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Unusual Chromosome Configurations: Evaluation of Sex Univalent and Trivalent Chromosome Models

Ashley B. Borseth
Bucknell University, abborseth@gmail.com

Unusual Chromosome Configurations: Evaluation of Sex Univalent and Trivalent Chromosome Models

by

Ashley Borseth

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Approved by:

Dr. Leocadia Paliulis, Research Adviser

Dr. Chris Martine, Department Chair & Committee Member

Dr. Steve Jordan, Committee Member

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Abstract

Aneuploidy, or abnormal number of chromosomes in a haploid set, in XY/XX organisms has consequences that can impede physical and cognitive development. To prevent aneuploidy, cellular division relies on the correct position of paired chromosomes and subsequent segregation in the meiotic program. If connections between paired chromosomes sever or errors in chromosome contraction towards spindle poles arise, aneuploidy occurs. Many arthropod species have chromosomes that naturally do not pair, or that pair differently than typical autosomes. Through the evaluation of such systems, novel insights into chromosomal coordination and positioning may be revealed. The objective of this study is twofold: (1) investigate univalent X chromosome behavior in the two-striped planthopper *Acanalonia bivittata* (X0/XX) and (2) explore a sex trivalent model (XXY/XX) in the giant shield mantis *Rhombodera megaera*. Here, we will investigate both systems using live-cell and immunofluorescence imaging to reveal mechanisms regulating chromosome coordination and segregation to further understand translational implications across all species.

Chapter 1: Introduction

Chromosomal pairings and movement are highly regulated throughout cell division. Human disorders characterizing congenital developmental delay are often associated with the failure of such coordinated events during meiosis. To further elucidate the mechanisms driving viable gametic cell division, free of adverse phenotypes, model arthropod organisms with characterized atypical chromosome configurations can be evaluated.

Meiosis generates haploid gametes from diploid precursors (Figure 1). During this specialized cell division, chromosomes must locate and recognize their homologous pairing partners to ensure the proper contribution of genetic material. The first meiotic division creates two haploid daughter cells from which homologous chromosomes are separated. Meiosis I is initiated as pairs of homologous chromosomes, each comprised of two sets of sister chromatids, condense and recombine with one another to form a bivalent tetrad during prophase I. The bivalent, held together by a cohesin complex in addition to the chiasma of at least one DNA cross over, allows for future alignment and segregation of the chromosomes (Moore and Orr-Weaver 1997, Paliulis and Nicklas 2000). Later in prophase I, a spindle apparatus comprised of microtubules is formed from the centrosome. Each cell has two spindle poles, while each chromosome has two opposite facing kinetochore attachment points. As kinetochores face in opposite directions, one chromosome will associate with microtubules from one pole, while its partner will associate with connections from the other pole. After the nuclear envelope disintegrates, the bivalents migrate toward the metaphase plate under tension from the

mitotic spindle. The positioning of the partner kinetochores and partner chromosomes allow for correct alignment on the metaphase plate in metaphase I. During anaphase I, connections in each cohesion complex of the bivalent tetrads separate and allow for homologous chromosomes to be contracted towards their prospective spindle pole to which their kinetochore is attached (Figure 2). If connections between paired chromosomes are severed incorrectly, or contraction towards the spindle pole is incorrectly regulated, missing or extra chromosomes in the cell may result (Nicklas 1997). Telophase I marks the decondensation of chromosomes and formation of the nuclear envelope around each set of chromosomes. Once independent nuclei form, cell division, or cytokinesis, ensues to form daughter cells. The second meiotic division is characterized by the formation of four haploid gametes from the sister chromatids within the two daughter cells which completes the meiotic program.

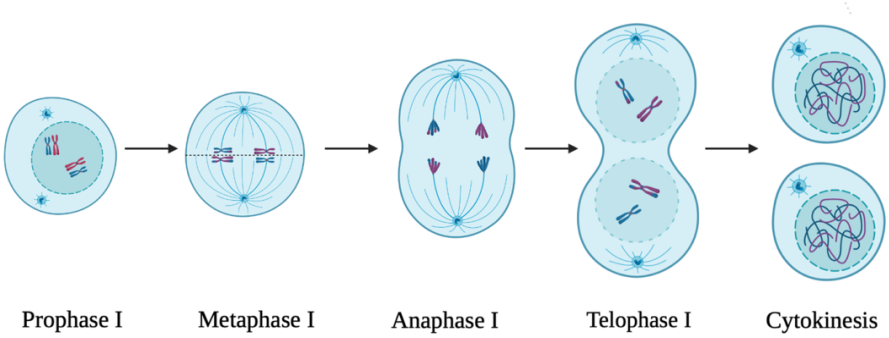

Figure 1: Meiosis I. Prior to meiosis I, DNA replication occurs. Prophase I is comprised of paired homologs physically linked. Homologous chromosomes recombine and form bivalent tetrads. A spindle apparatus forms in late prophase I. Early metaphase or late prophase is marked by the disintegration of the nuclear envelope and formation of microtubules that attach to the kinetochores of the chromosome. In metaphase I, bivalents move to the center of the spindle. Homologues are attached to opposite spindle poles. In anaphase I, spindle fibers contract and split the bivalents, and homologous chromosomes move to opposite poles of the cell. Finally, the cell begins to divide in telophase I until cytokinesis, or full cellular division.

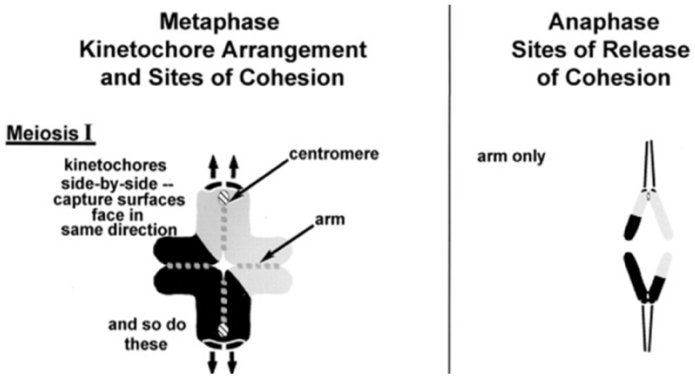

Figure 2: Chromosome attachment to spindle poles is mediated by connections between kinetochores and chromosome cohesin. In meiosis I, homologous chromosomes, each with a pair of sister chromosomes are connected. Kinetochores orient towards spindle poles in a direction that opposes that of the opposing sister chromatids in metaphase I. In anaphase I, sister chromatids move to opposite poles and are held together by cohesin present along the chromosome arm and between centromeres. Cohesin is released during homologue separation. (Reprinted from Paliulis and Nicklas, 2000. Originally published in *The Journal of Cell Biology*. 150:1223-1231).

Connection between homologous chromosomes from cohesin and/or chiasmata cross-over points aid in regulating chromosome movement throughout meiosis. Errors in the release of the cohesin protein complex or connection to the spindle apparatus during anaphase I can result in aneuploidy, or abnormal chromosome number in a haploid set. Notable human conditions of aneuploidy include Down syndrome (trisomy-21), Patau syndrome (trisomy-13), Edwards syndrome (trisomy-18), Turner syndrome (monosomy of X sex chromosome in females), and Klinefelter syndrome (trisomy of Y chromosome in males). These syndromes range in severity impacting both physiological and cognitive development. Aneuploidy presents many health consequences and, thus, drives a need to

further investigate the mechanisms regulating the coordination of chromosomes during cellular division.

Interestingly, in some species, non-homologues chromosomes can have coordinated movements and progress through the meiotic program with little to no consequence. While the formation of bivalent tetrads is usually essential for proper alignment and subsequent segregation in humans and other XY/XX organisms, this method of chromosome reduction is not always necessary, providing the opportunity to reveal subtle contributors in chromosome distribution across other species. This study examined these unusual cases by exploring the behavior of (1) segregation of the univalent X chromosome in the two-striped planthopper *Acanalonia bivittata* (X0 instead of XY in males) and (2) meiotic sex trivalent in the giant shield mantis *Rhomodera megaera* (XXY in males). Each case provides insight into the mechanisms that allow for proper positioning and subsequent segregation of chromosomes.

Chapter 2: Segregation of the univalent X Chromosome in the two-striped planthopper *Acanalonia bivittata*

Introduction

The aim of meiosis is to create haploid gametes from a diploid parent cell. Success of this reduction of chromosome number requires that while in prophase I, chromosomes (each composed of a replicated pair of sister chromatids) pair with their homologues to form a bivalent tetrad. The bivalent tetrad proceeds to undergo two rounds of cell division: in anaphase I separating homologues from one another, and then in anaphase II separating sister chromatids from one another. The two meiotic divisions lead to the production of four haploid gametes, each with a single chromatid of each chromosome.

Formation of bivalents is required for the reduction of chromosome number—homologues can separate correctly from one another in anaphase I as they were previously connected. While formation of bivalents during meiosis is essential to the formation of gametes with the correct number of chromosomes, there are interesting exceptions; that is, cases where one or more chromosomes in a cell do not form a bivalent. Here we study one example, the two-striped planthopper *Acanalonia bivittata* (Hemiptera, Auchenorrhyncha), in which the X chromosome in the male does not have a pairing partner and remains univalent throughout meiosis I.

Univalent X chromosomes have unique behaviors in different species contexts, as described in detail by Fabig *et al.* (2016). Univalent X chromosomes, with their two sister

chromatids and two sister kinetochores can either form an amphitelic attachment to the spindle and align with the other chromosomes at the metaphase I plate (Fabig *et al.* 2016, Brady and Paliulis 2015, Schrader 1935), or they can form a syntelic attachment to the spindle and associate with just one spindle pole (Fabig *et al.* 2016, Ault 1984). Those univalent X chromosomes that form amphitelic attachments, with one kinetochore facing one pole and its sister facing the other pole, can have different behaviors depending on the species to which they belong. Schrader (1935) studied the univalent X chromosome in the broad-headed bug *Protenor belfragei* (Hemiptera, Heteroptera). *Protenor belfragei* have holocentric chromosomes and a univalent X chromosome that forms an amphitelic attachment to the spindle in metaphase I. In anaphase I, the sister chromatids separate from one another. Similar behavior of a univalent X chromosome in a species with holocentric chromosomes has been observed in other true bugs, and in the nematode worm, *Rhabditis* sp. (Rhabditida) (Fabig *et al.* 2016, Shakes *et al.* 2011). In turn, in other Hemipteran insects, including some aphids, most of the studied members of the suborder Auchenorrhyncha (a small number of species have a neo-XY system), and in the nematode worm *Caenorhabditis elegans* (Rhabditida), all of which have holocentric chromosomes in mitosis, the univalent X chromosome first aligns on the metaphase I plate with sister kinetochores associated with opposite poles, and then does not separate sister chromatids, but moves intact to one spindle pole (Blackman 1985, Kuznetsova *et al.* 2013, Maryańska-Nadachowska *et al.* 2012, Felt *et al.* 2022).

Several members of Hemiptera, suborder Auchenorrhyncha, have been studied. Early work in one member of suborder Auchenorrhyncha, the spittle bug *Philaenus*

spumarius, revealed the univalent X chromosome remained undivided and progressed to one spindle pole (Boring 1913). A subsequent report studying the same species showed that the univalent X chromosome aligns at the metaphase plate with all the bivalents in meiosis I (Felt *et al.* 2017). In anaphase I, the homologous chromosomes of each bivalent separate from one another and move toward their associated spindle pole. The univalent X chromosome remains at the spindle midzone until all the autosomal homologues have approached their associated spindle pole, and then initiates movement toward one spindle pole. The movement is associated with the loss of microtubule connections to the spindle pole from which the X chromosome is moving away (Felt *et al.* 2017).

This report aims to expand upon previous work by detailing the behavior of the X chromosome in another member of order Hemiptera suborder Auchenorryncha, the two-striped planthopper *Acanalonia bivittata*. Previous studies reported the chromosome number and sex determining mechanism for *A. bivittata* (Boring 1907, Maryańska-Nadachowska *et al.* 2013), however behavior of the univalent X chromosome in living cells and reasons for that behavior remained to be elucidated. Here we confirm previously reported karyotype and sex determination mechanism for *A. bivittata*. We use live cell-imaging and immunofluorescence staining to reveal that *A. bivittata* spermatocytes display independent and delayed segregation similar to previous reports of behavior of the univalent X chromosome in the spittlebug *Philaenus spumarius*. We show that the ultimate segregation of the X chromosome to one spindle pole in *A. bivittata* is due to loss of microtubule connections to one pole and retention of connections to the opposite pole.

Materials and Methods

Collection and Identification

Mature *Acanalonia bivittata* were collected from a field site at the Bucknell University Farm (Lewisburg, PA). Planthoppers were identified by their green body (length ~6.6 mm) with two prominent brown stripes that extend dorsally from behind the eye and along the lateral margins of the thorax. Males were sexed best by identifying an outward slanting pygofer (Freund and Wilson 1995).

DNA Barcoding

DNA barcoding was completed as described in the BIO RAD Explorer DNA Barcoding Kit (catalog #17007432EDU). The amplification of cytochrome c oxidase subunit 1 was performed using the primers supplied by the BIO RAD. Sequencing was conducted at Genewiz (South Plainfield, NJ) using M13 reverse and M13-20 forward sequencing primers. The provided sequence was analyzed using the Sequencher v5.4.6 and trimmed to 632 bp. Alignments were produced using ClustalOmega (https://www.ebi.ac.uk/Tools/msa/clustalo/). A source sequence was referenced for final alignment (Gwiazdowski *et al*. 2015).

Orcein Staining of Spread Chromosomes

Acanalonia bivittata testes were fixed in a 6:3:1 ratio of ethanol:chloroform:acetic acid mixture. After 10 min, the testes were macerated on a microscope slide submerged with 60% acetic acid. A cell suspension was produced, spread onto a slide, and placed onto a slide dryer at 45°C until dried. Chromosomes were stained with aceto-orcein for

10 min, rinsed in distilled water, mounted in glycerol, and observed using a Nikon inverted phase contrast microscope (Felt *et al.* 2017).

Living Cell Preparations

Acanalonia bivittata testes were removed and submerged into a culture chamber containing a layer of Kel-F Oil #10 (Ohio Valley Specialty Company, Marietta, Ohio). Using lab forceps, spermatocytes were spread on a coverslip under a thin layer of oil. Live-cell imaging of the spermatocytes during meiosis I was completed using a Zeiss inverted microscope outfitted with a 100X, 1.25 NA phase-contrast oil immersion objective, an Infinity 1 camera, and Infinity Analyze software, or with a Nikon Eclipse TS 100 outfitted with a 100X 1.25 NA phase-contrast oil-immersion objective and a Spot RT monochrome camera (Diagnostic Instruments Inc.) with Spot Basic 3.5.7 software. Adobe Photoshop CC was used to take advantage of the full range of pixel values and to crop and rotate images (Lin *et al*. 2018).

Immunofluorescence

Testes of *A. bivitatta* were removed from the abdomen and contents were spread under a thin layer of Kel-F Oil #10. Living cells at different stages of meiosis were fixed following application of 4% glutaraldehyde in PEM buffer (80 mM PIPES pH 6.9, 2 mM $MgCl_2$, and 0.5 mM EGTA) directly adjacent to the cell of interest with a glass microneedle, bathing the cell with fixative. Following a 3 min incubation, the oil covering the cells was removed and secondarily fixed in a solution of 1% glutaraldehyde

in PEM buffer for 15 min (Nicklas *et al*. 1979). Preparations were rinsed in 1x PBS (Phosphate Buffered Saline) and the cells were then permeabilized in a lysis buffer of 3% Nonidet P-40, 2% Triton-X, 2% saponin, 6% DMSO, and 0.5% sodium deoxycholate for 30 min followed by wash in 1x PBS. Samples were incubated in 0.02g $NaBH_4$ for 15 min, followed by a 30 min incubation at 37 °C in a blocking solution of 5% fetal bovine serum/0.2% Tween 20/1x PBS. Cells were stained for 24 h at room temperature in TU-27 (anti-β tubulin) diluted 1:25 with 1% BSA (Bovine Serum Albumen) in 1x PBS. Following incubation, samples were washed in a 1% BSA/PBS solution and labeled with Cy3-conjugated goat anti-mouse IgG diluted 1:25 with 1% BSA/PBS. After an additional wash in 1% BSA/PBS, cells were rinsed in distilled water, mounted onto a glass slide with Vectashield, and sealed using nail polish along the edges of the coverslips (Felt *et al.* 2017). At least 10 cells from metaphase I, early anaphase I, late anaphase I, and telophase I were imaged. These specimens came from 20 individual planthoppers. Imaging of stained cells was done as follows: stained specimens were viewed using a 100X, 1.4 NA oil-immersion objective on a Leica SP5 Spectral Systems confocal microscope. Z-stacks were collected with 0.25 μm intervals. Images were rotated, cropped to a fixed size, colored, and merged using Adobe Photoshop CC (King and Nicklas 2000, Felt *et al.* 2017).

Results

DNA Barcoding

Primary identification of living insects as *Acanalonia bivittata* was executed through examination of morphological characteristics as described in Freund and Wilson (1995). This identification was confirmed by performing a DNA barcoding analysis of tissue from those specimens. The sequence of one specimen was submitted to Genbank (sequence accession number is OM986830). The partial COI gene sequences were analyzed using blastn, with default parameters which identified two sequences: one associated with MG398956.1 and a second associated with KR032540.1. The full sequence of OM986830 was used in ClustalOmega to recreate the alignment (Figure 3). The OM986830 isolate was 99.37% identical to MG398956.1 and 99.24% identical to KR032540.1 (Figure 3). This level of identity is consistent with the isolates belonging to members of the same species, as a blastn search using default parameters of the COI sequence reveals a range between 99% and 100% identity between all the 59 specimens identified as *Acanalonia bivittata*. No identified specimens of any other species have identity >84%, supporting that our identification of the specimen based on morphological characteristics is correct.

Figure 3. ClustalOmega alignment of cytochrome oxidase 1 gene in *Acanalonia bivittata*. The first and last two sequences represent specimens with the closest identity to our specimen according to blastn analysis. Our specimen, OM986830, is 99.37% identical to sequence MG398956.1 and 99.24% identical to sequence KR032540. Asterisks indicate areas of similarity across the sequences and are associated with residues that are highly conserved. Differences among the aligned sequences are highlighted in yellow. Numbers to the far right of the sequences represent nucleotide position.

Karyotype Analysis

Acanalonia bivittata spermatocytes were observed in prophase, prometaphase, and metaphase of meiosis I to determine chromosome number and sex determination mechanism. Testes contents from 15 individuals were used to determine chromosome structure and number. *Acanalonia bivittata* chromosomes contain 2*n*=25 chromosomes in males; 12 bivalent chromosomes and one univalent chromosome, which we have identified as the X chromosome based on previous work by Boring (1907) and Maryańska-Nadachowska *et al*. (2013) (Figure 4).

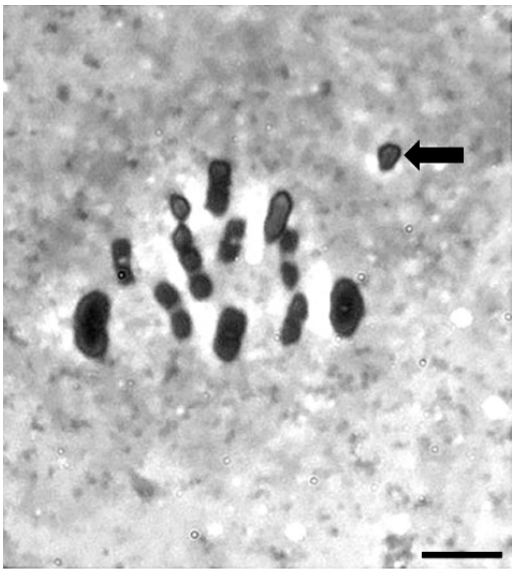

Figure 4. *Acanalonia bivittata* orcein-stained chromosome spread produced from a meiosis I prometaphase spermatocyte. 12 bivalents are presented in the spread. The arrow indicates the univalent X chromosome. Bar = 5 μm.

Sex Determination and Sex Chromosome Behavior

Live-cell imaging of *A. bivittata* revealed that the autosomal bivalents and the univalent X chromosome align on the metaphase plate during metaphase I (Figure 5, 0 min.). Interestingly, in all (>50) observed examples (both living and fixed) of metaphase I spermatocytes, the univalent X chromosome was located at the periphery of the spindle (i.e. off to one side of the spindle). At anaphase I onset, half-bivalents separate from one another while the X univalent remains at the spindle midzone (Figure 5, 10, 20 min.), as observed in 10 living cells. The X univalent then initiates movement toward one spindle pole, after all of the half-bivalents have separated from their partners. This was observed in five examples of living cells that could be followed from metaphase I through late anaphase I.

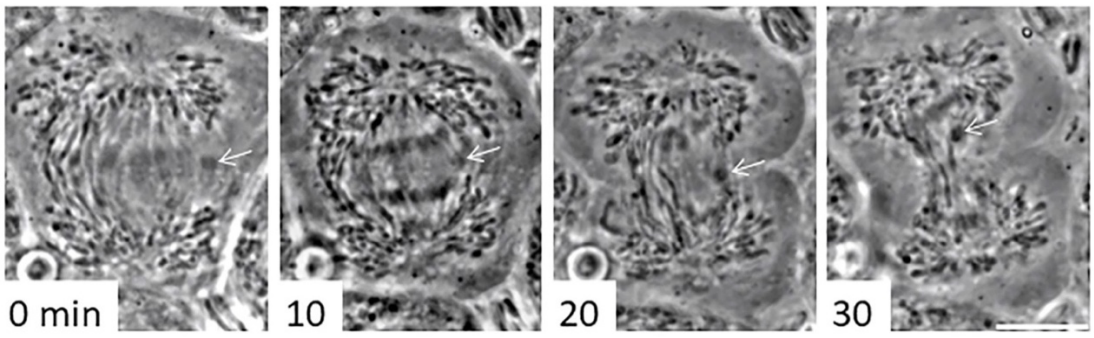

Figure 5. Metaphase I-late anaphase I in a meiosis I spermatocyte of *Acanalonia bivittata*. In metaphase I, the autosomal bivalents and the univalent X chromosome (arrow) align on the metaphase plate (0 min). At anaphase I onset, autosomal half bivalents begin to separate from their homologues (10 min), but the univalent X chromosome (arrow) remains at the spindle midzone until the autosomal half bivalents are well separated (20 min). The univalent X chromosome (arrow) eventually initiates movement toward one spindle pole (30 min). Bar =10 μm.

Immunofluorescence of *A. bivittata* revealed that the ultimate segregation of the X chromosome to one spindle pole was due to loss of microtubule connections during the first meiotic division (Figure 6). Microtubule connections from the X chromosome to both spindle poles were present in both prometaphase and metaphase. The X chromosome remained at the periphery of the spindle through metaphase I (Figure 6A-B). Immunofluorescence images of anaphase I cells revealed that, in early anaphase I cells there were connections to both spindle poles (Figure 6C) but in late anaphase I cells, the X univalent only had microtubule connections to one spindle pole (Figure 6D-F). Observations made from the immunofluorescence images reveals the eventual loss of microtubule connections to one side of the spindle leads to segregation of the X univalent towards the side with retained microtubule connections. Furthermore, we also observed that the X univalent was separated from the autosomes in the spindle periphery in metaphase I and early anaphase I (Figure 6B-E) but moved toward the central spindle in later anaphase I (Figure 6F). After segregation was complete, cleavage furrow ingression occurred as the X chromosome was located near the pole with the retained microtubule connections (Figure 6F).

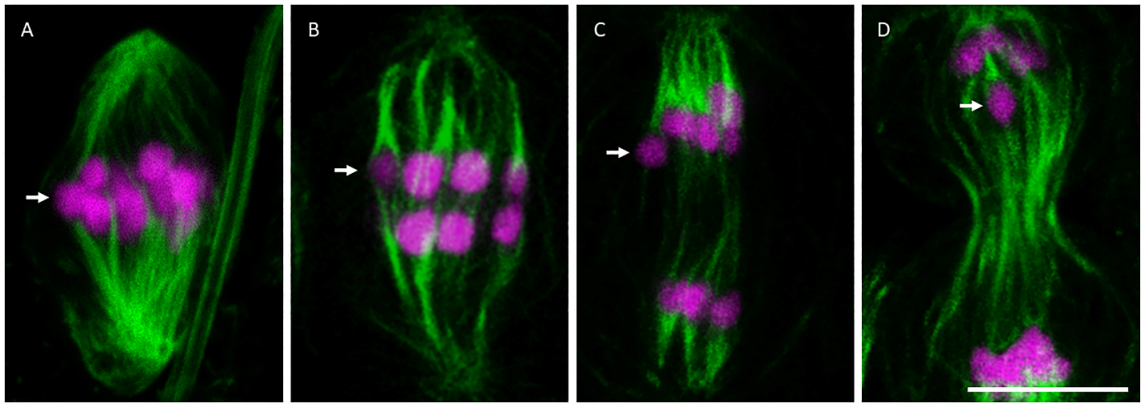

Figure 6. Independent and delayed segregation of X chromosome in *Acanalonia bivittata* results from attachment to a peripheral spindle and loss of microtubule connections. Immunofluorescence staining of microtubules (green) and DAPI staining (magenta) depict *A. bivittata* spermatocytes in the first meiotic division throughout the stages of metaphase (Figure 6A), early anaphase (Figure 6B), late anaphase (Figure 6C), and telophase (Figure 6D). The arrow tracks the X chromosome. Bar =7.5 μm.

Discussion

Results from the study confirm previously observed chromosome numbers and sex determining mechanisms in *Acanalonia bivittata* (Boring 1907, Maryańska-Nadachowska *et al.* 2013).

Live-cell imaging and immunofluorescence in *A. bivittata* has revealed that autosomal bivalents and the univalent X chromosome align on the metaphase plate during meiosis I (Figure 5, 0 min; Figure 6A). The univalent X chromosome appears to have a bipolar attachment to the spindle and resides in the periphery of the spindle (Figure 6A-B). After the separation of the half bivalents from their partners during anaphase I, the univalent X chromosome begins to move towards one spindle pole (Figure 5, 10, 20 min; Figure 6 C). Delayed movement of the univalent X chromosome may be associated with

its peripheral position along the metaphase plate and could reside in a separate spindle compartment as observed in the spermatocytes of the flea beetle *Alagoasa* (Oedionychus) (Wilson *et al.* 2003). Separation between univalent X chromosomes and autosomes may be due to a mitochondrial sheath that allows for microtubule connections beyond the central spindle. By studying microtubule and kinetochore interactions, Wilson *et al.* (2003) observed the attachment of sex chromosomes to a mitochondrial spindle which resulted in sex chromosome movement only during anaphase due to spindle elongation. These findings characterize chromosome behavior that may be reflected in *A. bivittata*. Regardless of the mechanism behind the delayed movement, it is evident that *A. bivittata* univalent X chromosomes experience a loss is microtubule connections to one spindle pole as anaphase progresses (Figure 6D).

The progressive loss of microtubule connections beginning in early anaphase in *A. bivittata* may be the result from the several different protein interactions. We propose Kif18A, a Kinesin-8 family microtubule depolymerase, as a candidate protein associated with the controlled degradation of microtubule connections on one side of the univalent X chromosome. Cells deficient in Kif18A undergo a prolonged period of prometaphase characterized with oscillating chromosomes (Mayr *et al.* 2007, Stumpff *et al.* 2007). Amplitudes of these chromosomes are proportional to Kif18A concentration (Stumpff *et al.* 2007). Furthermore, deficits of this protein have been linked to failed alignment at the metaphase plate, lagging chromosomes in anaphase, loss in tension across siter kinetochores, and uncoordinated kinetochore movement (Mayr *et al.* 2007; Stumpff *et al.* 2007). Differences in Kif18A activity on microtubules on opposite sides of the univalent

X chromosome could contribute to the molecular mechanism for the microtubule loss *A. bivittata* demonstrates in early anaphase.

In addition, the odd behavior of the univalent X may be explained by chromatin modifications. *Caenorhabditis elegans* has a univalent X chromosome in males that forms an amphitelic attachment to the spindle in metaphase I and moves intact toward one spindle pole after all the autosomes have segregated. The univalent X chromosome has a specific imprint (methylation of Histone H3 at Lys9) which is associated with silencing (Bean *et al.* 2004). Such imprints and silencing are associated with an absence of checkpoint signaling (Jaramillo-Lambert and Engebrecht 2010). It is possible that these imprints also impact attachment and segregation of a univalent X chromosome and could be an area of future experimentation.

Delayed segregation by univalent X chromosomes, relative to autosomes, is also demonstrated in other primary spermatocytes of hemipteran insects like several species of the family Cicadellidae in addition to the primary spermatocytes of male *Caenorhabditis elegans* (Fabig *et al.* 2016, John and Claridge 1974, Halkka 1959, Kuznetsova *et al.* 2013, Rebollo *et al.* 1998, Rebollo and Arana 1998). A prevalent characteristic of hemipteran insects and *C. elegans* is holocentric chromosomes in mitosis. Hemipterans, like *A. bivittata*, can experience restricted kinetochore activity that influences the behavior of bivalents and that can impact chromosome segregation (Fabig *et al.* 2016, Melters *et al.* 2012). Our previous examination of univalent behavior in other systems has revealed that many systems that contain holocentric chromosomes and undergo non-

inverted meiosis have similar patterns of sex chromosome segregation as *A. bivittata* and members of other phyla (Fabig *et al.* 2016; Felt *et al.* 2017, Fabig *et al.* 2020, Felt *et al.* 2022). Univalent segregation behavior appears to be regulated by meiotic system and not phylogenic relatedness.

Together, the results from this study corroborated with established knowledge within suborder Auchenorrhyncha and revealed novel univalent chromosome behavior within *A. bivittata*. This study confirmed the previously published chromosome number and sex determination mechanism of the two-striped planthopper *A. bivittata* (Boring 1907, Maryańska-Nadachowska *et al.* 2013). Furthermore, we have also revealed that the univalent X chromosome aligns at the metaphase plate, peripheral to the bivalent autosomes, forming a bipolar attachment to the spindle. Finally, we illustrate that the univalent X chromosome maintains microtubule connections to one spindle pole while losing connections to the opposite pole. The mechanistic underpinning of delayed movement of the univalent and cause of microtubule loss throughout anaphase remain an area of future investigation. Translational implications of this study have high relevance in organisms that have a lost chromosome pairing partner, or the diminution and potential loss of a Y chromosome in mammals.

Chapter 3: Chromosome number, sex determination, and meiotic sex trivalent behavior in giant shield mantis *Rhombodera megaera*

Introduction

Meiosis is a fundamental process for successful gamete formation and sexual reproduction. Successive events, such as the formation of bivalents (pairing of two homologous chromosomes, each composed of two sister chromatids), followed by two rounds of division separating homologues in anaphase I and sister chromatids in anaphase II, ensure the correct reduction of chromosome number. Failure of subsequent chromosome separation, or non-disjunction, leads to the duplication, loss, or other change in chromosome number (Nicklas 1997).

In general, bivalent formation is required for successful meiotic completion; however, unusual combinations of chromosomes occur routinely and lead to correct segregation for that organism. One notable example is the giant shield mantis *Rhombodera megaera* (Mantodea, Mantidea). Germ cells of males of this species have two X chromosomes (X_1 and X_2) and a Y chromosome that combine to form a sex trivalent (Figure 7). Each of the three chromosomes possess individual kinetochores where the two X chromosomes face one spindle pole and the one Y chromosome faces the opposing spindle pole (Hughes-Schader 1943, Hughes-Schader 1950). Thus, the configuration of the sex trivalent proposes an interesting question regarding how it can successfully navigate the meiotic program given seemingly unequal connections to spindle poles.

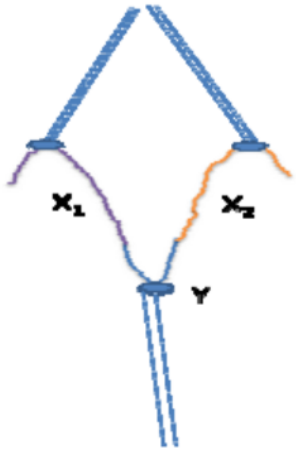

Figure 7. Chromosome arrangement of the sex trivalent. The sex trivalent is composed of three chromosomes, X_1 (purple), X_2 (orange) and Y (blue). Chromosomes possess individual kinetochores. X_1 and X_2 face one spindle pole and Y faces the opposing spindle pole. Reprinted from Felt (2017), Bucknell thesis.

Little is known about the behavior of naturally occurring trivalents. Sex trivalents have been documented across phylogenic kingdoms in plants and arthropods (Ostergren 1945, Hughes-Schader 1950, Nicklas and Arana 1992, Sember *et al*. 2020, Král *et al*. 2006, Král *et al*. 2022); however, evaluation of the mechanisms that dictate chromosome alignment and segregation have been limited. In most organisms in which meiosis I has been studied, chromosome alignment along the metaphase plate is required to prepare for correct chromosomal segregation (Gorbsky 2015, Potapova and Gorbsky 2017). It has been proposed that bipolar kinetochore connections between homologous chromosomes and complementary spindle poles aid in the alignment and subsequent distribution of chromosomes through the regulation of balanced forces (Fabig *et al*. 2020). The configuration of the sex trivalent, with three kinetochore attachment sites, initially

appears to contradict this mechanism of segregation. Published work characterizing sex trivalents in praying mantids has only begun to evaluate the alignment of chromosomes and the potential balance of forces on chromosomes in metaphase I and anaphase I (Paliulis *et al*. 2022). With a previously established sex-determining system of $X_1X_1X_2X_2/X_1X_2Y$ (White 1941, Hughes-Schrader 1950, Hughes-Schrader 1943), Nicklas and Arana (1992) found, within fixed stained specimens, that sex trivalents in many mantid species do align alongside autosomes in metaphase. Nicklas and Arana (1992) further proposed that metaphase alignment may be a regulatory step for successive, successful, segregation in anaphase I. Furthermore, studies evaluating the praying mantid *Hierodula membranacea* (Mantodea, Mantidea) revealed that not only does the sex trivalent align with autosomes, but it also simultaneously segregates alongside them during anaphase I as expected in bivalent configurations (Paliulis *et al*. 2022). Despite the additional pairing partner of the sex trivalent, progression through the meiotic program occurs unhindered, and metaphase alignment appears to have a functional significance likely regulated by balanced forces.

The unique configuration of the sex trivalent offers the opportunity to study the meiotic mechanisms regulating the position of chromosomes during metaphase I and segregation of chromosomes during anaphase I. This report aims to expand previous knowledge of sex trivalents in the praying mantid species *Rhombodera megaera* by quantifying these mechanisms. Here, we uncover a $2n=27$ karyotype for this species. Live-cell imaging reveals that sex trivalents align with autosomes in metaphase I in this species, and that sex trivalent segregation occurs concurrently with autosome segregation.

Quantification of immunofluorescence staining suggests that the alignment of the sex trivalent in metaphase is due to balanced microtubule connections, identifying a regulatory step that ensures proper, subsequent, distribution of chromosomes. This evaluation leads to a deeper understanding of the forces that regulate metaphase alignment that occurs in many species and these results may reveal translational implications associated with congenital aneuploidies that arise in mammals.

Materials and Methods

Collection and Identification

Individuals of *Rhombodera megaera* were obtained from Arthropod Ambassadors. A colony was generated through mating adult giant shield mantids. The adults and progeny matched the traditional characteristics of an enlarged thorax resembling a leaf or shield like structure associated with the *Rhombodera megaera* species.

Orcein Staining of Chromosome Spreads

Rhombodera megaera testes were fixed in a 3:1 ratio of ethanol:acetic acid mixture. After 10 min, the testes macerated on a microscope slide submerged with 60% acetic acid. Cells were spread onto a slide and placed onto a slide dyer at 45°C until dried. Chromosomes were stained for 10 minutes in 3% Giemsa stain. Following a 3X5 min rinse in water, 20 µl of glycerol was added to mount the chromosomes. A cover slip was added to the slides, sealed using nail polish, and observed using a Nikon inverted phase contrast microscope (Felt *et al.* 2017).

Orcein Staining of Chromosome Squashes

Rhombodera megaera testes were removed and placed in a 3:1 ratio of ethanol:acetic acid fixative mixture for 10 minutes. After a 30 second rinse in distilled water, testes were transferred into aceto-orcein stain for 5 minutes and then onto a slide containing 45% acetic acid for testes distribution using laboratory forceps. Testis follicles were left in acetic acid for 30 sec. A coverslip was placed over top and was squashed using absorbent paper for assistance. Slides were sealed with nail polished and observed using a Nikon inverted phase contrast microscope once dried.

Living Cell Preparations

Rhombodera megaera testes were removed and submerged into a culture chamber containing a layer of Kel-F Oil #10 (Ohio Valley Specialty Company, Marietta, Ohio). Using lab forceps, spermatocytes were spread on a coverslip under a thin layer of oil. Live cell imaging of the spermatocytes during meiosis I was completed using a Nikon Eclipse TS100 inverted routine microscope (catalog #2CE-MQLH-6) outfitted with a View4K HD camera (S/N: c2109030031), and View4k software. Adobe Photoshop CC was used to take advantage of the full range of pixel values and to crop and rotate images (Lin *et al*. 2018).

Immunofluorescence Staining and Analysis

Testes of *R. megaera* were removed from the abdomen and contents were spread under a thin layer of Kel-F Oil #10. Cells were treated with 4% glutaraldehyde in PEM buffer (80 mM PIPES pH 6.9, 2 mM $MgCl_2$, and 0.5 mM EGTA) directly adjacent to living cells at different stages of meiosis with a glass microneedle for fixation. Following

a 3-minute incubation, oil converging the cells was removed and cells were given a secondary fixative solution of 1% glutaraldehyde in PEM buffer for 15 min (Nicklas *et al*. 1979). Prepared cells were rinsed in 1x PBS (Phosphate Buffered Saline) and permeabilized in a lysis buffer of 3% Nonidet P-40, 2% Triton-X, 2% saponin, 6% DMSO, and 0.5% sodium deoxycholate for 30 min followed by wash in 1x PBS. Samples were incubated in 0.02g $NaBH_4$ for 15 min, followed by a 30 min incubation at 37 °C in a blocking solution of 5% fetal bovine serum/0.2% Tween 20/1x PBS. Following incubations, cells were stained in TU-27 (anti-β tubulin) diluted 1:25 with 1% BSA (Bovine Serum Albumen) in 1x PBS for 24 h at room temperature. Cells were then washed in a 1% BSA/PBS solution and labeled with Cy3-conjugated goat anti-mouse IgG diluted 1:25 with 1% BSA/PBS. Cells were given an additional wash in 1% BSA/PBS, rinsed in distilled water, mounted onto a glass slide with Vectashield, and sealed using nail polish along the edges of the coverslips (Felt *et al.* 2017).

Analysis of Chromosome Microtubule Bundles

75 cells from metaphase I were imaged. Imaging of stained cells was preformed using a 100X, 1.4 NA oil-immersion objective on a Leica SP5 Spectral Systems confocal microscope. Z-stacks were collected with 0.25 μm intervals. Images were scaled, colored, and analyzed using Adobe Photoshop CC. Photos were rotated so that each microtubule bundle associated with each chromosome of the sex trivalent was vertically centered and changed to greyscale for integrated density analysis. A standardized pixel box with the dimensions of 2X5 was created for all measurements and placed 3 pixels above the tip of each trivalent chromosome to capture fluorescence intensity. Three measurements of

background intensity were taken for each cell and average background intensity was subtracted from trivalent measurements. Integrated density for Y, X1, X2, and autosome chromosomal microtubule bundles were averaged with standard deviations for 12 cells.

Quantification of Chromosome Position

Positions of cellular spindle poles were estimated by identifying the vertex of the cleared areas within cells. Using Adobe Photoshop CC, a line was drawn approximating the horizontal and vertical axes of the spindle pole. The distances between the chromosome edges (estimated kinetochore attachment sites) and associated spindle midline were measured for the chromosomes of the trivalent and two additional autosomes in the same plane of the cell. The ratio of these distances was calculated (Paliulis *et al*. 2022).

Results

Karyotype Analysis

Rhombodera megaera spermatocytes were observed in prophase, prometaphase, and metaphase of meiosis I to determine chromosome number and sex determination mechanism. Observed cells (>50) generated from three individuals were used to determine chromosome structure and number. *Rhomobodera megaera* have a chromosome number of $2n$=27 in males; 12 bivalent chromosomes and one sex trivalent containing an additional chromosome pairing partner (Figure 8). To support these findings, *R. megaera* spermatocytes were also observed in meiosis II (>20 observed cells). Male-determining displayed 13 sister chromatids (Figure 9A) while females displayed 14 (Figure 9B).

Together, these findings are consistent with previously reported karyotype analysis of the *Rhombodera* genus (Hughes-Schader 1950).

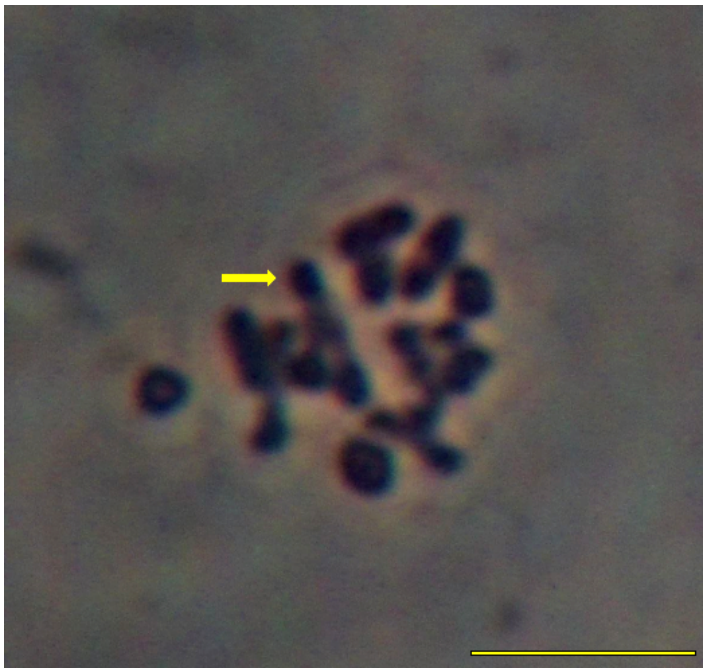

Figure 8. *Rhombodera megaera* orcein-stained chromosome spread produced from a meiosis I prophase spermatocyte. 2*n*=27 are presented in the spread. The arrow indicates the sex trivalent. Bar = 5 μm.

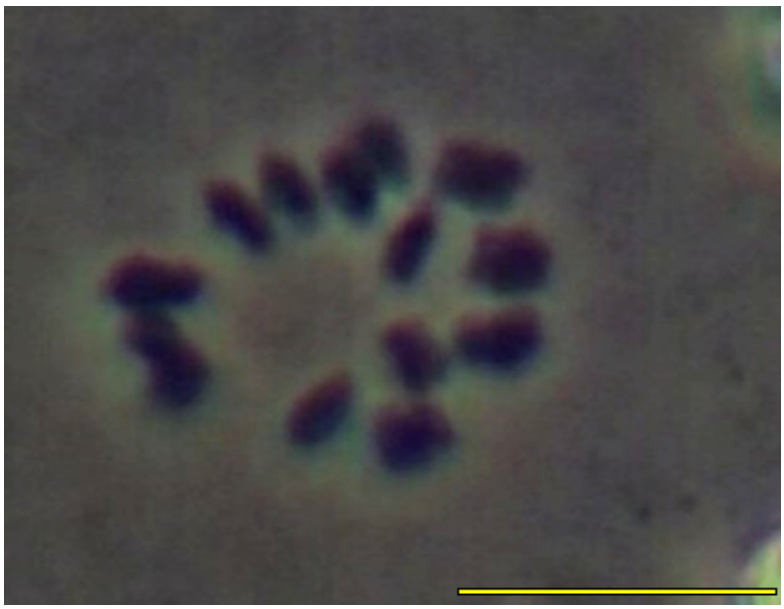

Figure 9A. *Rhombodera megaera* orcein-stained chromosome squash produced from meiosis II (Male). 13 sister chromatids (male) are presented in the spread. Bar = 5 μm.

Figure 9B. *Rhombodera megaera* orcein-stained chromosome squash produced from meiosis II (Female). 14 sister chromatids (female) are presented in the spread. Bar = 5 μm.

Sex Trivalent Behavior

Live-cell imaging of *R. megaera* revealed the alignment of autosomal bivalents and the sex trivalent on the metaphase plate during metaphase I (Figure 10, 0 min). Most observed samples (>75), both living and fixed, displayed the orientation of the sex trivalent in the center of the metaphase plate with autosomal bivalents flanking both sides, and alignment at the spindle midline with autosomes. Throughout early anaphase I and anaphase I onset, the sex trivalent and half bivalents segregated near simultaneously (Figure 10, 9, 12 min). The Y chromosome migrated towards one spindle pole while the two X chromosomes were pulled towards the opposing pole until anaphase I completion (Figure 10, 24 min). Sex trivalent chromosomal behavior was characterized by observing two living cells followed from metaphase I through late anaphase I.

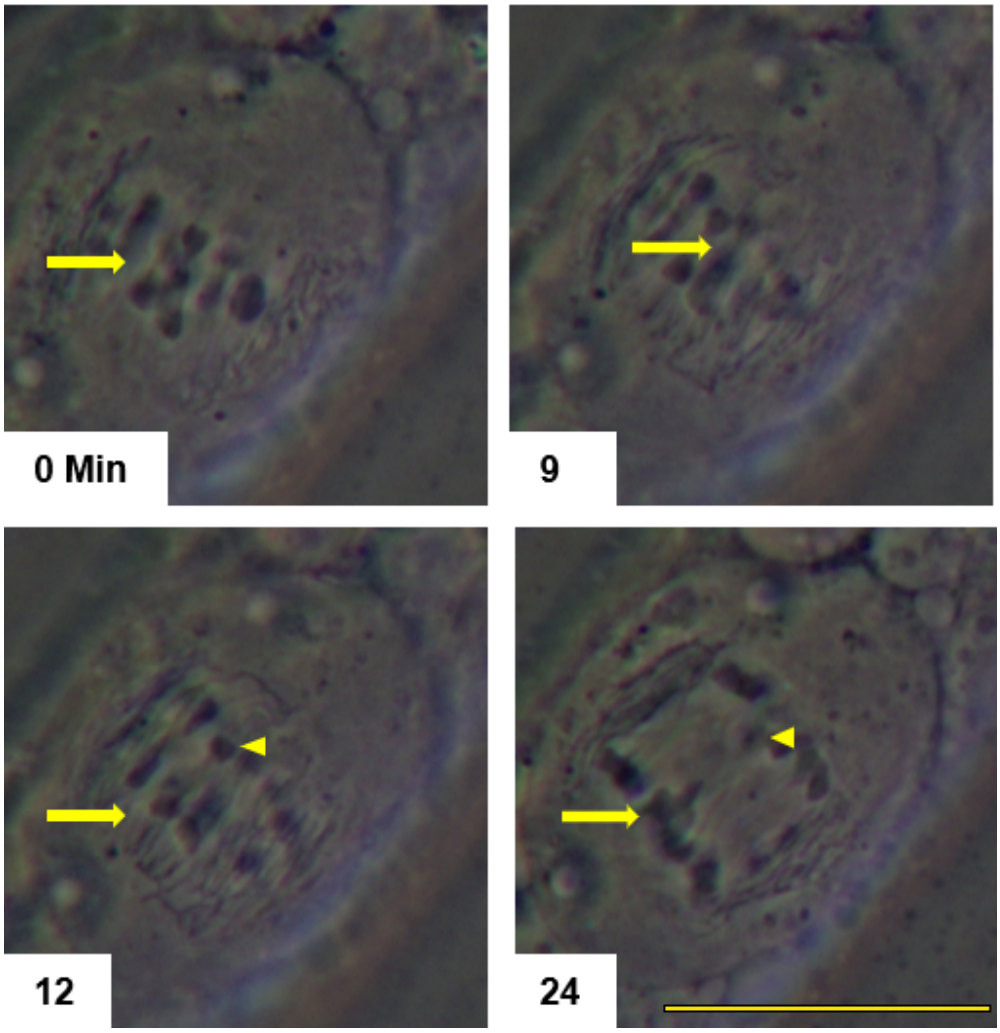

Figure 10. Progression throughout meiosis I metaphase to late anaphase in *Rhombodera megaera* spermatocyte. The sex trivalent (arrow) aligns on the metaphase plate with autosomes through metaphase up until early anaphase (0, 9 min). At anaphase onset (12 min), the sex trivalent Y chromosome (arrowhead) separates from the X_1 and X_2 chromosomes (arrow) alongside the segregation of autosomes towards respective spindle poles. The X_1 and X_2 chromosomes move towards one spindle pole opposite of the Y chromosome (arrow—12, 24 min). The sex trivalent fully segregates throughout late anaphase (24 min), displaying no abnormality to the meiotic program. Bar = 5 μm.

Analysis of *R. megaera* immunofluorescent stained cells in metaphase I found balanced alignment of the trivalent and autosomes along the metaphase plate. Alignment observations from both live-cell and immunofluorescence imaging were confirmed by quantifying distances between chromosome kinetochores and the metaphase plate as ratios. Trivalent chromosomes measured a ratio of 0.965±0.031 (calculated from n=12 observations) and autosomal bivalents measured a ratio of 1.027±0.044 (calculated from n=24 observations) indicative of balanced alignment (Figure 11, Table 1, p-value=0.383). Quantification of immunofluorescent cells also revealed that the sex trivalent Y chromosome bound twice as many microtubule bundles as one X chromosome in the trivalent, or approximately the sum of both X chromosome bundles combined together. Compared to autosomes, the sex trivalent bound similar amounts of microtubule bundles (Figure 12, Figure 13, p-value=5.674E-09).

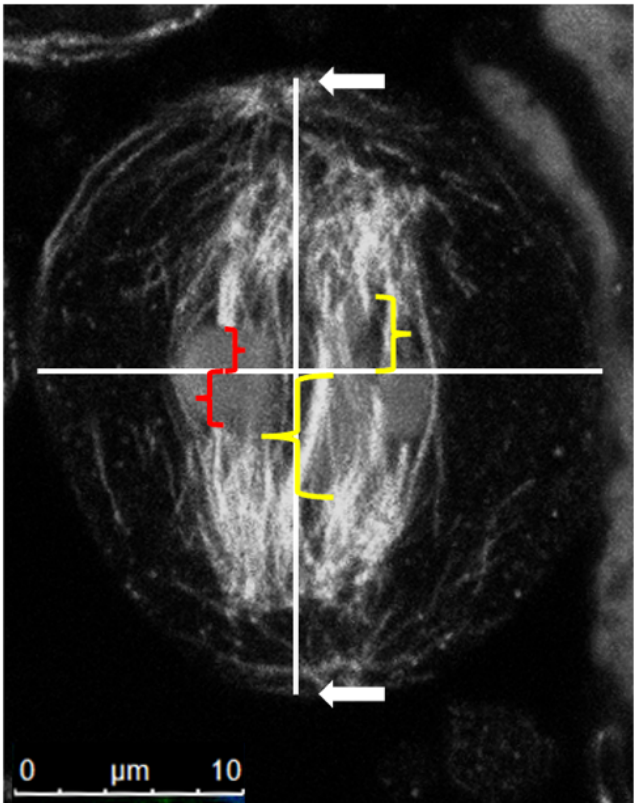

Figure 11. Analysis of the positioning of *Rhombodera megaera* sex trivalents in relation to autosomes in Metaphase I. A vertical line was drawn to estimate spindle poles (white arrows). A horizontal line was drawn to create a spindle axis. The distance of between the edges of trivalent (yellow brackets) and autosomes (red brackets) from kinetochore attachment to midline were measured in pixel length. The ratio of these distances was taken for 12 spermatocytes (*n*=12 trivalents; *n*=24 autosomes). Bar = 10 µm.

Ratio of Distance Between X Kinetochores and Center Line/ Distance between Y Kinetochores and Center line	Ratio of Distance Between Autosomal Kinetochore and Centerline/ Distance Between Partner Autosomal Kinetochore and Center Line
0.965±0.031 (*n*=12 trivalents)	1.027±0.044 (*n*=24 autosomes in 12 cells)

Table 1. *Rhombodera megaera* sex trivalents and autosomes align together along the metaphase plate. Distances between kinetochores and metaphase plate for both trivalents and autosomes reveal approximate ratios. A two-tailed t-test assuming equal variances was preformed to compare distance ratios between kinetochores and midline in sex trivalents and autosomes of *Rhombodera megaera.* The test revealed that there was no statistically significant difference in distance ratios (t-Stat=-0.884, df= 34, p-value=0.383). *n*=12 trivalent observations; *n*=24 autosome observations in 12 cells.

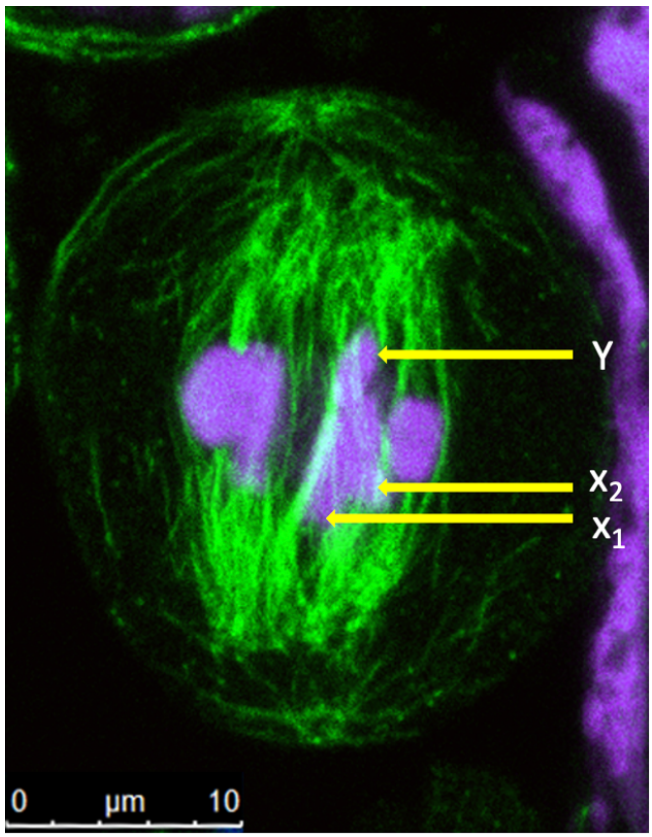

Figure 12. Immunofluorescence imaging of the *Rhombodera megaera* sex trivalent. Immunofluorescence staining of microtubules (green) and DAPI stained chromosomes (magenta) depict *R. megaera* spermatocytes in the first meiotic division in metaphase I. Components of the sex trivalent are labeled. Bar = 10 µm.

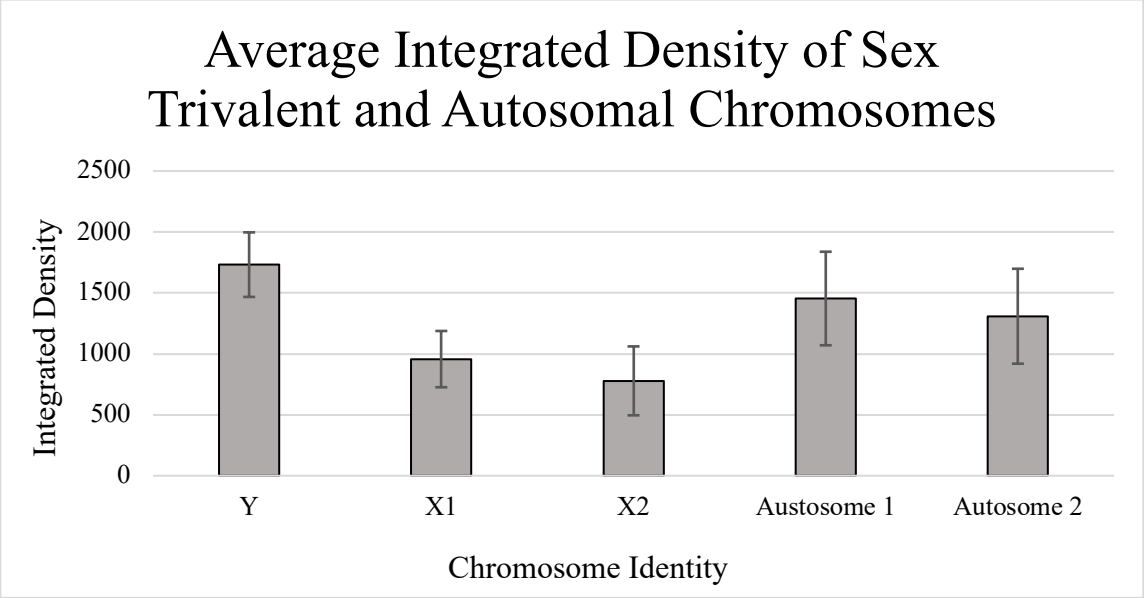

Figure 13. Average integrated density of sex trivalent and autosomal chromosomes in *Rhombodera megaera* spermatocytes reveal equal and balanced microtubule fluorescence intensities. Average integrated density of the Y chromosome microtubule bundle is comparable to those of autosomes in the same focal plane and is approximately twice the sum of its associated X chromosomes. A one-way ANOVA was preformed to compare the effect of the chromosome identity on the integrated density. The test revealed that there was a statistically significant difference between chromosomes (F-value=16.588, p-value=5.674E-09). *n*=12 trivalents observed; *n*=24 autosomes observed.

Discussion

This is the first description of the chromosome number and sex-determining system in *Rhombodera megaera*. The chromosomes of other members of the genus *Rhombodera* have been characterized by Hughes-Schrader (1950). Based on the characterizations of chromosome number within this genus, we have concluded a chromosome number of $2n$=27 in males with a X_1X_2Y (male)/$X_1X_1X_2X_2$(female) sex determination for this species.

Significance of the Alignment of the Sex Trivalent in Metaphase I

Chromosome alignment along the metaphase plate is a highly conserved phenomenon, suggesting it is an essential function in most cell types for chromosome segregation (Potapova and Gorbsky 2017). Mistakes during metaphase and anaphase I of meiosis can generate changes in chromosome content, producing aneuploid cells and abnormal expression of transcriptional regulators dictating development. The consequences of these perturbations often depend on which chromosomes are affected, but undoubtably influence physiological health. In mammalian models, improper chromosomal segregation is often associated with cancers, disrupted fertility, and human birth defects (Potapova and Gorbsky 2017). In mammals, chromosomal aneuploidies of somatic cells result in severe congenital diseases such as Down syndrome (trisomy 21), Edwards syndrome (trisomy 18), and Patau syndrome (trisomy 13). Sex chromosome aneuploidies like Turner syndrome and Klinefelter syndrome result in less severe phenotypes, yet still impair development and physiology. The study of abnormal chromosomal pairings in other models offer an opportunity to further understand human-health related consequences.

Within the mantid sex trivalent system, the significance of chromosome alignment has been considered through the evaluation of evolution by natural selection in several species of praying mantids. Nicklas and Arana (1992) observed the persistent congression of Mantidae trivalents along the metaphase plate and attributed that to the biological advantage of maintaining a quality-control mechanism that ensures proper spindle formation and chromosome orientation to ensure viable offspring. Indeed, when there is an

absence of correct alignment of Mantidae chromosomes, anaphase is blocked with cell degradation (Callan and Jacobs 1957, White 1965). This behavior is consistent with other systems reliant on precise chromosome arrangements in metaphase where loss of alignment leads to defects during anaphase (Stumpff *et al*. 2007, Czechanski *et al*. 2015, Fonseca *et al*. 2019).

Quantification of the Alignment of the Sex Trivalent in Metaphase I

Chromosome alignment along the metaphase plate for praying mantid species appears essential for the progression of the meiotic program, yet our understanding of the mechanisms regulating this process are limited. However, studies have shown the involvement of poleward forces to have a proportional relationship with the number of microtubule kinetochores present (Hays and Salmon 1990, Fabig *et al*. 2020). The sex trivalent proposes an interesting model to test this observation given one Y chromosome is associated to one spindle pole while two X chromosomes are associated with the opposing pole. Previous reports showed sex trivalent alignment on the metaphase plate; however, the alignment was only observed in fixed specimens, and spindle microtubules were not stained (Nicklas and Arana, 1992). Here, we show in living mantid spermatocytes that congression of all chromosomes to the metaphase plate occurs (Figure 10, 0min). Furthermore, this observation was quantified by creating a ratio that measured the distances between the X and Y kinetochores of the sex trivalent to the spindle midline and distances between autosomes to the spindle midline. The ratios of the sex trivalent (0.965±0.031) and autosomes (1.027±0.044) are approximately equal and suggestive of balanced alignment confirmed by statistical testing that did not find a difference between ratio

distances (Table 1). Following metaphase alignment, the Y chromosome and two X chromosomes of the sex trivalent segregated simultaneously with autosomes in anaphase I (Figure 10, 9, 12, 24 min) corroborating with previously published reports in varying mantid species (Li and Nicklas 1995, Paliulis *et al*. 2022).

Tension is generated through proper attachment between chromosomes and their associated spindle poles. It is known that tension plays an essential role in cell cycle progression (Li and Nicklas 1995) and that tension depends on the number of kinetochore microtubules (Hays and Salmon 1990). Hayes and Salmon (1990) identified that net poleward movement relied on both the number of kinetochore microtubules and distance from the pole. This work was expanded by King and Nicklas (2000) who found direct evidence of this in *Melanoplus* sanguinipes (Orthoptera, Caelifera). King and Nicklas (2000) micromanipulated spermatocytes and revealed that the release of tension at kinetochores decreased the number of kinetochore microtubules, while restoration increased microtubules back to their original number. Understanding kinetochore microtubule connections thus becomes essential to the evaluation of a regulatory step that directs proper attachment between chromosomes and their associated spindle poles. This current study evaluated such a regulatory mechanism using immunofluorescence staining of *R. megaera* cells in metaphase I for the first quantification of kinetochore microtubule bundles. Microtubule brightness, or integrated density, can relate proportionality of microtubule number and overall tension provided in poleward movement. Within the sex trivalent, the Y microtubule bundles appeared to have equitable tension compared to autosomes and approximately the sum of the two X chromosomes combined (Figure 13).

This quantification confirms the significance of metaphase alignment in this species that is regulated by balanced tension forces between chromosomes.

This study found a chromosome number of $2n=27$ and a X_1X_2Y (male)/$X_1X_1X_2X_2$(female) sex-determination mechanism of the praying mantid *Rhombodera megaera*. We demonstrate the sex trivalent alignment on the metaphase plate and segregation alongside autosomes throughout meiosis I using live-cell and immunofluorescence imaging. For the first time, immunofluorescence was used to quantify fluorescent microtubule bundles to assess microtubule strength with the goal to better understand metaphase alignment mechanisms. The Y chromosome of the sex trivalent displayed approximately the same amount of force as autosomes and about the sum of the two X chromosomes combined.

Conclusions

Studying the univalent X chromosome in the two-striped planthopper *Acanalonia bivittata* allowed for the confirmation of the sex determining system of X0 /XX, chromosome number, and characterization of univalency throughout meiosis I. Live-cell imaging and immunofluorescent stained cells from *A. bivittata* revealed the alignment of the univalent X chromosome alongside autosomes on the metaphase plate. The univalent X chromosome resided to the periphery of the cell and appeared to have bipolar attachment sites to the spindle during metaphase I that transitioned to a unipolar connection in anaphase I. Delayed univalent segregation was observed in anaphase I and was associated with the apparent loss of microtubules. The loss of microtubule connections has been proposed to be due to the result of several protein interactions. Kif18A, a Kinesin-8 family microtubule depolymerase, is a candidate protein correlated with degradation of microtubule connections. Studying proteins such as Kif18A in this species, or other organisms like *Caenorhabditis elegans* may confirm a molecular mechanism contributing to the lagging segregation displayed in *A. bivittata* in anaphase I. Other areas of possible investigation include the examination of chromosomal imprints (methylation of Histone H3 at Lys9) associated with delayed or erroneous checkpoint signaling impacting the attachment and segregation of chromosomes. Continued characterization of univalent behavior, and further understanding of the exact mechanism causing delayed segregation, may have implications for other organisms of this unique chromosome pairing, or within organisms where there is the potential loss of a Y chromosome.

Studying the giant shield mantis *Rhombodera megaera* with a X_1X_20/ $X_1X_1X_2X_2$ sex determining mechanism allowed for the evaluation of the meiotic mechanisms regulating the alignment of chromosomes during metaphase I. Chromosome alignment along the metaphase plate, for many species displaying bivalent chromosome configurations, is essential for progression through the meiotic program. The sex trivalent of *R. megaera* provided a unique model to evaluate the functional significance of alignment associated with the balanced of microtubule forces. Using live-cell and immunofluorescence imaging, sex trivalent positioning was documented and found to align on the metaphase plate and subsequent segregation occurred simultaneously alongside autosomes, indicative of equal partitioning of microtubule forces. To quantify this observation, immunofluorescent microtubule bundles, hypothesized to be proportional to microtubule strength, were analyzed. The Y chromosome microtubule bundles appeared to have equal strength compared to autosomes and were approximately twice the combined sum of the two X chromosomes. Results revealed insight into a mechanism regulating chromosome alignment, an orientation significant for proper chromosome distribution in many organisms.

Evaluation of unique chromosome pairings in different models offer insight on the varying regulatory steps that ensure proper chromosome segregation. Characterization of univalent chromosomes and trivalent pairings throughout meiosis I may produce translational implications with chromosome errors, or aneuploidies, that arise in human development. A congenital condition caused by the partial or complete loss of the X chromosome in women, analogous to a univalent chromosomal pairing, is a genetic

disorder referred to as Turner syndrome. Women with this syndrome experience a variety of developmental problems including failure of ovary development. Conversely, the addition of chromosomes in a trivalent autosomal pairing is exemplified in many conditions such as Down syndrome (trisomy-21), Patau syndrome (trisomy-13), and Edwards syndrome (trisomy-18). Down syndrome is a viable genetic condition impacting brain and body development. Patau syndrome and Edward syndrome include a combination of birth defects that ultimately result in the loss of life by age one. Given the severity of phenotypes caused by abnormal chromosome pairings in humans, it is essential to understand the mechanisms driving segregation and continue the work pioneered in other organismal models.

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