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Silver staining of the coleopteran Xy_p sex bivalent

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Abstract

In zygo- to pachytene of male otiorrhynchine weevils, the long arm of the acrocentric X chromosome associates with the y chromosome. Both sex chromosomes appear as positively heteropycnotic and argyrophilous cores. Bending of X turns the X-y chain to configurations resembling question marks. Bending is apparently due to one-sided puffing of lamp-brush-like loops out of the cores. In late diplotene, the Xy profiles already display a parachute shape. The distal half of the originally associated X arm suffers condensation changes resulting in a gap of variable expression. From diakinesis on, the interspace between X and y becomes filled with an argyrophilous substance which persists until A I and may participate in keeping the bivalent. Extraction and staining experiments suggest that the substance is not nucleolar but might be of a proteinous nature.

Introduction

The prevailing male sex bivalent of Coleoptera Polyphaga is the Xy_p. The subindex p comes from parachute (Smith, 1950), and for a good reason, because in its profile views the bivalent has the appearance of a parachute, with a large canopy (X) and a tiny load (y). In the Adephagan beetles, the Xy bivalent is usually arranged by some means of single contact, although Xy_p has been reported in some cases (Smith and Virkki, 1978). A parachute-like sex bivalent was also found in the only Myxophagan beetle cytologically studied (Mesa and Fontanetti, 1985). Hughes-Schrader (1980) has encountered an Xy_p in Megaloptera, a neuropteroid Order related to Coleoptera. Such a distribution suggests a common evolutionary ancestor, since it is unlikely that Xy_p could arise several times independently.

In general, disappearance of the Xy_p during the course of evolution can be taken as irreversible. We know of only one case, where the taxonomic evidence suggests a reappearance of Xy_p which vanished as a consequence of chromosomal rearrangements (Lanier, 1981). However, essential parts of X_p and y_p seem to have survived to ensure the formation of Xy_p *de novo*.

The mode of association of the parachute sex chromosomes has been under discussion since Stevens (1906) depicted the bivalent. The most notable opinions concerning agents of association are 'plasmosome' or nucleolus (Stevens, 1905, 1906); chiasmata (Smith, 1951); nucleolus, probably with a dense surface layer (Suomalainen, 1947 unpublished; Smith and Virkki, 1978); nucleolus (John and Lewis, 1960); and terminal contacts (Postiglioni and Brum-Zorrilla, 1981, 1988; Wettstein, 1981; Drets *et al.*, 1983).

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Drets *et al.* (1983) have published a well-documented account on the structural changes of the sex bivalent of a coccinellid, *Epilachna paenulata*. According to their interpretation, only in the latest metaphase I (M I), just prior to anaphase I (A I), do the 'canopy' and 'load' correspond to the X and y chromosomes, respectively. In the earlier stages, the canopy corresponds to the heterochromatically paired segments of X and y, and the load, to the euchromatic segments of both chromosomes. The question arises as to whether this course of events is general throughout the Coleoptera Polyphaga. Probably not, in view of the variation in the initial contacts between X and y (Smith and Virkki, 1978). However, observations concerning these events are still very scarce. Other beetle cytologists agree with the two arguments which the Uruguayan school raises against the nucleolar association in Xy_p: *viz* (1) the lack of localized NOR in the sex chromosomes (Virkki and Mazzella, 1984; Virkki *et al.*, 1990); and (2) the presence of NOR in an autosomal pair (Virkki, 1983; Virkki *et al.*, 1984; Virkki and Denton, 1987).

Activity of the regular autosomal NORs in early prophases of meiosis seems to be common in male Coleoptera, but it is short-lived, declining soon to quiescence. The latest regular, silver-marked NORs are from mid-diplotene (Virkki *et al.*, 1984). A secondary production of nucleolar substance, often in the form of droplets, can follow, especially in species where the spermatocytes show a notable diplotenic growth (Virkki and Denton, 1987). Using silver staining and acridine orange fluorescence, Postiglioni and Brum-Zorrilla (1988) demonstrated the separatedness of the early prophasic sex bivalent from the autosomal NOR + nucleolus in spermatocytes of a chrysomelid, *Chelymorpha variabilis*. In contrast, Virkki and Sepúlveda (1990) and Virkki *et al.* (1990) found a short-lived, racemose nucleolar substance adjacent to the early sex bivalent in the spermatocytes of several curculionids.

Although the possibility of a loose association of an autosomal NOR with the sex bivalent could not be excluded, the racemose or droplet structure of the substance tends to suggest the lack of a functioning NOR. Presumably, the nucleolus depicted in *Chelymorpha* represents the ordinary autosomal nucleolus, whereas that of the weevils forms slightly later and seems to be of a different origin. None of these nucleoli survive to participate in the final Xy_p structure. From diplotene to M I, when the parachute shape of the sex bivalent becomes established, the lumen (interspace between X and y) can be vividly marked in the Xy_p of the weevils by use of AgNOR techniques, although the silver seems to mark substances other than the nucleolus (Virkki *et al.*, 1990).

Our recent investigations by light-microscopy of argyrophily of the curculionid Xy_p bivalent from its beginnings to its breakdown at A I are described and discussed in this report.

Materials and methods

During 1981—89, weevils were collected in Puerto Rico and brought back to the laboratory, where their testes were processed to squash or air dried preparations as described earlier (Virkki *et al.*, 1990). The principal species was a

destructive agricultural pest weevil, *Diaprepes abbreviatus*. Five other species, from the relatively primitive Brachyderinae to the highly derived Cossoninae were also included in the material (Table 1), mainly because the sex bivalent was exceptionally easy to analyse in certain phases of spermatogenesis. For the same reason, a still unidentified Otiorrhynchine from nearby Mona Island was added to the sample.

Preparations were generally made without hypotonic pretreatment. A solution of 0.075 M KCl was used to study the effects of hypotony in the argyrophily of the Xy_p lumen. Usually the fixation was in Kahle-Smith fluid (KS: 1 part of glacial acetic acid to 3 parts of formalin to 7.5 parts of 95% ethanol). Acetic alcohol (ethanol) (1:3) and acetic acid (45%) were sometimes used as fixative.

Silver staining for nucleolus organizer (AgNOR) was used in accordance with Pathak and Elder (1980). In some experiments this procedure was preceded by Feulgen staining. Silver was washed off by applying 7% potassium ferricyanide for up to 5 h. To extract RNA, ethanolic barium hydroxide (Geyer and Schreibner, 1970) and perchloric acid were used. Treatment in warm 5% perchloric acid was generally insufficient (Virkki *et al.*, 1990), but modifications in argyrophily were obtained when the warm treatment was followed by immersion in 10% solution at 4°C for 18 h.

Table 1 Systematics, host-plant association, and insular Puerto Rican collection sites of the examined weevil materials

Weevil identification	Host-plant affiliation	Insular PR sample location
Brachyderinae: Barynotini		
Lachnopus curvipes (F.)	*Dalbergia ecastaphyllum* (L.) Taub.	Dorado, Road 165
Lachnopus sp.	*Rapanea coriacea* (Sw.) Mez	Toro Negro, Road 143
Otiorrhynchinae: Phyllobiini		
Compsus luquillo Wolcott	*Cecropia peltata* L.	Guavate, Road 7740
Diaprepes abbreviatus (L.)	*Swietenia macrophylla* G. King	El Yunque, Catalina
Exophthalmus quindecimpunctatus (Olivier)	*Guapira fragrans* (Dum.-Cours) Little	Vega Alta, Road 675
Baridinae: Peridinetini		
Peridinetus concentricus (Olivier)	*Piper aduncum* L.	Patillas, Road 3
Cossoninae: Cossonini		
Cossonus near *hamiltoni* Slosson	*Erythrina poeppigiana* (Walp.) Cook	Peñuelas, Road 378

For staining protein, an overstaining was performed up to 80 h in 0.5 to 2% Remazol brilliant blue R (Sigma Chemical Company), at pH 1.2 to pH 8. The slides were first treated in 0.9% NaCl. Giemsa stain was used with pale silver stained preparations, as a counterstain.

Proteases were applied to slides for periods of up to 30 min, 0.25% pepsin at pH 2.8, and 0.00125% trypsin at pH 6.8, both from Sigma Chemical Company. In addition, testis follicles were treated *in toto* in 0.012% trypsin solution (pH 7.0) for up to 20 min, with or without hypotonic pretreatment, and with or without fixation in 45% acetic acid. Photomicrography employed 35 mm Kodak plus-X pan film and a Zeiss photomicroscope II throughout.

Results

Argyrophily of the sex bivalent

Casual observation of AgNOR-stained metaphasic Xy_p bivalents suggests the presence of a nucleolus in the interspace (lumen) between X and y. As in nucleoli, the silver deposit of the lumen looks black in bright field, but brilliant white under phase contrast optics. More meticulous observations show, however, that the AgNOR-reducing agent of the lumen is not homogeneous in its capacity to precipitate and to retain silver, nor is it convincingly stainable with other nucleolus stains. A detailed presentation of the development of the Xy_p argyrophily was therefore considered necessary.

Up to diplotene

The most conspicuous heteropycnotic and argyrophilous elements of the earliest prophase of spermatocyte I are the sex chromosomes (Figure 1). Procentric autosomal heterochromites soon appear, approximating in number to the autosomal 2n = 20, or showing variable aggregations. In *Peridinetus concentricus*, the sex chromosomes appear either separate, or in close juxtaposition, which may indicate an end-to-end pairing by an invisible link (Figures 1a and 1b). Both the free and the associated ends of the X chromosome are so similar that it is impossible to determine whether it is always the same end which associates with the y chromosome. In *Exophthalmus quindecimpunctatus*, the X chromosome has a prominent heteropycnotic knob at one end, and it is the opposite end which associates with the y chromosome (Figures 1d to 1f). As the X chromosomes of the Puerto Rican Phyllobiini are acrocentric (Virkki unpublished observation), it is likely that the knob consists of procentric heterochromatin.

A common tendency in all species we studied is a bending of the X chromosome until its free end approximates or touches the other end, associated with the y chromosome. Ring figures are the result (Figures 1c and 1f). This is a phase of very rapid contraction of the sex chromosomes. Thus, there is a great variation in the Xy configurations even within a single spermatocyst. It seems possible that the ring association is not obligatory (see Xys of different condensation degrees in Figures 1c to 1e; the straightened Xs of Figures 1e and 1f are presumably squashing artefacts). The end result of these changes is a rather bulky, polarized

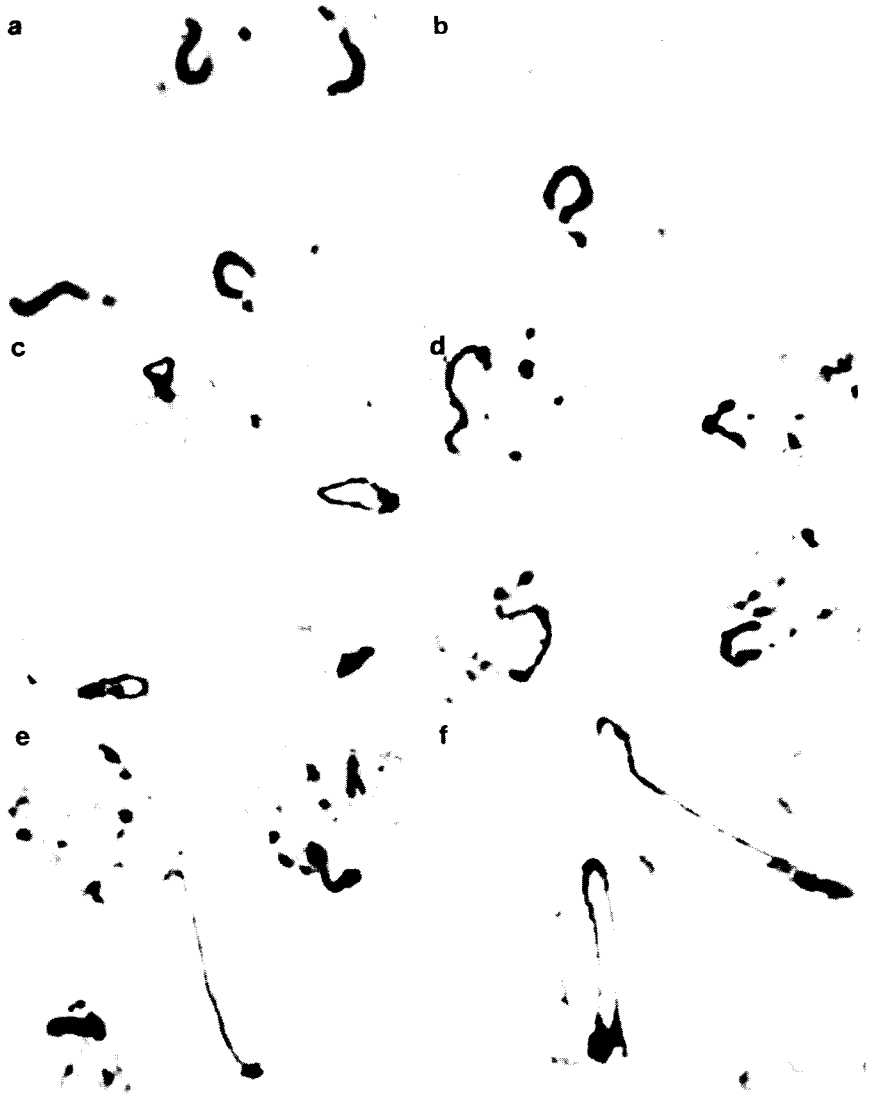

Figure 1 Early (up to pachytene) association of X and y chromosomes in *Peridinetus concentricus* (Figures 1a to 1c) and *Exophthalmus quindecimpunctatus* (Figures 1d to 1f). Squash preparations after KS fixation, without hypotonic pretreatment.
Figure 1a The heteropycnotic and strongly argyrophilous sex chromosomes are either separate and juxtaposed, or joined(?) end-to-end. x1,695.
Figure 1b Pachytene bouquet showing the Xy chain and incipient condensation of pericentric heterochromatin in the autosomal loops. x1,695.
Figure 1c Early pachytene showing a rapid succession of events: bending of the X chromosome, its free end touching the y chromosome, and subsequent condensation of both chromosomes. x1,695.
Figure 1d Comparable to Figure 1a. Note how a knob, presumably pericentric, marks one end of the X chromosome in this species. x1,570.
Figure 1e Pachytene as in Figure 1d. Squashing has stretched the centromeric end of one X chromosome far from the bouquet bottom. x1,570.
Figure 1f Comparable to Figure 1c. Note the variation of the sex bivalent shape in one and the same spermatocyst (Figures 1d to 1f). x1,570.

sex bivalent of pachytene bouquets. The y chromosome is often recognized adjacent to the X chromosome (Figures 2a and 2b). Thereafter, a short diffuse phase follows, during which the autosomal heterochromites decondense, and the sex chromosomes lose much of their bulky substance (Figures 2c and 2d). Staining and extraction experiments have shown that the substance lost is nucleolar in nature (Virkki *et al.*, 1990). The sex chromosomes denuded from this short-lived nucleolus show essentially the same mutual relation as in their earliest association; the y chromosome in a juxtaposition, if not joined end-to-end by an invisible link, with the X chromosome (*cf* Figures 1a, 1b, 2c and 2d).

Figure 2 Pachytene bouquets of *Diaprepes abbreviatus* (Figures 2a to 2c) and *Peridinetus concentricus* (Figure 2d). Squash preparations after KS fixation. Arrow marks the y chromosome.
Figure 2a Maximum argyrophilia and condensation of the sex chromosomes and of the pericentric heterochromatin of the ten autosomes. x1,950.
Figure 2b The metacentric structure of the autosomes is obvious where the bouquet loops are visible. x1,950.
Figure 2c Diplotene approximating: recession of condensation and of argyrophilia of all elements. x1,950.
Figure 2d Latest pachytene, or perhaps already diplotene. x1,950.

Diplotene to M I

The true end-to-end contact becomes obvious when the condensation of the chromosomes proceeds. We term this the 'question mark phase' of the Xy (Figures 3a and 3b; for corresponding whole nuclei, see Figures 3e and 3f in Virkki and Sepúlveda, 1990). Two changes follow almost simultaneously: *viz* a decondensation in the associated arm of the X chromosome, leading to a block separated by a gap (or little stained segment) from the main part of the X chromosome (Figures 3c and 3d). It seems that the entire distal half of the X chromosome is subject to decondensation, with the block appearing when a recondensation starts. Indeed, the block is too large to be part of the y chromosome. When the undercondensed gap closes, and a link between y and the free end of X becomes established, the typical parachute profile emerges (Figure 3e). The character of the new link is obscure.

Once the double contact between X and y is established, the interspace (lumen) of the bivalent is invaded by an argyrophilous substance (Virkki *et al.*, 1990). We use the large Xy_p of *Lachnopus* spp. to illustrate the beginning of this process (Figures 4a to 4f). First, in late diplotene to diakinesis, a limited argyrophily appears at both extremes of the lumen, as bands bordering the X and y chromosomes (border-band stage). These bands may be connected by a bridge and several thinner argyrophilic strains (Figure 4c). The proximity of these phases to the preceding question mark phase suggests that the bands are just a continuum of the question mark figure, the largest connecting bridge representing varying degrees of the gap-and-block formation in the X chromosome (*cf* Figures 3c to 3e and 4b, 4c). If this is so, then the non-argyrophilous parts of the parachute chromosomes are neo-formations. They might consist of lamp-brush-like loops puffed out eccentrically from the argyrophilous core (Figure 4e). Such a one-sided puffing might be the reason for bending of the X chromosome in the question mark figure.

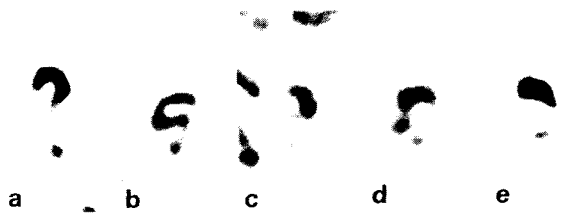

Figure 3 Formation of the Xy_p bivalent in *Diaprepes abbreviatus*. Squash preparations after KS fixation. Silver stain in Figures 3a and 3b; others unstained and under phase contrast.
Figures 3a to **3c** Question mark figures from early to mid diplotene. x2,970.
Figure 3d Recently formed parachute. x2,970.
Figure 3e A stable M I parachute. x2,970.

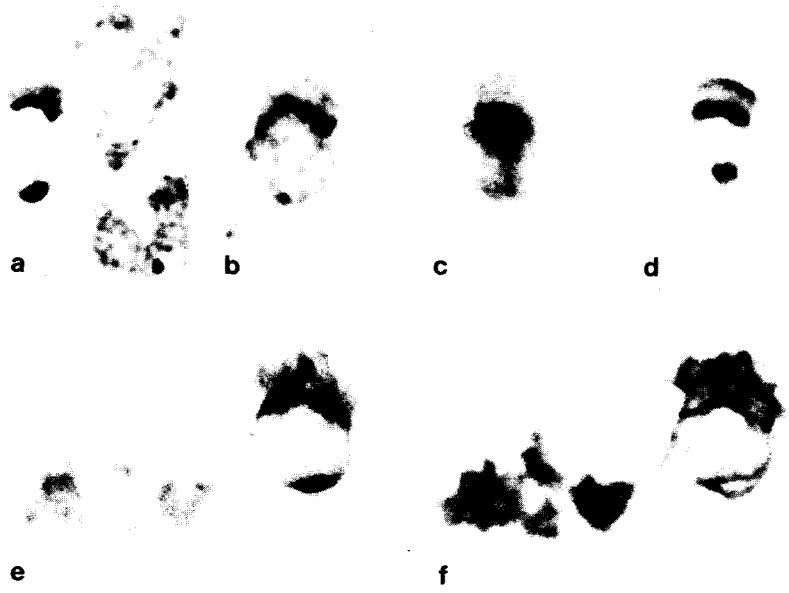

Figure 4 Silver staining of Xy_p in diplotene and diakinesis of *Lachnopus* sp. (Figure 4a) and *Lachnopus curvipes* (Figures 4b to 4f). Squash preparations after KS fixation.
Figure 4a Border band stage: Argyrophilous substance at the inner borders of both sex chromosomes. x2,300.
Figure 4b There seems to be a blister-like periphery, and a post-like structure in the centre. x2,300.
Figure 4c A lateral view of another Xy_p. Note that there is one thin and another thicker argyrophilous bridge joining the sex chromosomes. x2,300.
Figure 4d Comparable to Figure 4a. x2,300.
Figure 4e Contacting autosomal bivalent has pulled a pellicle or a string out of the Xy_p body. x3,050.
Figure 4f The same, photographed under phase contrast. Note the puffed appearance of X chromosome especially in Figures 4e and 4f. x3,050.

In the absence of ultrastructural studies it is still debatable whether the parachute lumen is surrounded by a pellicle. What an autosomal contact is pinching off from the parachute in Figures 4e and 4f, could be a membrane as well as a string. There can certainly be several argyrophilous strings per parachute (Figure 4c), but, on the other hand, the surface of the lumen looks well delimited in 'anterior' (Figures 4b, 4e and 4f) as well as in 'lateral' views (Figure 4c) of the parachute.

As we know from *Diaprepes abbreviatus* (Virkki and Sepúlveda, 1990), the argyrophily will be extended by M I to cover the entire parachute lumen. We present Xy_ps of a further three species to illustrate this point (Figure 5). Under phase contrast illumination, the late diakinetic parachute of *Cossonus* near *hamiltoni* reveals a gapped X and an empty lumen, but a prolonged AgNOR staining produces a totally black parachute (Figures 5a and 5b). A similar reaction is obtained in the large Xy_p of *Compsus luquillo*, shown here in 'anterior'

(Figure 5c) and 'lateral' (Figure 5d) views. Although these figures show somewhat overstained parachutes, the X chromosomes are not totally black, which suggests that the main focus of the silver deposit has been the lumen. Finally, Figures 5e and 5f show two ideally silver stained PM I parachutes in a bright field (lumen black, X pale yellow) and under phase contrast illumination (lumen glowing white with black borders, X black). The y chromosome appears as a small puff at the lower tip of the parachute of Figure 5f.

First meiotic division

The argyrophilous substance filling the Xy_p lumen disappears rapidly in late A I. We have seen it detach from the X chromosome of a recently formed XO system ($Xy_p \rightarrow XO$) in M I of *Aulacoscelis melanocera* (Santiago-Blay and Virkki, 1989). In A I of *Diaprepes abbreviatus*, it is stretched and divided, each sex chromosome sharing a piece proportional to its size (Figure 6a). At late A I it has disappeared, the pericentric blocks of all chromosomes having assumed the argyrophily (Figure 6B). This strategy of subsisting until the separation of X from y, and a rapid disappearance thereafter, suggests a role of the substance in the control of the sex chromosome segregation. At telophase I (T I) the X chromosome of *Diaprepes abbreviatus* is again distinguished by its heteropycnosis and argyrophily (Virkki and Sepúlveda, 1990).

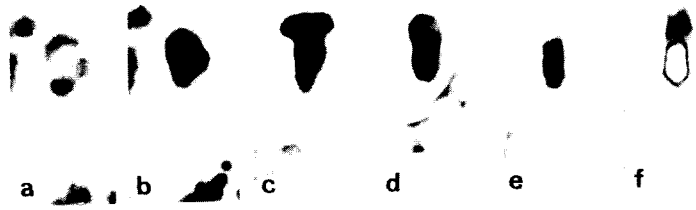

Figure 5 Argyrophily of the parachute lumen, and shape and dimensions of the sex chromosomes in diakinesis to M I cells of three species of weevils. Squash preparation after KS fixation.
Figure 5a *Cossonus* near *hamiltoni*, late diplotene, unstained and under phase contrast. Note the empty lumen and large y chromosome. x2,400.
Figure 5b The same parachute as above, silver stained and in bright field. x2,400.
Figure 5c *Compsus luquillo*, diplotene, silver stained and in bright field. Large X chromosome in anterior view. x2,400.
Figure 5d As above, but in lateral view (*e.g.* the angles of observation differ by about 90° in Figures 5c and 5d). x2,400.
Figure 5e Unidentified otiorrhynchine from Mona Island, M I, silver stained and in bright field. x2,400.
Figure 5f As above, but under phase contrast. Note the brightness of the silver deposit, surrounded by a black brim. x2,400.

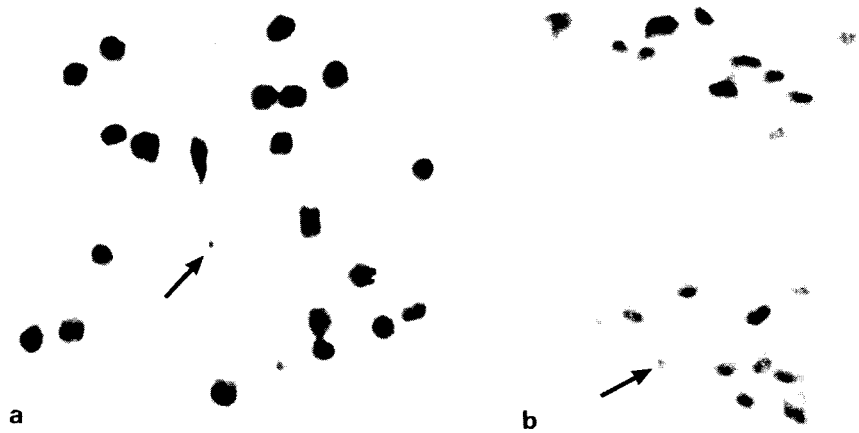

Figure 6 First meiotic anaphase of *Diaprepes abbreviatus* (Figure 6a) and *Lachnopus curvipes* (Figure 6b). Silver stained squash preparations after KS fixation. Arrow points to y chromosome.
Figure 6a Separation of X and y just begun, with argyrophilous substance still adjacent to them. x2,200.
Figure 6b More advanced separation of X and y. Only pericentric heterochromatin shows argyrophilia. x2,200.

Modifications in the argyrophily of Xy_p

Fixation

If the fixation time is short, from 1 to 2 min, there is a drastic difference in argyrophily of the Xy_p, depending whether KS fixative or acetic ethanol is used. KS fixed Xy_ps are argyrophilous as just described, but acetic ethanol fixed Xy_ps remain unstainable. If the latter fixation is prolonged to 2 h, the Xy_ps argyrophily appears similar to that produced by KS fixation (Virkki and Sepúlveda, 1990; Virkki *et al.*, 1990). An argyrophily shown after acetic ethanol fixation can be enhanced by refixing in KS (Figures 9b and 9c).

Thus fixation seems to induce the argyrophily, with the formalin-containing KS fluid acting many times more rapidly than acetic ethanol. According to Hubbell (1985), formalinic fixatives yield nonspecific AgNOR staining, which means that not only nucleolus-related proteins, but also some other proteins might become marked. Because all the components of the fixatives used are water-soluble and volatile, the presence of fixative residues in the preparations at the time of AgNOR staining is not possible. The argyrophily is the same in freshly made preparations and in preparations left dry for 6 months before staining. Thus it is a modification of some substance by the fixing fluids which produces the argyrophily. Such a substance might possibly consist of acid proteins, because formaldehyde is known to enhance their argyrophily through induction of aldehyde and carboxyl groups (Horobin, 1982).

Extraction of RNA and proteins

RNA should not be the cause of the Xy_p argyrophily, because the AgNOR staining is not affected by treatments known to extract RNA, such as aqueous solutions after Carnoy-type fixatives (Horobin, 1982; Figures 7a, 7b, 9b and 9h). Nor is it affected by acid hydrolysis (Virkki *et al.*, 1990; Figures 7c and 7d), and ethanolic barium hydroxide (Figure 10c). Acid hydrolysis prior to Feulgen staining (Virkki *et al.*, 1990) as well as perchloric acid (Figure 7d) can, however, induce coarse fibrous or flocculate structures in diakinetic Xy_p lumens. These structures are too pronounced to be just a leftover grid after extraction of some adjacent substance, except if the very elimination of the substance enhances the argyrophily of the grid. A more likely possibility is that the grid is due to concentration or coagulation of a previous thinly-dispersed substance.

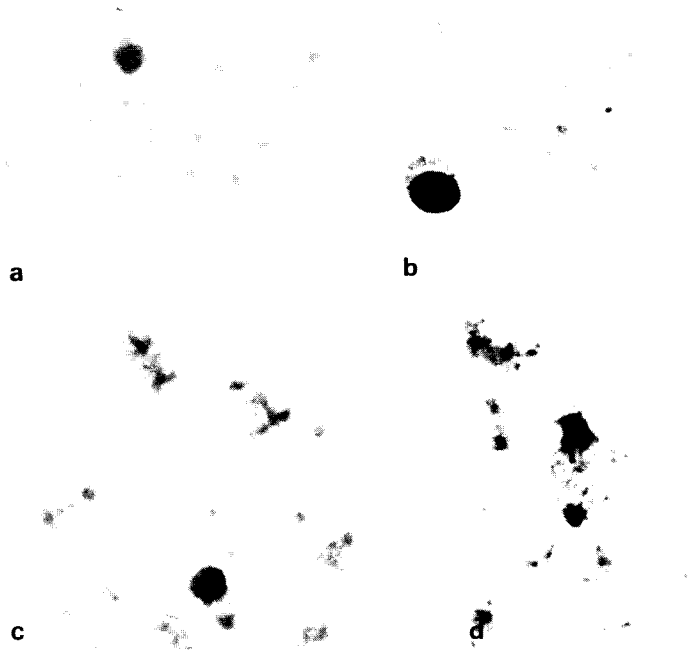

Figure 7 Effects of hypotony followed by acetic acid fixation (Figures 7a and 7b), air drying from acetic acid followed by acid hydrolysis (Figure 7c), and acid hydrolysis plus perchloric acid after KS fixation (Figure 7d), in argyrophily of late bivalents of *Diaprepes abbreviatus*. Bright field.

Figure 7a M I, 0.075 M KCl for 50 min. Procentric regions and synaptonemal complexes of autosomes, and lumen of Xy_p marked by silver. x2,100.

Figure 7b M I, 0.075 M KCl for 4 h. A very notable swelling has not weakened the argyrophily of Xy_p lumen. x2,100.

Figure 7c Diakinesis. Feulgen stain (4 min hydrolysis) before silver staining. Procentric regions and synaptonemal complexes of autosomes, and border bands of Xy_p marked by silver. x2,100.

Figure 7d Diakinesis. Feulgen stain (4 min hydrolysis), then 5% perchloric acid for 1 h at 65°C + 10% perchloric acid for 18 h at 4°C, followed by silver staining. In addition to procentric regions of autosomes, fibrous structures extending from the sex chromosomes to the notably swollen lumen are marked by silver. x2,100.

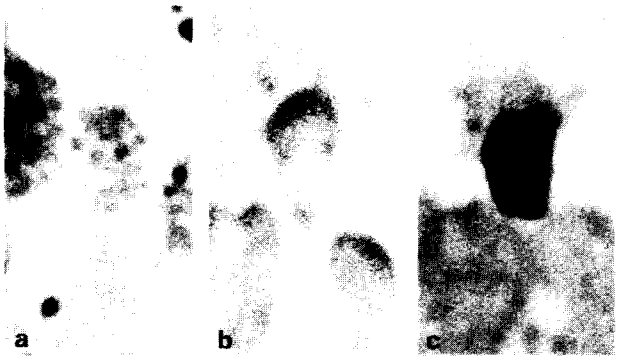

Figure 8 Experiments with M I parachutes of *Lachnopus curvipes*. Squash preparations after KS fixation. Bright field.
Figure 8a Testis pretreated *in toto* for 4 min in 45% acetic acid, for 5 min in 0.0125% trypsin (pH 7.0), then fixed, squashed, and silver stained. No silver in the parachute lumen. x4,530.
Figure 8b Slide treated in 0.9% NaCl for 5 min, then for 15 min in 2% Remazol (pH 5.0); as no stain was obtained, treated for 15 min more in 0.5% Remazol (pH 1.2). No stain in the parachute lumen. x4,530.
Figure 8c The same parachute as above, after prolongation of the Remazol (0.5%, pH 1.2) staining to 72 h (with no stain in the parachute lumen), then silver staining and counterstaining in Giemsa solution. The grossly swollen lumen has retained its argyrophily. x4,530.

Prolonged hypotony (Figure 7a) and air drying from 45% acetic acid (Figure 7c) help to mark synaptinemal complexes and centromeric regions of autosomes, but do not eliminate argyrophily of the Xy_p lumen. Even a grossly extended hypotony which destroys chromosomes through swelling, does not affect the silver staining of Xy_p (Figures 7b and 8c).

Except for a slight swelling, the proteases pepsin and trypsin do not change the parachute's appearance (Figures 9a to 9e and 9h). This does not necessarily signify a lack of proteins. Fixation may have masked the peptide bonds to which the proteases have specificity. Extension of the treatment time and/or enzyme concentration may produce swelling and flocculate/granulous staining of lumen in late diakinesis and M I parachutes (Figures 9f and 9g). Treatment of the testis *in toto* with 0.012% trypsin, with or without a preceding hypotony, does not affect the argyrophily of the Xy_p lumen, but if the pretreatment is in 45% acetic acid, no silver is deposited in the lumen (Figure 8a). Since the acetic acid fixation does not affect the argyrophily (see above; *contra* Hubbell, 1985), these results could be interpreted as follows. The lumen may contain protein which is not accessible in its natural configuration to trypsin molecules, even when swollen hypotonically, but becomes accessible if first penetrated by the small CH_3COOH molecules. Attempts at marking the lumen with a sensitive protein stain (Remazol) have failed, however, even after pretreatment in acetic acid. At pH 5.0 to pH 8.0, the slides remain colourless, while at pH 2.1, the chromosomes stain mainly superficially, the Xy_p lumen remaining stainless (Figure 8b). Using 0.9% NaCl solution as a mordant prior to the stain, and a prolongation of staining time, do not improve the result, nor lessen the argyrophily of the swollen (a hypotonic effect) lumen (Figure 8c).

Gradual removal of silver

The brightness of the silver stained lumens of M I under phase contrast (Figure 5f) suggests a high density silver deposition. It could be that the original question mark figure, or its derivative the border-band stage, are still embedded in it. This seems possible in view of the holes occasionally still seen in the M I lumens (Figure 10b), and taking into account how the full argyrophily of the lumen develops, adding substance to the question mark or border-band figure. Thus we expected to see density differences when eliminating the silver gradually. Washing off the silver from the parachutes requires more drastic measures than those given in the literature (Warburton and Henderson, 1979; Mayr *et al.*, 1985). A 1.5% solution of potassium ferricyanide does so completely in an overnight treatment, a 7.5% solution in about 4 h. We avoided thiosulphate, because it was destructive.

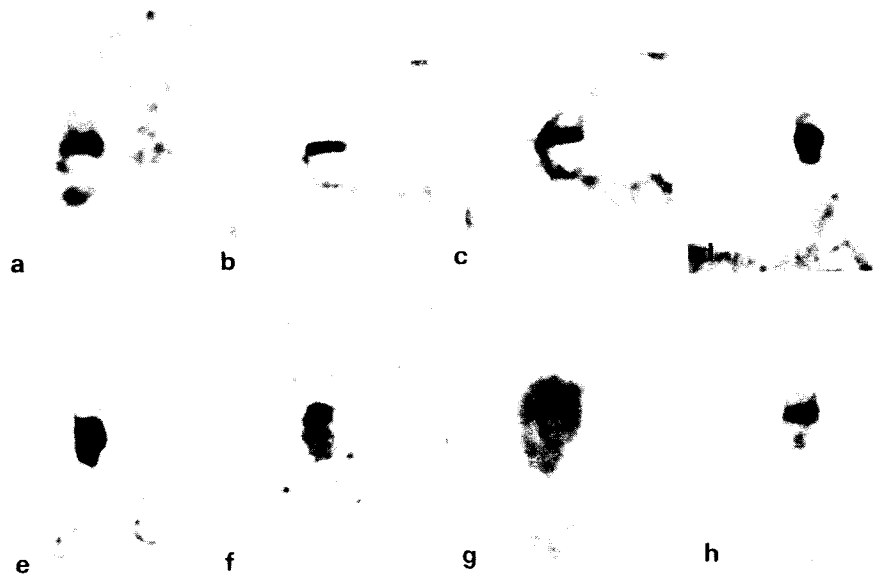

Figure 9 Effect of proteases in the argyrophily of Xy_p in *Diaprepes abbreviatus*. Bright field.
Figure 9a Diplotene, KS fixation, 0.25% pepsin (pH 2.8) for 20 sec, then silver stain. Some swelling is evident. x3,000.
Figure 9b Diakinesis, acetic alcohol fixation, 0.00125% trypsin (pH 6.8) for 20 sec, then silver stain. Slight swelling can be seen. x3,000.
Figure 9c The same Xy_p as above, Ag eliminated, refixation in KS, then new silver staining. Argyrophily (border bands) of Xy_p notably enhanced. x3,000.
Figure 9d M I, KS fixation, 0.00125% trypsin (pH 6.8) for 30 sec, then silver stain. x3,000.
Figure 9e M I, KS fixation, 0.00125% trypsin (pH 6.8) for 30 min, then silver stain. Slight swelling can be observed. x3,000.
Figure 9f Diakinesis, KS fixation, 0.00125% trypsin (pH 6.8) for 30 min, then silver stain. Swelling of Xy_p and flocculate argyrophily in the lumen. x3,000.
Figure 9g M I, KS fixation, 0.25% pepsin (pH 2.8) for 16 min, then silver stain. Very notable swelling of Xy_p and granulate argyrophily occur in the lumen. x3,000.
Figure 9h Diakinesis, acetic alcohol fixation, 0.0125% trypsin (pH 6.8) for 60 sec, then silver staining. Very little swelling (*cf* Figure 9b) can be seen. x3,000.

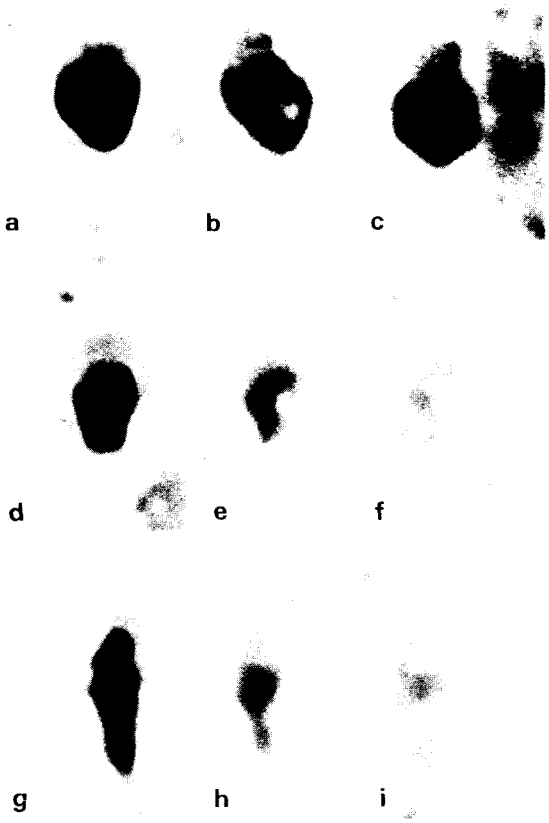

Figure 10 Experiments with silver staining and destaining of M I parachutes in *Lachnopus curvipes*. KS fixative, bright field. Figures 10b, 10d to 10i (as well as Figures 8b and 8c) are from one and the same slide, stained twice with Remazol, then silver and Giemsa stained. Parachutes in Figures 10d to 10i were then washed in 7.5% potassium ferricyanide.
Figure 10a Control: KS fixation and silver staining. x4,550.
Figure 10b As above, but incomplete staining of Xy_p lumen. x4,550.
Figure 10c Ethanolic $Ba(OH)_2$ for 20 min at 56°C, to eliminate RNA (Geyer and Schreibner, 1970), then silver staining. Lumen fully argyrophilic. x4,550.
Figure 10d Parachute in anterior view. Washing time 15 min. x4,550.
Figure 10e The same parachute as above. Washing time 220 min. x4,550.
Figure 10f The same parachute as above. Washing time 240 min. x4,550.
Figures 10g to **10i** Parachute in lateral view. Washing times as in Figures 10d to 10f. In the preparation, the distance between these parachutes (Figures 10d to 10f, and 10g to 10i) is only 0.1 mm. x4.550.

The large Xy_p of *Lachnopus curvipes* was selected to illustrate this process. In the anterior views (Figures 10d to 10f), the silver deposit is first reduced to an S-like body, and, finally, to a spot located in the 'upper' bend of S. Washing off lateral view deposits can be similarly interpreted (Figures 10g to 10i). Thus, it seems to be the question mark figure, especially the interstitial block of the originally associated end of X which in M I has the densest deposit of silver, or the highest capacity to retain it. Similar results were obtained with other Otiorrhynchinae parachutes of the material, although the half-reduced silver figure sometimes assumed more complicated shapes than S, corresponding to multiple bridges seen in Xy_ps of diplotene and diakinesis (Figure 4c).

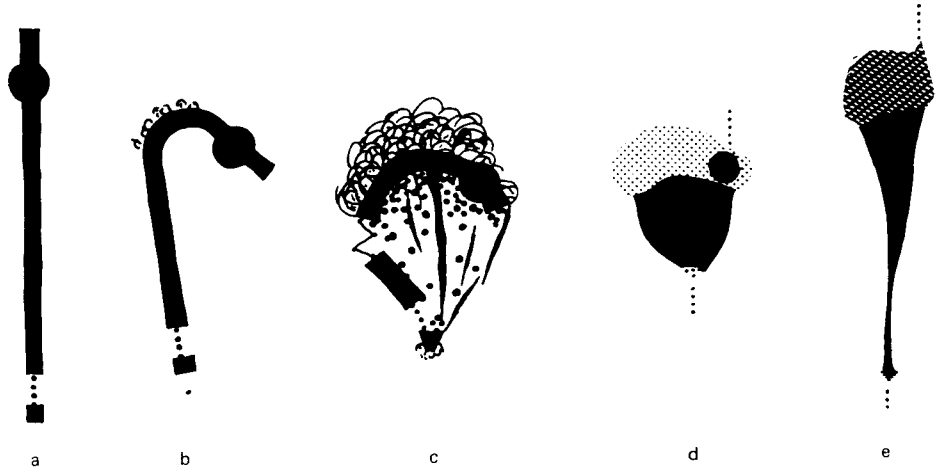

Figure 11 Development and breakdown of the parachute association starting from the initial end-to-end contact between X and y chromosomes, as observed in the weevils of the present material. Initial association marked by a dotted line in Figures 11a to 11c, procentric heterochromite of X by a rounded knob in Figures 11a to 11d. Solid black marks argyrophily.
Figure 11a Earliest prophase. End-to-end association of X and y.
Figure 11b Beginning of the question mark stage. Bending of the X chromosome supposedly due to lamp-brush-like loops extruding one-sidedly from the proximal half of the X chromosome.
Figure 11c Advanced question mark stage showing profuse one-sided looping of both sex chromosomes, and invasion of the space (lumen) between them by a fibrous-flocculate argyrophilous substance. The distal half of the X chromosome decondenses (or loses its argyrophily), then becomes stainable again, beginning from the end, and finally integrates with the main part of X.
Figure 11d M I. Both sex chromosomes, except for the procentric heterochromite of X, have lost their argyrophily. Lumen homogeneously argyrophilic.
Figure 11e A I. Argyrophily of the sex chromosomes increasing. The lumen substance becomes stretched, then strangled to pieces proportionate to the size of the adjacent sex chromosome. By T I the substance has disappeared.

Discussion

The impression obtained on the development of argyrophily and association of the Xy_p in weevils is summarized in Figure 1. The basic element, lasting until A I, consists of an association of X and y, end-to-end, in very early prophase, when both chromosomes are heteropycnotic and fully argyrophilous. Their pairing does not appear completely non-specific, because, at least in *Exophthalmus quindecimpunctatus*, the distal end of the long arm of X is always involved (in variations *cf* Smith and Virkki, 1978). This basic Xy retains its argyrophily until A I, but suffers morphological changes in the form of bending and puffing of the proximal half of X, and of condensation-decondensation events in the distal half of the long (associated) arm of the same chromosome. From diakinesis on, it becomes embedded in an additional argyrophilous substance.

Many of our illustrations support the interpretation of Drets *et al.* (1983) on the development of Xy_p. There are, however, disagreements with their interpretation and with that of Postiglioni and Brum-Zorrilla (1988). We found the early Xy chain a natural structure, not an artefact caused by hypotony. Furthermore, we could not confirm any early, persistent double-end contact between X and y. Although Xy rings are common in the bouquets, they subsequently open to form question mark figures where the free ends are far from one another. Only a very thin and long link, invisible in the light microscope, could be expected to join them. Finally, we have discovered the strongly argyrophilic lumen substance in the late parachute.

One of the most characteristic features of the silver deposit of metaphasic Xy_p lumen is its brilliant whiteness under phase contrast. We have seen similar brightness in silver stained centromeres, presumably marking the adjacent proteinaceous assembly plates of microtubuli (Figure 2a in Virkki, 1989), and in nucleoli attached to autosomal NORs (Figures 6 and 7 in Virkki, 1988). Two possible interpretations of this phenomenon come in mind. A prerequisition for the first interpretation is that the deposit is not a solid metallic body, but allows some transmission of light. Then retardation of transmitted light by one-half wavelength would achieve a fully constructive interference between source waves and diffracted waves, producing thus a shadowless illumination. Light just tangenting the deposit would be less retarded, producing a black pellicle-like effect (Figure 5f). The other possibility is that the brightness is due to a mirror-like reflection from a dense metallic surface. We have seen in overstained silver preparations a similar brightness spread on all chromosomes, and so we are inclined to support the last interpretation, especially because similar reflection effects have been obtained with gold in other laboratories (S. Inoué, personal communication).

Bending of the prophasic X chromosomes seems to occur in various organisms (Ohno *et al.*, 1958, 1959; Miklos, 1974). We have attributed it to an eccentric loop formation. Incipient loops of an early question mark stage cannot be seen by light microscopy, but subsequently the loops are recognizable in large parachutes (Figure 4e). These loops are comparable with the simultaneous lampbrush loops of diplotenic autosomal bivalents. The loops extrude from the

chromosome without notable changes (despiralization) in its length. In diplotenic autosomal bivalents, the loop formation is necessarily one-sided, because the parallel apposition of the homologues and the synaptonemal complex between them impedes inward looping. The question arises whether the one-sided looping of the parachute sex chromosomes could be taken as a vestigial process from a period when the bivalent still consisted of parallel homologues in diplotene. The delicate material invading the parachute lumen thus could be akin to synaptonemal complex proteins, which indeed are argyrophilous simultaneously with the lumen substance, if properly prepared (Figure 7a).

Prolonged AgNOR staining finally renders all chromosomes black (Virkki *et al.*, 1984; Hubbell, 1985), and so the deposition of silver is accumulative and not necessarily in a stoichiometric relation with the reducing cellular substance. Empty lumens of unstained M I parachutes observed under phase contrast, or when stained for proteins, show that even the heaviest silver deposit is on a very delicate structure which, in addition, can be notably expanded without a visible loss of argyrophily. Thus silver exaggerates the presence of such structures. This is comparable to staining of chromosomal scaffolds, delicate proteinous structures hard to visualize without silver staining (Howell and Hsu, 1979; Earnshaw and Laemmli, 1984).

In some preparations, an acid hydrolysis suffices to modify the delicate lumen substance in such a way that it becomes slightly argyrophilous and thus well recognizable under phase contrast in otherwise 'empty' border-band parachutes of early diakinesis (Figure 5 in Virkki *et al.*, 1990). Perchloric acid produces similar effects at the same stage of Xy_p (Figure 7d). We suggest that the fibrous to flocculate lumen herein detected consists of denatured proteins. In any case, RNA cannot be present after such treatments. Indeed, such a flocculate structure is probably related to a similar structure of swollen Xy_ps overtreated with pepsin and trypsin. The resistance of formalin-fixed lumen substances to trypsin has its parallelism in scaffold proteins (Goyanes *et al.*, 1980).

Persistence of the argyrophilous substance until the breakdown of Xy_p suggests that the substance might function as an adhesive, thereby serving to control the sex chromosome segregation. The strength of terminal contacts of meiotic sister chromatids can vary from nil to extremely persistent (Virkki, 1989). Such a variation might affect the persistence of pairing by end contacts of chromosomes. If an Xy_p contains only one weak chromosomal contact point, subject to stress during the decondensation-recondensation events of the X chromosome, and during the premetaphase stretch, the role of even a weak adhesive in keeping the bivalent could be decisive. In this respect Polyphaga differs from Adephaga, where the end-to-end Xy association seems to be sufficient for keeping the bivalent until A I.

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