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Consecutive endopolyploidy levels in cells of *Branchipus schaefferi* Fisher, 1834 (Branchiopoda: Anostraca)

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ABSTRACT

Endopolyploidy is a well-documented phenomenon in the natural world, yet its biological significance remains poorly understood. A multitude of effects and consequences have been described in the literature as being attributable to this phenomenon, yet none of them have been subjected to rigorous and well-established confirmation. The measurement of ploidy level is typically conducted using flow cytometry or densitometry techniques. Conversely, direct chromosome counting is a less frequently employed method. As a result, all published ploidy-level counts have been presented as even numbers. We describe the first ploidy level chromosome count in somatic cells of *Branchipus schefferi* Fischer, 1834, a common and one of the most abundant branchiopod crustaceans (Branchiopoda, Anostraca) widely distributed across Europe. The range of observed ploidy levels was found to vary from diploid to octoploid cells, including odd numbers (3n, 5n, and 7n), as well as different aneuploid numbers. It was furthermore observed that chromosomes at higher ploidy levels undergo an apparent telomere-binding process, leading to the appearance of interphase cells with a large nuclear volume. The possible origins of ploidy levels and the consequences of chromosome joining are discussed here.

KEY WORDS: chromosomes; Crustacea; endomitosis; ploidy levels

INTRODUCTION

Endopolyploidy is a biological phenomenon characterised by alterations in the genetic content of an organism's cells, resulting in an increase in nuclear volume, which has been traditionally associated with the growth and size of organisms (Øvrebø & Edgar, 2018). This phenomenon can be induced by two distinct mechanisms: endocycling (polyteny), where the chromosome number remains constant and DNA strands remain concatenated following replication, and endomitosis, which entails a doubling of the chromosome count due to the incomplete final stages of mitosis (Lee *et al.*, 2009). The functional implications of endopolyploidy remain unknown, although it is believed that its function may be very broad and important from an evolutionary and ecological point of view, affecting characteristics such as gene expression, cell and body size, cell differentiation, or growth rates (Neiman *et al.*, 2017). For example, in certain tissues such as the salivary glands of *Drosophila*, endopolyploidy allows the production of large and specialised cells with polytenic chromosomes, with a high protein synthesis capacity. Other biological significances proposed to endopolyploidy are metabolic adaptations, because endopolyploid cells often exhibit altered metabolic characteristics, such as increased metabolic activity and greater energy storage capacity (Neiman *et al.*, 2017).

Detection and quantification of endopolyploid cells are achieved through methodologies such as flow cytometric or densitometric approaches (Rasch & Wyngaard, 2008), as chromosome counting has not been employed to date for this purpose. Endopolyploidy has been documented in diverse biological domains, including plants, fungi, and some animal taxa. Its exploration in the latter remains nevertheless relatively understudied, with arthropods emerging as one of the noteworthy animal groups in which this phenomenon has been observed. Among arthropods, crustaceans, particularly species of *Daphnia* Müller, 1785 (Cladocera) (Neiman *et al.*, 2017), and *Artemia franciscana* Kellogg, 1906 (Anostraca) (Freeman & Chronister, 1988) have undergone extensive scrutiny concerning the presence of endopolyploid cells. These cells have been identified across various tissues in both taxa. In *Daphnia* sp., tissues of ectodermal origin and the digestive tract manifest cells with varying degrees of polyploidy during adulthood (Beaton &

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Hebert, 1999). Similarly, in early stages of the development of *A. franciscana*, tissues derived from the ectoderm exhibit cells with differing levels of polyploidy, including diploid and tetraploid cells in the epithelial tissue of the nauplius and metanauplius larvae, octaploid cells in the developing silk, and tetraploid and octaploid cells distributed throughout the digestive tract. These cells arise through chromosomal endoreduplication during the developmental process (Freeman & Chronister, 1988).

Branchiphus was the first described genus of Anostraca. *Branchiphus* Schaeffer, 1766 is represented in the Iberian Peninsula by two species: *Branchipus schaefferi* Fischer, 1834, and the endemic, *Branchipus cortesi* Alonso & Jaume, 1991. Cytogenetic studies of Anostraca have been sporadic and limited; however, these crustaceans were considered at the outset of this type of study by some as models to describe chromosomal behaviour during mitosis (Baker & Rosof, 1927, 1928). As outlined by Abatzopoulos *et al.*, 1986, anostracans exhibit distinctive cytogenetic attributes, including: 1) the challenging acquisition of complete chromosome spreads, particularly in tetraploids; 2) frequent chromosome linkage by delicate filaments; 3) the absence of a primary constriction in chromosomes; and 4) intricate coiling of sister chromatids, rendering their examination a formidable task.

We analyzed for the first time the phenomenon of endopolyploidy in *B. schaefferi*, as revealed by the relative size of their nuclei and by their chromosome count. The different levels of polyploidy detected in this species are described, and a possible mechanism of origin of these cells is discussed.

MATERIALS AND METHODS

Biological samples

Forty individuals of *B. schaefferi* were collected from four temporary rainwater ponds in Padul, Granada, Spain (UTM 5 × 5 30SVF39). Each pond was sampled with five males and five females, which were kept and cultivated in a laboratory setting in 60=l aquaria at room temperature and under natural light. They were identified to species after a morphological analysis based on the first and second antennal segments of antenna 2 and antennal appendages, following the dichotomous key of Alonso (1996), and using a ZEISS Stemi 508 stereo microscope (Carl Zeiss, Oberkochen, Germany).

Mitosis in nauplii larvae

Nauplii larvae were collected from the culture within 24 h after hatching and fixed in a 1.5 ml tube with absolute ethanol:glacial acetic acid (3:1) fixing solution. Thirty to forty fixed larvae were processed by placing them on a glass slide in a 10 µl drop of 45% acetic acid, where they were photographed and disintegrated. For disintegration, the slide was placed on a hot plate at 65 °C, continuously pipetting drops of acetic acid containing the larvae with a 0.5–10 µl micropipette.

The homogenate was spread over the entire length of the slide until the acetic acid evaporated completely. The slide with the fixed sample was then stained with Giemsa-phosphate buffer (1:9) for 15 min. This dye is frequently employed for the purpose of staining chromosomes due to its ability to bind to the phosphate groups of DNA. Fixed slides were also treated with a commercial solution of DAPI (4′,6-diamidino-2-phenylindole) following the DAPI Protocol for fluorescence imaging developed by Thermo-Fisher Scientific [https://www.thermofisher.com/es/es/home/references/protocols/cell-and-tissue-analysis/protocols/dapi-hca-protocol.html]. Both were observed under a ZEISS Axioskop 40 fluorescence microscope (Carl Zeiss) using 40× and 100×-plus immersion oil lens, and photographed with a digital camera (Motic Group, Xiamen, China).

Meiosis in juveniles

Meiosis was studied in ten juvenile males preserved in absolute ethanol:glacial acetic acid (3:1) fixing solution, by extraction of gonads onto a glass slide using a scalpel, taking care to remove all non-gonadal tissue. The dissected gonads were placed on another clean slide, together with 10 µl of 45% acetic acid, where they were homogenised using the clean base of a lancet. The slide was placed on a hot plate at 60 °C, continuously spreading the homogenate using a micropipette until complete evaporation and fixation. The slide with the fixed sample was immersed in Giemsa-phosphate buffer (1:9) for 15 min. These samples were observed under the optical microscope, and the meiotic cells photographed.

RESULTS AND DISCUSSION

Cytogenetic analysis of the mitotic cells showed that the specimens had a basic chromosome number of 2n = 20 (Fig. 1A). The chromosomes are small (2–3 µm) and predominantly metacentric (Fig. 1B). The chromosome count was confirmed through meiotic studies, in which the presence of 10 bivalents in diakinesis was detected (Fig. 1C). This chromosome number and sizes are coincident with those previously determined for *B. schafferi* by Bianchi-Bullini *et al.*, 1968. B or accessory chromosomes, as previously described by Beladjal *et al.* (2002), however, were not identified in the population we analysed, which comprised more than 30 samples.

DAPI-stained diploid cells showed the presence of two signals on one pair of the component (Fig. 1D), with no additional positive bands on any other chromosome, an. indication that the observation of heterochromatic bands in such small chromosomes is challenging, as mentioned by Kořínková & Gołdyn (2011). An alternative hypothesis is that this observation indicates the presence of DNA repeats that are specific to this chromosomal pair. This phenomenon has been linked to specific chromosomal regions, including satellite DNA (Jamilena *et al.*, 1993) and special chromosomes, such as sex chromosomes (Goday *et al.*, 2006; Palomeque & Lorite, 2008) and B chromosomes (Hanlon & Hawley, 2018).

The presence of polyploid cells has been identified in all samples analysed through the utilization of mitotic metaphase chromosome counting and the relative measurement of cell nuclei size in interphase cells. There was no evidence of polyploid cells during meiotic division. Mitotic cells exhibited a range of degrees of polyploidy and corresponding chromosome endowments (Fig. 2), including triploidy (3n = 30) (Fig. 2A), tetraploidy (4n = 40) (Fig. 2B), pentaploidy (5n = 50) (Fig. 2C), hexaploidy (6n = 60) (Fig. 2D), heptaploidy (7n = 70) (Fig. 2E), and octoploidy (8n = 80) (Fig. 2F). Aneuploid cells

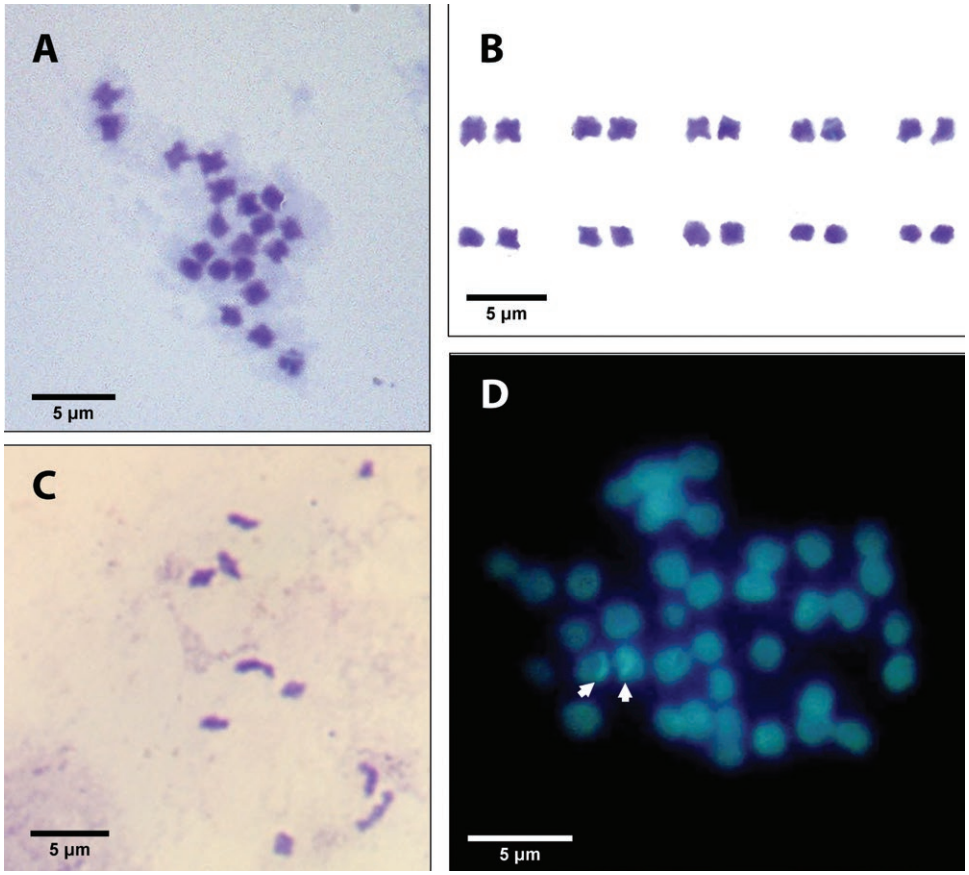

Figure 1. Giemsa-stained diploid cell of the nauplius of *Branchipus schafferi* in mitotic metaphase showing 2n = 20 chromosomes (A). Karyotype 2n = 20 of a Giemsa-stained diploid mitotic cell of nauplius *B. schafferi* (B). Giemsa-stained meiotic cell of juvenile *B. schaefferi* in diakinesis-metaphase I showing ten bivalents (C). DAPI-stained mitotic cell of *B. schaefferi* in metaphase showing two positive bands (white arrows) (D). Scale bars = 5µm.

were also observed at varying degrees of polyploidy (Fig. 3). In interphase polyploid cells, a significant variance in nuclear size was observed (Fig. 4), thereby confirming the presence of distinct levels of polyploidy. Nuclei with radii between two and ten times larger than the more prevalent diploid nuclei were observed (Fig. 4).

The occurrence of endopolyploid cells in nature is a relatively common yet noteworthy phenomenon (Lee *et al.*, 2009). These cells are produced through endomitosis, which distinguishes them apart from endoreduplication, a process prevalent in specialised chromosomes, such as polytene chromosomes, which are found in particular tissues and larval stages (Lee *et al.*, 2009). Endopolyploidy arises from incomplete anaphase divisions during mitotic processes and has been observed in various organs of numerous species of animals and plants (Neiman *et al.*, 2017). Flow cytometry techniques have been employed to examine endopolyploid cells, which entail fluorochrome staining and the measurement of nuclear volumes in interphase cells. In particular, these studies consistently demonstrate that the nuclear size increases exponentially in base two, resulting in even numbers. We nevertheless identified the endopolyploid levels by the precise quantification of the number of chromosomes and chromosome sets present in each cell. This enabled the novel discovery of endopolyploid levels that correspond to both even and odd chromosome numbers.

The cellular origin of endopolyploidy is generally attributed to the reduction of the cell cycle, whereby different phases can be eliminated or shortened (Øvrebø & Edgar, 2018). The most plausible explanation for the phenomenon of endopolyploidy in *Branchipus* is the existence of endomitosis cycles. Following DNA synthesis during the S phase, the separation of the two chromosomal poles during the M phase is therefore replaced by non-canonical cell cycles in which cells progress through all phases of the canonical cell cycle, but exit the M phase prior to the onset of cytokinesis (with the absence of anaphase and telophase). This phenomenon has already been observed in mammalian and other megakaryocytes and osteoclasts (see review by Øvrebø & Edgar, 2018). Our observation in *Branchipus* thus demonstrates that endopolyploid cells are capable of entering new reduced cell cycles, thereby generating new cells with an elevated chromosome number. This phenomenon has been documented in other branchiopods, including species of *Daphnia* (Beaton & Hebert, 1999), *Branchipus*, and *Artemia* (Freeman & Chronister, 1988), as well as in *Drosophila* (Fox *et al.*, 2010). The process may account for the presence of 4C nuclei and the subsequent even number of endopolyploid cells.

The process, however, does not explain the aneuploid cells and nuclei with odd numbers of chromosomes (triploid, pentaploid or heptaploid) we found (Fig. 2). In the first case, as noted by Edgar *et al.* (2014), it is possible that a programmed reduction of

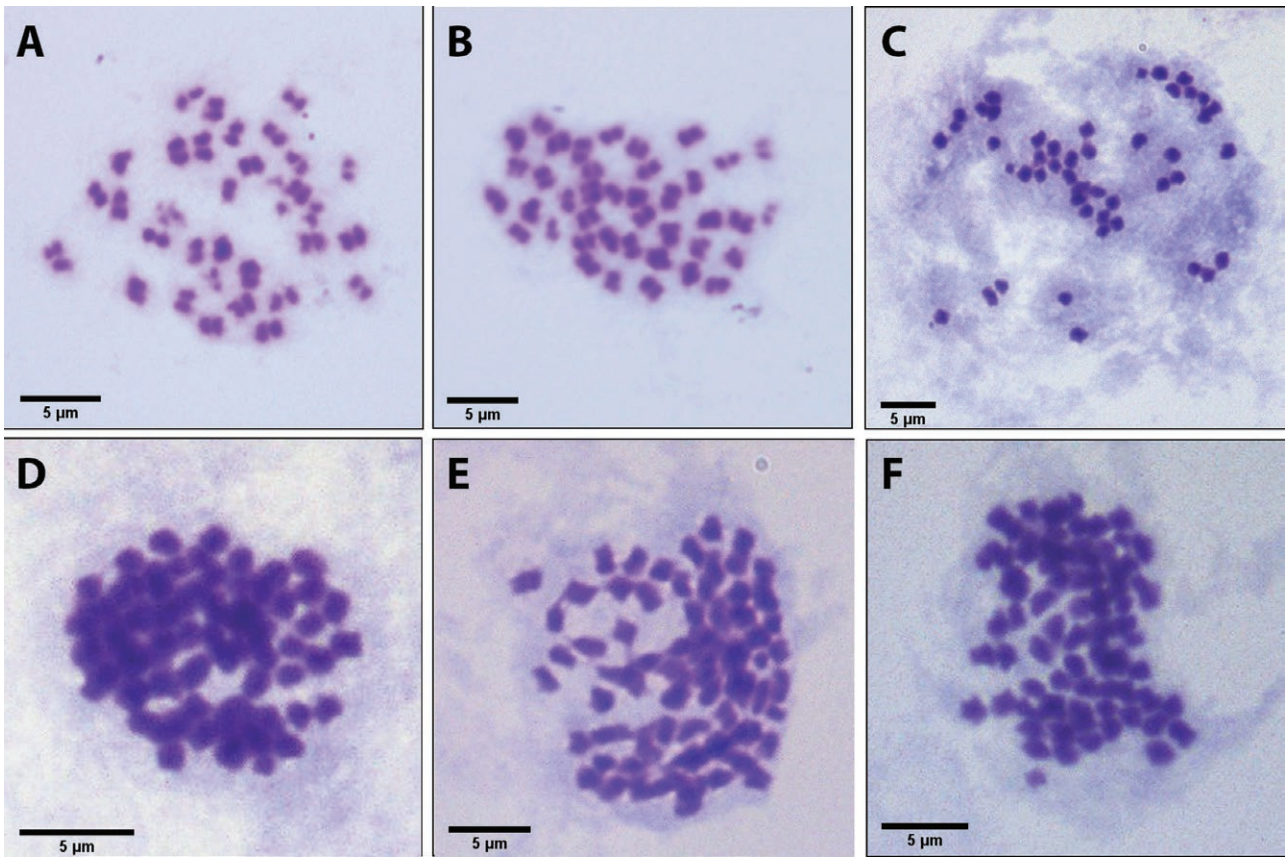

Figure 2. Giemsa-stained mitotic cells of the nauplius of *Branchipus schaefferi* in metaphase with different polyploidy levels. Triploidy (3n = 30) (**A**), tetraploidy (4n = 40) (**B**), pentaploidy (5n = 50) (**C**), hexaploidy (6n = 60) (**D**), heptaploidy (7n = 70) (**E**), octoploidy (8n = 80) (**F**). Scale bars = 5 µm.

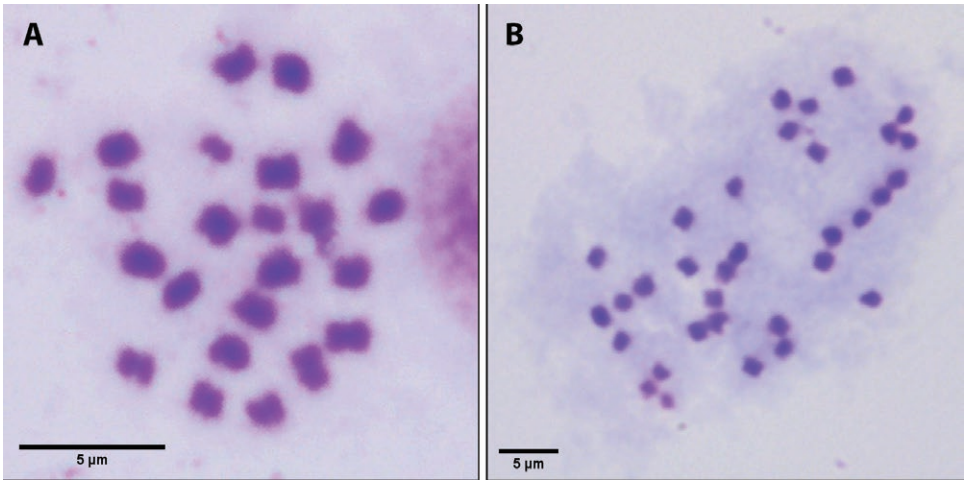

Figure 3. Giemsa-stained mitotic aneuploid cells of the nauplius of *Branchipus schafferi* with different polyploidy levels: 2n = 20+3 (**A**), 3n = 30+3 (**B**). Scale bars = 5 µm.

polyploidy occurs, leading to chromosome mis-segregation and aneuploidy production. This hypothesis would be supported by the remarkable presence of aneuploid cells in *B. schaefferi*. This process has been associated with an increase in genetically divergent daughter cells in *Drosophila,* which can lead to increased genetic variation (Edgar *et al.*, 2014). But it is unlikely that odd ploidy levels are determined solely by the gradual loss of individual chromosomes. Other possible explanations for the absence of complete sets of chromosomes include the loss of a centrosome from one of the poles (Ganem *et al.*, 2009), or an alternative mechanism involving asynchronous pronuclear replication.

Different levels of polyploidy are also supported by the observation of interphase cells, which show a marked variation in nuclear volume, which can be up to ten times that of a diploid

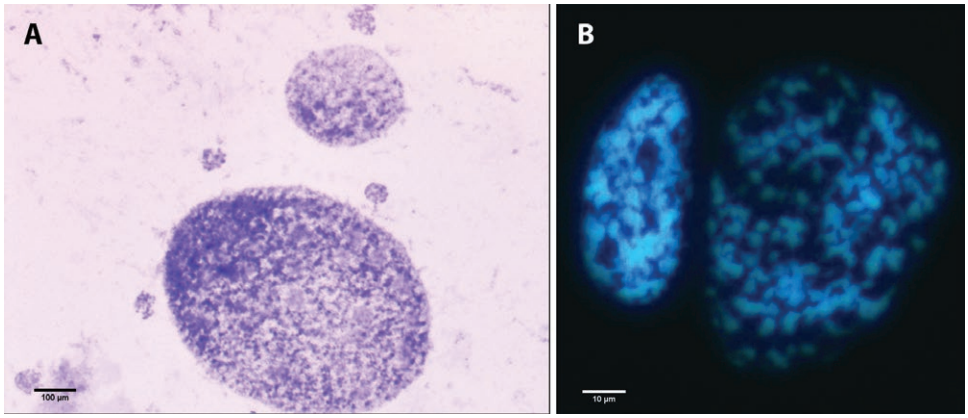

Figure 4. Mitotic cells of nauplius *Branchipus schaefferi* in interphase showing several nuclei sizes: Giemsa-stained cells (**A**) DAPI-stained cells (**B**). Scale bars = 100 µm (A), 10 µm (B).

cell (Fig. 4). This definitive interphase could be explained by the association between telomeres, which destabilise the chromosome structure and favor this process of chromosome fusion. We observed the phenomenon of chromosome association in endopolyploidy cells in *B. schafferi* by joining the chromatin mainly of their telomeres through the formation of filamentous DNA bridges between homologues and non-homologues, producing an ectopic fusion (Fig. 5). This phenomenon has been observed in plants and animals (Coates & Smith, 1984). It was first described by Baker & Rosof (1927, as *Branchipus vernalis*) in the classic study of spermatogenesis in *Eubranchipus vernalis* Verrill, 1869 and later by Abatzopoulos *et al.*, 1986 in *A. franciscana*. Telomeric fusion during the developmental endocycle processes in *Drosophila* has also been linked to an insufficiently replicated telomeric heterochromatin (Lilly & Duronio, 2005). The successive replications of hereditary material that occur in polyploid cells undergoing successive replication processes would therefore lead to a stagnation in the endopolyploidy process, making them enter a definitive interphase, which would be reflected in the presence of these observed giant interphase nuclei in *Branchipus*, in which the chromatin state is diffuse and highly decondensed (Fig. 4).

Endopolyploidy has been linked to several different factors. A first factor is growth and size of the individual. Endoreplication represents an efficient strategy for growth, as it increases the DNA content of cells, which often correlates with larger cell size (Kondorosi *et al.*, 2000). Our findings demonstrate the occurrence of endopolyploidy events in nauplii, a period of accelerated growth; however, endoreplication is not considered a mechanism for controlling the size of the organism when associated with specialised cell types that perform specific biological functions. No endopolyploid cells were observed when *B. schaefferi* gonads were analysed. A second possible explanation is genome size. A reduction in genome size has been linked to endopolyploid events (Neiman *et al.*, 2017). During specific phases of the cell cycle and in particular tissues, endopolyploid cells contribute to increased gene expression levels, as observed in the polytene chromosomes of salivary glands of the silkworm, *Bombyx mori* (Linnaeus, 1758) (Lee *et al.*, 2009). In the case of crustaceans like *Daphnia* and *Artemia*, however, the relevance of

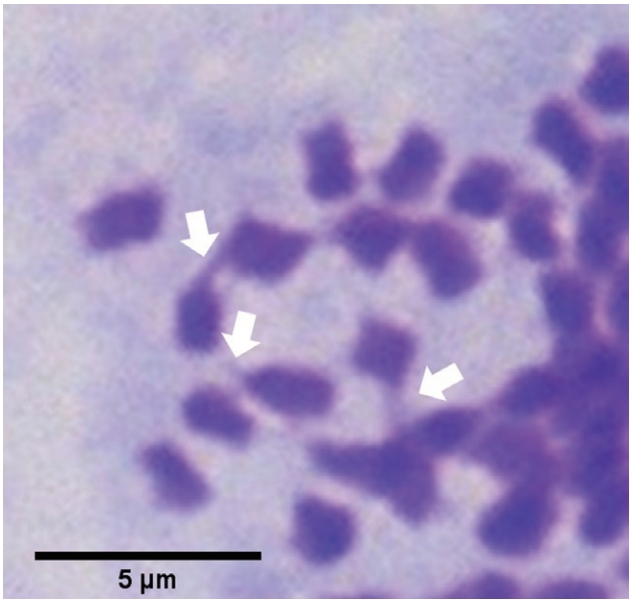

Figure 5. Giemsa-stained mitotic cell of nauplius *Branchipus schaefferi* showing DNA bridges between chromosomes (white arrows). Scale bars = 5 µm.

genome size is questionable, because the genome of *Daphnia* is 10 times smaller than that of *Artemia* (Korpelainen *et al.*, 1997), but endopolyploidy has been extensively documented in both groups. The currently available evidence does not permit the establishment of a correlation between the genome size of *Branchipus* (which is supposed to be reduced with a reduced number of chromosomes, 2n = 20 and small-size chromosomes, 2–3 µm) and endopolyploidy.

A third explanation of endopolyploidy is biotic and abiotic stress as a potential driver of ecological and evolutionary processes. *Branchipus* inhabits ephemeral waters and thus has a short life cycle, being exposed to extreme environmental factors, such as drought and high solar radiation. These situations, where energy sources are limited or rapid growth is necessary, could drive the emergence and evolution of endopolyploidy (Neiman *et al.*, 2017).

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