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CHROMOSOMA

Insect Sex Chromosomes

VI. A Presumptive Hyperactivation of the Male X Chromosome in *Acheta domesticus* (L.)*

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Abstract. The functional status of the X chromosome in *Acheta domesticus* has been analysed at the whole chromosome level on the basis of (1) ^{3}H-thymidine autoradiography, (2) 5-BrdU/AO fluorescence microscopy, (3) in vivo 5-BrdU incorporation and (4) ^{3}H-UdR induced aberrations. The rationale of these techniques in relation to the functional aspect of the X chromosome is that the inactive X chromosome would (1) show asynchrony in DNA synthesis, (2) show differential fluorescence, (3) respond differentially to in vivo 5-BrdU treatment and (4) the active X chromosome would show aberrations when treated with ^{3}H-Uridine. From the results, it appears that the X chromosomes in both male (XO) and female (XX) somatic cells of *Acheta* are euchromatic (active). Further, the single X in the male is transcriptionally as active as the two X chromosomes in the female. In other words, the single X in the male is hyperactive when compared with the single X in the female. From this it is inferred that the male X chromosome is differentially regulated in order to bring about an equalization of it's gene product(s) to that produced by both Xs in the female. *Drosophila melanogaster* has a comparable system of dosage compensation. Thus, *Acheta* is yet another insect showing evidence for an X chromosome regulatory mechanism of dosage compensation. Additionally, it is surmised that sex determination in *Acheta* is based on an autosomes/X chromosome balance mechanism.

Introduction

In a majority of eukaryotes, chromosomes are characterized by the presence of constitutive heterochromatin which is genetically inactive. The active portion of the genome is termed euchromatin. However, in some eukaryotes, euchromatic material may become facultatively heterochromatinized and temporarily inactive (Brown, 1966). This occurs in mammals (Lyon, 1961; Russell, 1961) and the mole cricket *Gryllotalpa* (Rao and Arora, 1978, 1979;

* Dedicated to Professor S.R. Ray Chandhuri on his 75th Birth Auniversary

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Arora and Rao, 1979). One of the two X chromosomes in the female soma is facultatively heterochromatinized. Similarly, in the Coccids (Hemiptera) and Sciaridae (Diptera), the entire paternal autosomal set is facultatively heterochromatinized (Brown and Chandra, 1977).

Ever since the discovery of the X chromosome (McClung, 1902), attempts have been made to decipher its role in the genome. As early as 1922, Bridges reported that certain sex linked genes of *Drosophila* are identically expressed in both sexes despite the difference in sex chromosome constitution. This equalization of the expression of the gene products was subsequently referred to as "dosage compensation" (Muller et al., 1931). So far, dosage compensation for X linked genes has been established only in mammals and *Drosophila*. In *Drosophila*, the dosage compensation apparently operates at the level of RNA synthesis in the chromosomes (Mukherjee and Beermann, 1965); the single X of the male is transcriptionally hyperactive and equivalent to the two X's of the female. Further, the X chromosome in male *Drosophila* completes replication faster (Mukherjee and Chatterjee, 1975, 1976) probably concomitant with hyperactivation. In mammals, dosage compensation involves genetic inactivation (facultative heterochromatinization) of one of the two X's in the female (Lyon, 1961) which occurs before implantation. In all placental mammals, the phenomenon is random, i.e., either the paternal or the maternal X is inactivated, while in metatheria, there is a preferential paternal X inactivation.

In mammals, the inactivation involves the entire X chromosome presumably with one (or more?) controlling center(s) (Russell, 1964), whereas in *Drosophila,* there are independent sets of dosage compensators distributed throughout the X and possibly also in the autosomes (Cock, 1964). Each X-linked gene is influenced by specific compensators in such a way that they are activated to an identical level in the two sexes (Lakhotia and Mukherjee, 1969). It is of interest to note that *Drosophila* and mammals have adopted entirely different mechanisms to achieve the production of equal levels of their gene products.

There is little information upon dosage compensation from other groups of organisms. One of the major reasons for this is the absence of genetic studies in these groups. This has been overcome in our laboratory by an alternative approach whereby the functional aspect of the X chromosome is studied at a whole chromosome level. The following findings prompted this alternative approach:

1. Genetically inactive X chromosomes invariably show asynchrony in DNA replication (Gartler and Andina, 1976).

2. Studies on 5-Bromodeoxyuridine induced alterations indicated that only inactive chromosomes are affected (Buhler et al., 1977).

3. ^{3}H-Uridine induces aberrations to a large extent, if not exclusively, in the euchromatic (active) chromosome (Klevecz and Hsu, 1964).

Utilizing a combination of these protocols Rao and his associate (Rao and Arora, 1978, 1979; Arora and Rao, 1979, 1980) established a presumptive X inactivation mechanism in the Orthopteran *Gryllotalpa* which appears to be the first case described apart from mammals. In furtherance of our

studies on X chromosome regulation, we have examined the situation in another Orthopteran, *Acheta domesticus*, adopting the same methodology. In addition, 5-BrdU/AO fluorescence microscopy was employed to identify whether or not a late replicating X chromosome was present (Mikkelsen, 1976).

Materials and Methods

The experimental insects, with a body weight ranging from 105–112 mg, were kept at $30 \pm 1°$ C in a B.O.D. incubator and were fed with bread and milk.

DNA Replication

1. ^{3}H-Thymidine Autoradiography. 4 μCi of ^{3}H-thymidine (specific activity 10.006 Ci/mM, Bhabha Atomic Research Center, Trombay, Bombay, India) in 0.02 ml of 0.75% insect saline was injected into each animal. Three animals of each sex were examined at two hour intervals, covering a period from 2 to 26 h. 0.02 ml of 0.08% colchicine was injected into each animal two h before sacrifice. Chromosome preparations were made by the air drying method, and treated with 5% TCA (trichloroacetic acid) for 10 min at 0°–2° C. The slides were washed in running tap water for about 30 min, rinsed in 70% alcohol and air dried. The slides were then coated with Kodak NTB-3 Nuclear Track Emulsion, dissolved in distilled water (1:1) at 42° C, and exposed for 10–12 weeks. After exposure, the slides were developed in Kodak D-19b at 10°–12° C for 10 min, rinsed in distilled water, stained with 4% buffered Giemsa at pH 6.8 for 5–8 min and made permanent. For the estimation of the various phases of the cell cycle, the method of Sisken (1964) was employed. The rate of loss of ^{3}H-thymidine from the haemolymph was monitored by withdrawing haemolymph samples at different time intervals, 0 h, 15, 30, 45 min, 1, 2, 3, 4, 5 and 6 h after injecting the label. The technique of Fox et al. (1974) was followed. 2 μCi of ^{3}H-thymidine in 0.02 ml of insect saline was injected into the animals. After the desired time intervals, 10 μl of haemolymph was withdrawn from the antennae of each animal, dropped onto a one inch diameter millipore filter and dried. The filters were submerged in toluene based scintillator fluid and counted using a Packard scintillation counter. The filters were subsequently washed with four changes of toluene, dried and extracted with excess (about 220 ml/filter) cold 5% TCA and 0.2% sodium pyrophosphate ($Na_4P_2O_7 \cdot 10\ H_2O$). After washing in water and drying the filters were counted again. Counts remaining on the filters were presumed to be due to the haemolymph cells and other tissue debris taken in with the haemolymph samples. The actual counts of the tritiated thymidine in haemolymph were obtained by subtracting the radioactivity in the cell debris from the initial values. Under the conditions used, the counting efficiency was 37%.

2. 5-BrdU/AO Fluorescence Microscopy. 5-BrdU (200 μg/animal) dissolved in 0.02 ml of insect saline was injected into the animals for labelling the late S cells and the animals were sacrificed after 6, 7 and 8 h. The timing is based on the cell cycle data. 0.02 ml of 0.08% colchicine was injected into each animal 2 h prior to sacrifice. Chromosome preparations were made by the air drying method. The slides were passed through alcohol grades to distilled water and stained with an 0.005% acridine orange solution, dissolved in Sorensen's buffer pH 6.8, for 10–15 min in the dark. The slides were thoroughly rinsed in distilled water and mounted in McIlvaine's buffer pH 6.8. Observations were made on an Olympus fluorescence microscope and photomicrographs were taken on Kodak plus X pan film (125 ASA).

3. Effects of 5-BrdU on the Chromosomes. In order to determine the effect of the dosage of 5-BrdU on the chromosomes three different doses (150, 200 and 250 μg of 5-BrdU dissolved in 0.75% insect saline) were injected into three different sets of animals for 4, 6, 8, 10, 12, and 14 h. Three male and female nymphs each were chosen for each time interval and for each set of experiments. 0.02 ml of 0.08% colchicine was injected into each animal 2 h before sacrifice. Chromosome preparations were made from hepatic caecae and testis by the air

drying method. Metaphases from both treated and control series were photographed on ORWO NP-15 (25 ASA) film using a Leitz microscope fitted with an Orthomat camera.

4. ^{3}H-Uridine Induced Aberrations. Twenty two animals of each sex, with a body weight ranging from 105–112 mg, were selected for the experiment. 20 μCi (in 0.02 ml of 0.75% insect saline) of ^{3}H-uridine (specific activity 36.6 Ci/mM, New England Nuclear) was injected into each animal for 14 h (early S phase). Cold uridine, 50 μg/animal, was injected one h after the injection of the labeled precursors. 0.02 ml of 0.08% colchicine was injected into each animal 2 h prior to sacrifice. The choice of the above mentioned timing is based on the fact that most of the ^{3}H-uridine induced aberrations seem to occur at early S as observed by Natarajan and Sharma (1971) in *Microtus agrestis*, and more recently in *Gryllotalpa* by Rao and Arora (1979). Chromosome preparations were made by the air drying method and stained as mentioned earlier. Desired metaphases were photographed on ORWO DK-5 high contrast copy film. The classification of breaks and gaps and the scoring procedures used are based on those employed by Natarajan and Sharma (1971) and Rao and Arora (1979). Only chromatid type aberrations (chromatid and isochromatid events) were found in the present study. Autosomal aberrations were also scored for comparison.

Results

Chromosomes

The diploid chromosome number in *Acheta domesticus* is 21 in ♂♂ and 22 in ♀♀ with an orthodox XO male and XX female sex determining mechanism. Among the autosomes, one pair is acrocentric, five pairs are telocentric, three pairs submetacentric and the smallest 10th is the metacentric. The X chromosome, being the largest metacentric, comprises about 20.28% of the total haploid genome (Fig. 1).

^{3}H-Thymidine Autoradiography

1. Chronology of the Cell Cycle and the Pattern of DNA Replication in Hepatic Caeca. The rate of loss of thymidine from the haemolymph is summarized in Figure 2. The maximum number of counts was obtained immediately after the injection of the label, followed by a sharp decrease. After 1 h the level remains fairly constant. We concluded that the injected thymidine is rapidly lost from the haemolymph. This is analogous to a short pulse.

The percentage of labelled mitosis was analysed during the post-treatment period. The intervals between the beginning of labelling and the half maximum on the ascending slope of the first peak of the labelling provides an estimate of G_2+P. The first peak of the labelling index curve indicates the maximum number of cells which are in the S period. The ascending limb and the descending limb are related to cells which are at late and very early S phase, respectively. The mean duration of S is calculated by the time interval between the one half maximum labelling index on the first ascending and descending limbs of the curve respectively, minus the treatment time. The time interval between half maximum on the ascending slope of the first and second labelling peaks gives the total generation time (T). The value of G_1 is calculated by the formula, $G_1=T-(M+G_2+S)$.

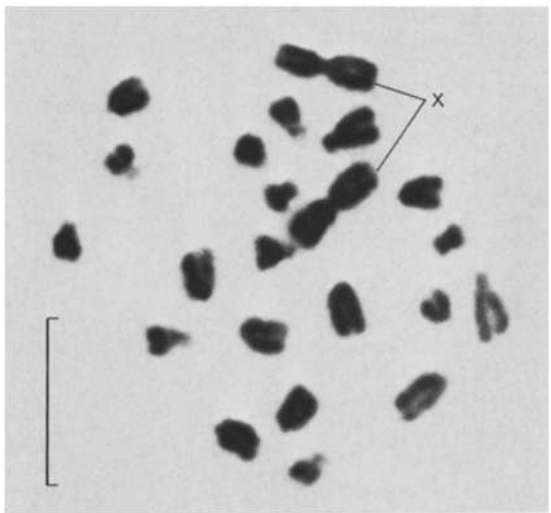

Fig. 1. Normal female metaphase showing 22 chromosomes. The X chromosomes are the largest and are metacentric[1]

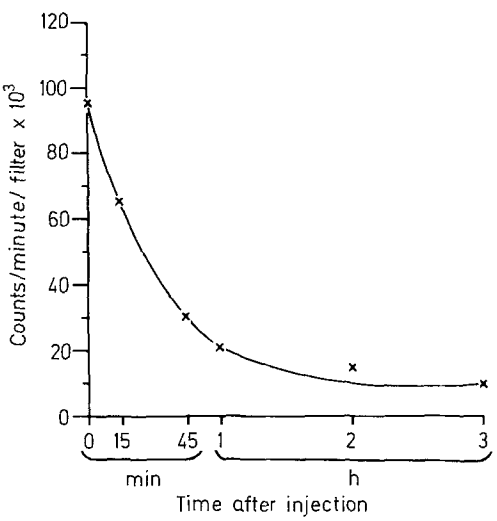

Fig. 2. Rate of loss of ^{3}H-thymidine from the haemolymph

A nucleus showing a minimum of eight grains is considered as being labelled. No labelled metaphases are observed earlier than 4 h. After 4 h, 32% are found to be labelled. The labelling index increases to 45% and 73% at 6 and 8 h respectively. After reaching the maximum of 87% at 10 h, it declines to 60%, 10% and 6% at 12, 14 and 16 h, respectively (Fig. 3). Thereafter, a second increase in the labelled metaphases was observed, reaching 30% at 18 h and 47% at 20 h, followed by a peak of

[1] Bar in all figures: 10 μm

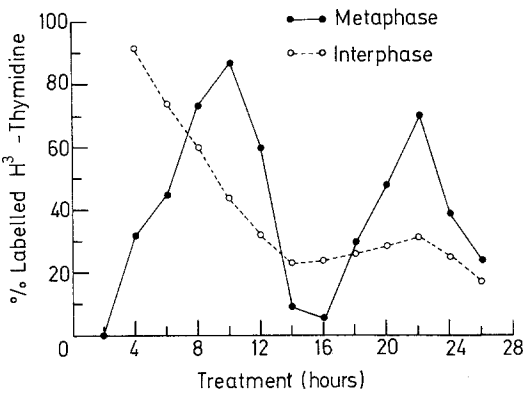

Fig. 3. The cell cycle in the hepatic caeca. Percent labelled metaphases (o——o) and interphases (o——o), treated with ^{3}H-thymidine for different time intervals (h)

70% labelled metaphases at 22 h and the eventual decline, where 38% and 24% metaphases were labelled at 24 and 26 h respectively. Samples from 20 h onward showed chromatid label and are considered to be in the second cycle following treatment.

From the above data, G_2+P is computed to be about 6 h and S about 7 h with a total generation time T of some 13 h. From the results obtained, it seems that G_1 period is negligible or absent (Fig. 3). There are very few reports available which provide estimates of the chronology of the cell cycle in the Gryllidae (Orthoptera) and so far as we are aware this is the first report on the in vivo cell cycle of hepatic caecae. Cleaver (1967) obtained similar estimates on grasshopper spermatocytes and neuroblast cells labelled in vivo. So too have Dolfini et al. (1970), using primary in vitro somatic cell cultures of *Drosophila melanogaster*, Fraccaro et al. (1976) on *Anopheles atroparvus* spermatogonia and brain cells in vivo and Arora and Rao (1979) with hepatic caeca cells of *Gryllotalpa* in vivo.

2. Chromosome Replication. On the basis of the labelled metaphases, the following replication patterns can be defined:

Pattern A: Cells caught shortly after the initiation of DNA synthesis, i.e., in early S phase (12–14 h sample). Significantly, both the X's in the female and the single X in the male start DNA synthesis synchronously with the autosomes (Fig. 4a, b).

Pattern B: Cells which are in the middle of the S phase at the time of tritiated thymidine incorporation and are observed in 10 h samples. All chromosomes show a uniform heavy labelling (Fig. 4c).

The centromeric regions of the X chromosome of both male and female show a very scanty labelling whereas the terminal regions are heavily labelled. This indicates that the centromeric regions start replication later than the terminal regions.

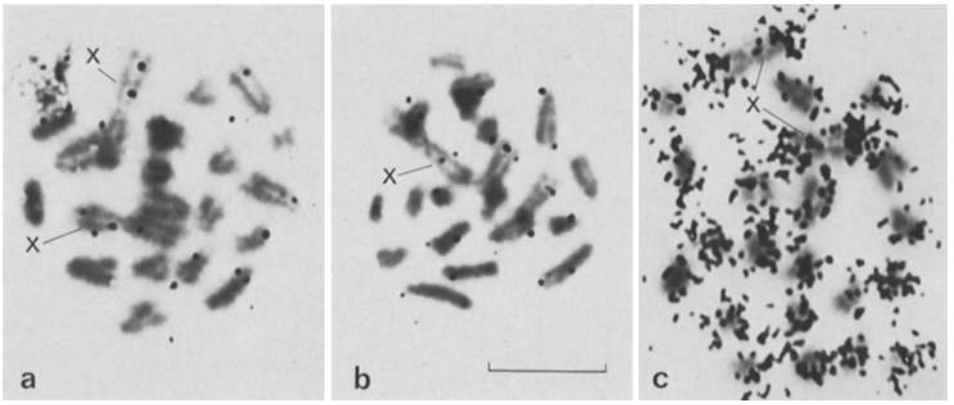

Fig. 4a–c. DNA replication. **a** Female and **b** male metaphases from early S (12–14 h samples). Note the two Xs in the female and the single X in the male all show sparse labelling as do the autosomes. **c** Female metaphase from mid S (10 h sample) showing heavy and uniform labelling on all the chromosomes. Note the identical labelling pattern in the two X's

Pattern C: Both the Xs in the female are still heavily labelled whereas the male X shows comparatively reduced labelling. Autosomes, both in male and female, are uniformly but less heavily labelled. This pattern represents the later part of mid S or the beginning of late S and is observed in 8 h samples (Fig. 5a, b). Thus, whereas the X chromosome in the male appears to be on the verge of completing DNA synthesis, both X's in the female are still synthesizing DNA.

Pattern D: This corresponds to the late S phase (6 h) of DNA synthesis (Fig. 5c, d). In the female, both of the X chromosomes and some of the autosomes are still labelled whereas most of the autosomes have completed DNA synthesis. Significantly, the X chromosome in the male has virtually completed its DNA synthesis at this time. There is uniformity in the sparse labelling pattern of both male and female autosomes.

Pattern E: Cells caught during the very late S phase of DNA synthesis, observed in 4 h samples. By this time, most of the autosomes in both male and female have completed their DNA synthesis. The male X chromosome is unlabelled whereas both the Xs in the female are still sparsely labelled. This indicates that the male X chromosome finishes DNA synthesis earlier (i.e. replicates faster) as compared with the two X's of the female and the autosomes.

Pattern F: Involves second division metaphases, where chromosomes show chromatid labelling, and is seen in 20 h samples (Fig. 5e).

Thus, it is clear that the X chromosome(s) both in male and female start DNA synthesis simultaneously along with the autosomes. However,

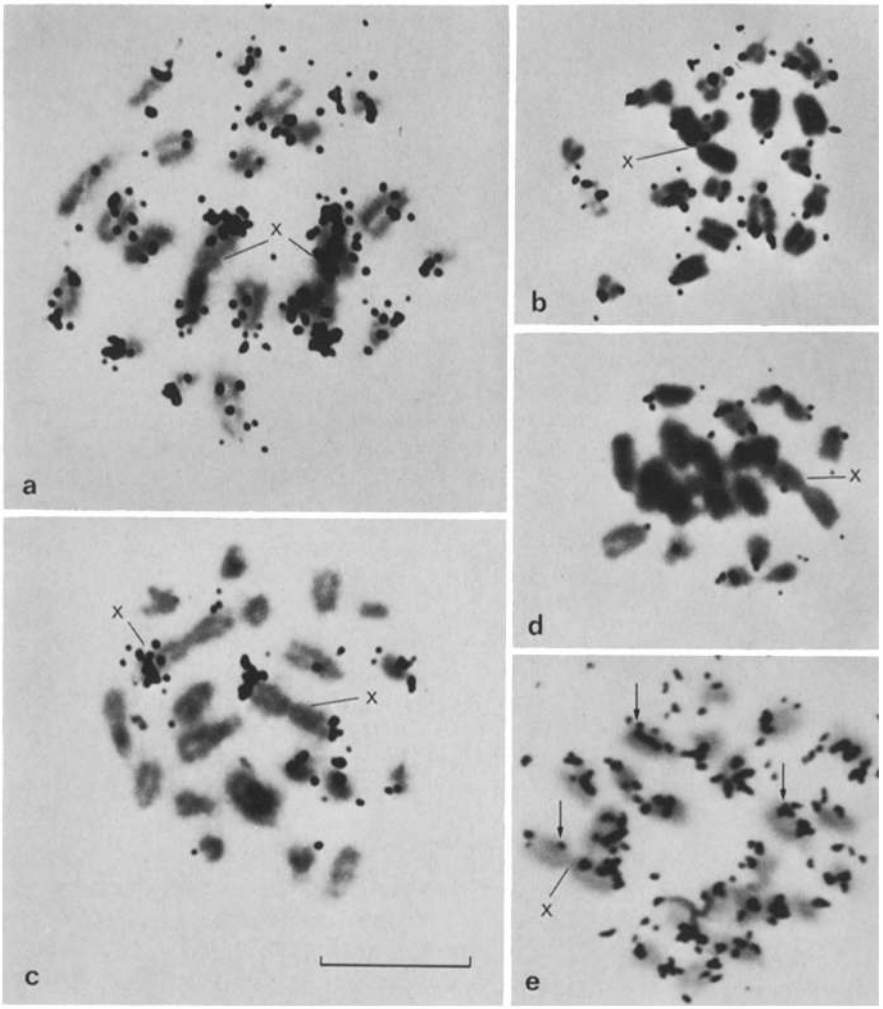

Fig. 5a–e. DNA replication. **a** Female and **b** male metaphases from late S (8 h sample). Note that both Xs in the female are heavily labelled as compared with the single X in the male which shows less grains. **c** Female and **d** male metaphases from very late S (4–6 h samples). Note the lightly labelled X's in the female and unlabelled single X in the male. **e** Male metaphase of the second division after treatment. Note the chromatid labelling (*arrows*)

the X in the male completes replication earlier than the two Xs of the female.

3. 5-BrdU/Acridine Orange Staining. 5-BrdU/Acridine orange fluorescence studies failed to differentiate the late replicating X chromosome in female somatic tissues when late S cells were treated with 5-BrdU. There was no differential fluorescence nor was there any weak staining of the X chromosomes (Fig. 6a, b). This observation is in accordance with the absence of

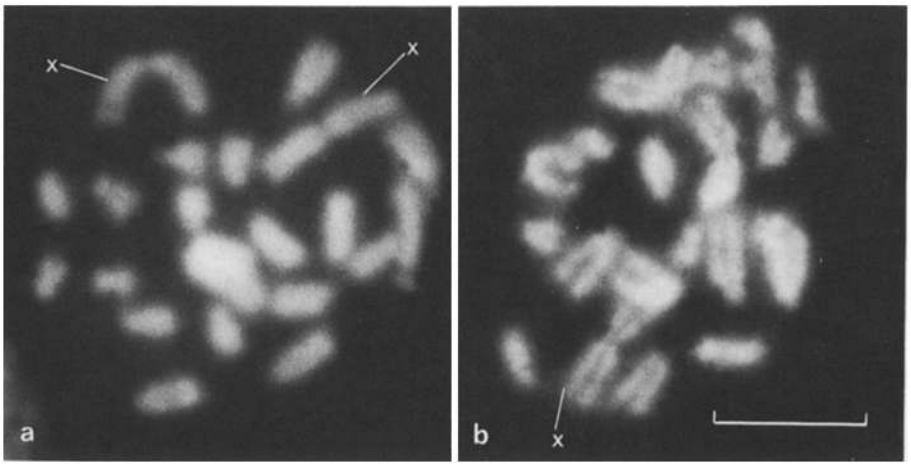

Fig. 6a–b. a Female and male **b** metaphases after 5-BrdU in vivo treatment followed by acridine orange staining. Note the uniform fluorescence of the two Xs in the female and that of the single X in the male

asynchronous replication between the two X chromosomes of the female as proved by ^{3}H-thymidine labelling.

Effects of 5-BrdU on the Chromosomes

Chromosomes were treated in vivo with different doses of 5-BrdU and 25–30 metaphases each were analysed for various time (4, 6, 8, 10, 12 and 14 h) intervals. In accordance with the previous results, there was again no differential response to 5-BrdU of the two Xs in the female as compared to controls (Fig. 7a–d). In the absence of any differential response of the X chromosomes to 5-BrdU in the female, it is inferred that both the Xs in the female like the single X in the male are active. If so, such active X chromosomes should be susceptible to the ^{3}H-uridine induced aberrations (Rao and Arora, 1979).

^{3}H-Uridine Induced Aberrations in the Chromosomes of Male and Female Hepatic Caecae Cells

The number of aberrations scored are from equal numbers of metaphases in both sexes. ^{3}H-uridine produces both chromatid and isochromatid type aberrations. For the purpose of scoring, achromatic lesions (gaps) have also been taken into consideration since these are also implicated in genetic damage (Brinkley and Hittleman, 1975). Chromatid type aberrations have been found to be more frequent in *Acheta* and these are about three times more common than are the isochromatid type aberrations.

1. Aberrations in the Autosomes. The mean number of autosomal aberrations in the male and female are 6.11 and 6.22, respectively (Table 1). This clearly

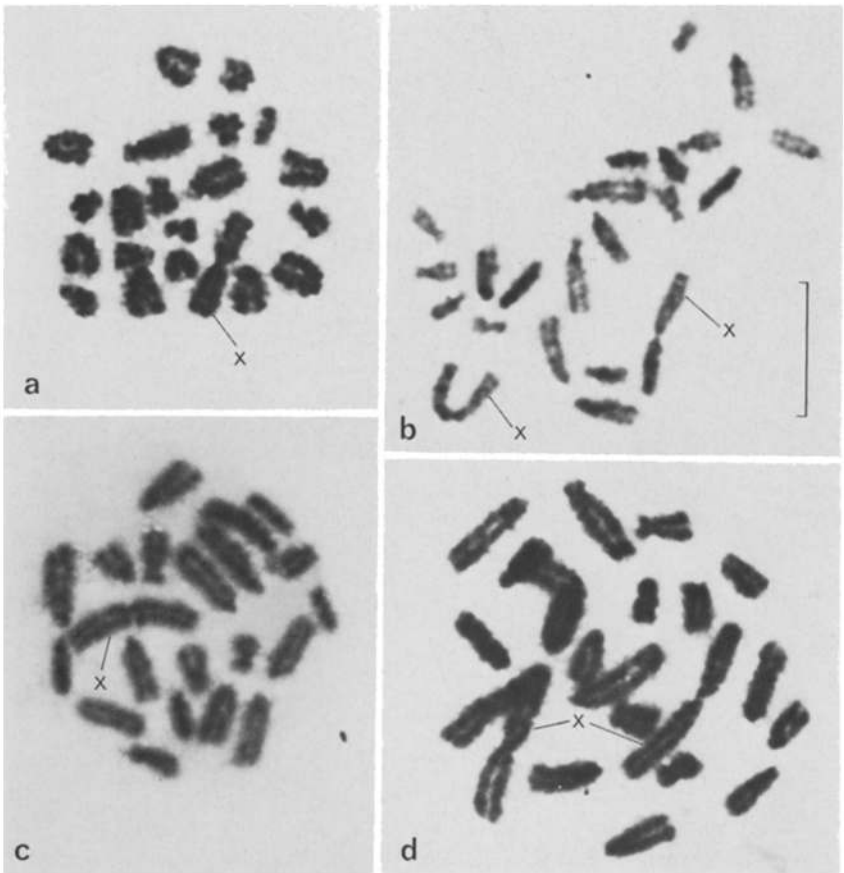

Fig. 7a–d. In vivo 5-BrdU treatment. **a** Male and **b** female control metaphases. **c** Male and **d** female metaphases treated during late S (8 h sample). Note the Xs in both male and female remain unaffected indicating that they are euchromatic in both sexes

Table 1. Frequencies of tritiated uridine induced aberrations in the male and female somatic cells of *Acheta domesticus* (L.) at the early S phase

Type of aberrations	Sex	Total number of metaphases	Chromatid	Isochromatid	Total	Mean ± SE	P* value
Autosome aberrations	♂	90	412	136	548	6.11 ± 0.20	0.50
	♀	90	423	137	560	6.22 ± 0.27	
Sex chromosome aberrations	♂	90	232	104	336	3.73 ± 0.15	0.10
	♀	90	207	162	369	4.10 ± 0.15	

* Significance level – Student's t-test

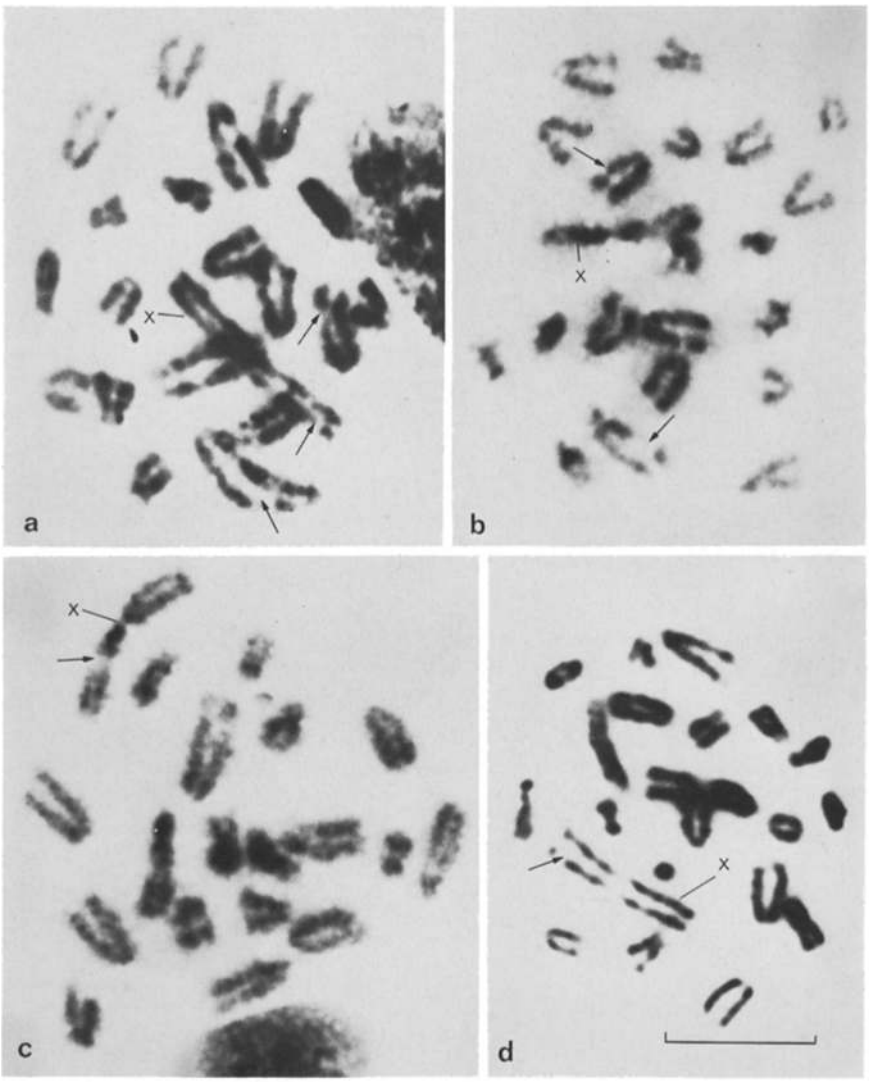

Fig. 8a–d. ^{3}H-uridine induced chromosome aberrations in the male. **a, b** Hepatic caeca metaphases showing aberrations in both autosomes and the X chromosome (*arrows*). **c** mid arm aberration, **d** terminal aberration in the X

indicates that the frequency of autosomal aberrations is the same ($P=0.5$) in both the sexes (Fig. 8a, b). Moreover all regions of the chromosome (terminal, distal and proximal) were found to be susceptible.

2. Aberrations in the X Chromosomes. The male X shows both the chromatid and isochromatid aberrations. The mean frequency of aberrations in the male X is 3.73, though, in some of the metaphases, more than four aberrations are found (Fig. 8c, d). Interestingly, in a limited number of polyploid

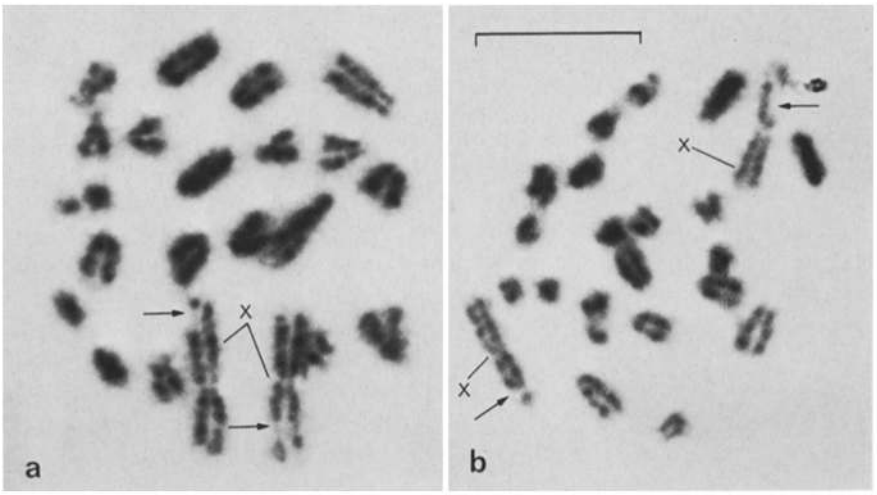

Fig. 9a, b. ^{3}H-uridine induced aberrations in the X chromosomes of female hepatic caeca. Note both X chromosomes are affected (*arrow*) indicating their euchromatic nature

cells that have been scored from hepatic caecae, all the Xs showed aberrations.

In females both X chromosomes show aberrations (Fig. 9a, b). The mean frequency of aberrations was about 4.10 for both X chromosomes. This value approximates that of the single X in the male. Statistically, there is no significant difference in the mean frequencies of the X chromosome aberrations between the two sexes ($P=0.10$). Thus, the single X in the male is affected almost to the same extent as both the Xs of the female taken together (Table 1). In other words, the single X in the male shows almost twice the number of aberrations as compared with a single X in the female.

Discussion

On the basis of the results obtained in *Acheta* from DNA replication (^{3}H-thymidine autoradiography), it is reasonable to infer that the single X chromosome in male somatic cells is euchromatic and genetically as active as the two Xs in the female. This inference is based on the fact that the X chromosomes in females do not show any asynchrony in DNA replication and both the Xs start and terminate DNA synthesis coincident with the autosomes. The male X chromosome shows a similar replicative pattern except that it completes replication earlier; i.e., that it replicates at a faster rate. Thus, the DNA replication in *Acheta* follows a similar pattern to that observed in *Drosophila* (Mukherjee and Chatterjee, 1975, 1976).

In order to confirm the euchromatic nature of the two Xs in female *Acheta,* the 5-BrdU/AO technique was applied which is known to differentiate the active X chromosome from the inactive one. It has been established

that in mammals after 5-BrdU incorporation during late S, followed by AO staining, the inactive X chromosome is found to be either segmented or stretched (decondensed) and shows dull fluorescence as compared with the active one (Dutrillaux, 1976; Mikkelsen, 1976). Interestingly, in *Acheta* there was no differential fluorescence nor was there any weak staining of the X chromosomes of either sex, all showed a uniform staining pattern. In *Gryllotalpa,* as expected, the Xs showed a distinct differential fluorescence. These results clearly indicate that the X chromosome in both sexes of *Acheta* is euchromatic and active. This is in accordance with the results obtained from tritiated thymidine autoradiography.

Essentially the same picture emerged from the studies on in vivo 5-BrdU effects on the X chromosomes. In cases, where the X chromosome is inactive it is affected differentially by 5-BrdU (Zakharov and Egolina, 1972; Buhler et al., 1977; Rao and Arora, 1978). As expected, in *Acheta,* there was no differential response of the X chromosome(s) to 5-BrdU treatment indicating that the X chromosome(s) in both sexes are entirely euchromatic and genetically active.

If, indeed, the two X chromosomes in female *Acheta* are euchromatic and active, they should show aberrations when treated with tritiated uridine. It has been established from our previous studies on *Gryllotalpa* that the active (euchromatic) regions of the chromosomes are more susceptible to tritiated uridine-induced aberrations than the inactive (heterochromatic) regions. The fact that chromosomes are not affected by ^{3}H-uridine at G_2 phase but are susceptible during the remainder of the cell cycle is in accordance with the assumption that the aberrations are due to the template activity of the chromosomes at the time of treatment (Rao and Arora, 1979). Furthermore, it has been shown that the tritiated uridine-induced aberrations are indeed due to transcriptional activity (Arora and Rao, 1980).

From the information obtained with tritiated uridine (Table 1) it is apparent that the frequency of aberrations in the autosomes of both males and females is almost the same. Equally apparent is the fact that the frequency of aberrations in the single X in the male and both Xs in the female is almost equal. From this it follows that the male X shows almost twice the number of aberrations as compared with a single X of the female. This is interpreted to mean that the male X is transcriptionally hyperactive. The *Acheta* system, therefore, bears a close resemblance to *Drosophila* in the manner of X chromosome regulation.

Dosage Compensation vis-a-vis Sex Determination

The phenomenon of dosage compensation has some relevance to the problem of sex determination. In this context, White (1973) has remarked that a "dosage compensation system should have been built up, by natural selection, in groups where the phenotypes of the two sexes are basically similar, evolving *pari passu* with the sex determining mechanism itself".

It is generally believed that among insects with male heterogamety, sex determination is essentially based on the autosome/X chromosome balance

(White, 1973). A particularly well established case is *Drosophila* where the dosage compensation mechanvsm is based on an effect of both the X linked and autosomal compensator genes which modulate the expression of various X-linked Mendelian genes (Lucchesi, 1973, 1977). In the light of this and on the basis of a presumptive male X chromosome regulation by hyperactivation in *Acheta,* it appears most likely that the sex determination in *Acheta* also is based on a balance of autosomes and X chromosomes.

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