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Sperm DNA in grasshoppers: structural features and fertility implications

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Abstract

This contribution briefly reviews the importance of grasshopper chromosomes in evolutionary studies and in achieving a comprehensive view of cytogenetics, emphasizing that meiotic chromosomes have been the traditional means for analyzing the nature of such problems. Structural changes occurring in the sperm, as a direct consequence of some chromosomal changes, have received less attention. We propose that grasshopper sperm could be a good model for the study of: 1) aspects of genome territoriality, and 2) consequences in the sperm DNA molecule, derived from a compromised meiotic process, linked with naturally forced genome hybridization. The results presented here, by using appropriate DNA probes for genome regionalization, allow the proposal of a grasshopper sperm chromosome organization. Centromere regions are distributed and concentrated at the proximal end of the sperm head where the flagellum is inserted. Also, chromosome arms are longitudinally organized from the proximal to the distal end, where the telomere of the longest autosome is the most distant genome region from the flagellum insertion. Subsequently, and because the length of the chromosome arms is different among non-homologous chromosomes, the telomeres are distributed along the length of the sperm head. Sperm DNA quality was analyzed using the sperm chromatin-dispersion test, demonstrating that in sperm nuclei of early spermatids containing fragmented DNA, there exist large haloes of dispersed chromatin, while those nuclei remaining compact, contain an intact DNA molecule. Interestingly, and as a main difference with respect to other analyzed species, the DNA loops remain attached to a central proteic core. The aforementioned observation is clearly visible in partially elongated spermatozoa and the DNA loops forming the halo expand from this central region. This aspect of sperm chromatin organization found in grasshoppers is a new contribution to orthopterology. Further, it provides a unique insight and allows questioning the possible existence of this kind of chromatin organization in other noninsect species, where reproduction is based on gamete production.

Key words

Orthoptera, sperm, male factor, DNA damage, insect cytogenetics

The sperm as an accidental carrier of DNA damage

The sperm is a privileged cell, autonomous throughout a part of its existence, involved in maintaining and transmitting intraspecific genetic identity to offspring and disseminating a certain degree of genetic variability. Each spermatozoon contributes 50% of the nuclear genetic information that can be considered as unique, since it is the product of a meiotic process in which the parental genetic information is combined to generate new genetic assemblages. A sperm cell is considered to have a balanced haploid genome, with a DNA molecule organized according to a Watson-Crick model, maintaining a proper chromatin arrangement that must be competent for transmitting the full genetic payload into the next generation with a high level of fidelity.

However in cell differentiation that occurs during spermatogenesis and spermiogenesis, single events, such as meiosis or the replacement of histones by protamines, can lead to errors in the DNA sequence and/or sperm protein alterations, which may be nonreparable after fertilization. Following fertilization, these changes can be very important to the fate of the nascent embryo cell cluster and therefore to the offspring (Agarwal & Allamaneni 2004, Zini & Libman 2006). This fact, together with other factors that act as causative agents for producing circumstantial cell damage, can affect the germ line and the production of normal sperm. Thus, oxidative stress, pollutants, bacterial infections or even other multifactorial stressing circumstances (Fernández *et al.* 2008, Cortés-Gutiérrez *et al.* 2007) can interfere with the development of the germ cell's ability to form a normal sperm.

The aforementioned reality results in an immutable situation whereby all ejaculates analyzed thus far have a certain proportion of normal sperm, while the other proportion of sperm contains a damaged DNA molecule. This occurs whether or not the samples are associated with clinical or pathological individuals or are from those individuals considered as being normal. The abnormal sperm fraction can potentially compete for fertilization with sperm carrying a normal DNA molecule and thereby reduce the fertilizing capacity of the individual. Thus, when sperm DNA damage is considered, it might be expected that those individuals carrying a higher proportion of sperm with a damaged DNA molecule would be potentially less fertile.

However, as occurs with other sperm characteristics, such as morphology or motility, the direct relationship between sperm DNA quality and fertility is not clearly established (Zini & Sigman 2009). Scientific reports fail and succeed in almost equal porportion to demonstrate that a high level of damaged sperm is associated with decreased fertility or the ability of a individual to generate a pregnancy (Zini & Libman 2006). In some cases, individuals or patients with higher levels of sperm DNA damage can still produce a pregnancy (Payne *et al.* 2005).

In biology, such ambiguities arise when trying to understand complex situations with an inherently multifactorial nature. The trend in science is to try and simplify the problem, thereby creating an unambiguous hypothesis which produces a satisfactory explanation to our questions. Being that sperm DNA damage can be multifactorial in origin, the question that should probably be posed, in order to understand the real role of sperm DNA damage in fertilization, is whether a sperm with a highly fragmented DNA molecule could produce a normal and viable embryo after forced fertilization, using methodologies such as intracytoplasmic sperm

injection. If establishing a correlation between high sperm DNA fragmentation and failed fertilization is not so straightforward, an experimental demonstration supporting the previous question would be almost impossible.

Nonorthodox sperm DNA configurations in the form of detectable DNA breaks in the sperm can have a double nature: 1) damage produced after sperm chromatin remodelling for histone-protamine replacement, and 2) damage produced by external or environmental causal agents. While the first has been largely unexplored in mammalian species due to lack of information regarding sperm chromosome arrangements, more information is available concerning the external or environmental damage. However, in grasshoppers most of these sperm alterations have been largely unexplored in both situations, mainly because of the absence of techniques that provide consistent results. The explanation for the absence of this type of information is very simple: 1) little knowledge about how the chromatin is organized in the sperm, and 2) the difficulty of adapting the current technologies for studying sperm DNA damage, primarily because of the particular morphology of the long-headed mature spermatozoa in grasshoppers.

Grasshopper chromosomes versus grasshopper sperm

The importance of grasshopper chromosomes for understanding genome organization, for conducting evolutionary studies and for obtaining a more comprehensive view of cytogenetics, is clear and should not be understated. West-Eberhard (2003) in examining the genetic theory of evolution, discusses the flexible phenotype as the product of development and the object of selection. The developmental plasticity of grasshoppers, of Orthoptera for that matter, is an important example of 'flexible phenotype'. Support for this statement arises from the vast amount of scientific literature and the quality of the scientist, Michael James Denham White (MJD White), who dedicated most of his life to this endeavor, by placing grasshopper chromosomes into an evolutionary context and making a primary contribution to the emerging neoDarwinian evolutionary synthesis. Even today, with most biologists immersed in the almost unimaginable advances of molecular biology, grasshoppers are a good experimental model wherein advanced tools and techniques can be tested. The *leitmotiv* of this special section, which represents a homage to the scientific contribution of Professor White, conveys insight regarding the most recent advances promoted from the use of grasshopper species.

Meiotic chromosomes in grasshoppers have been the traditional target for analyzing biological problems of a singular nature. Multiple genome rearrangements have been the primary focus for understanding mechanistic aspects of the meiotic process in itself. An example is genetic consequences of large genome rearrangements and the role they play in hybrid sterility, this even suggesting possible barriers to gene transfer from one individual to another within the same taxon (Hewitt 1979, Gosálvez *et al.* 1997). But the sperm is probably the most important cell for testing the fate of genetic consequences of meiosis and it is the only one which contains the direct genetic consequence of the meiotic process itself, albeit this having received less attention.

The single most-studied group of relationships involving genome configurations as related to their biogeographical distributions, has probably been the thelytokous wingless *Warramaba virgo* and its bisexual relatives, as researched by MJD White and coresearchers (White *et al.* 1973, White & Contreras 1978, White & Contreras 1982) in Australia. The presence of translocations, paracentric inversions or fusions and differences in C-banding patterns of their chromosomes by amplification or shrinking of heterochromatic segments, reveal a complex multispaced genome configuration where about 30 different clones of *W. virgo* can easily be identified. In general, local populations could be considered as a single clone, although in some cases two clones may exist at the same locality. This clonal diversity seems to be maximized in the Kalgoorlie region of Western Australia where *W. virgo* is believed to have arisen.

The evolutionary pathway seems to indicate that on two separate occasions, *W. virgo* resulted from a hybridization process between two bisexual species (White 1978) and the diploid parthenogenetic (parthenote) probably arose as a hybrid between two bisexual forms, closely linked to the modern taxa known as P169 and P196 (Hewitt 1975a) . Interestingly, when hybrids of both sexes were produced in the laboratory from reciprocal crosses (White & Contreras 1978), all males with a Y-chromosome derived from P196 died before reaching the adult stage. However, some males from the reciprocal cross, bearing a Y chromosome from P169, reached the adult stage. These hybrids appear to be entirely sterile because their testes were small and at the first meiotic division mostly univalents were formed. Within this scenario it is assumed that normal sperm production is abolished resulting in sterility. However, within this magnificent evolutionary and biological enigma of chromosome diversity, sex determinism, hybridization and biodiversity, the intrinsic aspects of sperm DNA and the consequences for chromatin organization emerging from incompatible meiosis resulting from hybridization have never been thoroughly investigated.

The Australian grasshopper *Caledia captiva* is another prominent example linking chromosome rearrangements, genome instability, hybridization and sterility (Shaw *et al.* 1976, Moran 1979, Shaw *et al.* 1982). *C. captiva* is characterized by a chromosome reorganization, displacing all centromeres from terminal to medial locations, but exhibiting a standard karyotype 2n = 23 ♂ / 24 ♀. The population is distributed over 1500 km north to south of East Australia and shows fixed differences in chromosome structure across this range, where more than 600 different chromosomal rearrangements have been identified (Shaw *et al.* 1980, Shaw *et al.* 1986). Between the two extremes, populations are characterized by complex chromosomal polymorphisms in which the entire genome changes gradually from metacentric in northern populations to acro/telocentric in the south. Latitudinal clines in the area of distribution are clearly established. These genome differences produce different patterns of fertility and sterility among the different allopatric or parapatric populations of *Caledia* distributed over its entire range. The Moreton and Torresian races of *C. captiva* are parapatrically distributed in southeast Queensland and have been analyzed mainly because they form very narrow hybrid zones, of width about 1 km. Although the fertility consequences of hybridization among different races has been the subject of careful investigation (Moran 1979), studies concerning the impact of sperm integrity remain to be conducted in detail.

The grasshopper *Dichroplus pratensis*, like *Caledia*, is a highly polymorphic and polytypic species. Intrachromosomal genetic recombination and genetic variability seem to be linked to the extensive production of centric fusions, where complex Robertsonian fusions are widely distributed in South America, reaching Patagonia and producing a high level of genome plasticity (Bidau *et al.* 2004; Bidau & Marti 2004, 2005). An increasing chiasma frequency has been found toward the margins of the range; and additionally, there is a positive and significant correlation with increasing levels of morphological variability. The decrease in fusion polymorphism has a direct consequence on both an inter- and intrachromosomal increase in genetic recombination in the marginal areas. It seems

that natural selection favoring higher levels of variability could have an adaptive value in these environments, which are prone to extreme changes.

Warramaba, *Caledia* and *Dichroplus* represent just three systems of immense potential for examining the consequences to sperm of highly rearranged genomes; they could be used to test how the sperm experience these changes as a consequence of meiotic imbalance when hybrid genomes are naturally or synthetically formed.

A fourth example we have chosen to discuss includes the two subspecies of the grasshopper *Chorthippus parallelus* which mate and hybridize in the Pyrenees. This is a good example of how information derived from sperm has provided evidence of the genetic differences existing between two taxa. The *C. parallelus* hybrid zone is formed as a consequence of a recent population expansion of two different subspecies *(C. parallelus parallelus*, the European subspecies and *C. parallelus erythropus*, the endemic Iberian subspecies) which originated with isolation during Quaternary glaciations (Hewitt 1988, Virdee & Hewitt 1992, Cooper *et al.* 1995). Both subspecies differ, aside from morphological and behavioral characteristics (Hewitt *et al.* 1988, Butlin *et al.* 1991, Butlin 1998), because of the distribution of constitutive heterochromatin linked to the sex chromosome and because of differences in gene activity linked to rDNAs, also localized within this chromosome (Gosálvez *et al.* 1988, Hewitt *et al.* 1988a). It is believed that the hybrid zone, localized in some contact areas between the two species in the Pyrenees, formed following range expansion at the end of the last glaciation period and has been maintained by a balance between gene flow and natural selection against hybrids.

One of the first experimental approaches, by crossing the two races, confirmed that synthetic F1 hybrid males are sterile and characterized by severe testis dysfunction, which provides a good example of Haldane's Rule (Hewitt *et al.* 1988, Hewitt 2008). However, in the field, individual males found in the hybrid zone are fully viable and fully fertile (Hewitt *et al.* 1987a, Bella *et al.* 1990), and clines have been found pertaining to mean male offspring follicle length from crosses between males and females on a transect through the hybrid zone (Shuker *et al.* 2005). Interestingly, sperm numbers vary between inter- and intrapopulation matings (Reinhardt 2006) and Virdee and Hewitt (1994) reported a certain rate of hybrid dysfunction across the hybrid zone. The sperm chromatin organization in the unbalanced hybrid genomes formed by these two species, still remains to be analyzed.

Another intriguing example of different interacting genomes is the montane grasshopper *Podisma pedestris*, which exists in the Alps in two chromosomally distinct populations (John & Hewitt 1970, Hewitt 1975b). A new distinct chromosome population is also reported in the Pyrenees (Gosálvez *et al.* 1988b). In the Alps, where this species is analyzed to a greater extent, the diploid chromosome number is a classic of 23 male, 24 female chromosomes and a XO/XX sex-chromosome mechanism, while the Pyrenees population cluster presents a neo-XY/neo-XX system and a diploid number of 22 in each sex. The neo-sex system resulted from an X/L3 fusion which has converted the acrocentric X element into a metacentric neo-X, while the unfused L3 autosome behaves as a neo-Y element. This kind of chromosome rearrangement could initially emerge from a single individual with a high level of fitness in a small isolated population. This particular Robertsonian fusion produces a considerable restriction of chiasma formation in the region proximal to the centromere in the L3 arm of the neo-X. The persistence of this chromosome change would have been facilitated considerably by either a modification in sex ratio or some form of meiotic drive. Notably, females of two races of *P. pedestris* were mated sequentially with a male of each race in one or the other reciprocal combinations, producing four types of double matings. When embryos were karyotyped for both types of chromosome rearrangements, an excess of racial homozygotes and a predominance of the first male's sperm in fertilization was observed. This indicates the presence of assortative fertilization, thereby producing homogamy (Hewitt *et al.* 1989). Knowing how the sperm chromatin structure is affected would facilitate a more precise knowledge of the structure of this hybrid zone.

B chromosomes have been the subject of analysis in grasshoppers in many studies. These extra DNA autonomous units interfere with the standard genome set and produce meiotic deficiencies with subsequent consequences in sperm production (Bella *et al.* 1985, Bidau 1986, Confalonieri & Bidau 1986, Hewitt *et al.* 1987b, Bidau & Confalonieri 1988, Camacho *et al.* 2004, Teruel *et al.* 2009). The effect of B chromosomes on the process of spermatogenesis was studied in the mottled grasshopper *Myrmeleotettix maculatus* using light and electron microscopy. Dissections of the whole male reproductive system were stained in Feulgen and scored for a variety of abnormalities, particularly in the process of sperm bundle organization. Many ultrastructural abnormalities were observed in the process of spermatid elongation (Hewitt *et al.* 1987a,b). These problems were much more frequent in individuals with B-chromosomes and produced a loss of sperm from the sperm bundles. It is suggested that B chromosomes may preferentially cause the dysfunction of spermatids carrying them, thereby producing the loss of gene inheritance observed in crosses (Hewitt *et al.* 1987 a,b).

Another extensively analyzed example where B chromosomes exhibit a high level of polymorphisms is the grasshopper *Eyprepocnemis plorans (*Camacho *et al.* 1980, Camacho *et al.* 1997, Perfectti *et al.* 2004*)*. The geographical distribution of different B chromosome variants of this species has been extensively studied on the southeast Mediterranean coast of Spain and the presence of B chromosomes reported in almost all natural populations analyzed (Camacho *et al.* 1980, Henriques-Gil *et al.* 1984, Bakkali *et al.* 1999). The only populations without B chromosomes have been located in an inland region of the Segura River basin (Cabrero *et al.* 1997). Even in North Africa, samples from this species showed the presence of a B chromosome very similar to those found in the Iberian Peninsula (Bakkali *et al.* 1999) and B chromosomes were also found in the Mediterranean island of Cerdegna, Italy (López-Fernández *et al.* 1992).

The morphology and size of the spermatids have been analyzed in this species. Normal and abnormal sperm in the form of macro- and microspermatids, according to the different size and number of centriolar adjuncts, were identified. The frequency of macro- and microspermatids showed an odd-even pattern with respect to the number of B chromosomes, with a higher frequency of abnormal spermatids associated with odd B-chromosome numbers (Teruel *et al.* 2009).

The South American genus *Dichroplus* exhibits diverse forms of supernumerary heterochromatin; this mainly affects three chromosome pairs: M6, S9 and additionally a mitotically unstable B chromosome (Bidau & Marti 2004, 2005) in the case of *Dichroplus pratensis* and an enthralling neo-XY system in *Dichroplus vittatus* (Bidau & Marti 2000). In *D. pratensis* individuals carrying the B chromosome also present an increased frequency of abnormal spermatids. In this case, the B-chromosome polymorphism is stable, and this points to an advantage of B-chromosome carriers and/or the occurrence of accumulation mechanisms counterbalancing their negative effects on fertility (Bidau *et al.* 2004, Bidau & Marti 2004). The intra- and interchromosome effects of both elements may be important for the

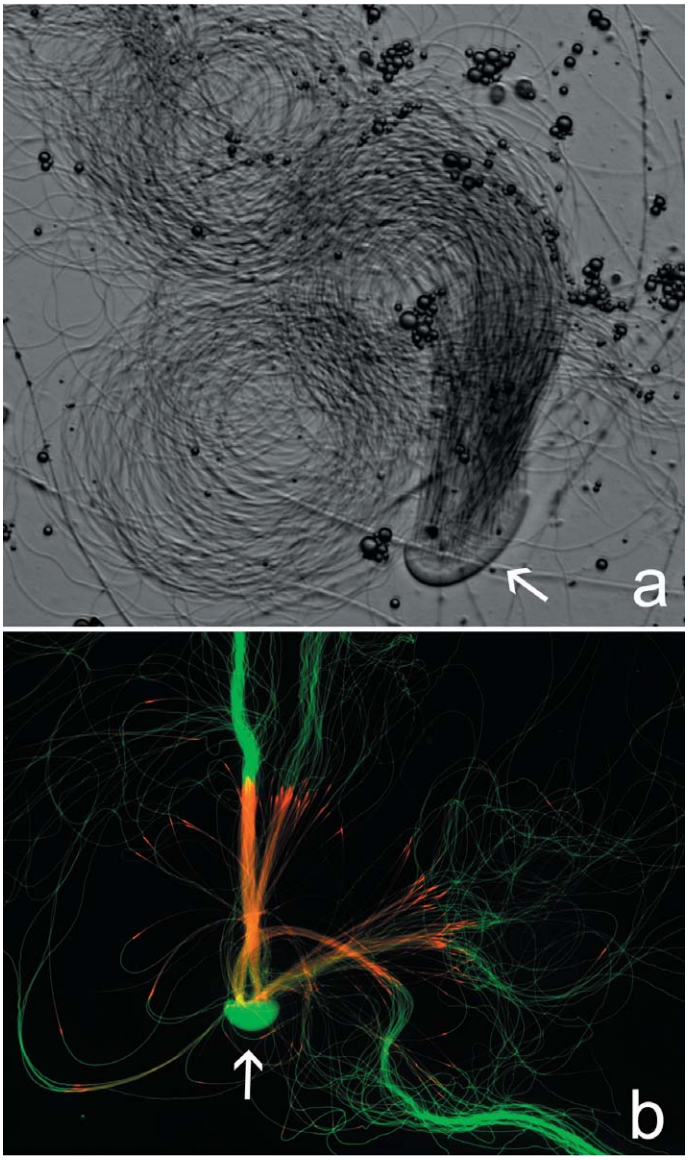

Fig. 1. Sperm bundles from *Melanoplus* species. a. Showing swirling motion of two groups of sperm anchored to a cresent-shaped base (spermatophore matix, arrow). b. Fluoresence of sperm using mercuribromofluorescein as primary green fluoresence for proteins, propidium iodine in red for DNA. For color version, see Plate II.

(developmental or phenotypic) plasticity of populations in terms of changing the standard pattern of recombination for exploring new gene combinations resulting in alternative phenotypes.

Importantly, the studies concerning population cytogenetics performed in the Australian grasshoppers *Cryptobothrus chrysophorus* (John & King 1977 a,b) and *Atractomorpha similis* (John & King 1983, John *et al.* 1986) merit citation within the context of genetic plasticity. The large chromosome variability reported in these species, and the possibility of producing synthetic hybrids between different races, where the presence of univalents leads to a failure of anaphase separation during the first or second meiotic divisions, present magnificent and unexplored systems for analyzing sperm-chromatin organization, since sperm instability can be biologically driven (John & King 1980). It has been reported that from 20 to 40% of the sperm is detected as diploid or polyploid (John & King 1980).

These examples described above rely on solid experimental models to provide new insights for understanding the consequences of a disrupted meiotic process in the sperm DNA molecule. This is important since each alteration derived from an unstable meiotic system needs to be considered as a potential mutation producer, which may be transmitted to the next generation or may decrease the fertility rate of the individual due to the production of sperm with a lower fitness. As we have previously mentioned in this section, while exhaustive research has been conducted on the chromosome system of grasshoppers, the most complete studies performed in sperm only describe minor changes in morphology, behavior of the sperm bundles or incidence of polyploidy and indirect inferences about the presence of macro- and microspermatids. A new research endeavor to link meiosis, consequences in sperm-chromatin organization and fertility is needed.

Structural features of grasshopper sperm

The sperm in grasshoppers is quite characteristic, both in terms of major organization of mature bundle formation and in the internal organization of the chromatin within long-headed sperm. While individual sperm are still anchored to the crescent-shaped sperm-bundle base (arrows in Fig. 1 a and b), they swirl wildly in a rotational manner, like a whirlwind (Fig.1a). That the sperm are anchored leads one to hypothesize that perhaps each sperm detaches in a successive manner, analogous to a pack of firecrackers that explode in succession after the fuse is lit, or in this case after a sperm is released, a chemical signal or enzyme detaching the sperm. Sperm might be released one after the other, with perhaps even a time delay between each one, so that as each oocyte passes through the oviduct a sperm, or several sperm, are able to encounter and attempt to fertilize it.

The ovary of the females consists of ovarioles that converge upon the two oviducts. These produce a final common oviduct which carries ripe eggs having a pronounced nucleus (Fig. 2 a-c). Each of the ovarioles consists of a mass of cells that form oocytes, nurse cells, and follicular cells and a series of follicles. The oocytes are nourished by the nurse cells during early growth stages. In the case of panoistic ovaries, the oocytes do not have nurse cells, but are instead surrounded by maternal somatic (follicle) cells (Fig. 2d). Panoistic ovaries, typical of grasshoppers, produce eggs with less euplasm and are associated with slow embryogenesis when compared to other insect ovary types (West-Eberhard 2003).

In reproduction, the male grasshopper transfers spermatozoa into the spermatheca through insertion of a spermatophore. An interesting question is how the sperm are released from the spermatophore matrix, *i.e.*, what is the chemical cue? Is it the female that is responsible for this, as it is known that sperm can either be stored or digested? Due to the sperm-storage properties of the spermatheca, a female may collect enough sperm in one mating to last until adulthood (Hinn 1999). Snodgrass describes a spermathecal duct with well-developed muscle tissue which could be used to pump sperm out of the spermatheca for use in fertilizing eggs (Snodgrass 1935). Curiously, it would appear that individual females can have varying rates of sperm-processing speeds: where sperm can be found throughout the spermathecae in as early as 5 h in some females, it can also be found highly concentrated right where it is deposited, as late as 26 h into mating (Hinn 1999). In order to be used for the purpose of fertilization, sperm must be split out from their bundles. However, sperm do not need to be split apart from their bundles to be stored (Hinn 1999).

Answers to many of evolutionary biology's biggest questions lie in understanding the production and interactions of sex-specific,

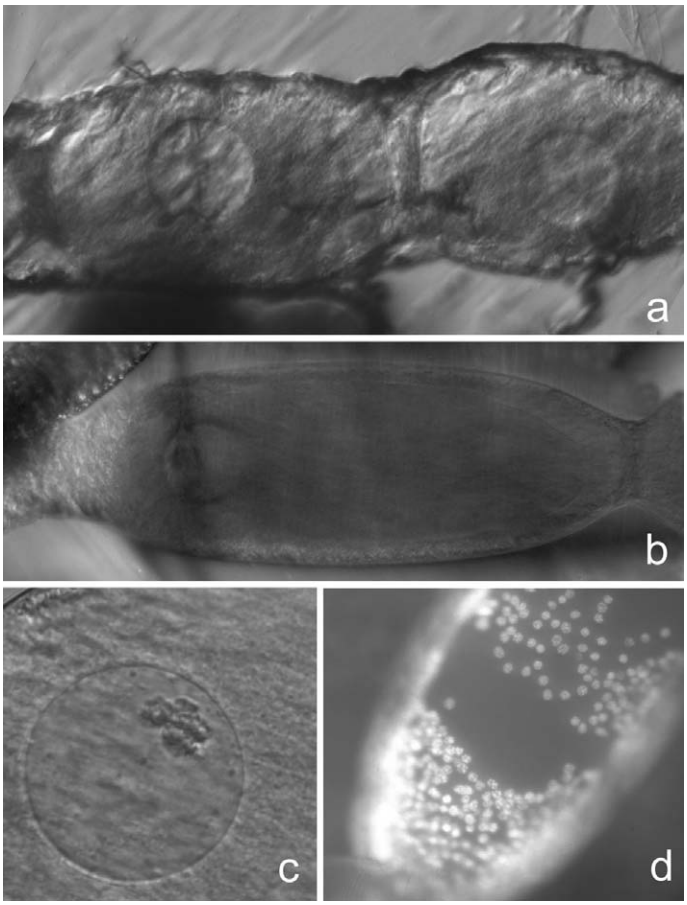

Fig. 2. *Melanoplus* species female gamete. a,b. Images of individual developing oocytes within panoistic ovarioles showing different nucleus positions during maturation. c. Prediapause embryo development. d. Visualization under fluorescence of the maternal somatic cells.

reproductive-tract proteins (Marshall *et al.* 2009). Melanopline grasshopper males make an investment in reproduction in the form of a nuptial gift. In a study by Friedel and Gillott (1976), radiolabeled proteins from the male accessory reproductive glands were found in the females' hemolymph within 24 h of mating in *Melanoplus sanguinipes* (Marshall *et al.* 2009). RNAi technology, in combination with studies on ejaculate and reproductive-tract proteins in insects such as grasshoppers, *e.g.*, *Schistocerca americana* (Dong & Friedrich 2005), offers an opportunity to assess the function of individual proteins and their role in mediating reproductive physiologies (Marshall *et al.* 2009).

Unfortunately, it is often difficult to study ejaculate proteins in nonvertebrate systems with internal fertilization, due to the difficulty of collecting whole ejaculates just prior to insemination and so avoiding the exposure to female proteins that occurs just after insemination (Marshall *et al.* 2009). However, in certain insect species like grasshoppers, the males produce a spermatophore, an external ejaculate that is surrounded by a protective protein coat and allows for internal insemination during copulation via a small duct (Marshall *et al.* 2009). Spermatophores thread up the ductus seminalis and into the proximal chamber, where the sperm is released in well-defined bundles (Hinn 1999). These bundles of sperm primarily remain in the proximal chamber during all mating durations, but can be seen in both the apical and distal chambers within 5 h of the initiation of mating, in equal numbers. According to Hinn (1999), by 8.5 h, sperm can be found in abundance throughout the proximal and distal chambers, and also at the bulbous end of the apical chamber. As mating proceeds, less sperm is actually found in bundles, rather it is loose, often showing as individual spermatozoa in the lumen (Hinn 1999).

In many orthopteran mating systems, nutrient transfer and sperm transfer are inextricably linked in the same package: the spermatophore. Melanoplines do not oviposit immediately after mating, delaying an average of 4.7 d from mating to oviposition (Hinn 1999). It has been suggested that a part of a male's sperm contribution may be used for nourishment. Using sperm as a source of nutrients is not unheard of in insects; *e.g.*, hemocoel insemination in bedbugs is presumed to nourish the female (Hinn 1999). Among lepidopterans, some of the sperm transferred is apryene or anucleate, and these are also suggested to be used for female nourishment (Boggs 1995). It is known that melanoplines can absorb proteins transferred during mating, and the absence of the spermatophore matrix from the distal chamber in all observations suggests it is quickly absorbed.

While the mechanical and kinetic aspects of the sperm transfer from male to female are more or less understood, the internal organization of sperm chromatin is quite obscure. The use of *in situ* hybridization techniques to properly localize specific genome domains has provided evidence that an ordered architecture of chromosomes exists in the interphase cells; and this 3-D organization is involved in a variety of nuclear functions (Manvelyan *et al.* 2008, Cremer & Cremer 2001, van Driel *et al.* 2003, Cremer *et al.* 2004). This new concept of genome organization gives rise to the concept of chromosome territories as a relative spatial arrangement within the interphase nucleus. Subsequently, the elucidation of higher-order chromatin structures and chromosome paths within the chromosome territory, is a topic of major interest because it is claimed that epigenetic changes depend upon the organization logic of these territories (Cremer & Cremer 2001, Chevret *et al.* 2000, Belmont *et al.* 1999, Lowenstein *et al.* 2004).

The spermatozoon, presenting an exceptional chromatin organization within each individual, attracts much interest. However, because of the sperm's peculiarities, the genome architecture, structural organization and chromosome positioning remain largely unresolved. It is well established that basic chromosomal proteins in mammalian sperm (protamines) mostly replace the somatic histone proteins. Investigations have been conducted to understand the fundamental structure formed when genomic DNA is packaged by protamines (Balhorn 1982, Oliva & Dixon1991, Gaucher *et al.* 2010). The basic nucleoprotamine structural unit of DNA packaging has been described as a donut-shaped toroid (Balhorn 1982, Allen *et al.* 1997, Brewer *et al.* 2003). In the higher structural level of organization for mammalian species it is assumed that chromatin in sperm is organized into loop domains, these attached at their bases to a nuclear matrix (Ward & Coffey 1991, Ward & Ward 2004). Multicolor chromosome-banding techniques and 3-D reconstruction of sperm positioning were used to characterize relative localization and the orientation of all human chromosomes. It was concluded that chromosomes are organized not only in a nonrandom way, but also this territoriality is driven by gene density and chromosome size (Manvelyan *et al.* 2008). However, it has been reported that chromosome size has not been found to be a determinant of the overall chromosome positioning in boar sperm (Foster *et al.* 2005).

Although it is largely undetermined how the chromosomes are arranged, the sperm in grasshoppers could be used as a good model to study aspects of genome territoriality. This is mainly because the

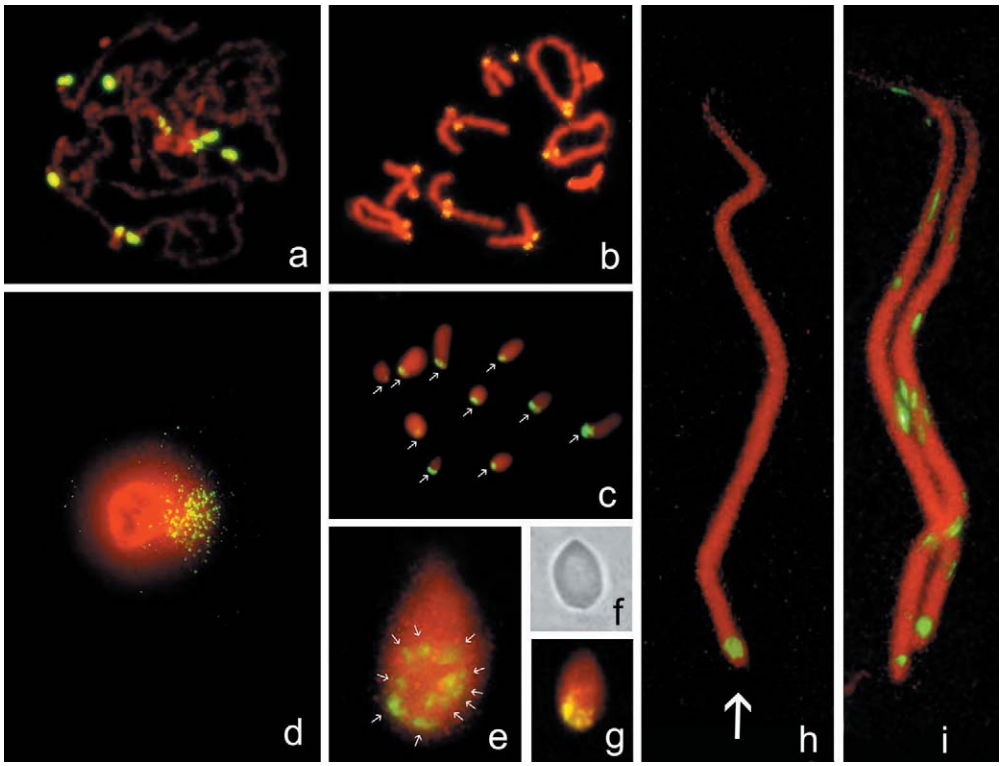

Fig. 3. *P. conica* sperm centromere localization in meiosis. a. Pachytene. b. Diplotene. c. Anaphase II. d-g. Early developed spermatids (d) and partially elongated spermatozoa (e-g). Arrows in e indicate position of centromeres. Note that all centromeres in anaphase II (c) are positioned toward one of the poles and concentrated in the blunt end of the young roundish sperm (d). More condensed sperm nuclei have a maximum number of 9 to 10 visible centromeres (e). Proximity of the different centromeres barred their resolution as separate units. h-i. Localization of telomeres in elongated sperm using a poly-TTAGG DNA probe for a comparsion with the centromere distribution (h). For color version, see Plate II.

sperm, when condensed, is long and provides a unique dimension attained with massive head elongation. This is not so evident in the round or fusiform sperm from most mammals. In what is likely one of the first tentative descriptions of how the sperm is organized in grasshoppers, J. Herbert Taylor (1964) described the mature sperm of these insects as a very elongated, needle-like structure. He suggested that "*the most likely arrangement of chromosomes would be either a parallel arrangement in a bundle or a tandem alignment with each chromosome occupying a short segment of the elongated head*". Using autoradiographic evidence, he concluded that the chromosomes were randomly positioned along the length of the nucleus.

Evidently, the random distribution is not the most likely hypothesis at present –although in grasshopper sperm territoriality has not been described. What does seem clear is that the chromosomes are longitudinally distributed within the sperm head, and are probably organized in an end-to-end spatial distribution. Evidence of this organization was obtained after analyzing the pattern of specific DNA probes of elongated sperm (López-Fernández *et al.* 2006).

In *Pyrgomorpha conica* fluorescence *in situ* hybridization, using a specific DNA clone (*PyrAlu-2p*), derived from a *P. conica* DNA library specific for pericentromeric heterochromatin, and a DNA probe with a poly-TTAGG sequence to label telomeres, was used to map these two genome domains. Conventional FISH using the DNA probe *PyrAlu-2p,* highlighted the pericentromeric constitutive heterochromatin (López-Fernández *et al.* 2006). These regions map all of the centromeres in the pachytene and diplotene chromosomes and are clearly positioned toward one of the poles at anaphase II (Fig. 3a-c). All of the chromosomes, except the X, are bivalents and in this species tend to have a chiasma close to centromeres. FISH signals for detecting centromeres show how they concentrate in the blunt end of the young roundish spermatids (Fig. 3d). In more condensed spermatid nuclei (Fig. 3e-g) a maximum number of 9 to 10 centromeres are detected (Fig. 3e). Frequently, the proximity among the different centromeres barred their resolution as separate units, which typically occurs in highly elongated sperm (Fig. 3g and arrow in 3h). Distribution of telomeres in the sperm can also be denoted by using a poly-TTAGG DNA probe for telomeric sequences (Fig. 3i).

In Orthoptera the chromosome organization consists of the centromere regions distributed along the proximal end of the sperm head and the chromosome arms longitudinally organized from the proximal to the distal end. Because the length of the chromosome arms is different among nonhomologous chromosomes, the telomeres are distributed all along the length of the sperm head (Fig. 3i).

The sperm of grasshoppers could also be processed using the Sperm Chromatin Dispersion (SCD) test to study the presence of DNA breaks along the sperm DNA molecule (Fernández *et al.* 2005, Cortés-Gutiérrez *et al.* 2007). The SCD test requires two steps. In the first, DNA strands that contain breaks or nicks are denatured to produce single-strand DNA motifs. In the second, partial protein depletion from chromatin results in a characteristic pattern of DNA loops, spreading around a nucleoid of DNA that remains attached to protein residues (Fernández *et al.* 2003, 2009).

In early spermatids just emerging from telophase II, and after applying the SCD test in grasshoppers, if the sperm DNA is intact, absence of a halo of dispersed chromatin, or just a small halo, will be observed around a compact core (Fig. 4b). In elongated and more mature sperm, no chromatin-dispersion halo or a characteristic compact halo of DNA loops, are formed around a dense central proteic core (Fig. 4a-c).

If the sperm nucleus contains fragmented DNA, the haloes are larger and spotty, because of the presence of dispersed DNA fragments (Fig. 4d-f). However in grasshoppers, the interesting difference with respect to other analyzed species, is that the DNA loops remain attached to a central proteic core, which is clearly visible in partially elongated spermatozoa (Fig. 4 a-f), and the DNA loops forming the halo expand from this central region. There is a proteic and continuous core running along the sperm (Fig. 4a). In some cases the core appears as discontinuous and the halo produced is

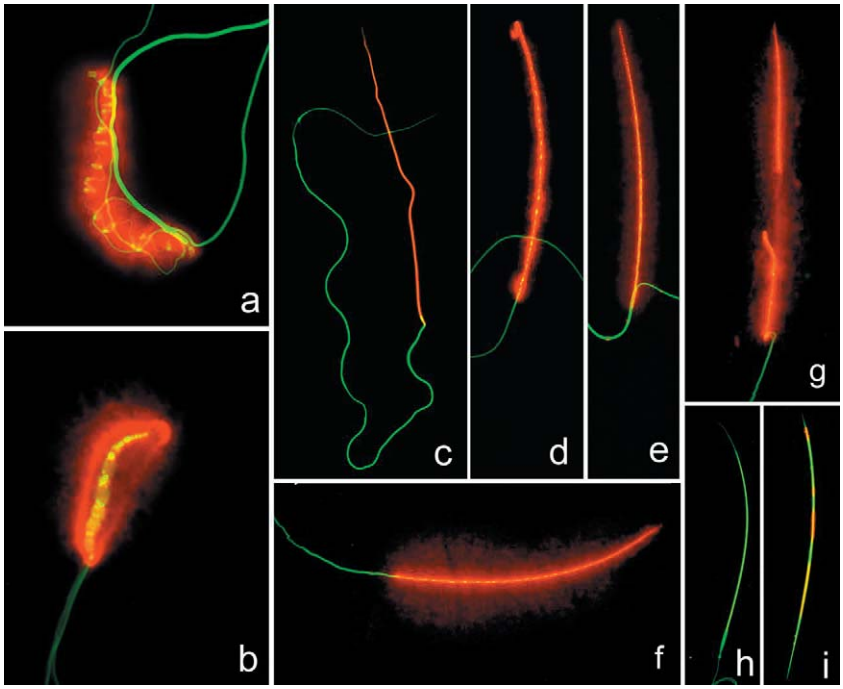

Fig. 4. Partially elongated grasshopper sperm after SCD tests showing DNA loops attached to a central proteic core. a-f. Double staining using bromofluorescein for proteins in green and propidium iodide for DNA in red. Central core is clearly visible as a continuous structure in partially elongated spermatozoa (a). From b to f the sperm core is becoming disrupted (except in c which does not show expanded DNA loops) while the sperm head exhibits expanded DNA loops emerging from the internal core. A sperm showing the central proteic core producing a physical longitudinal detachment along the length of the sperm head (g). h-i. Sperm viability assessment in grasshoppers after acridine orange (green)/propidium-iodide staining. Sperm showing differential and longitudinal loss of membrane quality. A normal sperm membrane (h) and regionally disrupted membrane (i) are shown. For color version, see Plate III.

larger (Fig. 4b). We are hypothesizing that this particular sperm morphology is related to the presence of fragmented DNA. In fact, when the sperm is incubated for 24 to 48h at 37ºC, DNA damage is produced in all sperm and after this incubation, the sperm core tends to appear disrupted in a longitudinal fashion (Fig. 4e-f), even producing a longitudinal detachment of the internal core (Fig. 4g).

We found in a previous experiment that a bundle of DNAs running coaxially to each chromatid length inside the sperm, constitutes the core of the spermatid head (Cerná *et al.* 2008). We can now confirm that this particular view offered by DNA dyes, correlates with the massive anchorage of DNA stretches attached to a true proteic core. The fragmented sperm nuclei appear as a huge halo of dispersed and spotty DNA (Fig. 5a,b). The DNA fragmentation can be selectively labelled using *in situ* nick translation and only those nuclei presenting haloes show extensive DNA labelling (Fig. 5c).

Previously, using specific antibodies against Triplex DNA configurations, we have proposed the presence of triplex DNA motifs in scaffold DNA and the accumulation of nicks in the emerging sperm DNA loops. These triplex DNA may bind a single-stranded DNA from a denatured duplex in a loop to proper duplexes in the scaffold or vice versa. According to the present observations, triplex DNA will be responsible for the anchoring of the expanded DNA loops to the chromatid proteic scaffold, also assuring a recognizable DNA rejoining to avoid undesirable DNA exchanges during histone-protamine replacement. All of these changes occurred in a proximal to distal orientation in the sperm head (Černá *et al.* 2008).

Subsequent to the putative longitudinal sperm mapping of sperm DNA breakage in grasshoppers, a parallel phenomenon was described in echidna (Johnston *et al.* 2009). In the echidna, the sperm is also organized in bundles and exhibits long and thin sperm heads as they become more mature. Using a combined methodology of comet assay and *in situ* nick translation, we reported that the DNA breakage occurs from the proximal to the distal end of the sperm head.

The longitudinal observation of sperm chromatin change is not only related to the chromatin and its particular organization, but also to the sperm membranes. Although we need to analyze this aspect in more detail, the loss of membrane quality, *i.e.*, the so-called sperm viability, is not in grasshoppers a continuous process affecting the entire membrane surface, as happens in mammalian species (Garner & Johnson 1995). The combined use of DNA dyes to

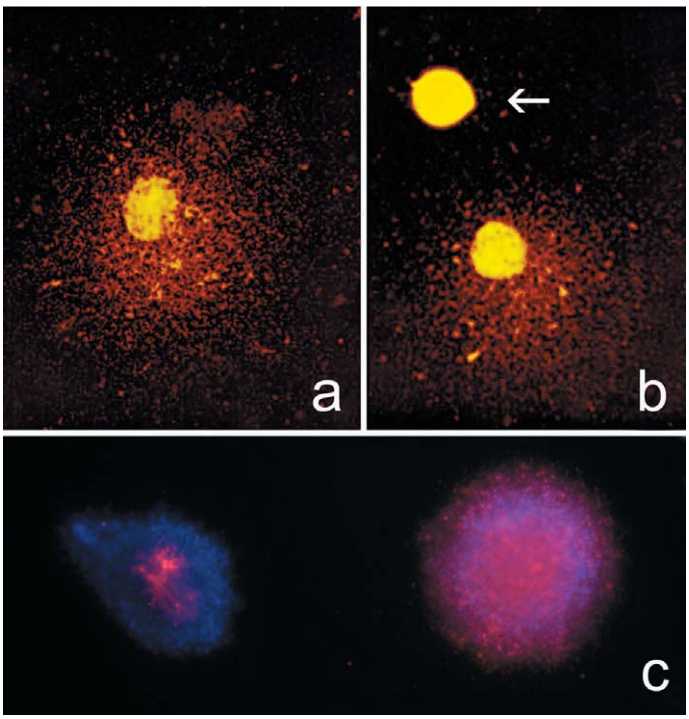

Fig. 5. Example of sperm chromatin dispersion test (SCD) applied to grasshopper sperm. a-b. Fragmented sperm nuclei appear as a large halo of dispersed and spotty DNA. Intact DNA with the absence of a halo of dispersed chromatin or a small halo observed around a compact core (arrow). c. DNA fragmentation having been selectively labelled using *in situ* nick translation (red for nucleotide incorporation and blue for counterstaining). For color version, see Plate III.

assess sperm viability such as SYBR-14/propidium iodide or acridine orange/propidium iodide, produces a differential and longitudinal loss of membrane characteristics (compare Fig. 4h with 4i).

These results point to the existence of a peculiar organization of chromatin in the sperm of grasshoppers where the chromosomes are displaced from a proximal to distal position, with the centromeres localized close to the flagellum insertion (see also diagram in Černá *et al*. 2008). The differences in the thickness of the entire sperm from the proximal to the distal end when elongated, is noticeable and it seems to be related to the different length of each chromosome. The distal end of the sperm nuclei would be occupied by the telomere of the largest chromosome and each telomere marks the longitudinal relative position for the end of each chromosome. All of the sperm chromatin is attached to a central proteic core running from the proximal to the distal end. Disruption of this basic structure, as evidenced with a partial protein depletion using methodological approaches such as the SCD, could be used to analyze the sperm DNA integrity.

Many people are unaware of the magnitude of the scientific contributions made using the grasshopper and view these insects as a commonplace, often proclaimed, "pest". However, given the extreme diversity in evolutionary reproductive systems and genetic plasticity found among grasshopper species, it is obvious why Professor MJD White chose them as an experimental model. The work that he initiated and performed continues to be built upon and provides important information and observation which can be compared and applied in other areas of evolutionary biology and reproductive study. We and many others, have continued advancing the knowledge base laid down by Dr. MJD White. That being said, the future likely holds many more important discoveries using grasshoppers as a model. We leave you with this thought: what might be revealed using RNA interference in terms of heterotopy with regard to adaptive evolutionary change?

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