The effect of X-radiation on spermatogenesis and the fertility of Schistocerca gregaria (Forsk.)

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SUMMARY

Normal spermatogenesis and the effects of X-radiation on the male locust germ cells have been studied with the aid of light and electron microscopy. Adult insects were irradiated with doses of 100–500 rad (i.e. 1–5 J kg⁻¹) and subsequently examined, at intervals, up to 55 days later. In secondary spermatogonia all doses caused nuclear fragmentation and necrosis, and in primary spermatogonia and a few spermatocytes caused a delay in division. Spermatids and the majority of spermatocytes developed into spermatozoa which were passed from the follicles to the seminal vesicles. The supply of sperm was, subsequently, temporarily stopped. The follicles were eventually repopulated by the division of the primary spermatogonia. Radiation-induced abnormalities, e.g. the formation of supernumerary centrioles, flagella and definitive mitochondria, were most common in the cytoplasm. The Golgi bodies and acrosome seemed to be unaffected. Abnormal outgrowths of the late spermatid and sperm nuclei appeared to be caused by the presence of the numerous centrioles and flagella. The bright yellow coloration of the cuticle, which is characteristic of sexually mature males, was not fully developed in irradiated males. Breeding experiments showed a significant reduction, when compared to the controls, in the number and percentage hatchability of eggs obtained from females mated to irradiated males. The doses of radiation employed did not appear to affect the longevity of the adults.

INTRODUCTION

The success of the sterile-male technique for the control and eradication of screw-worm fly, Cochliomyia hominivorax (Bushland & Hopkins, 1953; Knipping, 1955, 1959), has stimulated further interest in the detailed effects of radiation on insect germ cells, with or without reference to the use of their technique as a means of pest control.

The majority of investigations have involved the use of high doses of X- or gamma-rays, Porthetria dispar (L), 50–20000 R (Rule, Godwin & Waters, 1965); Cochliomyia hominivorax, 100–6200 R (Riemann, 1967); and Trichoplusia ni, 1000–50000 R (North & Holt, 1967). These, together with earlier work by Gatenby, Mukerji & Wigoder (1929), and Goldschmidt (1934), have shown insect spermatogonia to be radiosensitive in that they are destroyed by high doses but any that remain show a relatively low mutagenicity. The later stages,

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of spermatogenesis, exhibit greater resistance to breakdown but have a higher mutagenicity (Riemann, 1967).

Relatively low doses of X-rays can disrupt germ cells and particularly affect their cytoplasmic inclusions, e.g. 100–600 R (100 R = 2.58 × 10⁻² C kg⁻¹) produced cytoplasmic abnormalities in the germ cells of *Melanoplus differentialis*, irradiated as second instar nymphs (Tahmisian & Devine, 1961).

The recovery of germ cells in the screw-worm fly, which had been damaged by the irradiation (with 1500 R) of 2-day-old pupae, was described by Riemann (1967). None of the other previously cited reports describe this process.

The aim of this investigation was to study the effects of low dose X-radiation on the adult testis of *Schistocerca gregaria*, with particular reference to cytoplasmic inclusions. A series of breeding experiments, with irradiated males, was carried out to evaluate the effects of radiation on mating and reproductive capacity.

**MATERIALS AND METHODS**

The stock culture of *Schistocerca gregaria* was provided by the Centre for Overseas Pest Research, London, and maintained by the Hunter-Jones method (1961).

The X-ray source was a G.E.C. Maximar machine operated at 175 kV and 6.5 MA, without filters. The dose was measured with an ionization chamber linked to a Simplex Universal Dosimeter which had been calibrated with a standard radium source.

Young, 4- to 7-day-old, adult locusts, selected for irradiation, were placed in a shallow cardboard box 50 cm below the X-ray tube. In all a total of 54 male locusts were irradiated, receiving doses of 100–500 rad ± 10 % (100 rad = 1 J kg⁻¹) at a rate of 25 ± 2.5 rad/min.

**Examination of testes**

In the first series of experiments testes were examined, 24, 72, 144 and 264 h after irradiation, by both light and electron microscopy. In the second series, a group of insects, irradiated with 400 rad, was examined and the results recorded over a period of 55 days (Table 2).

The tissue for light microscopy was dissected, and cleaned of fat and tracheoles, under insect Ringer and then fixed in Champy or Zenker. Wax sections were cut 4 μm in thickness, and then stained with either Heidenhain's iron haematoxylin or Schiff's reagent (Feulgen technique).

For examination with the electron microscope the testes were removed from the specimens under 2.5 % glutaraldehyde solution, pH 7.3, containing 0.045 g/ml sucrose and maintained at a temperature ranging from 0 to 4 °C. The follicles were cleaned and replaced in the solution for 2–24 h. The tissue was post-fixed in a 2 % solution of osmium tetroxide for 2 h and then washed in a saturated aqueous solution of uranyl acetate. Single follicles were embedded in Epon 812.
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An LKB ultratome was used to cut grey or gold sections which were stained in uranyl acetate and/or lead citrate, the grids were subsequently examined with an A.E.I. EM 6B electron microscope.

Breeding experiments

Ten fledgling male locusts were exposed to 400 rad and placed with ten virgin females, of the same age, in a cage containing four sand tubes which were examined daily for egg pods, for a period of 60 days. Any eggs produced were incubated at 30 ± 2 °C. The number of hoppers hatching from each pod and the number of eggs remaining unhatched, after 17 days, was recorded. A control cage of non-irradiated males and virgin females was also set up, and similar counts made.

RESULTS

Control insects

Adult male Schistocerca gregaria have two testes, each consisting of 60–65 finger-like processes 4 mm in length and connected, by a vas deferens, to the seminal vesicles, accessory glands and ejaculatory duct. Longitudinal sections show the germ cells to be arranged, within the follicles, in a sequence that reflects the temporal changes involved in spermatogenesis. Each follicle can be divided into four zones (Fig. 1 A):

Zone 1. Germarium or zone of primary and secondary spermatogonia.
Zone 2. Zone of primary spermatocytes.
Zone 3. Zone of maturation, containing secondary spermatocytes and spermatids.
Zone 4. Zone of transformation comprised of late spermatids and spermatzoa.

The germ cells, from the secondary spermatogonia to the late spermatid stage, develop within cysts. This arrangement is typical of the Acrididae (Wigglesworth, 1965).

Irradiated insects

X-irradiation, at all doses, caused cellular abnormalities, each of which can be placed into one of five categories:

(1) Cell breakdown and disintegration

Nuclear fragmentation and cell necrosis were the most obvious and common effects of radiation. Secondary spermatogonia were particularly sensitive, being affected by all doses. The degree of breakdown and the number of cells affected were dependent on the dose, and above 300 rad most were completely destroyed. After irradiation at all dosages some, the number being dependent on the intensity of the doses, continued to divide but subsequently fragmented at interphase,
FIGURE 1

ABBREVIATIONS USED IN FIGURES

$Ac$ acrosome  
$Ax.f.$ axial filaments  
$c$ centriole  
$CA$ centriole adjunct  
$F$ flagellum  
$G$ germarium  
$MD$ definitive mitochondria  
$N$ nucleus  
$NB$ nebennkern  
$Spc$ spermatocytes  
$Spt$ spermatid  
$V$ cytoplasmic vacuole
others broke down almost immediately; these were almost certainly at interphase or early prophase at the time of irradiation. As a result, 72–264 h after treatment (Fig. 1B, C, D), the spermatogonial region consisted of intact primary spermatogonia and Feulgen positive cell debris, the whole zone was greatly reduced in size (Table 1).

(2) Delayed division of cells

All radiation doses caused a delay in the division of certain cells. Primary spermatogonia, while resistant to nuclear fragmentation and necrosis, after irradiation enter a period in which cell division is suspended. The length of this period is dependent on the dose, a delay of 72–144 h was caused by 100 rad and 23 days by 400 rad. The primary spermatogonia then recommenced division to produce apparently normal secondary spermatogonia. By 264 h, after irradiation at 100 rad, repopulation of the gerarium was almost complete but after 200 rad division had only just restarted and subsequent to a dosage of 300–500 rad the recovery period was even longer.

In general, spermatocyte division was unaffected by radiation. However, doses of 300 rad or more did stop the division of one or two and occasionally of all spermatocytes in a few cysts.

After irradiation therefore the spermatogonia either disintegrated (secondary) or temporarily ceased dividing (primary) while in the majority of the later stages spermatogenesis continued without interruption, the resultant sperms passing into the seminal vesicles. Subsequently, there ensues a temporary period of aspermia followed by the repopulation of the follicles by the products of the resumed division of the primary spermatogonia. To study this effect in greater detail the testes of a group of insects, irradiated with 400 rad, were examined over a period of 55 days. The results showed that abnormal spermatozoa alone were present (plus non-dividing spermatogonia) eleven days after irradiation.
Table 2. The breakdown and recovery of spermatogonia after irradiation with 400 rad

<table>
<thead>
<tr>
<th>Stage</th>
<th>Time (days)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1st spermatogonia</td>
<td>N</td>
</tr>
<tr>
<td>2nd spermatogonia</td>
<td>N</td>
</tr>
<tr>
<td>1st spermatocyte</td>
<td>N</td>
</tr>
<tr>
<td>2nd spermatocyte</td>
<td>N</td>
</tr>
<tr>
<td>Early spermatid</td>
<td>N</td>
</tr>
<tr>
<td>Late spermatid</td>
<td>N</td>
</tr>
<tr>
<td>Sperm</td>
<td>N</td>
</tr>
</tbody>
</table>

A, Cells showing one or more abnormality; B, cells breaking or broken down; N, cells not breaking down - apparently normal; S, cells which have stopped dividing - otherwise apparently normal; X, cells absent; NA, cells not breaking down but exhibiting one or more abnormality.

spermatozoa were absent from days 35 to 45 and once more present 55 days after the treatment. The full results are presented in Table 2.

(3) Cytoplasmic vacuolation and organelle displacement

Vacuolation of the cytoplasm was apparent 24 h after exposure to all doses, the spermatocyte being the most sensitive stage. In some cases the nucleus and other organelles were displaced so that they touched the cell membrane. Subsequent differentiation was apparently unaffected.

(4) Mitochondrial abnormalities

During the late spermatocyte and early spermatid stages, of control insects, the mitochondria aggregate close to the nucleus, swell and fuse together to form a single structure, the nebenkern (Fig. 2A). This later divides, the two halves elongating, one each side of the developing axial filaments, to form the definitive mitochondria of the mature sperm. These extend almost the whole length of the flagellum. All mitochondrial stages were very sensitive to all the doses of X-radiation used, the results first becoming apparent after 24 h. The most evident effect is seen at the early spermatid stage. After doses of 300–500 rad the mitochondria aggregate but do not swell or fuse; instead they remain as a diffuse cloud close to the nucleus. At lower doses some fusion may occur leading to the production of peculiarly shaped structures (Fig. 2B, C) which are presumably aberrant nebenkern, larger and less stainable than normal. However, although no typical nebenkern were formed, the definitive mitochondria characteristic of mature spermatozoa, i.e. showing no structural aberrations, were always produced. However they were often increased in number, some spermatozoa having up to twelve instead of the usual two (Fig. 3E, F).
Fig. 2. Electron micrographs.

(A) × 14,250. Normal nebenkern, formed by fusion of spermatid mitochondria.
(B) × 14,250. Abnormal nebenkern 24 h after receiving 100 rad.
(C) × 19,000. Late spermatid nebenkern, 144 h after 300 rad, which has divided into two unequal portions; three sets of axial filaments are present (arrowed).

(5) Multiplication of centrioles, centriole adjuncts and flagella

Spermatids have a single centriole, attached to the posterior region of the nucleus and consisting of nine fibrils embedded in an electron dense matrix to form a cylindrical structure, from which the axial filaments arise. This is surrounded by a relatively large body, the centriole adjunct, comprised of numerous fused granules (Fig. 3A). Gatenby & Tahmisian (1959) have suggested that the
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function of the centriole adjunct is to attach the flagellum to the nucleus. The adjunct is a typical feature of insect sperm.

The centriole/centriole adjunct complex, which will be referred to as the centriole in the text, is extremely radiosensitive. All dose levels have an effect but the number and extent of the abnormalities increase with an increase in dosage.

Two or three centrioles were occasionally found in a single spermatid or spermatozoon of control insects. Radiation increased the occurrence and extent of this type of abnormality. In any cyst of spermatids, which have developed from germ cells irradiated at an earlier stage, the vast majority of cells were affected 72 h after a minimum dosage of 400 rad.

Some late spermatids had a single extra-large centriole, in others the size was normal but the number had increased. The presence of numerous centrioles caused the posterior region of the nucleus to be drawn out of shape (Figs. 3B, C, 4E–4I). Up to six centrioles and flagella were observed in individual spermatids and spermatozoa (Fig. 3E). Transverse sections through these flagella showed the internal arrangement to be normal with the axial filaments lying adjacent to the definitive mitochondria (Fig. 3F). Spermatozoa with numerous centrioles and flagella retain their polarity as, in all spermatids and spermatozoa examined, only one acrosome was present, typically sited anteriorly with the flagella and centrioles originating posteriorly to the nucleus.

Effect of radiation on longevity

Irradiated insects showed no marked increase in mortality when compared with the controls. They were kept for 65 days after irradiation with 400 rad, without any relative increase in the death-rate. All those in the first series of experiments survived until examined.

Fig. 3. Electron micrographs.

(A) × 23 700. Normal early spermatid showing nucleus and attached centriole/centriole adjunct, axial filaments and nebenkern.

(B) × 15 200. Early spermatid, 24 h after 500 rad, centriole adjunct is affecting post-nuclear shape.

(C) × 9350. Early spermatid, 264 h after 200 rad, showing influence of centriole adjunct on the posterior region of the nucleus. Some cytoplasmic vacuolation is also present.

(D) × 12 000. Early spermatid, 144 h after 500 rad, showing double centriole adjuncts and cytoplasmic vacuolation.

(E) × 12 000. T.S. early spermatid bundle, 72 h after 200 rad, showing supernumerary axial filaments (arrowed) and mitochondrial derivatives. Some cytoplasmic vacuolation is also present.

(F) × 54 000. T.S. sperm bundle, 144 h after 300 rad, showing sperm with two flagella.
Fig. 4.

(A) Normal spermatid (early).

(B) Flagellate spermatid (early) 72 h after 300 rad showing three centriole adjuncts and flagella.

(C) Nucleus of early spermatid, 144 h after 200 rad, with four centriole adjuncts and flagella.

(D) Head of normal sperm.

(E) 72 h after 100 rad.

(F) 72 h after 200 rad.

(G) 144 h after 300 rad.

(H) 144 h after 400 rad.

(I) 264 h after 500 rad.

Nuclei of later spermatids showing supernumerary centriole adjuncts and flagella and their effect on the nucleus.
Table 3. Results from the breeding experiments (control insects)

<table>
<thead>
<tr>
<th>Pod no.</th>
<th>No. eggs</th>
<th>Day laid</th>
<th>No. hatched</th>
<th>Hatch (%)</th>
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<tr>
<td>1</td>
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<td>21</td>
<td>60</td>
<td>96.7</td>
</tr>
<tr>
<td>2</td>
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<tr>
<td>11</td>
<td>50</td>
<td>53</td>
<td>0</td>
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</table>

Average no. eggs/pod = 47.91.
Total no. eggs = 527.
Total no. hatched = 336.
Mean percentage hatchability ± s.e. = 67.51 ± 10.7.

Breeding experiments

The cuticle of the irradiated insects did not fully develop the bright yellow coloration, characteristic of sexually mature males, but remained yellowish-brown. The bright yellow normally appears, in males, 3–4 weeks after the final moult. Radiation did not, however, seem to affect mating and sperm transference, as irradiated males were seen to copulate and the spermathecae of several females, examined shortly after mating, were found to contain sperm. This was presumably stored in the seminal vesicles until copulation.

The number and percentage hatchability of eggs obtained from the control and irradiated insects, during the breeding experiments, were compared by the Analysis of Variance test. The difference between the two was found to be significant at the 95% level. Standard errors of mean, for percentage hatchability, were calculated as:

1. Control = 67.51% ± 10.7%.
2. Irradiated = 17.93% ± 13.4%.

These results are discussed on page 176.

DISCUSSION

The germ cells of insects, in particular the secondary spermatogonia, are generally agreed to be sensitive to X-irradiation, (Mandl, 1964; Marshall, 1965; Mathur, 1960; Riemann, 1967). Abraxas grossulariata is exceptional; Gatenby et al. (1929) found that in this species the spermatocytes are the most sensitive stage. The germ cells of Schistocerca gregaria are typical in that they fall into the first category.
Table 4. Results from the breeding experiments (irradiated insects)

<table>
<thead>
<tr>
<th>Pod no.</th>
<th>No. eggs</th>
<th>Day laid</th>
<th>No. hatched</th>
<th>Hatch (%)</th>
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<tr>
<td>1*</td>
<td>83</td>
<td>33</td>
<td>20</td>
<td>24.1</td>
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<tr>
<td>2</td>
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<td>7</td>
<td>48</td>
<td>60</td>
<td>7</td>
<td>14.5</td>
</tr>
</tbody>
</table>

* First eggs laid on day 10, but not in the sand tubes. Insects were irradiated on day 1.
Average no. eggs/pod = 48.7.
Total no. eggs = 341.
Total no. hatched = 64.
Mean percentage hatchability ± s.e. = 17.93 ± 13.4.

Before the effects of X-radiation on the germ cells of *Schistocerca gregaria* are discussed a brief summary of some known effects of ionizing radiation may prove helpful.

Riemann (1967), working on *Cochliomyia hominivorax* irradiated with 6200 R, described a delay in mitosis affecting the germ cells. Only small doses are required to produce this effect, mitosis being temporarily stopped by as little as 1 rad in grasshopper embryo neuroblasts (Thornburn, 1972) and by 50 rad in the case of human kidney cells (Casarett, 1968). In general, cells appear to be particularly sensitive during the G2 stage of interphase and up to a critical point in late prophase. Irradiation before this time causes cells to cease division or revert to the G2 condition; irradiation at a later stage of mitosis has no effect on division (Thornburn, 1972).

Division, in arrested cells, recommences after a period of time indicating that the mechanisms causing delay are repairable. Physiological damage is the most likely cause of this delay in division, e.g. chromosome 'stickiness' (Thornburn, 1972). Casarett (1968) describes the chromosomes as being most sensitive, to structural alteration, during the S-stage of mitosis. Bacq & Alexander (1961) extend the period of maximum sensitivity to all resting stages. Non-repairable radiation damage to chromosomes, e.g. dominant lethal mutations, chromosome breakages or deletions may explain the total breakdown of cells.

Repairable, and non-repairable, damage is not often a direct result of radiation, except at very high dosages. The production of highly reactive free-radicals can be induced by the action of ionizing radiation on certain cell constituents, e.g. water. These can react, together or with organic molecules, to produce peroxides which can 'poison' the cell. They can also affect the surface charge and colloidal properties of macromolecules which leads to denaturization of proteins, e.g. enzymes; and could cause blockages in metabolic pathways.
The membrane-damage or enzyme release hypothesis (Bacq & Alexander, 1961) postulates that radiation causes breakages or changes in the permeability of cell membranes. The nuclear membrane appears particularly liable to permeability changes (Thornburn, 1972), thus permitting the entry of toxic agents, e.g. peroxides, nucleases, proteases etc.

The division of primary spermatogonia in Schistocerca gregaria was temporarily stopped by X-irradiation. As the majority of these germ cells were at interphase 48 h after treatment it is unlikely that chromosome 'stickiness' would account for the arrest. The most probable causes are either chromosome breakages or a temporary repression of genes controlling cell division and/or synthetic processes vital to cell division, e.g. DNA and RNA synthesis. The mechanism is clearly reversible as all primary spermatogonia eventually recommenced division to produce apparently normal secondary spermatogonia.

The secondary spermatogonia are the most radiosensitive germ cells, in Schistocerca gregaria, being affected by even the lowest doses and completely destroyed by high doses, i.e. 300–500 rad. Cells in interphase are particularly sensitive, breaking down almost immediately. Dividing cells completed division and only degenerated when they reached interphase. The disruption of secondary spermatogonia begins shortly after irradiation. For example, after 400 rad cells in interphase began to degenerate almost immediately, this process being almost complete 1 day later. After 72 h little other than cell debris can be observed in this region of the testis. This very rapid action almost certainly excludes non-repairable chromosome or genetic damage as possible mechanisms causing degeneration. It is noteworthy that cell degeneration is preceded by nuclear fragmentation. This suggests that the permeability of the nuclear membrane may have been altered, thus permitting the entry of lytic agents to which the nucleus is particularly sensitive. These agents may be products of the cell, e.g. enzymes, and/or may have been produced during irradiation, e.g. peroxides.

Where cell breakdown does not occur, e.g. in spermatocytes and spermatids, abnormalities are frequently observed. These are characteristic of the cytoplasmic inclusions.

Production of supernumerary centrioles, mitochondrial material, flagella and nuclear outgrowths in the spermatids of X-irradiated insects have been previously described by Gatenby (1941) and Tahmisian & Devine (1961). Other workers report the Golgi bodies, of insect germ cells, to be particularly radiosensitive (Gatenby et al. 1929; Mukerji, 1929; Mathur, 1960). The presence of supernumerary acrosomes and centrioles around the spermatid nuclei of Melanoplus differentialis were shown, by Tahmisian & Devine (1961), to cause a loss of axial symmetry and they suggested that this might explain the abnormal outgrowths of the nuclei.

In Schistocerca gregaria supernumerary mitochondria and centrioles, up to six of the latter, are frequently observed in spermatids which have developed from irradiated spermatocytes and early spermatids. However, the Golgi bodies
in the spermatocytes of this insect appear quite normal and always produce a single acrosome in each spermatid, thus normal axial symmetry is retained.

The genes controlling centriole formation, in *Schistocerca gregaria*, appear normally to be rather unstable as centriole replication occurs, but on a smaller scale, in non-irradiated control insects. It seems probable that these genes are particularly sensitive to X-radiation, especially in the spermatocyte and early spermatid stages. It seems clear that it is only during this period that they are susceptible, since irradiated primary spermatogonia give rise to normal spermatids; and spermatozoa are quite unaffected.

The formation and subsequent fate of the nebenkern is under the control of the centrioles (Gatenby, 1941), consequently the genetic interference causing centriole replication would also have an effect on the mitochondrial content of the spermatid. The failure of the mitochondria to fuse to form the nebenkern may perhaps be explained by temporary interference with either the genes controlling this process and/or with the breakdown of the old or the formation of the new bounding membrane.

Any of these radiation-induced abnormalities might be expected to have an effect on the insects' fertility and reproductive capacity. As the insects received whole body irradiation, tissues, other than the testes, might also have been affected. Of particular interest here is possible damage to the corpora allata. Loher (1961) showed that the corpora allata of *Schistocerca gregaria* play a major role in the production of a maturation accelerating pheromone. If the corpora allata were removed from fledgling males they did not become sexually mature or show normal male sexual behaviour. Pener (1965) working on the same insect, demonstrated that if the anterior (dorsal) nerves of the corpora allata were cut then the cuticle of the adult males become light yellow and they showed sub-normal sexual behaviour. It is noteworthy that the males used in the present investigation did not develop fully the bright yellow coloration of the cuticle characteristic of sexually mature males. The secretion of the allatal hormone may well have been depressed in these insects.

The breeding experiments showed a reduction in the number of egg-pods laid by females mated to irradiated males and a significant reduction in the percentage hatchability of these eggs. The first eggs were laid 33 days after irradiation, i.e. at the onset of the period of aspermia in the follicle. Irradiated sperm, which certainly included those derived from irradiated spermatocytes and spermatids, must have been used to fertilize these eggs. The reduction in hatchability may well be explained by dominant lethal mutations, carried in the sperm nuclei and/or by a decrease in sperm motility due to the supernumerary flagella and hence a reduction in the number of eggs fertilized.

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REFERENCES


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