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Nucleolus size varies with sex, ploidy and gene dosage in insects

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Abstract. The nucleolus constitutes a cytologically visible phenotype for ribosomal DNA (rDNA). Nucleolar size, as determined by silver staining, is a good indicator of cell proliferation rate and biosynthetic activity. Nevertheless, the relationship between rDNA content and sexual dimorphism for nucleolar size is not well documented. In the present study, the impact of sex and ploidy level on nucleolar size is investigated in three haplo/diploid and three diplo/diploid species of insect. Nucleolar sizes are found to be proportional to ploidy level in the haplo/diploid hymenopterans *Trypoxylon albitarse* and *Nasonia vitripennis*. Conversely, in the ant *Messor barbarus*, nucleolar sizes are larger in haploid males (winged) than diploid females (apterous). Among the diplo/diploid species, evidence for gene dosage compensation on nucleolar activity is suggested by the absence of sex differences in *Drosophila simulans*, a species in which rDNA is limited to the X chromosome. By contrast, in the grasshopper *Stenobothrus festivus*, another species with rRNA genes restricted to the X chromosome, the size of the nucleolus is significantly larger in females than in males. Additionally, in the grasshopper *Chorthippus parallelus*, where rDNA is distributed evenly on several autosomes of males and females, the females also show larger nucleoli than males. In both grasshopper species, the magnitude of the female/male ratio for nucleolus area is very similar to the body size ratio, suggesting that body size, as well as sex, ploidy, gene dosage and physiological activity, may be an important determinant of nucleolus area.

Key words. Gene dosage, nucleolus size, ploidy, sex differences.

Introduction

The nucleolus comprises a membrane-free nuclear compartment where the pre-ribosomal components are synthesized from several classes of ribosomal RNA (rRNAs) and a multitude of proteins for subsequent export to the cytoplasm where the ribosomes are assembled (Carmo-Fonseca *et al*., 2000). In both plant and animal cells, the nucleoli are dynamic structures showing extensive variation in size. Variation in nucleolar size is dependent mainly on the activity of the organelle: fully-active nucleoli are larger, whereas inactive nucleoli tend to be small (Shaw & Jordan, 1995). The assembly of a nucleolus requires transcription of ribosomal DNA (rDNA) by the enzyme RNA polymerase, and takes place in the nucleolus organizer regions (NORs) (i.e. the chromosome sites where repetitive clusters of rRNA genes are located) (McClintock, 1934; Scheer & Weisenberger, 1994). However, not all copies of the rRNA genes within a NOR are continually active (Woolford & Warner, 1991). Nucleolus size may partly depend on the number of rRNA genes because deletions within the rDNA array of the *Drosophila melanogaster* Y chromosome decrease nucleolus size (Paredes & Maggert, 2009). The size of nucleolus increases in growing cells in proportion to the amount of rRNA synthesized (Caspersson, 1950; Nakamoto *et al*., 2001; Mosgoeller, 2004). Nucleolus area also correlates with the level of activity of rRNA genes (Kurata *et al*., 1978; Altmann & Leblond, 1982). Nucleolus area is used therefore

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as an indicator of the level of rRNA gene activity, with larger nucleoli being generally associated with high activity (Lahmy *et al*., 2004; Moghaddam *et al*., 2010). Nucleolar size can be affected by different factors, varying from environmental and physiological stresses (Rubbi & Milner, 2003; Olson, 2004) to hormonal changes (Herbener & Bendayan, 1988), as well as the presence of supernumerary chromosomes (Teruel *et al*., 2007, 2009).

The genetic control of nucleolus size is only beginning to be uncovered. In the nematode *Caenorhabditis elegans*, a gene *ncl-1* (for abnormal nucleolus) is shown to be involved in the negative regulation of rRNA synthesis, and to inhibit cell growth (Frank & Roth, 1998). The homologous gene in *D. melanogaster* is *brat*, which is able to replace *ncl-1* functionally in *C. elegans* (Frank *et al*., 2002). *Brat* mutants have enlarged nucleoli and excess rRNA (Frank *et al*., 2002). Overexpression of *Brat* inhibits organ growth and cell growth, and also slows cell division, through the regulation of ribosome synthesis (Frank *et al*., 2002).

Intraspecific variation in the size and number of NORs appears to be a common feature in several animal groups (Araújo *et al*., 2002). Similarly, the ability of some organisms to increase the content of rDNA as a mechanism of dosage compensation is reported for species as different as *Xenopus laevis* (Barr & Esper, 1963; Miller & Gurdon, 1970) and *D. melanogaster* (Tartof, 1971; Semionov & Kirov, 1986). Nevertheless, the biological relevance of such variation is scarcely studied. Dosage compensation can also take place through transcriptional changes (i.e. adjustment of gene expression rate to the dosage of other genes). Typically, this results in similar level of expression for sex-linked genes in both sexes, despite a double dosage in the homogametic sex, as first shown in *Drosophila* (Muller, 1932). Different species appear to have evolved different mechanisms to deal with dosage compensation. For example, in males of *D. melanogaster* (which are genetically XY), the X chromosome genes are hypertranscribed (Mukherjee & Beermann, 1965); in the nematode *C. elegans,* the X-linked genes in XX cells are hypotranscribed (Meyer & Casson, 1986); and, in mammalian females (XX), one of the two X chromosomes is inactivated (Lyon, 1961). However, a recent review concludes that sex chromosome dosage compensation is a rather rare phenomenon in animals, perhaps occurring only in *Drosophila* and *Anopheles* spp. (Mank *et al*., 2011).

Dosage compensation of rRNA genes in *D. melanogaster* appears to operate at the transcriptional level (Mukherjee & Beermann, 1965), allowing reduced amounts of rDNA to be overexpressed in males, amd generating as much rRNA and nucleolar material as needed to maintain homeostasis (Frank *et al*., 2002). Nevertheless, the factors that determine the amount of rDNA and nucleolar material required to maintain homeostasis in different species, and in insects in particular, remains to be determined. In the present study, the impacts of sex, ploidy level and rRNA gene dosage on nucleolar size are investigated in males and females, as well as at different developmental stages, of three haplo/diploid (Hymenoptera) and three diplo/diploid (Diptera and Orthoptera) insect species.

Materials and methods

Nucleus and nucleolus area were measured in three haplo/diploid hymenopterans, namely the wasps *Trypoxylon* (*Trypargilum*) *albitarse* Fabricius and *Nasonia vitripennis* Walker, and the social ant *Messor barbarus* L., as well as three diplo/diploid species, namely *Drosophila simulans* Sturtevant and the grasshoppers *Stenobothrus festivus* Bolívar and *Chorthippus parallelus* Zetterstedt (geographic race *erythropus*). Sampling localities, sample size and the tissues analyzed are summarized in Table 1. Sex was determined phenotypically in all species, except *T. albitarse,* where male and female pupae were indistinguishable, and sex was determined by ploidy level after chromosome count (Araújo *et al*., 2002). In *N. vitripennis*, ploidy level was determined by flow cytometry. For flow cytometry, cerebral ganglia were dissected from the head, and haemolymph was obtained as a drop emerging from an abdominal needle puncture. Tissues were immersed in 500 μL of Galbraith buffer [for 1 L: 4.26 g of MgCl, 8.84 g of sodium citrate, 4.2 g of 3-(*N*-morpholino)-propane sulfonic acid, 1 mL of Triton X-100, 20 μg mL^{-1} boiled ribonuclease A, pH 7.2] to clean the ganglia from undesired remains. The ganglia were homogenized in 500 μL of Galbraith buffer using a Kontes Dounce Tissue Grinder (Kimble Chase, Vineland, New Jersey) (approximately 15 strokes) and the resulting liquid was filtered into an Eppendorf micro tube carrying a membrane (20–25 μm) coupled to another Eppendorf tube. To ensure the filtering of all the material, 200 μL of Galbraith buffer was added to the tissue grinder and then poured into the Eppendorf

Table 1. Species, sample size, tissues and developmental stages analyzed.

Order	Species	Population	Number of		Tissue	Stage analyzed
			Males	Females		
Diptera	*Drosophila simulans*	Laboratory Stock	15	15	Ganglion	Adults
Orthoptera	*Stenobothrus festivus*	Sierra Nevada (Spain)	15	15	Gastric caeca	Adults
Orthoptera	*Chorthippus parallelus*	Sierra Nevada (Spain)	5	5	Gastric caeca	Adults
Hymenoptera	*Messor barbarus*	Granada	10	10	Ganglion and haemolymph	Adults
Hymenoptera	*Trypoxylon albitarse*	Viçosa, Minas Gerais (Brazil)	23	10	Ganglion	Prepupae
Hymenoptera	*Nasonia vitripennis*	STDR[a] laboratory strain	10	10	Ganglion and haemolymph	Pupae and adults

[a] STDR is a red-eye mutant laboratory strain obtained in 1950 (Whiting, 1954).
Ten silver-stained nuclei were analyzed per tissue and individual.

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tube. Then, after 1 min of centrifugation at 600 ***g***, the supernatant was stored at 4 °C in the dark. Before measurement in the flow cytometer, 100 μL of propidium iodide solution (for 500 μL: 100 μL of propidium iodide, 50 μL of 20 × SSC and 350 μL of ultrapure water; 20 × SSC = 175.3 g sodium chloride + 88.2 g sodium citrate L^{-1} water, adjusted to pH 7.0) were added to the isolated nuclei, which were incubated at 4 °C for 30 min without shaking. Samples were then analyzed using a FACS Vantage high speed cell sorter (Becton Dickinson U.K. Ltd, U.K.) fitted with Coherent Enterprise 621 laser (488 and 351–364 nm) in the 'Servicio de Citometría de Flujo del Centro de Instrumentación Científica de la Universidad de Granada'.

To obtain interphasic nuclei for nucleolus area measurements, tissues were fixed in acetic acid/ethanol 1 : 3 (v/v). Cell preparations were made by squashing tissues in 50% acetic acid using coverslips and glass microscope slides, and immersing the slides in liquid nitrogen to separate coverslips, before submitting the tissues to silver staining (Rufas *et al*., 1982). On average, 10 silver-stained nuclei per tissue and per individual were digitized using an Olympus DP50 digital camera (Olympus España, S.A.U., Spain) attached to a Nikon Optiphot microscope (Nikon Instruments Europe B.V., The Netherlands). The areas of the nucleus and nucleolus were measured (in arbitrary units) using IMAGEJ, version 1.20s [http://rsb.info.nih.gov/ij]. The measurements comprised area instead of volume as justified previously (Teruel *et al*., 2007). To estimate measurement errors, a subset of 30 cells per species was measured twice on different days. Measurement error was calculated according to Yezerinac *et al*. (1992). The low magnitude of errors (<3% in *D. simulans* and *S. festivus*, 0.22% in *T. albitarse*, and 0.38% for the nucleus and 0.71% for the nucleolus in *M. barbarus*), confirmed the precision of the method. Measurements were averaged for each individual. The variables measured included number of nucleoli per nucleus, the area of the nucleus and nucleoli, as well as the nucleolus/nucleus area ratio. Results were compared between sex, cell-types and developmental stages by means of Student's *t*-tests for metric traits (i.e. nucleus and nucleolus area, and nucleolus/nucleus ratio) and contingency chi-square tests for meristic trait (number of nucleoli per cell). The *t*-tests were performed with STATISTICA, version 6.0 (Statsoft, Inc., Tulsa, Oklahoma) and the chi-square tests with RXC software (G. Carmody, Carleton University, Ottawa, Canada). The latter software calculates chi-square values for the observed contingency table and for 10 000 simulated tables obtained by permutation, and also calculates *P*-values using Monte Carlo methods. Sequential Bonferroni tests were used to minimize type I errors in multiple tests within species. The *P*-values obtained are indicated as P_b.

Results

The solitary wasp T. albitarse

The haploid karyotype in *T. albitarse* consists of $n = 16$ chromosomes in males and $2n = 32$ in females (Araújo *et al*., 2002). Females had consistently more nucleoli per cell than males (Table 2). Similarly, the areas of the nucleus and the nucleolus in female cells were approximately two-fold higher than the male values. Therefore, no significant differences were observed when the proportion of nucleolar to nuclear areas was assessed. Independently of sex, nucleolus area represented 5% of the nuclear area. Therefore, in *T. albitarse*, the size of the nucleolus is proportional to the size of the nucleus, to ploidy and to rRNA gene dosage.

The parasitoid wasp N. vitripennis

Flow cytometry assessment of the ploidy level in ganglion cells and haemocytes of *N. vitripennis* showed an almost two-fold higher amount (×1.96) of DNA in ganglion cells of females compared with males. Notably, male haemocytes showed approximately 1.81-fold more DNA than male ganglion cells. Haemocytes thus showed significantly higher number of nucleoli than ganglion cells in male pupae ($\chi^2 = 12.18$, d.f. = 1, $P = 0.0006$) and adults ($\chi^2 = 13.33$, d.f. = 2, $P = 0.0003$). Furthermore, Student's *t*-tests showed that nucleus area, nucleolus area and nucleolus/nucleus ratio were significantly larger in male haemocytes than male ganglion cells (Table 3) at both pupal ($P_b < 0.0034$ in all three cases) and adult stages ($P_b < 0.0087$ in all three cases). Nevertheless, differences between cell types appear to be regulated developmentally because the nucleolus/nucleus ratio was over six-fold higher in haemocytes compared with ganglion cells at the pupal stage, but only approximately three-fold in adults (Table 3).

Developmental regulation of the number of nucleoli was also observed in female *N. vitripennis*. Although female ganglion cells had significantly more nucleoli compared with haemocytes at the pupal stage ($\chi^2 = 13.21$, d.f. = 1, $P = 0.0003$),

Table 2. Sex differences in nucleus and nucleolus size in prepupae of the solitary wasp *Trypoxylon albitarse*.

Item	Males Mean ± SD	Females Mean ± SD	χ^2/t	d.f.	*P*	P_b	F/M
Number of nucleoli per cell	1.23 ± 0.29	1.68 ± 0.32	49.74	2	<0.0001	**<0.0002**	1.36
Nucleus area (AU)	25.77 ± 9.74	51.10 ± 19.80	−4.97	31	0.000023	**0.000092**	1.98
Nucleolus area (AU)	1.32 ± 0.58	2.65 ± 1.01	−4.72	31	0.000048	**0.000144**	2.00
Nucleolus/nucleus (%)	5.27 ± 1.82	5.54 ± 3.16	−0.40	31	0.693667	—	1.03

AU, arbitrary units; F/M, female/male ratio; P_b, sequential Bonferroni corrected probability.
Significant sex differences are indicated in bold.

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Table 3. Sex differences in nucleus and nucleolus area in pupae and adults of the parasitoid wasp *Nasonia vitripennis*.

Stage	Organ	Item	Males	Females	χ^2/t	d.f.	P	P	F/M
			Mean ± SD	Mean ± SD					
Pupae	Ganglion	Number of nucleoli per cell	1.01 ± 0.03	1.21 ± 0.16	20.43	1	<0.0001	**<0.0004**	1.20
		Nucleus area (AU)	46.36 ± 5.69	50.17 ± 15.97	−0.71	18	0.486495	—	1.08
		Nucleolus area (AU)	0.85 ± 0.35	1.59 ± 3.16	−2.71	18	0.014282	**0.042846**	1.88
		Nucleolus/nucleus (%)	1.87 ± 0.85	3.21 ± 0.38	−2.42	18	0.026352	0.052704	1.72
	Haemolymph	Number of nucleoli per cell	1.14 ± 0.13	1.04 ± 0.09	6.11	1	0.0216	0.0648	0.91
		Nucleus area (AU)	86.77 ± 20.02	65.65 ± 21.82	2.26	18	0.036779	0.073558	0.76
		Nucleolus area (AU)	10.26 ± 3.86	11.41 ± 4.46	−0.62	18	0.544191	—	1.11
		Nucleolus/nucleus (%)	12.02 ± 4.43	17.27 ± 2.50	−3.26	18	0.004361	**0.017444**	1.44
Adults	Ganglion	Number of nucleoli per cell	1.01 ± 0.03	1.19 ± 0.13	18.00	1	<0.0001	**<0.0004**	1.18
		Nucleus area (AU)	28.61 ± 14.07	24.76 ± 12.62	0.64	18	0.527608	—	0.87
		Nucleolus area (AU)	0.88 ± 0.28	0.79 ± 0.41	0.55	18	0.589615	—	0.90
		Nucleolus/nucleus (%)	3.48 ± 1.33	3.32 ± 1.36	0.25	18	0.801991	—	0.95
	Haemolymph	Number of nucleoli per cell	1.16 ± 0.16	1.20 ± 0.19	0.60	2	0.7732	—	1.03
		Nucleus area (AU)	79.60 ± 34.85	69.27 ± 26.63	0.74	18	0.466075	—	0.87
		Nucleolus area (AU)	8.01 ± 4.30	11.12 ± 4.93	−1.51	18	0.148148	—	1.39
		Nucleolus/nucleus (%)	10.22 ± 2.91	16.29 ± 3.79	−4.01	18	0.000814	**0.003256**	1.59

AU, arbitrary units; F/M, female/male ratio; P_b, Sequential Bonferroni corrected probability. Significant sex differences are indicated in bold.

Table 4. Sex differences in nucleus and nucleolus area in adult *Messor barbarus* ants.

Organ	Item	Males	Workers	χ^2/t	d.f.	P	P_b	W/M
		Mean ± SD	Mean ± SD					
Ganglion	Number of nucleoli per cell	1.00 ± 0.00	1.28 ± 0.19	32.56	1	<0.0001	**<0.0004**	1.28
	Nucleus area (AU)	32.37 ± 5.15	37.26 ± 8.44	−1.56	18	0.135876	—	1.15
	Nucleolus area (AU)	1.77 ± 0.51	1.44 ± 0.25	1.82	18	0.085955	—	0.82
	Nucleolus/nucleus (%)	5.48 ± 1.36	3.99 ± 0.82	2.96	18	0.008440	**0.025320**	0.72
Haemolymph	Number of nucleoli per cell	1.10 ± 0.19	1.03 ± 0.06	4.03	1	0.0801	—	0.94
	Nucleus area (AU)	57.27 ± 8.29	52.95 ± 16.25	0.75	18	0.463384	—	0.92
	Nucleolus area (AU)	9.70 ± 2.97	5.80 ± 3.26	2.81	18	0.011683	**0.035049**	0.60
	Nucleolus/nucleus (%)	16.79 ± 3.79	10.73 ± 4.43	3.37	18	0.003393	**0.013572**	0.64

AU, arbitrary units; P_b, Sequential Bonferroni corrected probability; W/M, worker/male ratio. Significant sex differences are indicated in bold.

no significant differences were observed in adults ($\chi^2 = 1.03$, d.f. = 2, $P = 1$). Similarly, Student's t-tests did not reveal significant differences in the size of the nucleus between both cell types at the pupal stage ($P = 0.087$). However, the size of the nucleus in adults ($P_b = 0.0003$) and the absolute and relative (to the nucleus) nucleolar areas were larger in haemocytes than ganglion cells at both stages ($P_b < 0.00001$ in all four comparisons) (see Table 3).

No significant differences were observed in the size of the nucleus of haematocytes in the two developmental stages analyzed ($P > 0.05$), suggesting that these cells had already reached their full functional level at the pupal stage. By contrast, the nuclear sizes of ganglion cells decreased significantly from the pupal to the adult stage in both sexes (male $P_b = 0.0066$; female $P_b = 0.0038$). Moreover, the nucleolar area decreased from pupae to adult in female ganglion cells ($P_b = 0.0333$), whereas the nucleolus/nucleus ratio increased in male ganglion cells ($P_b = 0.0145$).

The number of nucleoli per cell was affected by ploidy level but not by developmental stage. Female ganglion cells had significantly more nucleoli per cell compared with males at both developmental stages (Table 3). Remarkably, haemocytes, which do not show ploidy differences between the sexes (Rasch *et al*., 1977; present study) showed no differences in nucleolus number between developmental stage or sex (Table 3). In agreement with females having a two-fold higher amount of DNA, and hence double the number of rRNA genes per nucleus compared with males, the nucleolus area of ganglion cells and nucleolus/nucleus ratio at the pupal stage was 1.88-fold and 1.72-fold higher, respectively, in females. Nevertheless, no sex differences for either trait were observed in adults (Table 3). Furthermore, no sex differences in the nucleolus area of heemocytes were observed. By contrast, the nucleolus/nucleus ratio was significantly higher in both pupal (1.44-fold) and adult (1.59-fold) female haemocytes.

The social ant M. barbarus

Adult workers (diploid) and males (haploid) were analyzed (Table 4). Ganglion cells in haploid individuals (males) always showed a single nucleolus, whereas haemocytes showed two nucleoli in 10% of cells ($\chi^2 = 10.53$, d.f. = 1,

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Table 5. Sex differences in nucleus and nucleolus area in *Drosophila simulans* adult flies.

Item	Males Mean ± SD	Females Mean ± SD	χ^2/t	d.f.	P	P_b	F/M
Number of nucleoli per cell	1.00 ± 0.00	1.02 ± 0.08	3.03	1	0.242	—	1.02
Nucleus area (AU)	37.43 ± 17.35	26.99 ± 7.55	2.14	28	0.041	0.167	0.72
Nucleolus area (AU)	1.83 ± 0.81	1.61 ± 0.54	0.85	28	0.404	—	0.88
Nucleolus/nucleus (%)	5.08 ± 1.59	6.05 ± 1.59	−1.65	28	0.109	—	1.19

AU, arbitrary units; F/M, female/male ratio; P_b, Sequential Bonferroni corrected probability.

$P = 0.0013$), Male haemocytes had a significantly larger nucleus area ($t = 8.06$, d.f. = 18, $P < 0.000001$), nucleolus area ($t = 8.35$, d.f. = 18, $P < 0.000001$) and nucleolus/nucleus ratio ($t = 8.91$, d.f. = 18, $P < 0.000001$) than ganglion cells. Similarly, in worker (diploid) ants, haemocytes had larger nucleus areas ($t = 2.71$, d.f. = 18, $P = 0.014$), nucleolus areas ($t = 4.23$, d.f. = 18, $P = 0.0005$) and nucleolus/nucleus ratio ($t = 4.86$, d.f. = 18, $P = 0.0001$) than ganglion cells. Conversely, ganglion cells in these same diploid individuals had significantly more nucleoli per nucleus than haemocytes ($\chi^2 = 23.86$, d.f. = 1, $P < 0.0001$).

Workers showed more nucleoli in ganglion cells than males, although similar numbers were observed in haemocytes (Table 4). The area of the nucleus did not differ between males and workers, despite the difference in ploidy level. Remarkably, the nucleolus area was significantly larger in male haemocytes compared with worker haemocytes. However, in ganglion cells, this difference did not reach significance. The area of the nucleolus relative to the area of nucleus was significantly larger in males compared with workers, for both types of cells analyzed.

The fruit fly D. simulans

In total, 150 ganglion cells were analyzed in male and female *D. simulans*. Overall, 98% cells in females and 100% in males showed only one nucleolus per nucleus. No statistically significant sex-related differences were observed in nuclear and nucleolar areas, nor in the nucleolar/nuclear ratio (Table 5).

The grasshopper S. festivus

Males showed a single nucleolus in all cells analyzed, whereas females had two nucleoli in most cells, averaging 1.79 nucleoli per cell (Fig. 1). A single nucleolus was observed in 21% of female cells, suggesting either somatic association of the two X chromosome NORs or the inactivity of one of them. Overall, the mean nucleolus area of female cells was 1.68-fold higher than that of male cells. Moreover, when differences in nucleus size were taken into account, the area of the nucleolus relative to area of the nucleus was 1.34-fold higher in females than males (Table 6).

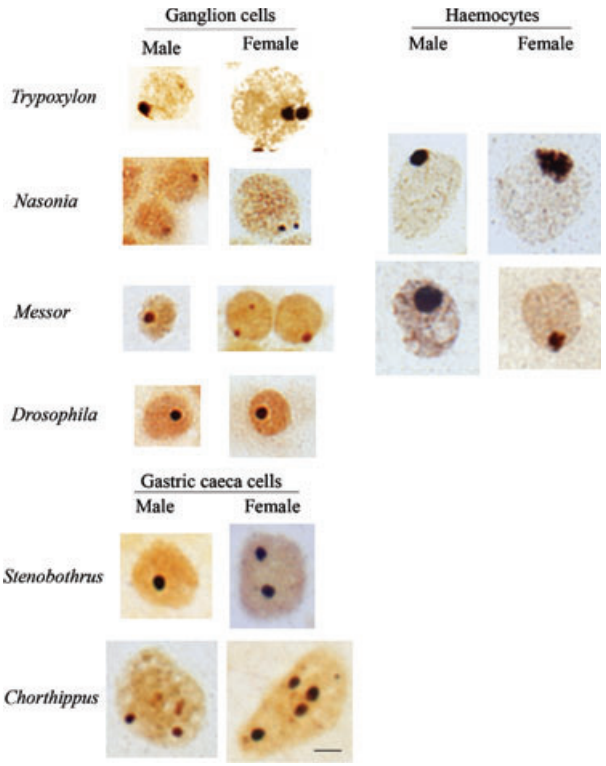

Fig. 1. Silver-stained interphase nuclei from ganglion cells and haemocytes of males and females from six insect species. From top to bottom, the solitary wasp *Trypoxylon albitarse*, the parasitoid wasp *Nasonia vitripennis*, the ant *Messor barbarus* (males and workers), the fruit fly *Drosophila simulans*, and the grasshoppers *Stenobothrus festivus* and *Chorthippus parallelus*. Scale bar = 10 μm.

The grasshopper C. parallelus erythropus

Female values were significantly higher than those for males in all traits analyzed (Table 7).

Discussion

Sex differences in nucleolus size can result from a variety of genomic features, such as ploidy level and rRNA gene dosage. Direct evidence comes from the results of the present study with respect to the three species of Hymenoptera analyzed.

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Table 6. Sex differences in nucleus and nucleolus area in adult grasshoppers *Stenobothrus festivus*.

Item	Males Mean ± SD	Females Mean ± SD	χ^2/t	d.f.	*P*	P_b	F/M
Number of nucleoli per cell	1.00 ± 0	1.79 ± 0.19	127.8	3	<0.0001	**<0.0004**	1.79
Nucleus area (AU)	55.68 ± 16.00	64.16 ± 15.84	−1.46	28	0.156022	—	1.15
Nucleolus area (AU)	1.97 ± 0.54	3.31 ± 1.74	−2.84	28	0.008385	**0.016770**	1.68
Nucleolus/nucleus (%)	3.61 ± 0.62	4.97 ± 1.16	−4.03	28	0.000392	**0.001176**	1.34

AU, arbitrary units; F/M, female/male ratio; P_b, Sequential Bonferroni corrected probability.
Significant sex differences are indicated in bold.

Table 7. Sex differences in nucleus and nucleolus size in *Chorthippus parallelus* adult grasshoppers.

Item	Males Mean ± SD	Females Mean ± SD	χ^2/t	d.f.	*P*	P_b	F/M
Number of nucleoli per cell	1.46 ± 0.31	2.30 ± 0.47	10.39	3	0.0124	**0.0248**	1.58
Nucleus area (AU)	75.57 ± 13.60	99.48 ± 10.71	−3.09	8	0.014921	**0.014921**	1.32
Nucleolus area (AU)	3.39 ± 0.92	6.36 ± 0.83	−5.42	8	0.000630	**0.002520**	1.88
Nucleolus/nucleus (%)	4.51 ± 1.16	6.41 ± 0.63	−3.22	8	0.012195	**0.036585**	1.42

AU, arbitrary units; F/M, female/male ratio; P_b, Sequential Bonferroni corrected probability.
Significant sex differences are indicated in bold.

Hymenoptera are haplo-diploid insects where gene dosage is constitutively balanced (1 : 1) for any ploidy level because a given gene in single dose in haploid individuals will interact with a number of genes also in single dose, and the same fits to diploid individuals. Therefore, no dosage compensation appears to be necessary in haplo-diploid organisms. The result of the present study indicate that this is so in the wasps *T. albitarse* and *N. vitripennis*, where sex differences in nucleolus size may be explained mostly by ploidy level. *Trypoxylon albitarse* appears to show a constitutive adjustment of nucleolus size to ploidy level because nucleolus size is proportional to the size of the nucleus in both sexes, thus resulting in nucleoli being larger in females. In the absence of transcriptional dosage compensation, a similar rate of rRNA gene transcription would logically lead to larger nucleolus area (approximately double-sized) in the diploid sex. Moreover, if nucleus size is a reflection of genome size (Gregory, 2002) and ploidy level, then the nucleolus area relative to nucleus area should be similar in both sexes, as is the case in *T. albitarse*.

In *N. vitripennis*, a similar situation is found for ganglion cells, which are haploid in males but diploid in females, although not for haemocytes, which are diploid in both sexes, as is indicated by the flow cytometry results in the present study that confirm a previous study by Rasch *et al*. (1977). Therefore, haplo-diploid tissues, such as ganglia, appear to show the nucleolus size being constitutively proportional to ploidy level and gene dosage. In tissues that become diploid in males (e.g. the haemolymph), the nucleolus/nucleus ratio is larger in females, and is similar to the finding in grasshoppers. Rasch *et al*. (1977) argue in favour of gene dosage compensation in haplodiploid organisms by means of an additional cycle of DNA replication in some somatic tissues that insures a minimum DNA amount per cell (e.g. in haemocytes that become diploid in haploid males of the wasps *Habrobracon juglandis*, *Habrobracon serinopae* and *N. vitripennis*). However, unless the additional DNA replication affects rRNA genes differentially, ploidy level does not change the intragenomic proportion among genes and cannot serve as a compensation mechanism. The higher nucleolus/nucleus ratio in female haemocytes of *N. vitripennis* (approximately 1.5-fold higher than that of males) could perhaps be explained by certain life-history traits (e.g. body size or longevity). King & Hopkins (1963) report a total body length of 1.92 mm in males and 2.22 mm in females for this species, and a longevity of 2.29 and 3.52 days for mated males and females, respectively. This yields female/male ratios equal to 1.16 for body size and 1.54 for longevity, suggesting that the longevity shows a sex bias similar to that of nucleolus/nucleus area and thus could be associated with the higher nucleolus size in females.

In the ant *M. barbarus*, the reverse situation exists because the nucleolus/nucleus ratio is significantly higher in males than in workers (i.e. it is higher in haploid than in diploid individuals). This difference cannot be explained by body size differences because workers are almost six-fold heavier than males (J. P. M. Camacho, unpublished data). Of course, the more relevant comparison would have been with queens, although these are difficult to find. However, the most remarkable phenotypic feature that differentiates males from workers is the presence of wings in males and their absence in workers. Because differential gene expression is reported between alate and dealate queens in the ant *Solenopsis invicta* (Tian *et al*., 2004), it is conceivable that the larger nucleoli in males may reflect higher transcriptional demands associated with flight. The fact that males show larger nucleoli with half the number of rRNA genes than workers suggests that it is presumably the result of the hyper-transcription of these genes. This is not dosage compensation but simply the transcriptional regulation of rRNA genes to satisfy the demands

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for translational machinery by other genes presumably showing a high expression level in males performing the nuptial flight.

In diploid organisms with heterogamety, sex-linked genes show different proportions between sexes with respect to the autosomal genes with which they interact. The homogametic sex has 1 : 1 proportions, whereas the heterogametic sex has 1 : 2 proportions. In *D. simulans*, males and females have nucleoli of similar size, as well as having a similar nucleolus/nucleus ratio. This could be interpreted as a result of gene dosage compensation, which might conceivably be visualized in this species through nucleolus size because it carries rRNA genes only on the X chromosome (Lohe & Roberts, 1990, 2000). The first report of dosage compensation in *D. simulans* is by Muller (1948) for the *bobbed* locus of the X chromosome, which actually refers to the rDNA locus, and is absent from the Y chromosome in this species. Lakhotia *et al*. (1981) observe that, in X0/XX hybrid mosaics of *D. simulans*, which are obtained by crossing to *D. melanogaster*, the X chromosome of the X0 nuclei displays an almost two-fold higher rate of transcription (as measured by the incorporation of ^{3}H-uridine) than each of the X chromosomes in XX nuclei within the same gland. A logical consequence of this is the similarity in nucleolus size between the sexes observed in the present study, despite the double dosage of rRNA genes in females. Dosage compensation for autosomal genes (somatic dosage compensation) also occurs in some organisms, such as in partial trisomics of maize (Birchler, 1979) and *D. melanogaster* (Devlin *et al*., 1982), and in triploids of *Bombyx mori* (Suzuki *et al*., 1999).

Because genes usually operate within expression networks, sex chromosome dosage compensation in diploid organisms is best viewed as a regulatory mechanism tending to balance the transcription level and product demands between sex chromosome and autosome genes in the heterogametic sex, where they are in an unequal (1 : 2) ratio. This limits the arena for sex chromosome dosage compensation to the heterogametic sex, and it operates through an increase in transcription level of the single X or Z chromosome to the level demanded from a diploid complement (Mank *et al*., 2011). On this basis, the hyper-transcription of sex-linked genes in *Drosophila* males is the paradigm for this phenomenon.

The present study provides evidence that nucleolar size may be related to rRNA gene dosage compensation in *D. simulans* but not in the grasshopper, *S. festivus*. In this last species, rRNA genes are limited to the X chromosome, so that the homogametic females (XX) carry double dosage of rRNA genes compared with heterogametic males (X0) (Cabrero & Camacho, 2008). Nucleolus size is significantly larger in females than males, suggesting that this trait is not sensitive to dosage compensation in this grasshopper species. Dosage compensation is shown for the sex-linked enzyme phosphoglucomutase in several orthopteran species, including the grasshopper *Goniaea australasiae* (Hebbert, 1984). In *S. festivus*, however, the observed differences between sexes in nucleolus size suggest that sex differences are probably a result of other factors, as suggested by the similar observations found in the other grasshopper species, *C. parallelus erythropus*, which carries two clusters of rDNA located in autosomes 2 and 3 (Cabrero & Camacho, 2008), so that males and females carry a similar number of rRNA genes. A possible factor explaining sex differences in nucleolus size in grasshoppers is body size, which is approximately 1.33-fold higher for females than males in both species (J. P. M. Camacho, unpublished data), and this figure is very similar to the 1.34-fold higher nucleolus/nucleus ratio for females of *S. festivus* and the 1.42-fold higher ratio in *C. parallelus erythropus*. The possibility that sex differences in nucleolus size might be associated with body size merits further research.

In summary, the findings of the present study show that, in insects, nucleolus size is a complex trait that is associated with a variety of genomic, physiological and environmental factors. In some cases (e.g. some Hymenoptera), nucleolus size may be proportional to ploidy level and thus rRNA gene dosage. In others (e.g. the fruitfly *D. simulans*), nucleolus size may be adjusted by mechanisms of sex chromosome gene dosage compensation, whereas in yet others (e.g. the grasshopper *S. festivus*), no signs of dosage compensation are apparent and nucleolus size is approximately proportional to body size.

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