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DESCRIPTION OF MEIOSIS IN FEMALE AND MALE PACIFIC WHITE SHRIMP *LITOPENAEUS VANNAMEI* (DECAPODA: PENAEIDAE)

Rodolfo Garza-Torres, Alejandro M. Maeda-Martínez, Danitzia A. Guerrero-Tortolero, Hortencia Obregón-Barboza, and Rafael Campos-Ramos

(RGT, rgarza@cibnor.mx; AMMM, almaeda04@cibnor.mx; DAGT, daguet04@cibnor.mx; HOB, hobregon04@cibnor.mx; RCR, correspondence, rcampos@cibnor.mx) Centro de Investigaciones Biológicas del Noroeste (CIBNOR), Mar Bermejo 195, Col. Playa Palo de Santa Rita, La Paz, Baja California Sur, 23096, México

ABSTRACT

The first meiotic prophase was analyzed in both genders of Pacific white shrimp *Litopenaeus vannamei*. Progression through meiosis, from the formation of the synaptonemal complex to diplotene is described during molting stages. Most of the meiotic cells in both genders were observed at pachytene, recognized by complete synapsis of bivalents. In both genders, the zygotene, pachytene, and diplotene stages occurred at inter-molt, pre-molt, and post-molt stages in all individuals, which suggests that there is no relationship between molting and the first meiotic prophase. Sperm counts from the vas deferens and spermatophores support a continuous production of male gametes. The nature of the synaptonemal complex in gonad cells shows that each pair of homologous chromosomes synapses end to end, revealing a high chromatin density and a complex tangled arrangement in the nucleus. Both genders have 44 bivalents, of which around 40 have an o-ring configuration, and the rest have a v-shape, meaning that most bivalents present chiasmata at both ends, each representing two crossing-over events per bivalent per meiosis.

KEY WORDS: chromosomes, *Litopenaeus vannamei*, meiotic prophase, synaptonemal complex

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INTRODUCTION

Mitotic chromosomes have been comprehensively studied in the Pacific white shrimp *Litopenaeus vannamei* (Boone, 1931), and other commercially penaeid species (Campos-Ramos, 1997). Most species have a diploid number of 88 chromosomes, where chromosomes are small, and with a progressively decreasing length, which is difficult for karyotype analysis. Nuclei at the diplotene stage were obtained from male testes in *Marsupenaeus japonicus* (Bate, 1888) (Hayashi and Fujiwara, 1988), *Fenneropenaeus chinensis* (Osbeck, 1765) (Jixun et al., 1989), *Farfantepenaeus aztecus* (Ives, 1891), *Farfantepenaeus duorarum* (Burkenroad, 1939), and *Litopenaeus setiferus* (Linnaeus, 1767) (Chow et al., 1990), and *L. vannamei* (Chow et al., 1990; Campos-Ramos, 1997; Alcivar-Warren et al., 2006), and there is only one report of this stage in the female ovary of *F. chinensis* (Jixun et al., 1989). Counting of highly condensed bivalents has led to establishing the haploid number of species and confirmed the diploid number that was previously obtained.

Currently, there are no comprehensive studies of meiosis in crustaceans. The long and complex first prophase of meiosis I (Strickberger, 1976; Bernhard, 1990; Loidl, 1994) involves three important processes: 1) pairing of the homologous chromosomes (synapsis), 2) genetic recombination, and 3) segregation of homologous chromosomes. The synaptonemal complex (SC) is a protein complex that forms during the first meiotic prophase, where chromosome synapsis and genetic recombination occur during the zygotene and pachytene stages, respectively (Moses, 1956; Fawcett, 1956; Carpenter, 1979; von Wettstein et al., 1984; Schmekel et al., 1993; Loidl, 1994).

In female decapods, neurosecretory and endocrine organs control the ovarian development (vitellogenesis), final maturation, and ovulation (spawning) according to the molt cycle (Qiu, 1986; Yano, 1988, 1995; Dall et al., 1990; Browdy, 1992). In penaeid species with a closed thelycum such as *Penaeus monodon* Fabricius, 1798 and *M. japonicus*, mature males insert their spermatophore into the soft thelycum of newly molted immature females, and oöcyte maturation begins until reaches final maturation and spawning. Therefore, in closed-thelycum species, mating with spermatophore transfer takes place before vitellogenesis (Yano, 1988, 1995; Dall et al., 1990). In contrast, in penaeid species with an open thelycum such as *L. vannamei* and *L. stylirostris* (Stimpson, 1874), mature males attach their glutinous spermatophore onto the hard thelycum of females having advanced gonad maturation at inter-molt stage, where mating-chasing behavior is a prerequisite for spermatophore transfer and successful fertilization. Therefore, in open-thelycum species, mating occurs around two hours before final oöcyte maturation and spawning, following vitellogenesis (Dall et al., 1990; Yano, 1995).

There is a discrepancy in reports of female decapods regarding when meiosis I begins. Some reports indicate that it begins at the onset of spawning, associated with final maturation, and hence, to the molt cycle. Examples are *M. japonicus*, where ovulation and meiosis I characterize the maturating stage of oöcytes (Yano, 1988), and the red flagellated shrimp *Acetes chinensis* Hansen, 1919 (Decapoda: Sergestidae), where meiosis I starts prior to spawning (Quiu, 1986). Some reports indicate that meiosis I is arrested at metaphase I and then resumes in mature females at the onset of spawning. In this case, the beginning of meiosis I is independent

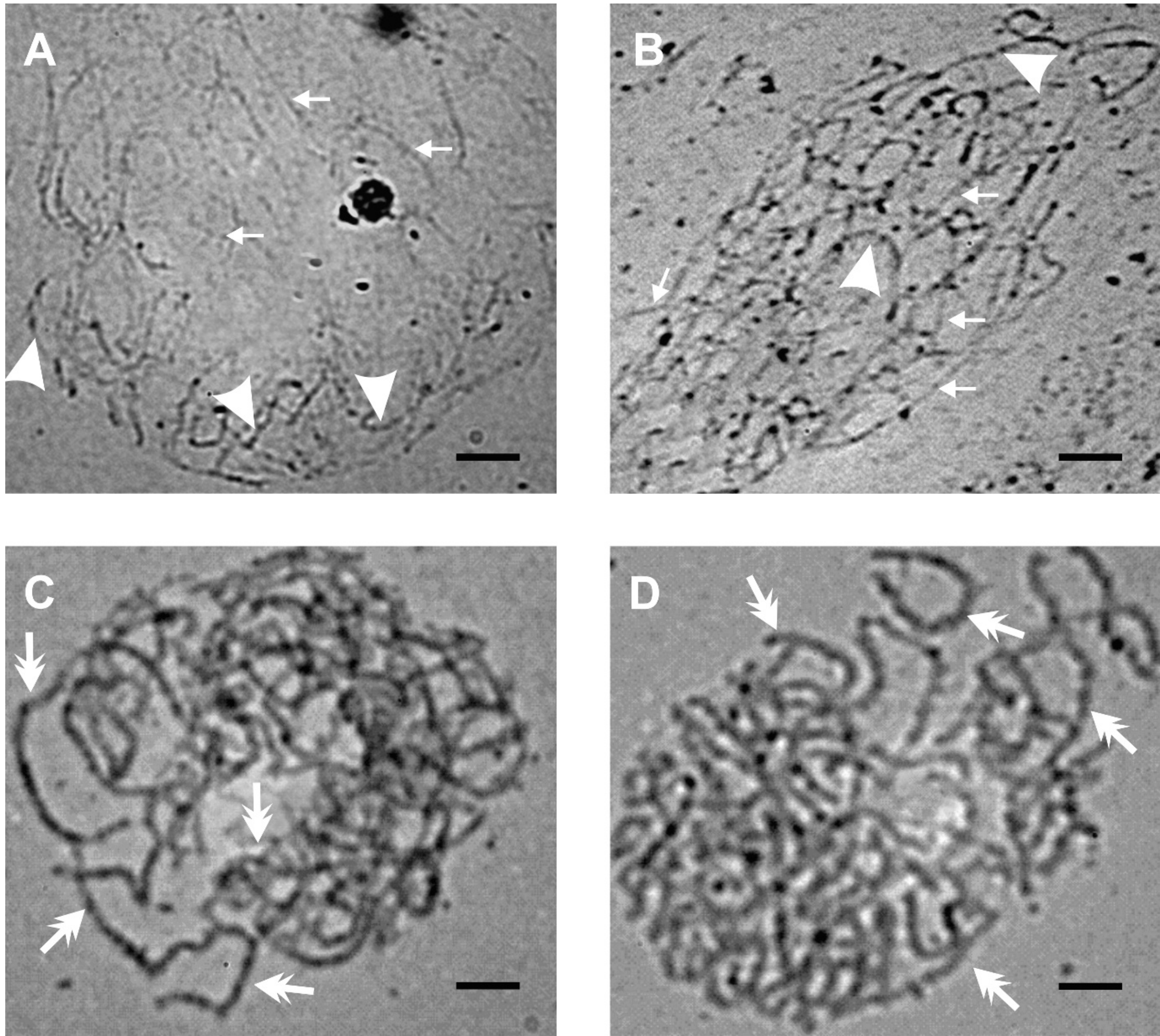

Fig. 1. *Litopenaeus vannamei*. Tangled-arranged synaptonemal complexes of female (A) and male (B) at zygotene stage, and female (C) and male (D) at pachytene stage stained with orcein. Bar represents10 µm. Zygotene, stage is distinguished from pachytene stage because homologous chromosomes have just synapsed and started to ''zip-up'' in one direction, apparently from one end to the other (big arrowheads). During zygotene, thinner and longer than pachytene bivalents are seen because of a less condensation stage of chromatin. Additionally, it is possible to observe chromosomes (lateral elements) that are still unpaired (small arrowheads), in contrast to the fully synapsed bivalents at pachytene stage (double arrowheads).

of final maturation and spawning; hence, the molt cycle. The arrested oöcyte stage at metaphase I is shown to occur in *L. vannamei* using fluorescent probes for DNA and tubulin and confocal microscopy (Hertzler, 2005), by histology of *F. aztecus* (Clark et al., 1980), a closed-thelycum species like *M. japonicus*, and in the freshwater prawn *Macrobrachium rosenbergii* de Mann, 1879 (Okumura and Aida, 2000).

In male shrimp, testes produce spermatids that are transported to and accumulate in the vas deferens. Spermatogenesis continues along the ejaculatory bulbs and the spermatozoa mass is stored in the terminal ampoule, where it is wrapped in secretions from glands forming the spermatophore (King, 1948). A histological study of *L. vannamei* shows that spermatogenesis is a continuous process, not related to the molt cycle, in contrast to the molt-related formation of the spermatophore (Heitzmann et al., 1993); Parnes et al. (2006) confirmed the timing of formation of the spematophore.

This study describes meiotic stages in gonads of both genders of *L. vannamei*, and elucidates whether meiosis is: 1) continuous and not dependent on the molt cycle, or 2) progresses as ''packages'' of meiotic cells from post-molt to pre-molt stages or at a specific molt stage.

Materials and Methods

Specimens of *L. vannamei* were obtained at the CIBNOR facilities. The molt stages were based on the description of de Oliveira-Cesar et al.

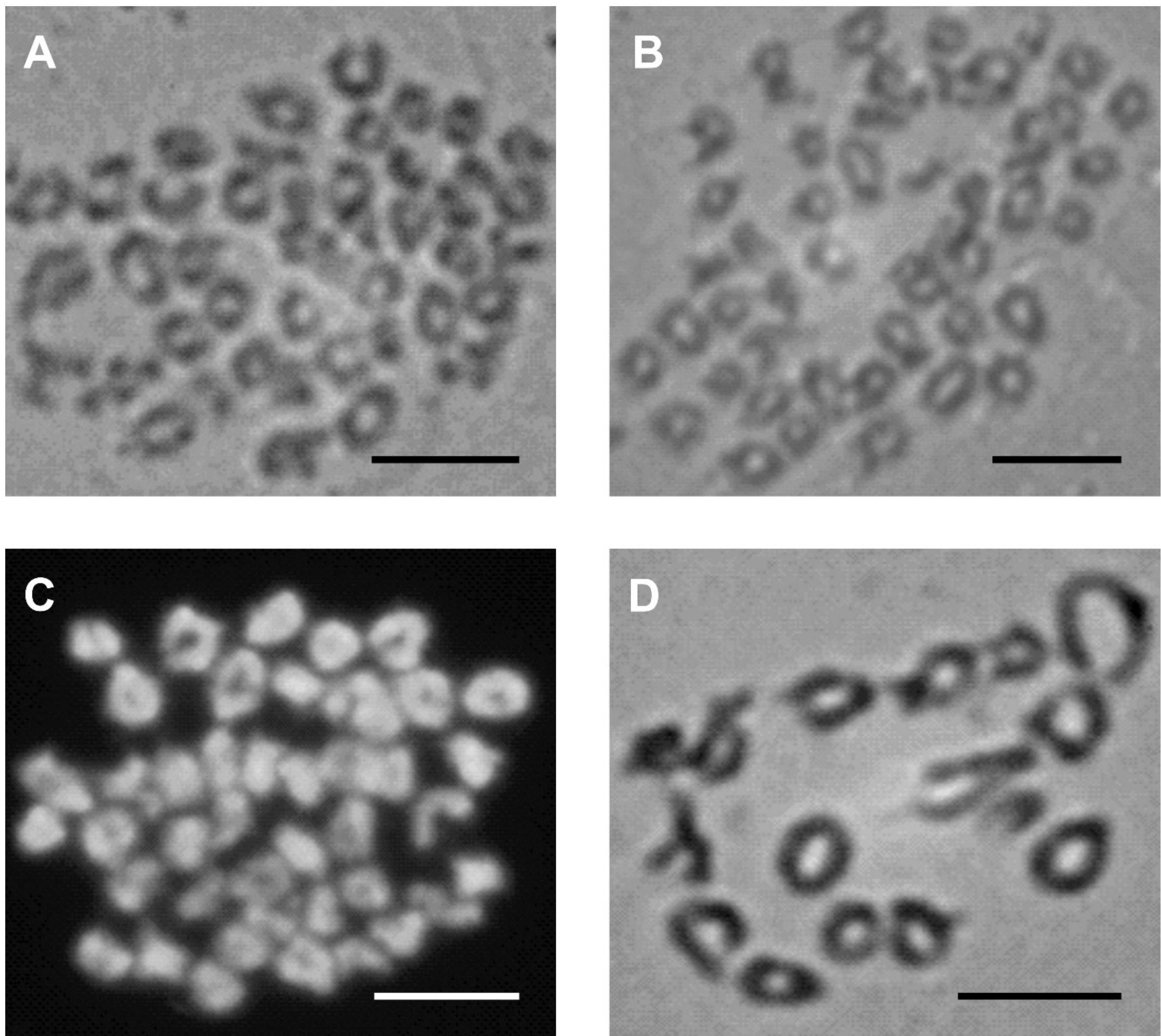

Fig. 2. *Litopenaeus vannamei*. Female (A and B) and male (C and D) at early diplotene stage, showing 44 bivalents. A, B, and D: orcein staining; C: DAPI staining. Bar represents10 μm.

(2006), and the criteria for identification of meiotic stages were based on the descriptions of von Wettstein et al. (1984) and Loidl (1994). The genital organs (Garza-Torres et al., 2009) of one group of immature adult shrimp (25-27 grams body weight) were dissected after identifying the molt stage without any treatment (control). Another group, after identifying the molt stage, was injected with 5-μg colchicine per body gram, and dissected 8 h after injection. Each group consisted of six ovaries and six testes in each of the following stages: late post-molt (Stage B), inter-molt (Stage C), early pre-molt (Stage D1), and late pre-molt (Stage D3). The lobes of gonads were dissected and processed using two techniques and three staining protocols: the air-dried technique, consisting of one hour of hypotonic shock in distilled water and then three changes lasting 15 min each, of freshly prepared and cold Carnoy fixer (3:1 methanol to acetic acid), followed by dissociation of tissue in 50% solution acetic acid.

Three slides per gonad were prepared according to Campos-Ramos (1997) by dropping and immediately absorbing the liquid in a pre-warmed slide around 30°C. Slides first were stained in 2% orcein in 45% solution acetic acid solution for 5 min, rinsed twice in distilled water, once in ethanol, and then air-dried. Slides then were stained with DAPI (4′-6-diamidino-2-phenylindole). A slightly modified technique to observe the SC in tilapia (Campos-Ramos et al., 2001) was used on the shrimp gonad, which consisted in cutting the gonad tissue with small scissors in 1 mL 0.2% Triton X-100 solution, buffered to pH 8.5 with 0.01 M sodium tetraborate. The resulting cell suspension was left for one hour in a 1.5 mL tube at 4°C, the upper 500 μL were transferred to another tube containing 500 μL 4% paraformaldehyde (dissolved with 1 N sodium hydroxyl and buffered to pH 8.5 with 0.2 M sodium tetraborate), gently shaken, and then incubated for 10 min at room temperature. About 100 μL of the fixed cell suspension was pipetted onto three microscope slides, and horizontally air-dried under a fume hood. Slides were washed gently with running tap water and then distilled water, followed by a rinse in ethanol and then air-dried. Finally, slides were stained with 50% silver nitrate (Howell and Black, 1980).

Meiotic cells were photographed with a compound light microscope equipped with fluorescence and attached digital camera. Measurements of the diameter of each nucleus (μm) were made in six nuclei at each meiotic stage with Image J software (http://rsb.info.nih.gov/ij/index.html). The relative abundance of nuclei of each molt stage was recorded as: 0 (nuclei not present); + (nuclei rarely present); ++ (few nuclei present); +++ (nuclei

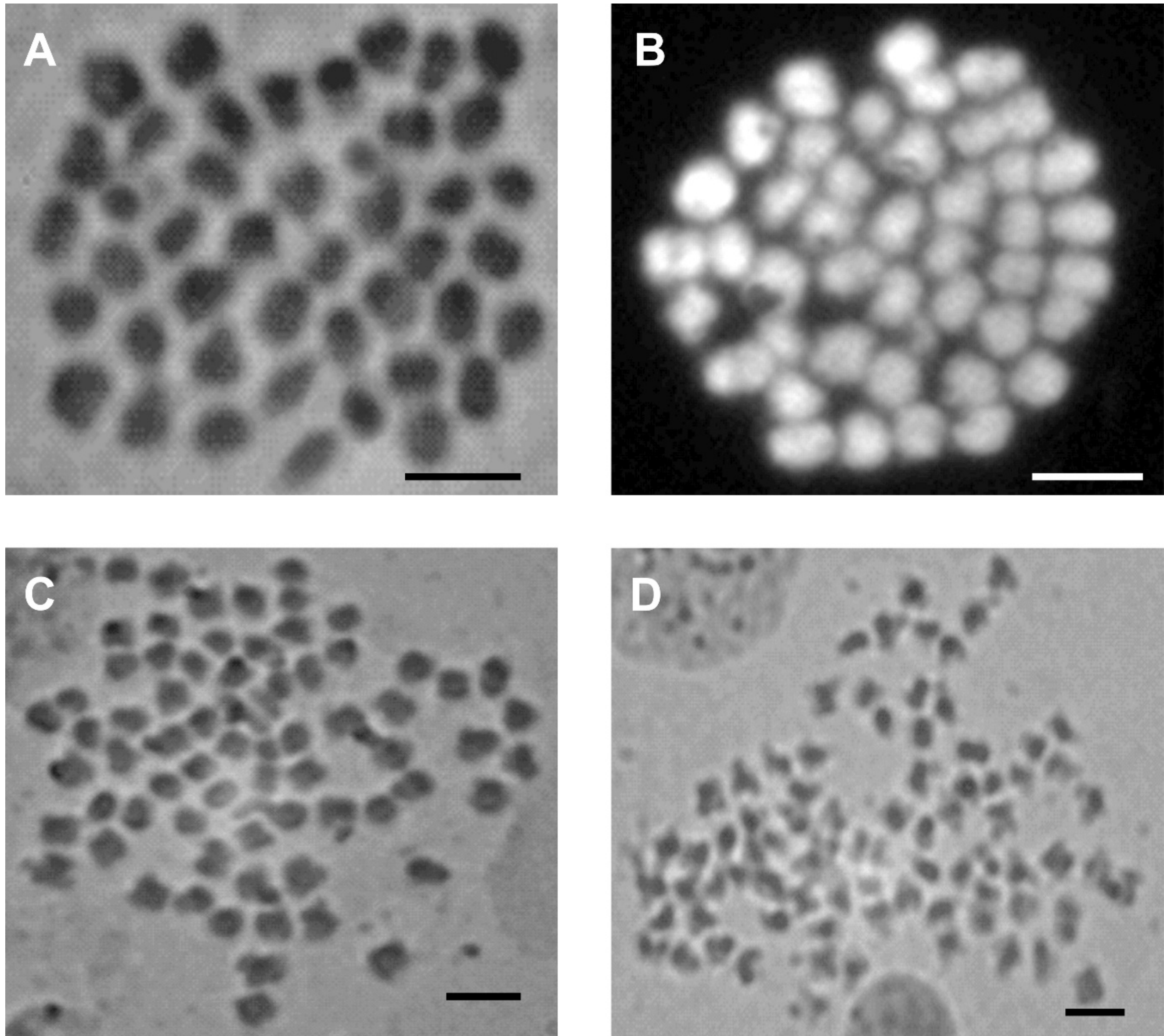

Fig. 3. *Litopenaeus vannamei*. Female (A) and male (B) at advanced diplotene stage, showing 44 bivalents; A, orcein staining; B, DAPI staining. Mitotic spreads (C and D) from spermatogonia staining with orcein. Bar represents10 μm.

commonly present), and ++++ (nuclei abundantly present). To contrast the qualitative information obtained from male germ cell meiotic stages, quantitative data of sperm cells were obtained from six males at each molt stage. Vas deferens and spermatophores from the terminal ampullae were dissected, cut in small pieces, and manually homogenized in 1 mL sterile seawater. Sperm counts were made with a hemacytometer (0.1 mm deep; Hausser Scientific, Horsham, PA). The average of counts from the bilateral vas deferens and from both spermatophores was obtained for each shrimp. Normality (Kolmogorov-Smirnov's test) and homogeneity of variances (Levene's test) of data were calculated. Mean nucleus diameter and sperm counts among the molt stages were compared with ANOVA using SPSS v. 16.0 (SPSS, Chicago, IL). Statistical significance was set at $P < 0.05$.

Results

The best technique for observing the SC was the air-dry method, followed by orcein staining. The SC was not resolved with DAPI staining and with tissue that was fixed with paraformaldehyde and stained with silver nitrate. Diplotene stage was resolved better with the orcein and DAPI protocols than with the silver nitrate protocol. Both genders showed all stages of meiosis I, with the exception of the leptotene stage (0). The control group showed zygotene (++) and pachytene nuclei (++++) only, but the group injected with colchicine showed zygotene (++), pachytene (++++), early diplotene (+), and advanced diplotene (++). Most of the meiotic cells in both genders remained at the pachytene stage, recognized by the complete synapsis of bivalents. The nature of the SC in our sample showed each pair of homologous chromosomes synapses end-to-end, revealing high chromatin density and a complex, tangled arrangement in the nucleus. In all shrimp of both genders, zygotene (Fig. 1A, B), pachytene (Fig. 1C, D), early diplotene (Fig. 2A-D), and advanced

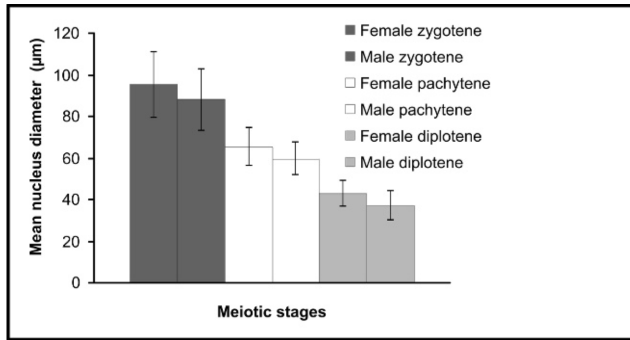

Fig. 4. *Litopenaeus vannamei*. Mean nucleus diameter (± SD) at zygotene, pachytene, and diplotene stages in females and males.

diplotene (Fig. 3A, B) were observed at post-molt, inter-molt, and pre-molt stages. Female oögonia (++) and male spermatogonia (+++) were observed during these molting stages, and only males showed a few mitotic nuclei containing 88 chromosomes (Fig. 3C, D). As the first meiotic prophase continued to the diplotene stage, a reduction and disabling of the SC occurred, leaving homologous chromosomes attached by chiasmata. At these stages, it was possible to obtain the haploid chromosome number of 44 because nuclei contained the highest degree of bivalent condensation. Early diplotene nuclei in both genders had, of the 44 bivalents, ~ 40 with an o-ring configuration and the rest had a ''v'' configuration (Fig. 2). There was no significant difference in the nuclear diameter at each meiotic stage between genders. The nuclear mean diameter gradually diminished from the zygotene (95 ± 16 µm in females, 88 ± 15 µm in males) to the pachytene (66 ± 9 µm in females, 60 ± 8 µm in males) and to diplotene (42 ± 10 µm in females, 38 ± 8 µm in males), more than a 50% decrease (Fig. 4). Spermatids (++++) and immature sperm (++++), as well as previtellogenic oöcytes (+++) were common during the molt cycle. Sperm counts from vas deferens and spermatophores were not significantly different during the molt stages (Table 1).

Discussion

The meiotic cells in gonad of *L. vannamei* showed the general features of prophase I that are observed in eukaryotes. Because of high chromatin density, the SC from gonads was best obtained through the air-dried technique using Carnoy as a fixative and stained with orcein. This was easier to process than the paraformaldehyde fixative and silver staining that is commonly used. In terms of spreading of nuclei, neither technique was able to unravel bivalents. The many bivalents that are tangled in the nucleus made it impossible to measure each bivalent and recognize any bivalent associated with a putative sex bivalent. From the arrangement of the SC and the low resolution of the technique, no other bivalent structures, such as kinetochores and attachment plaques were observed. Most of the meiotic cells in both genders remained at the pachytene stage, recognized by the complete synapsis of bivalents, which support the histological observation of Heitzmann et al. (1993), in male *L.*

Table 1. Mean sperm counts (± SE) from the vas deferens and spermatophorores during molt stages of *Litopenaeus vannamei*. No statistical difference was obtained.

$\times 10^3$	Stage B postmolt	Stage C intermolt	Stage D1 premolt	Stage D3 premolt
vas deferens	1581 ± 243	1235 ± 329	1356 ± 219	1437 ± 229
spermatophores	1837 ± 317	1906 ± 204	1765 ± 212	1862 ± 351

vannamei. When analyzing the SC in spermatocytes of diploid and triploid *F. chinensis* using transmission electron microscopy, Xie et al. (2008) observed tangled bivalents without revealing further characteristics, they found that it was extremely difficult to locate well-spread nuclei for analysis. Further improvement of the SC technique in gonads of shrimp is required, specifically, a method to obtain better spreading of bivalents.

We found that the beginning of meiosis I in female *L. vannamei* is continuous, and therefore, occurs independently from vitellogenesis, final maturation, and spawning, hence, the molt cycle. Hertzler (2005) has documented the arrested oöcyte stage at metaphase I in spawning females. According to Garza-Torres et al. (2009), previttelogenic primary growth occurs from day 70 as postlarva, which distinguishes female gonad differentiation. Therefore, we suggest that meiosis I occurs after differentiation of the gonad in juveniles and the ovary continues to accumulate arrested oöcytes in previtellogenic growth. At adulthood, and under reproductive conditions, secondary oöcyte maturation (vitellogenin) takes place, and by the time of spawning, the oöcyte resumes meiosis. The separation of the first meiotic polar body from the oöcyte is evident ~ 7 min after spawning (Dumas and Campos-Ramos, 1999). According to Bernhard (1990), most invertebrates, including arthropods, annelids, some mollusks, and the tunicates arrest the oöcytes in Metaphase I. Some reports of female decapods indicate that meiosis I in oöcytes begins at the onset of spawning (Qiu, 1986; Yano, 1988). This is still controversial and requires confirmation.

Our study also confirmed that spermatogenesis in male *L. vannamei* is continuous and is not related to the molt cycle, which agrees with Heitzmann et al. (1993). The production of gametes by meiosis (non-molt related) and the elimination of old sperm (molt-related) (Heitzmann et al., 1993; Parnes et al., 2006) are two separate physiological processes. In other decapods, such as the orange-claw morphotype of male freshwater prawn *M. rosenbergii*, DNA synthesis in testes at the pre-molt stage is at least three times higher than at the post-molt and inter-molt stages, and when compared to the inter-molt stage of the blue-claw morphotype (Sagi et al., 1988, 1991). Therefore, there is no a general rule that spermatogenesis should be continuous in all male species of decapods.

The diploid chromosome number in *L. vannamei* is 88 chromosomes (Campos-Ramos, 1997), and the haploid number is 44 bivalents (Chow et al., 1990; Campos-Ramos, 1997; Alcivar-Warren et al., 2006). According to Campos-Ramos (1997), the tentative karyotype is 4m + 10sm + 56st + 18t. Most of these chromosomes are subtelocentric and telocentric (37 pairs) that, apparently according to the present study, have an o-ring configuration during early

diplotene and represent two crossovers per bivalent per meiosis. The other seven chromosome pairs are either metacentric or submetacentric, which could represent bivalents having a "v" configuration and where only one crossover occurs. These observations suggest that there is recombination in most bivalents in both genders, a scenario that is common in gonochoric animals since achiasmatic meiosis is rather rare. Sex-linked markers mapped on the maternal genome in *M. japonicus* (Li et al., 2005) and *L. vannamei* (Zhang et al., 2007) suggest that there is a bivalent involved in sex determination, and imply that the female is the heterogametic sex in these species.

Sex determination in gonochoric crustaceans is primarily through sex chromosomes (Charniaux-Cotton, 1960; Ginsburger-Vogel and Charniaux-Cotton, 1982). Evidence of sex chromosomes is found in the eye-white phenotype in bisexual *Artemia franciscana* Kellogg, 1906, which is sex-linked to the female W-sex chromosome (Bowen, 1963, 1965; Bowen et al., 1966). The obligate recombination between sex chromosomes occurs during pachytene and the restoration of a diploid WZ zygote during the second meiotic division in *Artemia parthenogenetica* Bowen and Sterling, 1978 (Campos-Ramos et al., 2006b). In freshwater prawns and marine penaeid shrimp, it also appears that a WZ sex determination system is common (see Campos-Ramos et al., 2006a for a review).

In marine shrimp, triploid induction has been successfully achieved in *F. chinensis* (Li et al., 2003) and *M. japonicus* (Coman et al., 2008). Li et al. (2003) observed a skewed sex to female in a 4:1 proportion in triploid shrimp. Coman et al. (2008) observed all-female triploid shrimp and stated that it was not possible to explain the sex-determining mechanism, as described for fish. They proposed an over-dominant W-chromosome and a lethal ZZZ-genotype. This explanation seems controversial because triploid male shrimp do exist, as found by flow cytometry (Li et al., 2003) and SC analysis (Xie et al., 2008). An alternative to the hypothesis of dominant and lethal sex-genotypes, the recombination between sex chromosomes explains better why the gender proportion in penaeid triploids is skewed to females, as shown in fish species having a WZ/ZZ system, e.g., the blue tilapia *Oreochromis aureus* (Steindachner, 1864) (Penman et al., 1987; Mair et al., 1991). In any case, the gender proportion in triploids suggests that sex chromosomes do exist in penaeids and that further research is necessary in sex determination studies.

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