# High-resolution Chromosome Analysis in Lepidoptera[1]

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ABSTRACT

Improved methods for obtaining good cytological preparations from testicular tissues of Lepidoptera are described. Testes were pretreated with various hypotonic solutions, fixed in Carnoy's, and slides prepared using air dry-squash and cell suspension-air dry techniques. Air-dried slides were stained directly with Giemsa or treated with the C-band technique prior to staining. Well-spread chromosomes with good morphological characteristics were found in spermatogonial cells and spermatocytes of all stages. A detailed characterization of the mitotic chromosome complement and 1st meiotic division in males of the hesperiids *Pyrgus oileus* (L.), *P. albescens* (Plötz), and *Lerema accius* (Smith and Abbot) and the noctuids *Trichoplusia ni* (Hubner), *Argyrogramma veruca* (Fab.), and *Prodenia ornithogalli* Guenee are described.

Although chromosome numbers for more than 1000 species of Lepidoptera are reported, little is known of the morphological characteristics of the karyotype. The chromosomes of butterflies and moths usually are visualized at meiotic metaphase as a series of highly contracted minute spherical or ovoid bodies, and observations are traditionally made from sectioned or squashed material (Emmel 1969, Soumalainen 1969). As noted by Robinson (1971), there is a need for improvement in the techniques for the handling of Lepidoptera chromosomes. Improvements in cytological procedure involving pretreatment of tissues and air-dry methods of slide preparation allow a more detailed characterization of the karyotype. In the present study, a number of cytological techniques were tested on several species of Lepidoptera. Procedures are presented for obtaining good cytological preparations from testicular tissues as well as a detailed analysis of the karyotype of 3 species of Hesperiidae and 3 species of Noctuidae. The results demonstrate that greatly improved resolution of chromosome morphology, condensation, and orientation during meiosis can be obtained in these insects.

## MATERIALS AND METHODS

*Biological Material.*—Immatures of the hesperiids *Pyrgus oileus* (L.), *P. albescens* (Plötz), and *Lerema accius* (Smith and Abbot) and the noctuids *Trichoplusi ni* (Hubner), *Argyrogramma veruca* (Fab.), and *Prodenia* (=*Spodoptera*) *ornithogalli* Guenee, collected in Houston, Harris, Harris Co., TX, were used in this study. Field-collected specimens and laboratory-reared progeny of field-collected adults were examined. Testicular preparations of from 21–35 individuals of each species were examined. Chromosome counts were obtained from at least 10 intact 1st metaphase cells from 10 individuals of each species. Additional counts were made at diakinesis and in diploid spermatogonial mitoses. Chromosomes were paired according to length, and measured in greatly enlarged photographs of diploid colchicine arrerted mitotic (C-metaphase) cells.

Chromosome observations were mainly made on testes of 2nd, 3rd, and 5th instar larvae. Mitotic metaphase cells were most abundant in 2nd and 3rd instar larvae, while early prophase cells were found in the testes of 3rd instar, and late prophase and metaphase I cells were predominant in 5th instar larvae. Metaphase I cells were also found in young pupae of the species of Hesperiidae examined. Mitotic cells were not found in field-collected adults of any of the species examined. Spermatogenesis in moths and hesperiid butterflies is essentially completed at the time of adult eclosion (Sado 1961, Emmel and Trew 1973, Werner 1975).

*Pretreatment.*—Various hypotonic solutions were employed in order to evaluate their effect on chromosome morphology and spreading of chromosomes. Testes were dissected in insect-Ringer solution (Crozier 1970) and transferred directly to fixative or to one of the following solutions at room temperature: (1) Ringer-distilled water 1:1, 10–20 min; (2) 0.9% sodium citrate, 10–30 min; or (3) a combination colchicine-hypotonic of 0.05% colchicine in 0.5% sodium citrate solution, 30–45 minutes. The peritoneal sheath and the follicular epithelium of larger testes were carefully removed while the testes were submerged in insect-Ringer or immediately before submersing pretreated organs in fixative.

*Cytological Preparations.*—A conventional squash technique, a combination squash-air dry technique,

[1] Received for publication Oct. 28, 1975.

and cell suspension-air dry techniques were applied to all of the species examined.

Orcein squashes were made as suggested by Emmel (1969) except that fixed tissues were dispersed before squashing in 0.5% lactic aceto-orcein by repeated syringing through a 20-gauge needle.

The squash-air dry technique employed was as follows. Testes were fixed in acetic-ethanol (1:3) for 30 min, transferred to a drop of 40–45% acetic acid on a slide, quickly torn into pieces and squashed. The preparation was then placed on a block of dry ice for 20 min, the cover glass detached using a razor blade, and the frozen preparation thawed in acetic-ethanol for 5 min before air drying. Slides were stained immediately after drying or stored for up to 2 mo before staining and observation.

For cell suspension-air dry prepartions, tissues were fixed in acetic-ethanol for 5 minutes. Two methods were used to suspend and disperse the cells: (1) tissues were placed in a drop of 50% acetic acid on a prewarmed slide (ca. 40°C). The tissues quickly disintegrate in this solution and the cells are liberated to form a suspension. The cell suspension was dispersed over the slide by adding a few drops of acetic-ethanol, and the slide left to dry. (2) tissues were placed in a well slide holding 5–10 drops of 50% acetic acid. The cell suspension was dispersed over a warm slide using a micropipette as suggested by Stock et al. (1972).

The heterochromatin staining procedures (C-banding) of Vosa (1973) and Hsu (1971) were employed with slight modifications. Briefly, air-dry preparations were immersed in a saturated $Ba(OH)_2$ solution (5–10 min), incubated in 2xSSC (0.3M NaCl and 0.0M Na citrate) at 65°C for one hour, rinsed in DW, air dried and stained with Giemsa. Additional slides were treated with 2xSSC adjusted to *p*H 12 with NaOH (1–10 min) and then incubated overnight in 2xSSC at 65°C rinsed in DW, and air dried.

Air-dry preparations were stained with 2% Giemsa (Matheson) in Sorensen's phosphate buffer (*p*H 6.8) for 10–15 min at room temperature.

## RESULTS AND DISCUSSION

*Cytological Procedures.*—Spermatogonial cells and spermatocytes of all stages were found in both squash and air-dry preparations. The best results were obtained using hypotonic-solution pretreatment and the cell suspension-air dry methods of slide preparation. Pretreatment schedules that produced well spread chromosomes with good morphological characteristics are given for the various cell types in Table 1.

Figures 1–2 and 4–5 present direct orcein squash and squash-air dry preparations of 1st metaphase (MI) cells. The squash-air dry technique consistantly produced optically flat MI plates with contrastingly dark chromosomes. Although highly symetrical arrays of chromosomes in MI cells were readily obtained in material squashed without hypotonic treatment, satisfactory chromosomal detail was not seen. Hypotonic pretreatment, in combination with squashing, gave unsatisfactory results because the swollen cells were easily broken and chromosomes widely scattered.

Table 1.—Pretreatment used to obtain satisfactory chromosome spreading and good chromosome morphology of the various stages of spermatogenesis. Shorter treatment times were ineffective; longer treatments produced indistinct and overcontracted chromosomes and precocious separation of metaphase chromatids. Cell suspension-air dry preparations.

| Hypotonic solution | Minutes pretreatment | | |
|---|---|---|---|
| | 15 | 30 | 45 |
| Ringer:DW (1:1) | leptotene-[a] diplotene | | |
| 0.9% Na citrate | leptotene-diplotene | diakinesis, MI | |
| Colchicine-0.5% Na citrate | | diakinesis | mitotic metaphase, MI |

[a] Treatment times longer than 15 min were unsatisfactory due to rupturing of testicular follicles.

Figues 3, 6, and 21 present 1st metaphase to 1st anaphase cells from hypotonically treated cell suspension-air dry preparations. These cells show greater chromosomal detail than is seen in the squash preparations (Figures 1–2, 4–5). In favorable cells, the 4 chromatids of each bivalent are clearly visible (Fig. 3, arrows). Pretreatment times longer than optimal produced highly contracted chromosomes with separating chromatids.

Figures 7–12 present spermatogonial C-metaphase cells. The best results were achieved using colchicine-sodium citrate hypotonic treatment for 45 minutes. Longer treatment produced highly contracted chromosomes showing parallel separation of chromatids. Hypotonic treatment with either 0.9 or 0.5% sodium citrate alone produced fewer metaphases and chromosomes appeared elongated and tangled.

Figures 13–17 illustrate meiotic prophase cells from pretreated air-dry preparations. Brief hypotonic treatments gave the best results (Table 1). Overtreatment of early prophase cells resulted in rapid loss of chromosome morphology. For example, hypotonic treatment of pachytene cells longer than 10 min caused the loss of chromomere patterns; treatment longer than 20 min produced fuzzy chromosome outline. Pretreatment times necessary to achieve spreading of mitotic chromosomes and to show details of chromosome orientation at MI were too long for leptotene through diakinesis, and treatments suitable for pachytene were ineffective for mitotic metaphase and MI.

It was necessary to alter the procedure slightly for each of the species studied in order to obtain optimal results. Moreover, the various stages of spermatogenesis reacted differently to the different procedures. In general, brief hypotonic treatment produced satisfactory spreading of meiotic prophase; prolonged hypotonic treatment produced well-spread MI chro-

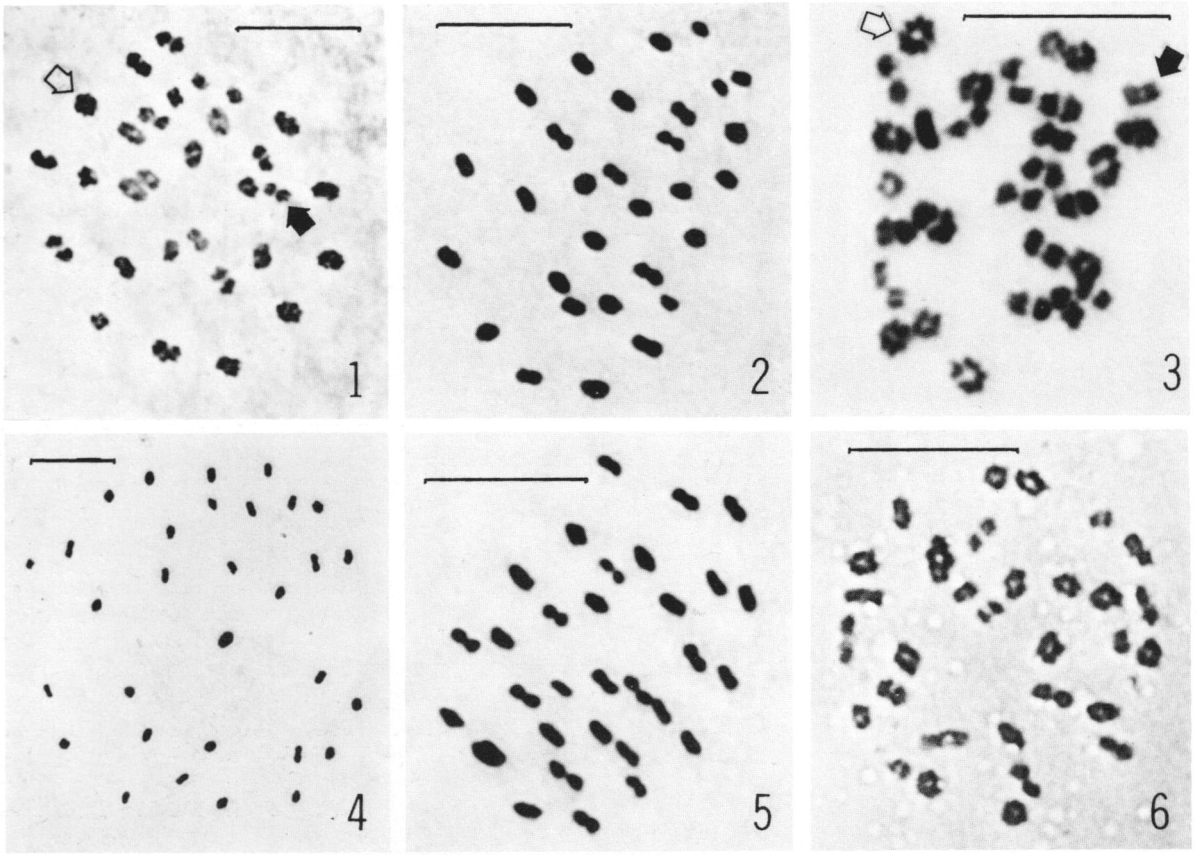



FIG. 1–6.—First metaphase-anaphase. End-to-end configuration (solid arrows), cross-shaped configuration (open arrows). Horizontal lines equal 10 micra. 1, *Pyrgus oileus,* N=30, orcein squash. 2, *P. albescens,* N=30, squash-air dry; Giemsa stain. 3, *Lerema accius,* N=29, cell suspension-air dry; Giemsa stain. 4, *Trichoplusia ni,* N=31, squash-air dry; Giemsa stain. 5, *Argyrogramma veruca,* N=31, squash-air dry; Giemsa stain. 6, *Prodenia ornithogalli,* N=31, cell suspension-air dry, Giemsa stain.

mosomes; and prolonged colchicine-hypotonic treatment produced excellent spermatogonial metaphase cells. The percentage of acetic acid used to suspend the cells was critical because at 50% acetic acid cells at diakinesis, MI, and mitotic metaphase quickly disintegrate. For metaphase cells, 45–50% acetic acid was most suitable; 50–55% acetic acid produced well-spread, mostly intact prophase cells. A relatively dilute cell suspension and slides prewarmed to ca. 40°C facilitated chromosome sperading. Higher temperatures made staining more difficult and caused distorted chromosome morphology.

Cell suspension-air dry methods have proved successful not only for lepidopteran testicular tissues but also for ovarian and neural tissues in other insect groups (Crozier 1968, Lespinasse 1973, Holmquist 1975). As noted by Stock et al. (1972), the success of these methods depends on hypotonic pretreatment, suspension of Carnoy-fixed cells in acetic acid, and dispersion and rapid drying of the cell suspension on slides. The suspension technique described above has given reproducible results of a high standard for testicular tissues in a number of lycanenids, hesperiids, noctuids, and pyralids.

Recently, a standard mammalian air dry technique was adapted to lepidopteran testicular tissues by Bigger (1975). Bigger suggested the use of colcemid injection of living adults, masceration of tissues in trypsin solution, acetic alcohol fixation, and dispersion of a cell suspension on to dry slides. The mammalian technique was then combined with acetic-saline-giemsa staining which enabled the construction of detailed karyotypes of 2 butterfly species. The simplier air-dry technique described in the present study is also compatible with acetic-saline-giemsa as well as with trypsin-giemsa banding of mitotic metaphase chromosomes (ms. in preparation).

*Chromosome Observations.*—Over 95% of the MI and diakinesis cells selected for chromosome number determinations contained the haploid count regarded as typical for any individual examined. Approximately 75% of the mitotic cells selected contained a diploid count. Deviant counts were usually lower due to loss during preparation.

Metaphase I-anaphase I cells of the species examined are illustrated in Figures 1–6. Mean length of chromosomes and total chromosome length (TCL) of 4 of the species are given in Fig. 22. Representa-

tive diploid cells from which chromosome measurements were taken are presented in Fig. 7–12.

In the hesperiid species examined, a chromosome number of N=30 was found in *Pyrgus oileus* and *P. albescens* (Fig. 1–2). Chromosome length measurements in these 2 species were essentially identical. A haploid number of 29 was found in *Lerema accius* (Fig. 3). The chromosomes of the 3 species are graded in size (Fig. 22a, b). In most cells, 3 pairs of chromosomes appear distinctly smaller than the rest. The smallest chromosomes are ca. ⅓ the length of the largest.

The present observations show that the karyotypes of *P. oileus, P. albescens,* and *L. accius* differ from previous descriptions given by Emmel and Trew (1973). These authors report a haploid number of 32 for *P. oileus* and 28 for *P. albescens,* and note that in *L. accius,* 6 of the 29 chromosomes of the haploid complement are notably smaller than the others. The differences in Emmel and Trew's results and the present observations are probably due to the greater accuracy obtainable with air dry methods as opposed to the conventional squash technique.

A chromosome number of N=31 was found in the noctuids *Trichoplusia ni, Argyrogramma veruca,* and *Prodenia ornithogalli* (Fig. 4–6). Chromosome length measurements were extremely similar in *T. ni* and *A. veruca.* The karyotype of the 3 species consists of one large chromosome and a graded series of smaller chromosomes (Fig. 22c, d). The length ratio of largest to smallest chromosome averaged 5.7 in *T. ni,* 5.5 in *A. veruca,* and 4.1 in *P. ornithogalli.* The largest chromosome bears a distinct subterminal constriction (Fig. 10–11).

The hesperiid karyotypes studied are clearly different from the noctuid karyotypes because of the large constricted chromosome and smaller total genome length in the 3 noctuid species. Although information on chromosome number is available for 82 species of Hesperiidae and 121 species of Noctuidae (Robinson, 1971; de Lesse 1970, Emmel and Trew 1973, Dong and Emmel 1975, Werner 1975), this is the first critical analysis of mitotic chromosome size and morphology in these insects. Most hesperiids studied so far have N=29, 30, or 31 chromosomes (ca. 51 of 82 species) and most noctuids have N=31 chromosomes (ca. 110 of 125 species).

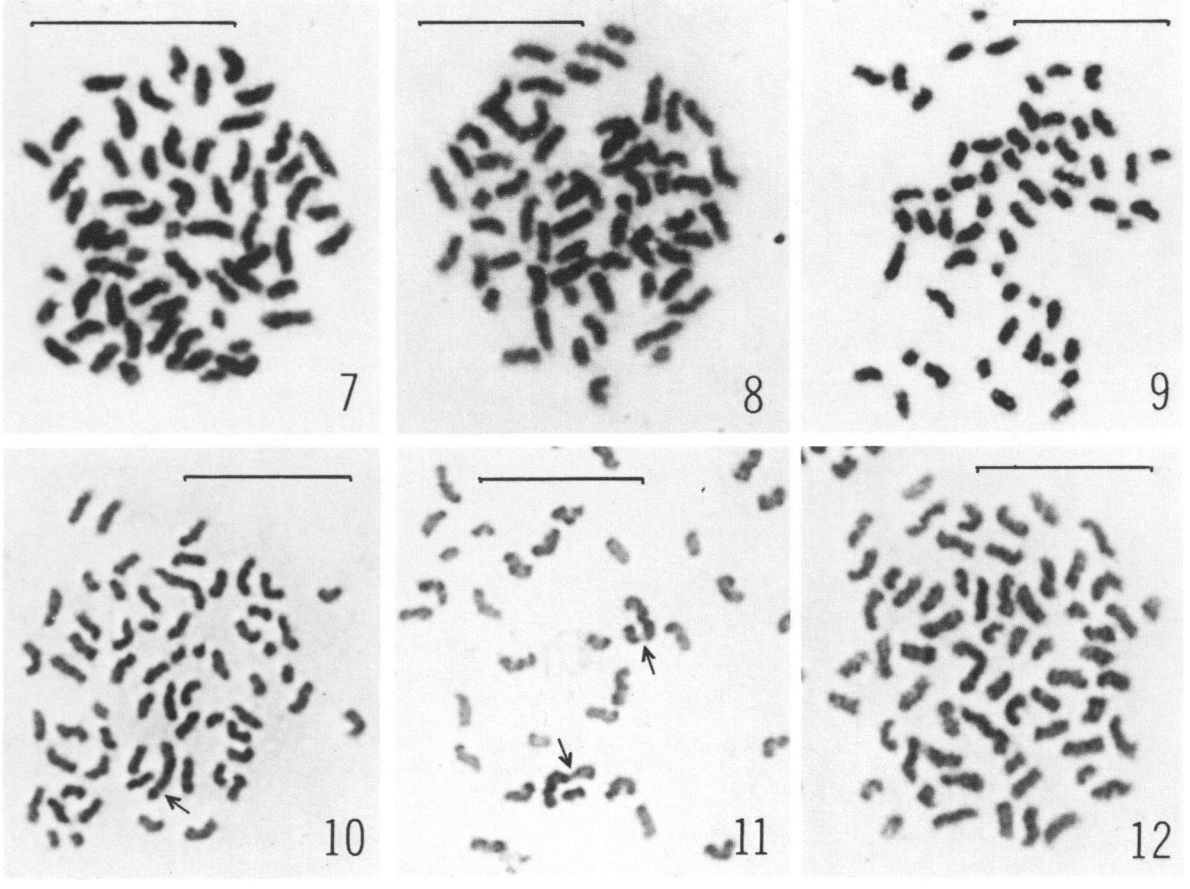
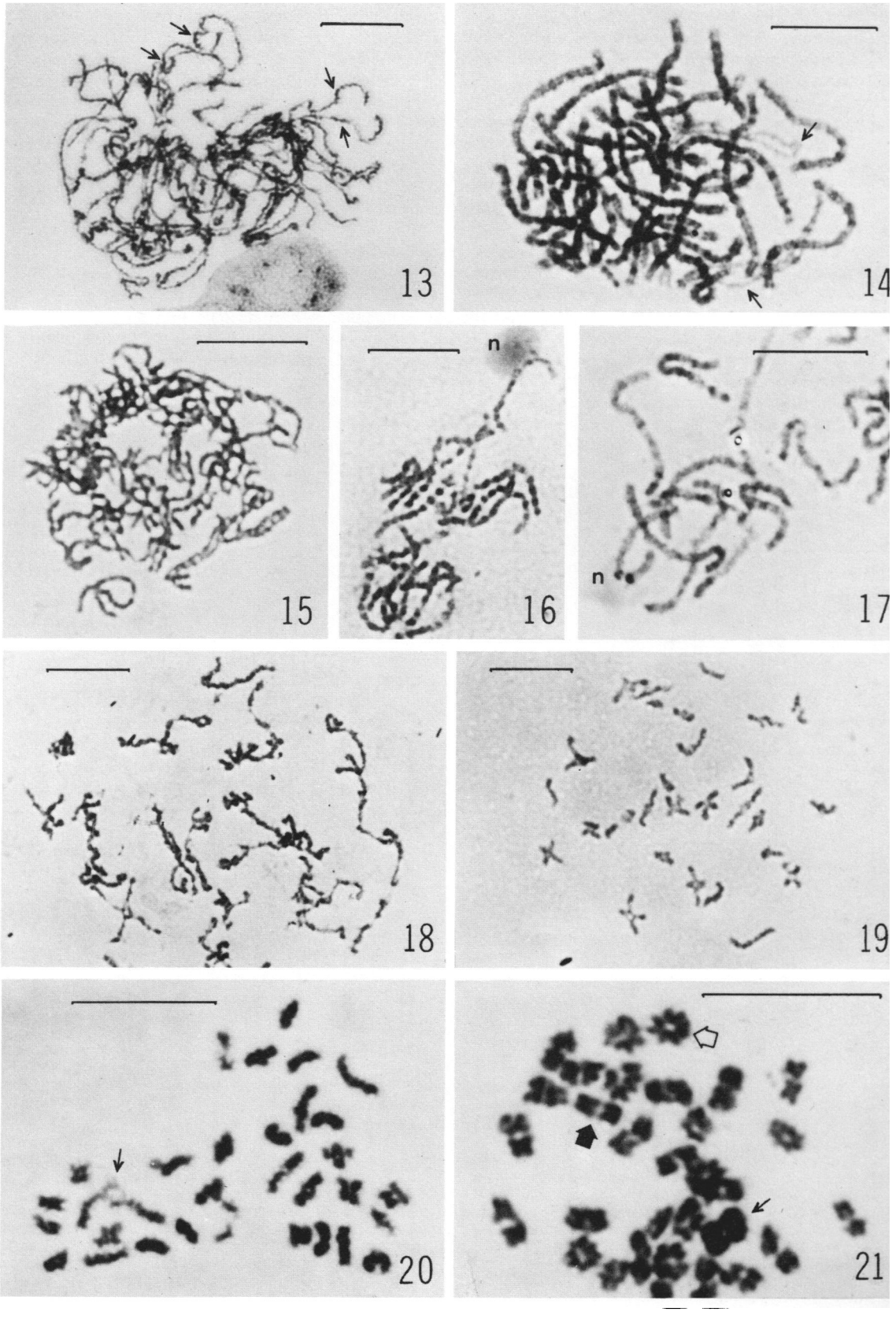



FIG. 7–12.—Mitotic C-metaphase cells. Cell suspension-air dry preparations. Subterminal construction at arrows. Giemsa stain. Horizontal lines equal 10 micra. 7, *Pyrgus oileus,* 2N=60. 8, *P. albescens,* 2N=60. 9, *Lerema accius,* 2N=58. 10, *Trichoplusia ni,* 2N=62. 11, *Argyrogramma veruca,* partial metaphase. 12, *Prodenia ornithogalli,* 2N=62.



The present data indicate that a karyotype of N=31 with a conspicuously large chromosome bearing a deep subterminal constriction may be common to several noctuid taxa. However, the mitotic karyotype of the noctuid *Heliocontia apicella* (Grt.) also with N=31 chromosomes, consists of a graded series lacking a relatively large chromosome (unpubl.). Although previous investigators have noted large size differences among meiotic metaphase chromosomes in several Lepidoptera, the chromosomes are highly contracted at this stage and appear approximately equal in size in most species (Soumalainen 1969). The great degree of karyotypic stability demonstrated in Lepidoptera, as indicated by the presence of identical chromosome numbers in widely separated members of most families emphasizes the need for a more detailed characterization of the karyotype. Additional noctuid and hesperiid species as well as other Lepidoptera should be examined before the differences in chromosome size and morphology can be interperated taxonomically and phylogenetically.

Meiosis appeared similar in the 6 species. Meiotic prophase is illustrated in Fig. 13–17 for *Pyrgus oileus*. This species provided the most favorable material for observations of meiotic prophase due to large size and good spreading of chromosomes. The most conspicuous feature of meiosis is the extreme degree of chromosome condensation. Chromosomes contract from an average length of ca. 20$\mu$ at zygotene to less than 1$\mu$ at MI.

Unpaired chromosomes at zygotene exhibit dark staining (presumably heterochromatic) terminal and interstitial regions. Heterochromatic regions are most conspicuous in partially paired or closely aligned homologues (Fig. 13 arrows). A distinct chromomere pattern is seen at pachytene. Observations of late zygotene-early pachytene cells suggests that at least some of the extended heterochromatic regions in early prophase chromosomes correspond to darkly staining chromomeres in condensed pachytene chromosomes.

The distribution of incompletely paired chromosome regions suggests that pairing of chromosomes and lapse of synapsis may be initiated at both terminal and interstitial positions. Furthermore, the bivalents in any single prophase cell usually show different degrees of completeness of pairing indicating that pairing may initiate at different times or may procede at different rates in different chromosomes.

A single chiasma was observed per bivalent in all species. Chiasmata are visible in diplotene cells at various positions along the chromosomes (Fig. 18). The number and positions of chiasmata inferred from bivalent morphology remain essentially unchanged throughout late prophase and MI. Chromosomes show either an end-to-end or a cross-shape configuration during diakinesis (Fig. 19–20) and at anaphase I (cf. Fig. 13 and 21, open and closed arrows).

The C-banding technique was applied to pachytene chromosomes in *P. oileus* and *P. ornithogalli* in order to further study the distribution and extent of heterochromatin. The NaOH C-band technique was unsatisfactory in that undifferentiated chromosomes with only faint chromomere patterns were obtained. The milder BaOH C-band technique produced an accentuated chromomere-like pattern in *P. ornithogalli* (Fig. 16). C-banding of *P. oileus* revealed a very dark staining interstitial region on one of the larger chromosomes and less darkly staining terminal and interstitial regions on most of the other chromosomes. The nucleolus is associated with the very dark C-band in *P. oileus* (Fig. 17). In *P. ornithogalli,* the nucleolus is associated with a lightly staining region on one of the larger chromosomes (Fig. 16).

Descriptions of meiosis in Lepidoptera are mostly confined to conventionally sectioned or squashed chromosomal material. Thus, a comparison of previous interpretations with the results from air-dry preparations of pretreated tissues is difficult. Miller and Miller (1966), using hypotonic saline treatment and a squash-air dry technique, found elongate beaded pachytene chromosomes in *Speyeria aphrodite* (Fab.). Traut and Rathjens (1973) studied pachytene chromosomes in ovarian cells of *Ephestia kuehniella* Zeller with fluorescent Feulgen staining of dispersion-air dry preparations, and they were able to identify the W-chromosome and describe a distinct pattern of chromomeres and interchromomeres on the autosomes. However, these authors did not analyze the meiotic sequence in spermatogenesis. Using an improved squash-air dry technique, Murakami and Imai (1974) investigated chromosome structure and orientation in meiosis of *Bombyx mori* L. In general, the morphological characteristics of the hesperiid and noctuid chromosomes noted here correspond to the previous descriptions from air-dry preparations of other species. A distinct chromomere pattern was found at early prophase in the species studied here, and certain of the chromosomes appear to be traceable from one cell to another based on this pattern. It is not known, however, whether the chromomeres correspond to the C-bands demonstrated at pachytene

FIG. 13–21.—Meiosis in hesperiid and noctuid species. Cell suspension-air dry preparations. Giemsa stain. Horizontal lines equal 10 micra. 13, *Pyrgus oileus,* zygotene. Heterochromatic regions on homologous chromosomes at arrows. 14, *P. oileus,* pachytene, incomplete pairing at arrows. 15, *P. oileus,* zygotene-pachytene. 16, *Prodenia ornithogalli,* pachytene showing chromosome-like C-bands and nucleolus (n) associated with a lightly stained chromosome region. 5 min BaOH C-band preparation. 17, *P. oileus,* pachytene showing nucleolus (n) associated with a dark interstitial C-band. 10 min BaOH. 18, *P. ornithogalli,* late diplotene. 19, *P. ornithogalli,* diakinesis. 20, *Trichoplusia ni,* diakinesis. Large bivalent with one near terminal chiasma (arrow). 21, *T. ni,* first anaphase, end-to-end configuration (solid arrow), cross-shape configuration (open arrow). Note end-to-end association and axial orientation of large bivalent at small arrow.

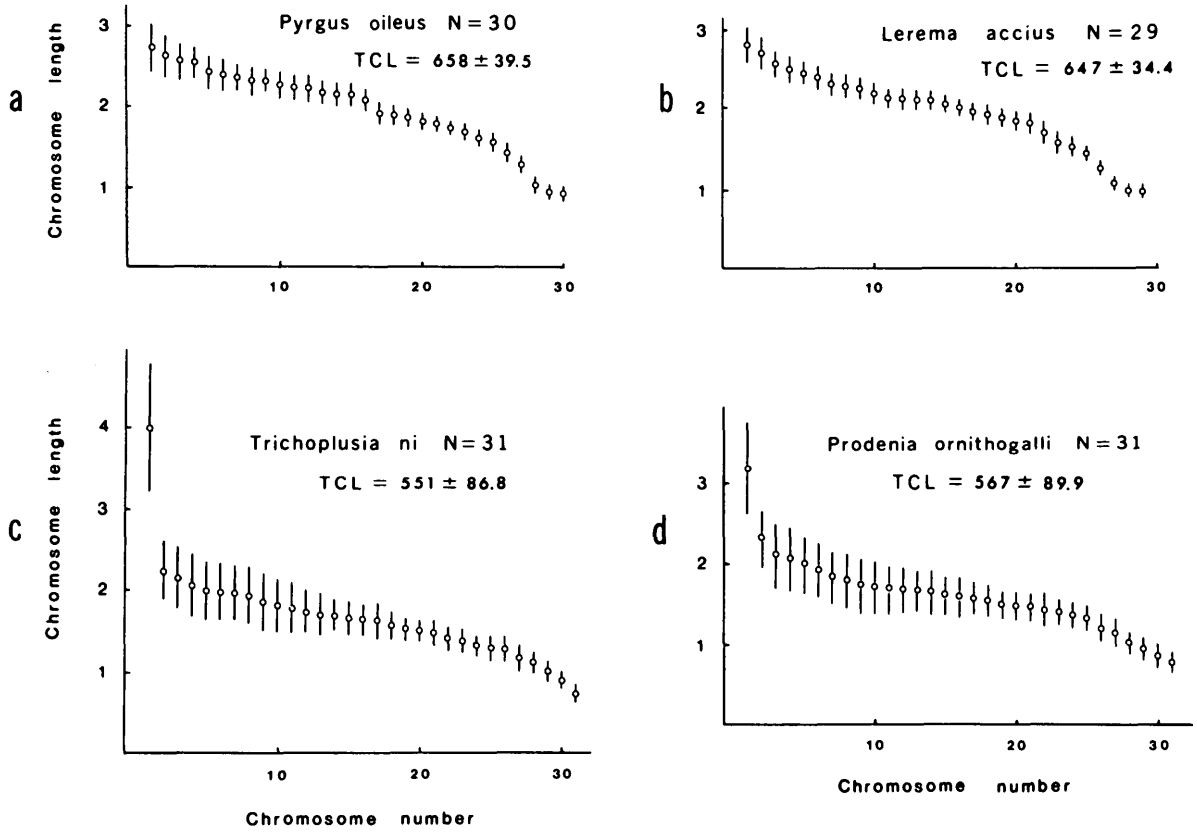



FIG. 22.—Mean chromosome lengths in micra ± SD in 2 species of Hesperiidae (a,b) and 2 species of Noctuidae c,d). TCL = relative total chromosome length. The curve formed by the open circles (mean chromosome lengths) illustrates that each species has a characteristic chromosome size distribution.

in these species. The function of the nucleolus-associated C-band heterochromtin described here also is unknown. The low number of chiasmata formed per bivalent and the incomplete terminalization of chiasmata are in marked contrast to the observations of Murakami and Imai. The holocentric bivalents in *Bombyx mori* undergo complete terminalization and thus the chromosome dyads are always connected end-to-end by a terminal chiasma at MI (Murakami and Imai 1974). In the hesperiid and noctuid species studied, anaphase disjunction apparently occurs without terminalization of chiasmata. These observations suggest that orientation of bivalents at MI may be influenced by chiasma position and terminalization as well as by the proposed holocentric properties of lepidopteran chromosomes.

ACKNOWLEDGMENT

I thank Mr. André Blanchard for aid with species identifications and Dr. A. D. Stock for helpful discussion and review of the manuscript.

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