room temperature. After blocking non-specific binding sites with 5% goat normal serum, the sections were incubated overnight with a primary anti-BrdU-serum (mouse anti-BrdU immunoglobulins; clone RPN202; Amersham Pharmacia) diluted 1:200 in TBS. Detection was enhanced with the Envision® (Dako) reagents. The sections were counterstained with haematoxylin, dehydrated and mounted in DPX.

Western blotting

Samples destined for western blotting were frozen in liquid nitrogen immediately after dissection and maintained at –80°C until processed. Extracts of locust gut tissue were prepared by thawing the tissue on ice and homogenizing accurately weighed samples in ice-cold homogenization buffer: 2× tricine buffer with 8% sodium dodecyl sulphate (SDS; Novex, San Diego, CA, USA) containing 1 mmol l^{-1} final concentration of the protease inhibitors pefablock (Centerchem Inc., Stanford, CT, USA), bestatin and phosphoramidon (Sigma Chemical Co., St Louis). The homogenate was centrifuged at 100 000 ***g*** for 30 min, and the supernatant was collected. Homogenate

supernatant protein content was measured using a BCA kit (Biorad Labs, Richmond, CA, USA) after trichloroacetic acid precipitation and NaOH resolubilizing of the extract protein.

Protein extracts were diluted to an approximate protein concentration of 1 μg μl^{-1}, heated to 95°C for 5 min and loaded into the sample well and electrophoretically fractionated on a 4–12% tricine SDS-PAGE gel (Novex) at 140 V for 45 min under reducing conditions (5% β-mercaptoethanol). Transfer blotting was accomplished in the same apparatus equipped with a titanium plate electrode insert, and proteins were affixed to a nylon membrane (Immobilon PVDF; Millipore, Billerica, MA, USA) at 30 V for 1.5 h. The membrane was blocked overnight in 5% non-fat milk-PBS, incubated for 1 h in a 1:500 dilution of mouse anti-PCNA and washed three times in 0.1% Igepal (Sigma, St Louis, MO, USA). The membrane was then exposed to biotinylated immunoglobulins (Dakopatts; 1:200 dilution) for 1 h and then to avidin–biotin peroxidase complex (Dakopatts; 1:500) for an additional hour. Peroxidase activity was revealed with the ECL + Plus chemiluminescence kit (Amersham, Arlington Heigths, IL, USA) following manufacturer's instructions. The negative control was performed by omitting the specific primary antibody.

Feulgen staining

In order to measure the relative DNA content in the nuclei of the midgut caeca, the Feulgen reaction was applied in 14 μm sections of locust midguts belonging to the first diet experiment (see Experimental procedure, experiment 1).

Sections were brought to water after removal of the paraffin with xylene and hydration through a graded series of ethanol (96%, 80%, 70%). The optimum Feulgen hydrolysis time was estimated after Kjellstrand (1977). The maximum stain was achieved after 1 mol l^{-1} HCl hydrolysis for 12 min at 55°C. After a rinse in distilled water, the slides were placed in Schiff's reagent (Merck, Darmstadt, Germany) at room temperature for 2 h 45 min. Next, the slides were transferred to sulphurous acid for 2 min. The sulphurous step was repeated twice (2 min each). Then the slides were washed in running water for 1 min, dehydrated in alcohol and mounted after clearing in xylene.

All slides were treated simultaneously in the same solutions.

Quantification of the Feulgen reaction

Measurements were made using the 100× objective, and a shading correction was employed in order to avoid optical aberrations. Mature nuclei of the caeca are oval shaped and their major axis is perpendicular to the epithelial layer. Only the nuclei sectioned through the medial plane parallel to the long axis showing well-defined and sharp limits were chosen for measurements in each field.

Scanning microdensitometry was performed to quantify the Feulgen reaction with the help of the Visilog 4 image analyser. The integrated optical density (IOD) values of the mature nuclei of the caeca in each treatment were measured in several random sections. 400 nuclei were counted in each specimen.

Results

PCNA-like immunoreactivity was found in the nuclei and cytoplasm of the cells in the three main regions of the locust midgut: the caeca (Fig. 1A), the main region or ventricle (Fig. 1B) and the ampullae through which the Malpighian tubules drain to the gut at the midgut/hindgut limit (Fig. 1C). PCNA-like immunoreactivity appeared as a granulated material within nucleoplasm and tended to be located in the vicinity of the nuclear membrane. In the caeca and the ventricle, the immunoreactivity is concentrated in groups of small, undifferentiated cells found in the basal region of the midgut epithelium (Fig. 1A,B), thus providing new confirmation of the regenerative nature of these nests of cells. In the ampullae of the Malpighian tubules, the PCNA-like immunoreactive nuclei are grouped, forming structures similar to the nests found in the ventricle (Fig. 1C). All regenerative nests in the ventricle and the caeca contained at least some nuclei immunostained for PCNA. Similarly, all the ampullae sections studied showed PCNA-like immunoreactive nuclei. Usually, the cells in the core of the nidi were strongly stained, and the immunostaining faded away in the more peripheral cells so that the differentiating enterocytes surrounding the regenerative nest were progressively less stained. No PCNA-like immunostaining could be detected in mature (completely differentiated) enterocytes (Fig. 1A–C). To further confirm the specificity of the PCNA-like immunoreaction, a western blot was performed using tissue extracts obtained from locust gut. A single defined band of ~30 kDa was immunoreactive for PCNA (Fig. 1E). In addition, we compared BrdU incorporation and PCNA expression by immunocytochemical methods in serial consecutive sections of the midgut. Both BrdU- and PCNA-labelled cells were always found within the regenerative nidi. In many of these cells, the BrdU- and PCNA-like immunoreactivity was colocalized in the nucleus, further supporting the DNA replication process associated with the observed PCNA-like immunostaining (Fig. 1D).

A microwave pre-treatment was needed to make the epitopes available to the anti-PCNA antibody. The optimisation of the microwave heating time clearly improved PCNA staining. Fig. 2 shows the effect of different microwave treatment times on PCNA-like immunoreactivity. In the locust midgut, a faint immunocytochemical signal was detected only after at least 20 min of microwave heating. The immunoreactive optimal signal was found after 40 min. Extended microwave pre-treatment time (>40 min) resulted in a dramatic decrease in immunoreactivity levels.

Regional differences in the midgut

Regional differences within the midgut of *Locusta* appear in relation to PCNA-like immunostaining (Fig. 3). The midgut caeca, located at the foregut–midgut junction, and the ampullae through which the Malpighian tubules drain, at the midgut–hindgut junction, are the two regions in which the immunostaining for PCNA (PCNA-OD) was most intense and the PCNA index (PCNA-I) was highest. In our quantitative studies, midgut caeca and ampullae showed statistically

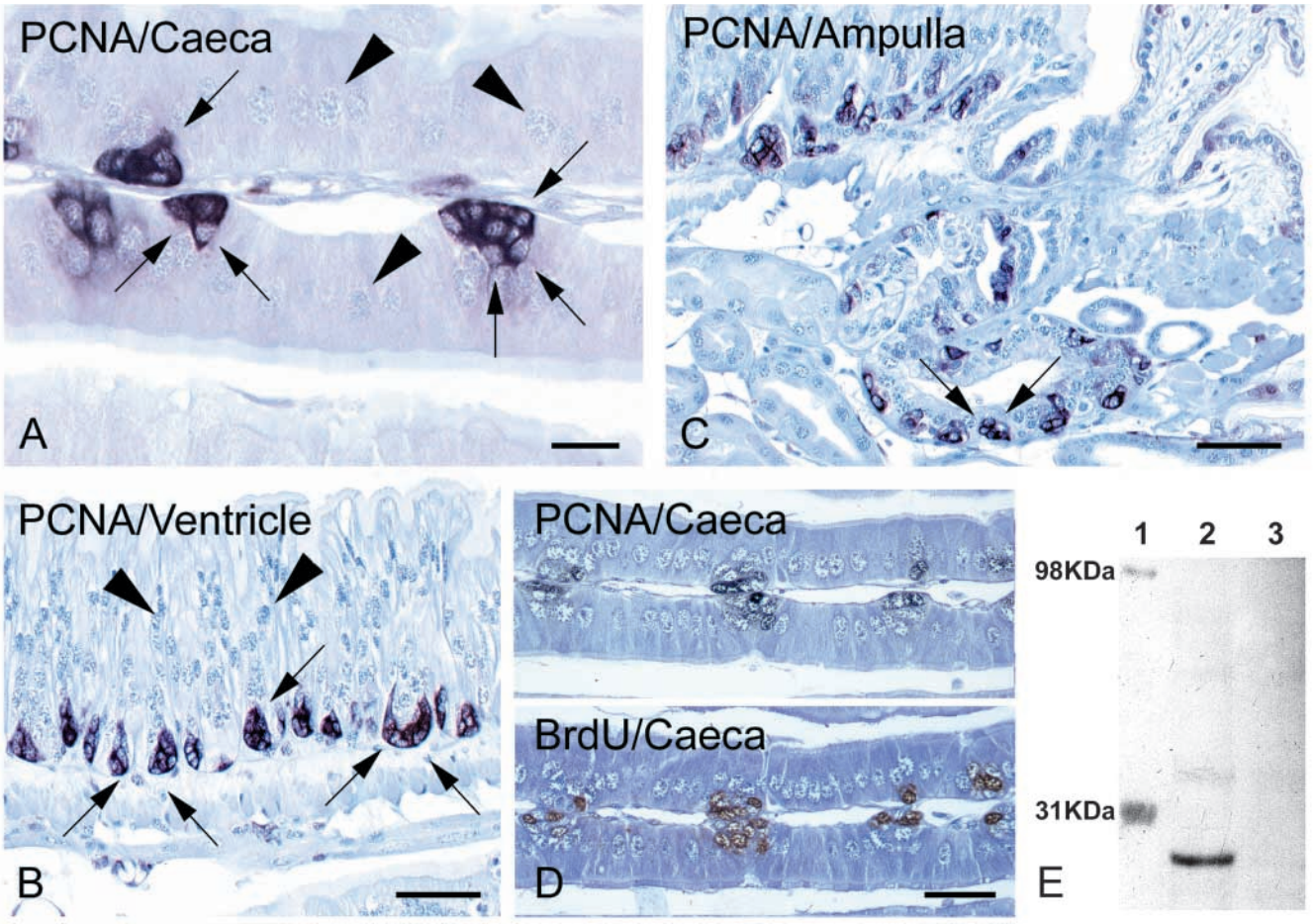

Fig. 1. PCNA-like immunoreactivity throughout the locust midgut. (A) Panoramic view of locust midgut caeca walls. Strong immunoreactivity appears mainly in the core of the regenerative nests (arrows). (B) Ventricle wall. PCNA-like immunoreactivity is exclusively present in the cells of the regenerative nests at the base of the epithelium (arrows). Enterocytes surrounding the regenerative nests are progressively less stained, and no immunoreactivity is found in mature enterocytes outside the nests (arrowheads). (C) PCNA-like immunoreactivity in the locust ampullae of Malpighian tubules. PCNA-like immunoreactivity is found in clusters of regenerative cells (arrows). (D) PCNA-like immunoreactivity and BrdU-immunoreactivity in serial consecutive sections of the midgut in the caeca region. (E) Western blot analysis of locust gut tissue extract using antiserum against PCNA. A single band of ~30 kDa is detected under reducing conditions. Lane 1, molecular mass markers; lane 2, locust gut extract; lane 3, negative control without antibody. Scale bars, 25 μm in A and 50 μm in B–D.

significant differences (see below) when compared with the other regions (Fig. 3).

Effect of age during the stadium, nutrient content of food and sex on PCNA-I

The diet supplied and the age within the stadium have a strong influence on both the PCNA-I and PCNA-OD levels (Fig. 4). The more balanced diet (21:21) elicited the higher PCNA-I and PCNA-OD values, and these fell as the diets became progressively unbalanced in their P:C ratio (diets 7:35 or 35:7). Additionally, locusts showed higher PCNA-I and PCNA-OD values on day 4 after ecdysis than on day 1. When PCNA-I values were included in an analysis of variance (ANOVA), the two factors (diets and day) showed statistical differences (Fig. 4A; d.f.=4, 50, F=4.984, P=0.002 for the effect of diet and d.f.=1, 50, F=29.736, P<0.000 for the effect of age/day within the stadium), and no interaction was detected between them (d.f.=4, 50, F=0.529, P=0.715). Similarly, PCNA-OD was related to locust age and the nutritional balance of their food (Fig. 4B; d.f.=1, 50, F=10.125, P=0.003 for the effect of day during the stadium, and d.f.=4, 50, F=6.953, P<0.000 for diet composition). The highest PCNA-OD values were found in insects fed a balanced diet (21:21) and on day 4 [note that PCNA-OD is expressed as a numerical value from 0 (white, representing no labelling) to 255 (black, indicating strongest labelling)].

These results suggest that the values of PCNA-I and PCNA-OD, and thus the proliferative index of the midgut epithelium, are related to the nutritional balance of the insect. The total percentage of protein and digestible carbohydrate was constant at 42% in all the diets used, so that the total amount of nutrients does not account for the differences found between diets. Therefore, these could be due to the ratio of protein to carbohydrate in the food and/or to the concentration of one or

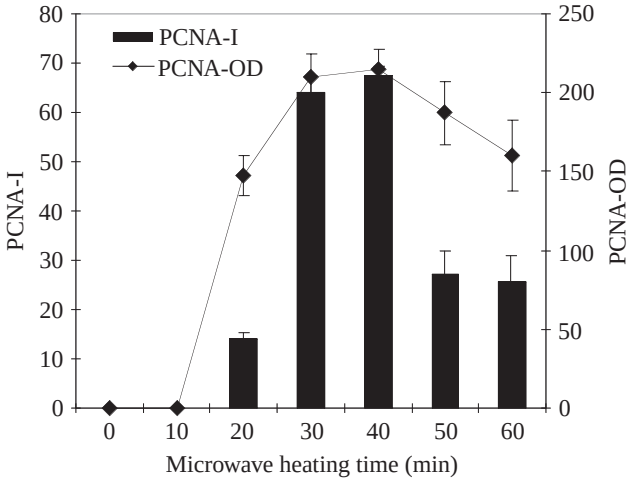

Fig. 2. Effect of microwave heating time on PCNA-like immunoreactivity in the nests of midgut caeca. Immunoreactivity is only noticeable after 20 min of microwave heating, and the optimum staining is reached after 40 min of microwave treatment. However, longer exposures to microwave heating dramatically drop the staining levels below the optimum.

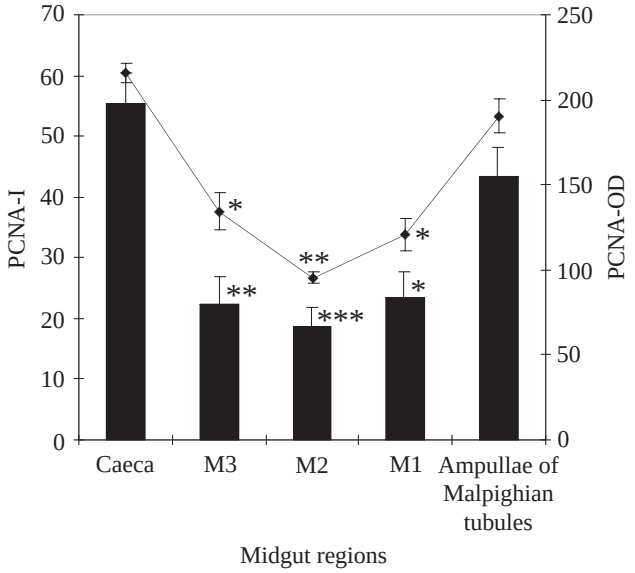

Fig. 3. Differences in PCNA-like immunoreactivity (bars, PCNA-I; line, PCNA-OD) between the five regions of the locust midgut. For statistical analysis, values of caeca have been compared to the rest of the regions. There are no significant differences between the values of caeca and ampullae. Caeca and ampullae of the Malpighian tubules show higher levels of staining compared with ventricle (*P<0.05, **P<0.01, ***P<0.001).

other macronutrient. If the first hypothesis is true, food 7:7, which has the same P:C ratio as the 21:21 food, albeit with both macronutrients diluted threefold, should give similar levels of PCNA-I and PCNA-OD to those observed with the 21:21 diet. No significant differences were found between diets 7:7 and 21:21 (d.f.=1, 51, F=0.077, P=0.783 for PCNA-I and d.f.=1, 51, F=0.655, P=0.423 for PCNA-OD), suggesting that

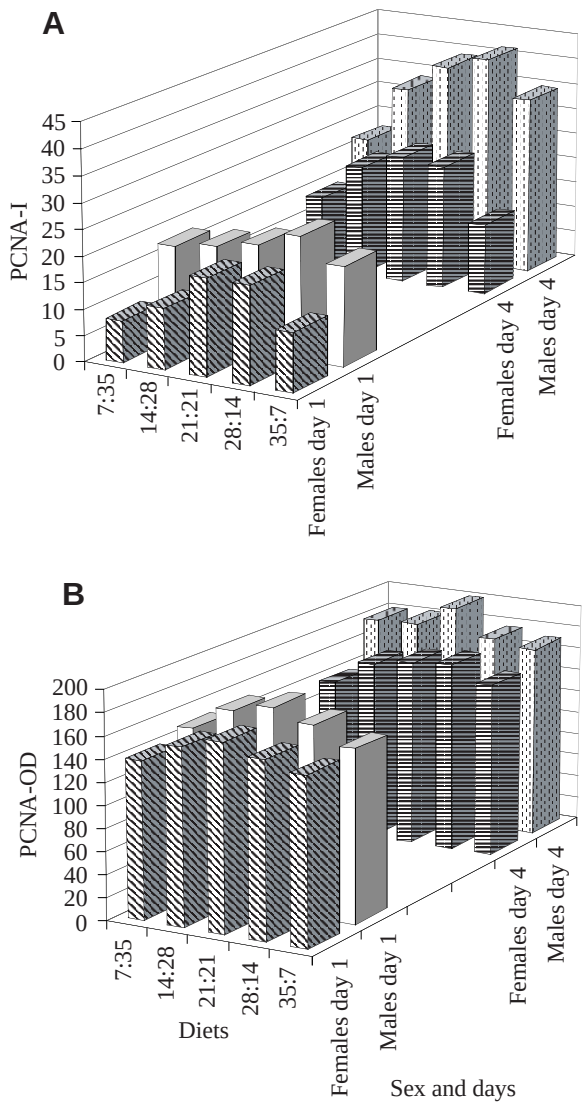

Fig. 4. Effect of diet composition, sex and time during the fifth instar on values for PCNA-I (A) and PCNA-OD (B). The balanced diet (21:21) shows the highest levels of PCNA, and as the diets became more unbalanced in their ratio of protein to digestible carbohydrate, the level of PCNA dropped. Males showed more PCNA than females, and PCNA levels were higher on day 4 than on day 1.

the differences observed are rather related to the ratio of protein to carbohydrate in the food. In addition, diet 21:21, which is known from earlier works to be close to the optimal ratio and concentration of protein and carbohydrate for fifth-instar locusts (19% P:23% C; Simpson and Raubenheimer, 1993; Chambers et al., 1995), elicits the highest PCNA-I and PCNA-OD values in our experiment, while diets 7:35 and 35:7, which are distant in P:C ratio from the optimum 19:23, induce the lowest values. The data of our experiments (day 1 and day 4) were regressed against a geometric estimation of the protein and carbohydrate nutritional quality (see Chambers et al., 1995), namely the distance in nutrient space of each of the two macronutrients from their optimal percentage in diet 19:23 (see Zudaire et al., 1998a). The regression was significant on both

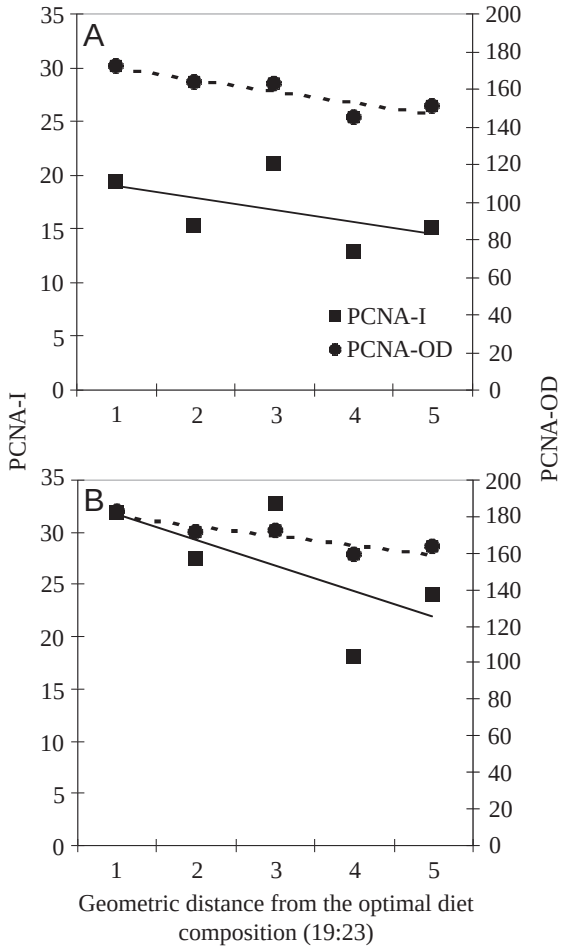

Fig. 5. Regression lines (PCNA-I, solid line; PCNA-OD, broken line) showing the relationships between both PCNA-I (squares) and PCNA-OD (circles) values in locusts fed on a given diet and the relative distance of this diet from a nutritionally optimal composition (see Chambers et al., 1995) on days 1 (A) and 4 (B). Regression lines: (A) PCNA-I, y=–0.5x+18.64, r^2=0.23; PCNA-OD, y=–3.33x+172.1, r^2=0.62; (B) PCNA-I, y=–1.26x+31.61, r^2=0.25; PCNA-OD, y=–2.94x+180.8, r^2=0.6.

days 1 and 4 [d.f.=2, 27, F=4.43, P=0.021, r^2=0.23 for PCNA-I on day 1 (Fig. 5A, with distance to optimal diet shown as relative categories) and d.f.=2, 27, F=4.48, P=0.021, r^2=0.25 for PCNA-I on day 4 (Fig. 5B) and d.f.=2, 27, F=21.524, P<0.000 for PCNA-OD on day 1 (Fig. 5A) and d.f.=2, 27, F=3.868, P=0.033 for PCNA-OD on day 4 (Fig. 5B)], further supporting the hypothesis that both PCNA-I and PCNA-OD are dependent on the P:C ratio of the food.

Changes in PCNA-I with insect age and influence of food eaten

In the second experiment, which investigated the effect of insect age during the fifth-instar in more detail, PCNA-I increased significantly from day 0 to day 3 after ecdysis, on which we found the maximum value (Fig. 6), and then decreased until day 8. To confirm the results found in the previous experiment, data were compared between days 1 and

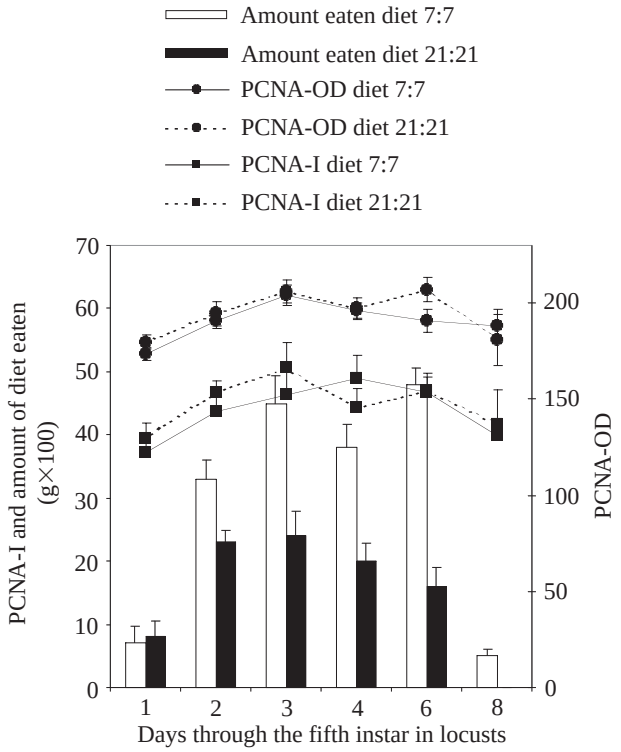

Fig. 6. Plot showing the values of PCNA-I (squares) and PCNA-OD (circles) related to the quantity of food eaten (diet 7:7, open bars; diet 21:21, solid bars) through the first 8 days of the fifth instar for both diet 7:7 (lines) and diet 21:21 (dotted lines). Both PCNA-I and PCNA-OD values increased between day 0 and day 3 and decreased thereafter. Notice the correlation between the amount of food eaten and the levels of both PCNA-I and PCNA-OD.

4. As expected, strong differences were found in PCNA-I between the two days (d.f.=6, 52, F=6.247, P<0.000 for PCNA-I and d.f.=6, 52, F=11.021, P=0.000 for PCNA-OD), while no difference was found between insects fed diets 7:7 and 21:21 (d.f.=1, 51, F=0.077, P=0.783 for PCNA-I and d.f.=1, 51, F=0.585, P=0.484 for PCNA-OD).

Fig. 6 also shows the amount of food eaten on each day. As expected from earlier work (Raubenheimer and Simpson, 1993), the quantity of food eaten by fifth-instar locusts differed with both age and diet. Intake reached a peak at around day 4 and declined thereafter, while consumption was higher on 7:7 than 21:21 diet, indicating compensatory feeding. When the PCNA-I values for diet 7:7 and 21:21 were regressed against the quantity of food eaten by the locust on each day, significant linear regressions were obtained (Fig. 7A,B) for both diets (d.f.=1, 4, F=19.17, P=0.0119 for diet 7:7 and d.f.=1, 4, F=11.16, P=0.0288 for diet 21:21). In order to compare statistically the slope and distance between both regression lines, we included diet as a factor in an analysis of covariance (ANCOVA) with the mass of food eaten as a covariate. The regression lines were parallel (d.f.=1, 43, F=5.03, P>0.05) but differed in intercept (d.f.=1, 43, F=11.05, P=0.010). As expected, both variables (PCNA-I and amount of diet eaten) were significantly correlated (d.f.=1, 43, F=12.12, P=0.008). Similar regression lines were obtained for PCNA-OD

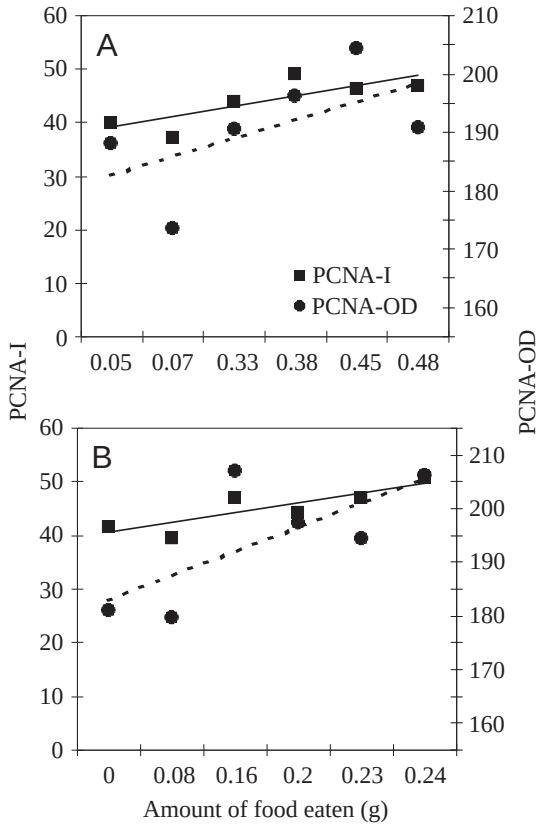

Fig. 7. Plot showing the regression lines between values for PCNA-I and PCNA-OD (A for diet 7:7 and B for diet 21:21) and the quantity of 7:7 and 21:21 diet eaten by locusts across the first 8 days of the fifth instar. Regression lines: (A) PCNA-I, y=21.47x+37.5, r^2=0.81; PCNA-OD, y=40.12x+173.8, r^2=0.55; (B) PCNA-I, y=34.1x+39.77, r^2=0.65; PCNA-OD, y=98.28x+179.3, r^2=0.62.

(Fig. 7A; d.f.=1, 43, F=9.2421, P=0.0384 for diet 7:7; Fig. 7B; d.f.=1, 43, F=4.569, P=0.0994).

Effect of the quality of the food on DNA content

To further understand the significance of the differences observed in PCNA levels, we carried out microdensitometric DNA measurements on the undifferentiated nuclei (UN) of the regenerative nests and the mature nuclei (MN) of the differentiated cells of the caeca of the locusts fed on different synthetic foods. Although in the present work we have used an indirect technique to calculate the DNA content of the nucleus, discrete peaks of integrated optical density (IOD) were obtained (Fig. 8). Several subpopulations showing different IOD peaks (different DNA content) were clearly distinguished. A marked difference was found between UN and MN. The UN showed lower IOD values when compared with the MN. The MN of the locusts that were fed the near optimal 21:21 diet showed the highest IOD values, including several peaks (at 1600, 1800 and 2100 arbitrary IOD units). The rest of the diets resulted in lower IOD values and only displayed one or two discrete peaks (at 1000 and 1200 IOD units). MN of animals fed with diets 7:35 and 35:7, the most distant from the optimal diet, had the lowest IOD values (at 1000 IOD units; occasionally a peak at 1200 IOD units was observed). Locusts fed diet 28:14 showed two different peaks at 1000 and 1200 IOD units, supporting the presence of two nuclear subpopulations. Diet 14:28 provided a single peak at 1200 IOD units, although another less conspicuous peak could be present at 1000 IOD units. In summary, locusts fed on diets with a P:C ratio close to the optimal 19:23 show higher IOD values than locusts fed on more unbalanced diets, supporting a correlation between the geometric distance to the optimal diet and the amount of DNA.

Discussion

Little is known about the rate of cell proliferation in insect tissues. Some isolated studies (e.g. Smith et al., 1987) provide data on cell proliferation in the insect central nervous system by means of immunocytochemical techniques that demonstrate the incorporation of BrdU. More recent studies have focused on the cell proliferative processes at the molecular level in *Drosophila* (Lehner and O'Farrell, 1990; Timmons et al., 1993; Park et al., 1998). However, few studies address cell proliferation in insect midgut cells (Endo, 1984). The present study shows that immunocytochemical detection of PCNA in histological sections is a simple, useful and straightforward tool to assess the cell proliferation status of insect cells, in particular of the digestive system. Our immunocytochemical and western blot data, in agreement with previous studies (Sukuza et al., 1989; Mathews et al., 1984; Waseem and Lane 1990; Yamaguchi et al., 1991), show the presence of PCNA in insect cells and support a widespread, evolutionarily conserved, PCNA-cyclin-related control mechanism in DNA replication.

The antigen retrieval process applied in this study is based on heating of the sections rinsed in a dilute solution of citric acid in a microwave oven. Similar heating protocols using heavy metal solutions are described by Shi et al. (1991) as an immunohistochemical enhancement method for formalin-fixed, paraffin-embedded tissues. The mechanism concerning microwave oven recovery of antigens is not yet fully understood, but in the case of formalin-fixed tissues it is possible that cross-linking of proteins caused by formaldehyde may be altered by microwave heating (Shi et al., 1991). In the present study, we have found that antigen retrieval by heating in a microwave oven also works successfully for Bouin's-fixed tissues. The citrate-based solutions used in our study were formulated following other protocols in which citrate buffers were used (Evers and Uylings, 1994; Brown and Chirala, 1995). We show that the antigen retrieval process is dependent on the microwave heating time and the pH of the citric buffer. Our data suggest that the specific parameters in relation to buffer composition and heating have to be adjusted from the protocol used for neutral, formalin-fixed tissues because of the particular composition and fixation characteristics of Bouin's fluid. Our results are in agreement with those of Evers and Uylings (1994) who found that pH

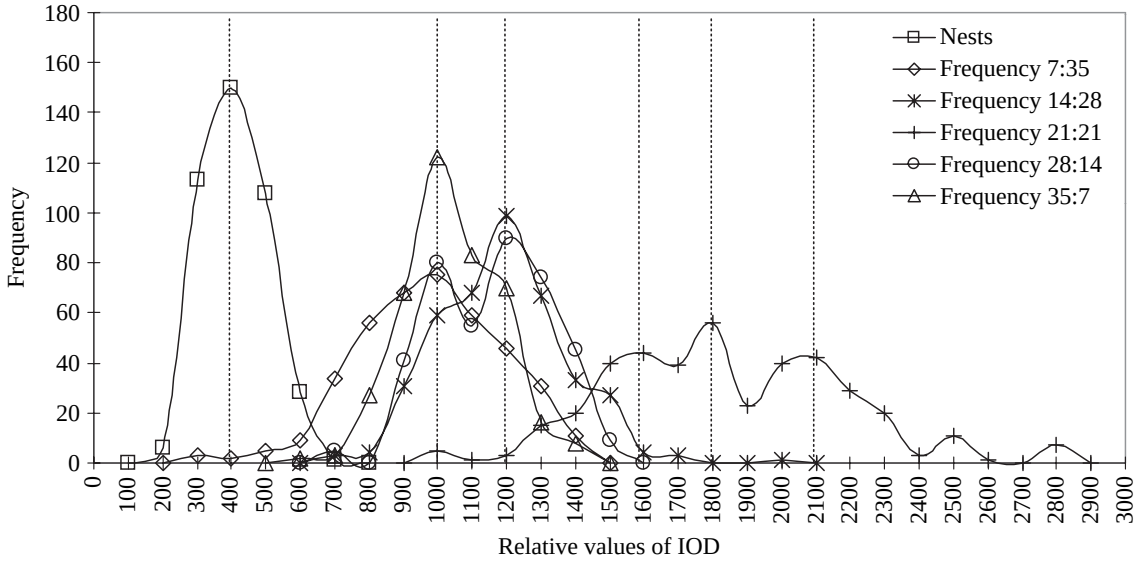

Fig. 8. A frequency spectrum showing the integrated optical density (IOD) values (in arbitrary units) for Feulgen stains of the mature and undifferentiated nuclei of the caecal epithelial cells of locusts fed on different synthetic foods (*N*=400 in each group).

and microwave heating time of the solution was of great importance for retrieval of antigen in formalin-fixed, paraffin-embedded tissues. They also found that these parameters may vary when different antibodies are used (Evers and Uylings, 1994). We have found that the optimal conditions for PCNA retrieval, using clone PC10, in Bouin's-fixed, paraffin-embedded tissues of insects are pH=6.0 and 40 min of microwave heating at 700 W. Clone PC10 has been successfully used in many studies of cell proliferation in mammals (Hall et al., 1990; Preziosi et al., 1995; Start et al., 1992). It has also been reported that clone PC10 gives the best results in PCNA detection in fish tissues (Ortego et al., 1994). The only study on immunological PCNA detection in insect tissue sections to our knowledge also used clone PC10 as the primary antibody (Yamaguchi et al., 1991).

Our data show PCNA-like immunoreactivity throughout the different regions of the midgut exclusively restricted to the cells within the nests, supporting the proliferative nature of these areas. Western blot analysis further confirmed the specificity of the immunoreaction. In agreement with previous studies in insects (Ng et al., 1990), PCNA-like immunoreactivity in locusts appears as a single band of ~30 kDa. Our densitometric data suggest profound differences not only in the number of PCNA-like immunoreactive cells (noted as PCNA-I in the text) but also in the levels of PCNA-like immunostaining (PCNA-OD) in the cells of locusts belonging to different experimental groups or located within different regions of the midgut. The midgut caeca has the highest levels of PCNA, followed by the regenerative cells in the ampullae at the midgut–hindgut junction. In addition, the mature nuclei of locusts fed on a balanced diet (21:21, closer to the optimal 19:23) show high PCNA-I and higher DNA contents than in those locusts fed on unbalanced diets, and values of both variables fell progressively as the diets became more unbalanced.

It has been well established that PCNA is a good marker of proliferative cells and that PCNA is a required element for DNA synthesis (Miyachi et al., 1978; Celis et al., 1987; Mathews et al., 1984; Bravo et al., 1987). Therefore, several cellular activities, such as DNA amplification, repair of damaged DNA, cycles of endopolyploidy, mitotic cell division or DNA excision repair (Shivji et al., 1992) could be, at least in theory, associated with the DNA synthesis that we have observed in the cells of the locust regenerative nests. By immunocytochemical techniques we show an association between BrdU incorporation and PCNA-like immunostaining in the cells of the regenerative nests, supporting the DNA replicative nature of the ongoing processes in these cells. Although important, this association between BrdU incorporation and PCNA does not discriminate between mitosis and the other above-cited processes involving DNA synthesis. However, the fact that we found only a small number of mitotic figures in all the 15 μm Feulgen-stained slides examined (data not shown) makes it unlikely that the DNA synthesis shown by PCNA and BrdU immunocytochemistry is exclusively associated with the S phase of the mitotic cell cycle. On the other hand, DNA amplification processes (i.e. extra replications of certain genes or DNA sequences) have been reported in a number of insects (Nagl, 1978). Our results on DNA measurements suggest that the nuclei of the caecal cells undergo a 'maturation' process from an undifferentiated status (low DNA content) within the nidi to a 'mature' form (higher DNA content) when they move away from the regenerative nests while growing in size and differentiation. The differences in PCNA-like immunoreactivity observed among locusts fed on different diets, together with the concomitant variations in the DNA content and the low number of mitotic figures in the regenerative nests, support a process of differential DNA amplification and probably cycles of endopolyploidy (or endoreduplication) involving genes

necessary for the correct completion of the feeding or moulting cycle for each particular diet. This is in agreement with Nagl's hypothesis (Nagl, 1978) that endopolyploidy and polyteny might be strategies by which, through the increase of the number of DNA templates, insect cells have a high synthetic capacity for selected proteins. This reasoning also provides the basis to understand the significance of the high proliferative index found in the caeca and the ampullae. It seems that ampullae may have an important role as a hitherto unappreciated 'endocrine organ' in the locust midgut (Montuenga et al., 1996). The higher PCNA staining found in these regions could be related to a higher metabolic activity required for their absorptive (in the case of caeca) and secretory (in the case of ampullae) functions.

As expected from earlier work (e.g. Raubenheimer and Simpson, 1993; Simpson et al., 1988), we found that the highest intake of food occurred near mid-stadium. This was the case in locusts fed both diets 7:7 and 21:21. Also, as shown previously (Raubenheimer and Simpson, 1993; Zanotto et al., 1993), the quantity of 7:7 diet eaten was greater than that of diet 21:21, indicating compensatory feeding for nutrient dilution. The PCNA-I and PCNA-OD levels in both groups (locusts fed 7:7 *vs* 21:21) were statistically similar and there was a highly significant correlation between the quantity of food eaten and PCNA-I values in both groups. Hence, these data strongly suggest that PCNA-I and PCNA-OD levels (and as a consequence DNA synthesis) reflect the nutritional status of the insect and that locusts fed on diet 7:7 were able to reach the same level of DNA synthesis as those fed on a 21:21 diet by altering the amount of food eaten. However, the same was not true for those insects fed the other diets (7:35, 14:28, 28:14 and 35:7). This is because the P:C ratio of these latter foods differed from the optimal (19:23), forcing insects to consume too much of one nutrient relative to the other. Such interference between nutrients leads to animals having to make a compromise between the metabolic and other costs of overeating one nutrient and undereating the other (Raubenheimer and Simpson, 1993; Simpson and Raubenheimer, 1993). Consequently, our results showed a correlation between the levels of PCNA expression and of nuclear DNA content and the ratio of protein and carbohydrates in the diet, with values falling as foods became more unbalanced in their ratio of P to C.

It will be of great interest to see whether the effect of nutritional status is due to a general stimulation of metabolic activity by ingested nutrients, whether it is differentially stimulated by specific nutrient groups and/or whether it involves regulation of particular DNA synthetic pathways. We demonstrated that the synthesis and release of some but not all regulatory peptides present in the diffuse endocrine system of the locust midgut is also related to the quality of the food, suggesting that selected synthetic pathways in the midgut epithelial cells are specifically upregulated by protein and carbohydrate contents in the diet. Further genetic and molecular studies will be necessary to better understand these mechanisms.

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