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Level of Heat Shock Proteins Decreases in Individuals Carrying B-Chromosomes in the Grasshopper *Eyprepocnemis plorans*

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Abstract
We analyzed the effect of B-chromosome presence on expression level of heat shock protein 70 (Hsp70) in cerebral ganglion and gonad in both males and females of the grasshopper *Eyprepocnemis plorans*. Two natural Spanish populations, Salobreña (Granada) and Torrox (Málaga) were assayed, the former harbouring a neutralized (non-driving) B-chromosome (B_2) and the latter a parasitic (driving) B-chromosome (B_{24}). The analysis was performed by Western blotting, immunostaining and densitometric measuring expression level of the Hsp70 family in adult individuals. The results showed that Hsp70 levels of testis were significantly higher in Salobreña than Torrox, and were significantly lower in testes of B-carrying males from both populations. A similar effect was observed in the ovary of females from Torrox. No effect was, however, observed in cerebral ganglia in any sex or population. B-chromosome effects in Torrox showed a dose-dependent pattern. The results point to an interesting interaction between B-chromosome and stress protein expression in reproductive tissue.

Heat shock proteins are a group of molecular chaperones involved in maintaining cellular homeostasis in response to stress [Feder and Hofmann, 1999]. Among them, the Hsp70 multi-gene family includes some of the most conserved of these proteins, i.e. the heat shock-inducible 70-kDa chaperone (Hsp70) that helps stabilizing and fold proteins during stress and recovery from stress. Hsp70 also participates in other aspects of cellular homeostasis, such as regulation of transcription, trafficking and degradation of proteins, and acquisition of thermotolerance [Lindquist and Craig, 1988; Feder and Hofmann, 1999; Sørensen et al., 2003]. Other members of the Hsp70 family, e.g. Hsc70, are constitutively expressed and respond only weakly to heat. Under normal conditions, Hsc70 plays a major role in endocytosis, and acts as a molecular chaperone facilitating protein trafficking among intracellular compartments, including translocation across membranes [for review, see Fishelson et al., 2001].

After a heat shock, Hsp70 passes from cytoplasmic or nuclear locations to preferentially concentrate in the nucleolus [Pelham, 1984], thus avoiding nucleolar disassembling caused by increased temperature. In addition to being associated with temperature stress, higher levels of Hsp70 are related to several other sources of stress, such as exposure to radiation, heavy metals, salinity, pesticides

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or parasitism [see Sørensen et al., 2003], and including stress of genetic origin, such as that produced by inbreeding [Wheeler et al., 1999; Kristensen et al., 2002]. Genetic-based stress can also be produced by genomic elements such as parasitic B-chromosomes [Camacho et al., 2002], which are additional dispensable chromosomes that are present in some individuals in a number of plant and animal species [Jones and Rees, 1982].

B-chromosomes are considered both selfish genetic elements, because they are transmitted at higher ratio than that predicted by the Mendelian laws of inheritance, and genomic parasites, because they usually decrease carrier fitness. As with any parasite, there are important interactions between B-chromosomes and their hosts. These interactions take place at several biological levels, including genes, genomes, cells, organisms and populations [Camacho, 2005]. Like other selfish genetic elements (e.g. transposons, segregation distorters and cytoplasmic factors), B-chromosomes could produce genetic stress in the individuals carrying them [Teruel et al., 2007, 2009]. Indirect evidence of this stress on carrier individuals is manifested by B-chromosomes depressing some fitness-related traits, such as egg fertility in the grasshopper *Eyprepocnemis plorans* [Zurita et al., 1998; Muñoz et al., 1998]. Another indication of the stress caused by B-chromosomes at cell level is the increase in the number of chiasmata per cell, since recombination appears to increase in stressful conditions [Parsons, 2008]. In *E. plorans*, B-chromosomes affect chiasma frequency depending on the degree of parasitism, implying that parasitic Bs cause higher increase in chiasma frequency than neutral Bs [Camacho et al., 2002]. This grasshopper harbours a B-chromosome system which has become a paradigm for the near-neutral model of B-chromosome evolution [Camacho et al., 1997, 2003]. Under this model, B-chromosomes invade populations by being selfish (i.e. showing drive), but the evolutionary response of A chromosomes leads to the neutralization (loss of drive) of B-chromosomes.

Most B-chromosomes are heterochromatic and rich in repetitive DNA of several types, although ribosomal DNA (rDNA), satellite DNA (satDNA) and mobile elements seem to be especially abundant in them [for review, see Camacho, 2005]. Although B-chromosomes seem to harbour few functional genes, one of the most interesting impacts of B-chromosomes is on gene expression of the A chromosome set. For example, the presence of B-chromosomes in the plants *Scilla autumnalis* [Ruiz-Rejón et al., 1980; Oliver et al., 1982] and *Allium schoenoprasum* [Plowman and Bougourd, 1994] have been shown to influence the expression of A genes for an esterase and endosperm protein, respectively. More recently, a study by means of differential display reverse transcription-polymerase chain reaction has revealed the differential gene expression of 3 cDNA fragments: Chaperonin containing TCP-1, subunit 6b (zeta) *(CCT6B),* Fragile histidine triad gene *(FHIT)* and hypothetical gene *XP* transcript, in B-carrying yellow-necked mice *Apodemus flavicollis* [Tanic et al., 2005]. These examples of gene expression affected by B-chromosomes entail the possibility that the expression of host's stress-response genes could be affected by the presence of these parasitic elements. In addition, other sensors of stress such as ribosomal biosynthesis [Rudra and Warner, 2004] and nucleolar size [Olson, 2004] appear to respond to B-chromosome presence and evolutionary stage, since we have found that the nucleolus area in several male meiotic stages was lower in a population of *E. plorans* showing a high frequency of the parasitic B_{24} than in a population where the predominant B (B_2) is neutralized [Teruel et al., 2007]. In the present paper, we aim to analyze whether the presence of B-chromosomes influences the amount of heat shock protein in individuals from 2 natural populations of the grasshopper *Eyprepocnemis plorans* harbouring these same 2 B-chromosome variants differing in the degree of parasitism.

Materials and Methods

Adult males of the grasshopper *Eyprepocnemis plorans* were collected in 2 Spanish populations at Salobreña (Granada) and Torrox (Málaga) in 2006. In 2007, a subsequent sample of females was collected in Torrox (table 1). These populations differ in the kind of B-chromosomes present in the populations. In Salobreña, B_2 is a neutralized B-chromosome showing usually weak deleterious effects [López-León et al., 1992; Muñoz et al., 1998], but the main B-chromosome at Torrox (B_{24}) is a parasitic B-chromosome showing meiotic drive and larger virulence on host fitness [Zurita et al., 1998; Perfectti et al., 2004].

The 2006 sample of male grasshoppers was analyzed at the Department of Biological Sciences, Ecology and Genetics (Aarhus University). The 2007 sample of female grasshoppers was analyzed at the Department of Genetics in the University of Granada, following slightly different procedures. In both laboratories, individuals were kept at room temperature (ca. 25°C) for several days before experimental procedures. Males were anaesthetized and dissected to take out cerebral ganglia and testes. Ganglia and most testis tubules were frozen by immersion in liquid nitrogen and stored at –80°C until Western blot analysis. The remaining testis tubules were fixed in ethanol-acetic acid (3:1) for cytological analysis. The number of B-chromosomes in each male was scored by squashing 2 testis tubules in 2% acetic orcein prior to light microscope observation (table 1). Females were anaesthetized and

dissected to take out cerebral ganglia and ovaries. Ganglia and most ovarioles were frozen by immersion in liquid nitrogen and stored at –80°C until Western blot analysis. For cytogenetic analysis, several ovarioles were immersed in 2% colchicine for 6 h prior to fixation in ethanol:acetic acid (3:1). The number of B-chromosomes per female was determined in squash preparations of ovarioles submitted to the C-banding technique [Camacho et al., 1991] (table 1).

For Hsp70 quantification, male testes and ganglia were homogenized in ice-cold phosphate-buffered saline solution (PBS) containing 2 mM phenylmethyl-sulphonyfluoride (PMSF) in ethanol and 1% (volume) antiprotease cocktail (100 μg/ml pepstain A, 50 μg/ml leupeptin, 10 mM benzamidine, 10 mM sodium metabisulfite). For females, the homogenization buffer consisted of RIPA Buffer (R 0278, Sigma), protease inhibitors cocktail (Sigma) and 10% SDS. Gonad tissues were homogenized in 1.0 ml and ganglion in 500 μl of buffer. The homogenate was centrifuged for 30 min at 13,000 rpm at 4°C, and the supernatant, containing total proteins, was transferred to another tube. Each sample was then divided into 3 aliquots and frozen at –80°C.

We next determined total protein amount by BCA™ Protein Assay (Pierce), according to the manufacturer's instructions. The level of Hsp70 protein was determined by Western blot (fig. 1), for which purpose we used the same amount of total proteins for each organ sample in order to compare among individuals: 20 μg of testis, 60 μg of ovary and 8 μg of ganglion, in both males and females.

Protein extracts from males were electrophoresed in 10% polyacrylamide gels (PAGEr Duramide Precast Gel, Cambrex) and transferred into PVDF membranes (Immobilom™ P Transfer Membranes, Millopore) by the humid system. In females, however, protein extracts were electrophoresed in 10% polyacrylamide gels (NUPGE® 10% Bis-Tri Gel, Invitrogen) and transferred onto Amersham Hybond™-P membrane, by using a semi-dry system (TRANS-BLOT® SR Semi-dry transfer cell, BioRad). All membranes were incubated at 4°C in a 1:5,000 dilution of anti-Hsp70 (MA3-006 3A3, Affinity BioReagents) for 16 h. This antibody recognizes a protein region involved in Hsp70 nucleolar localization after stress [Milarski and Morimoto, 1989]. Since, according to the manufacturers' Technical Specifications, MA3-006 is specific to both Hsp70 and Hsc70 protein family members, we assumed that the detected Hsp70 expression is a combined effect of both related proteins. However, considering the temperature treatments used here we expect constitutively expressed Hsc70 to be the protein detected. Finally, we incubated the membranes in a 1:1,000 dilution of the secondary antibody, stabilized goat anti-mouse HRP-conjugate (Pierce), for 1 h at room temperature.

Detection was performed with the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce), following the manufacturer's instructions, and using a VersaDOC™ Imaging System 1000 (Bio-Rad) image reader. The amount of Hsp70 was determined using Quantity One 4.3.1 software (Bio-Rad), using maximum optical density in bands (peak) for males, and the volume, i.e., total signal intensity inside a defined boundary drawn around each Hsp70 band after subtracting background, in the case of females.

For internal standardization, we followed 2 strategies. In the case of male samples, 3 shared samples were run onto each gel to allow comparison between gels. For female samples, we included a standard sample (a mix of all the samples) in each gel.

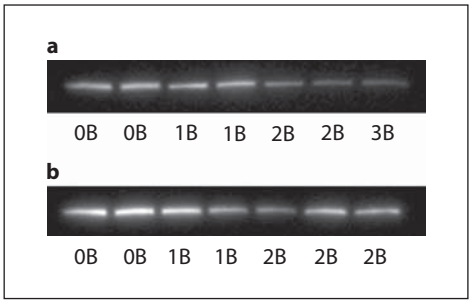

Fig. 1. Western blots for Hsp70 from testis (**a**) and cerebral ganglion (**b**) of *E. plorans* males from the Torrox population.

Table 1. Number of individuals analysed, arranged by population and number of B-chromosomes

Population	Sex	Number of B chromosomes				
		0B	1B	2B	3B	Total
Salobreña	Male	4	5	1	1	11
Torrox	Male	4	5	5	1	15
	Female	10	12	12	3	37

Each sample was measured twice and the 2 measures were averaged for each individual. We detected a systematic deflection in the transfer of proteins through the Western semi-dry protein transfer instrument used with the female samples. We ran control samples to quantify the intensity and direction of this systematic error and corrected the value of the female samples accordingly. Statistical analyses to test temperature, population and B-chromosome number effects were performed by one-way or 2-way ANOVAs. Due to the low number of individuals with 3 B-chromosomes, this category was grouped with 2B individuals. Independent statistical analyses were performed for male and female samples because the protein extraction protocols were different. Statistical analyses were performed by using STATISTICA® software.

Results

Western blots showed the presence of a single band corresponding to a protein of about 70 kDa, in all tissues assayed (fig. 1). After quantifying Hsp70 levels, we first analyzed the effect of population and B-chromosome presence on Hsp70 level in gonads and cerebral ganglia of both sexes, by means of 2-way ANOVA. In testis, we found significant differences for Hsp70 levels between populations ($F = 18.38$, d.f. = 1, 22, $p < 0.001$) and between B-

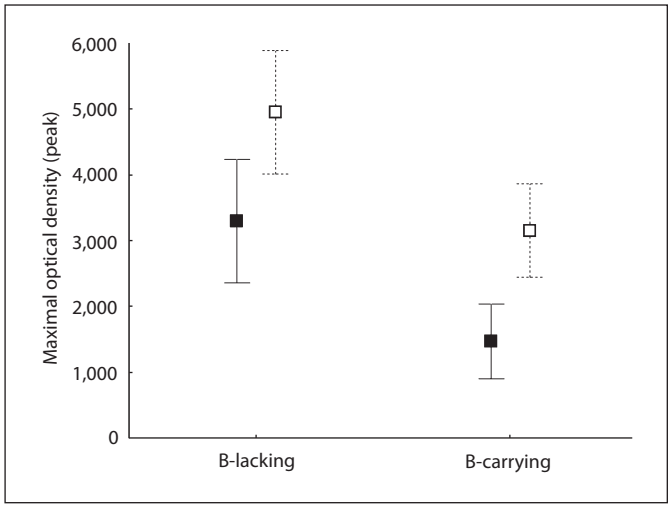

Fig. 2. Mean levels of Hsp70, expressed as maximal optical density (peak), in testes from males lacking and carrying B chromosomes from the Torrox (solid squares) and Salobreña (hollow squares) populations. Bars indicate 95% confidence interval.

carrying and B-lacking males ($F = 21.83$, d.f. = 1, 22, $p < 0.001$). Hsp70 levels were higher in Salobreña than Torrox, and higher in B-lacking than B-carrying males (fig. 2).

A similar result was observed in ovary, with B-lacking females showing about twice (mean = 3.05, SD = 2.78, N = 11) the amount of Hsp70 in B-carrying females (mean = 1.49, SD = 1.01, N = 27) ($t = 2.57$, d.f. = 36, $p = 0.0146$).

In cerebral ganglion, a 2-way ANOVA showed absence of significant effects of both population and B presence in males (population: $F = 0.0019$, d.f. = 1, 19, $p = 0.966$; B-presence: $F = 1.20$, d.f. = 1, 19, $p = 0.288$). Likewise, a Student t test showed no significant differences between Torrox females lacking B-chromosomes (mean = 5.75, SD = 8.92, N = 9) and those carrying them (mean = 4.23, SD = 7.28, N = 27) ($t = 0.51$, d.f. = 34, $p = 0.611$).

To analyze the pattern of decrease for Hsp70 levels in the gonads of B-carrying individuals, and given that individuals with 3 or more B-chromosomes are rare (see table 1), we classified individuals into 2 B-carrying categories, i.e. 1B and $2B^+$, including in the latter all individuals with 2 or more B-chromosomes. The Salobreña sample included only one 2B male and one 3B male, for which reason this analysis was not performed in this population. In Torrox, one-way ANOVA indicated significant effects of B-chromosome number in males ($F = 5.22$, d.f. = 2, 12, $p = 0.023$) and females $F = 4.91$, d.f. = 3, 35, $p = 0.013$), in a dose-effect decreasing pattern (fig. 3).

Discussion

Our experiments have shown a decrease of Hsp70 level associated with B-chromosome presence in male and female gonads (but not cerebral ganglion). In Torrox, the effect showed a dose-dependent pattern in both sexes, so that Hsp70 levels decreased with increasing number of parasitic B_{24} chromosomes. In Salobreña, the presence of the neutralized B_2 chromosome was also associated with a decrease in Hsp70 amount, but with higher levels than those observed in Torrox (see fig. 2). This suggests that decreased levels of this protein could be associated with the parasitic nature of the B_{24}-chromosomes, which could reduce the capacity of hosts to cope with stress, as these parasitic chromosomes affect other important cellular and organismic processes such as chiasma frequency [Camacho et al., 2002] and egg fertility [Zurita et al., 1998].

An intriguing observation is the fact that decreasing Hsp70 levels associated with the number of B_{24} chromosomes parallel the reduction of nucleolus area associated with the number of B_{24} chromosomes [Teruel et al., 2007]. The fact that an important function of Hsp70 is nucleolus maintenance under adverse conditions [Pelham, 1984; Morcillo et al., 1997] is suggestive of the possibility that the B-chromosome effect on reducing nucleolus area is mediated by a reduction of the expression of Hsp70 family proteins. This indirect effect would also explain why nucleolus area [Teruel et al., 2007] and Hsp70 level [this report] are higher in males from Salobreña than in those from Torrox, since the neutralized B_2 variant in Salobreña appears to have lower impact than B_{24} in Torrox on other traits such as egg fertility [Camacho et al., 1997; Zurita et al., 1998] or chiasma frequency [Camacho et al., 2002]. In addition, the fact that the antibody employed in our experiments recognizes a protein region involved in Hsp70 nucleolar localization [Milarski and Morimoto, 1989] reinforces the possibility that the B-chromosome effect on nucleolar size [see Teruel et al., 2007] would be mediated by its action on Hsp family members.

However, other explanations could also be postulated to explain the B-chromosome effect on Hsp proteins. Sørensen et al. [2003] have suggested that in natural populations frequently exposed to stress, the cost of increasing Hsp70 expression could be higher than the possible benefits. Therefore, those populations evolving in frequently stressful environments would show lower expression levels of Hsp70 and would adapt to stress by other means [Sørensen et al., 2003]. For instance, in the

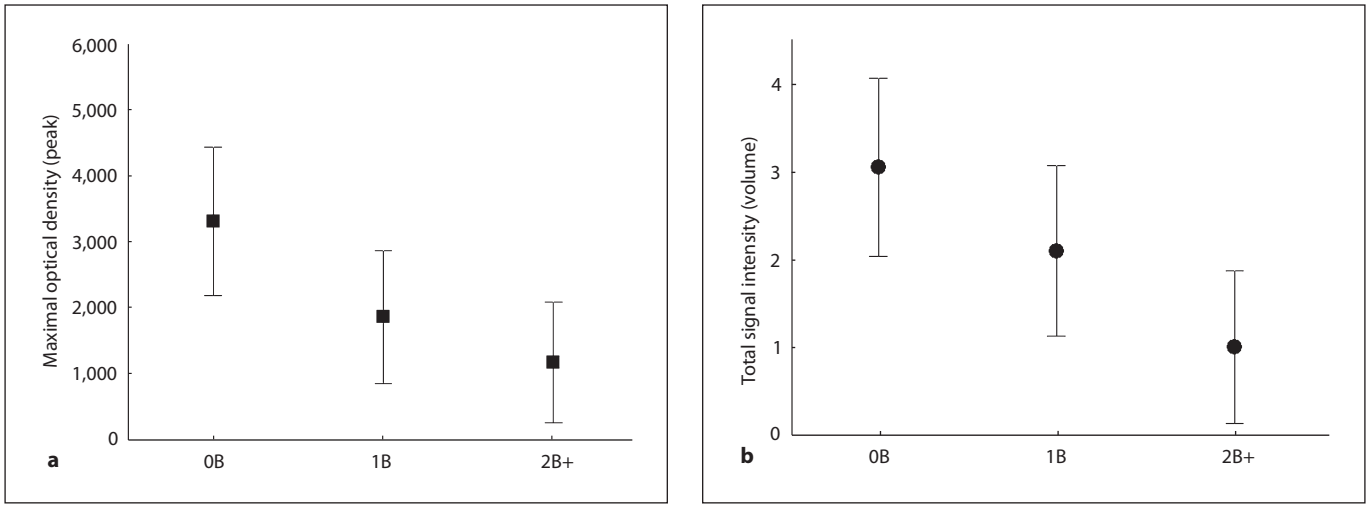

Fig. 3. Mean levels of Hsp70 in male (squares) and female (circles) gonads of the grasshopper *Eyprepocnemis plorans* from the Torrox population, expressed as maximal optical density (peak; **a**) and total signal intensity (volume; **b**), respectively. $2B^+$ = individuals with 2 or more B chromosomes. Bars indicate 95% confidence interval.

grasshopper *Tetrix tenuicornis*, a decrease in Hsp72 level has been observed in populations exposed to heavy metals [Warchalowska-Śliwa et al., 2005]. This finding is supported by the observation that populations selected for heat resistance usually show a reduced expression of Hsp70 in response to heat stress [Sørensen et al., 1999]. Therefore, the reduced expression of Hsp70 in the B_{24} carrying population could be a by-product of the long-term co-evolution of hosts and this parasitic chromosome. However, this does not explain individual differences in Hsp70 levels associated with B-chromosome number.

Our present analysis has also shown that B-chromosome effects were apparent in gonads but not in the analyzed somatic tissue (cerebral ganglia), in both males and females. It has been shown that Hsp expression levels usually show variation among tissues [Singh and Lakhotia, 2000; Lakhotia et al., 2002]. However, another possible explanation might be that somatic tissues are less sensitive to B-chromosome effects than gonadal tissues. These contrasting effects are expected for B-chromosomes, since their obligate vertical transmission implies that their fitness is closely linked to host (A chromosomes) fitness and thus some B-chromosome attenuation is expected [Camacho, 2005]. Previous experiments have provided evidence in *E. plorans* that the presence of the neutralized B_2 is associated with scarce effects on somatic traits at both morphological and physiological levels [Martín-Alganza et al., 1997]. The main effects of B-chromosomes are normally found on traits related with gonad function, such as, for instance, egg fertility [Muñoz et al., 1998; Zurita et al., 1998] and chiasma frequency [Camacho et al., 2002]. The absence of effects in the level of Hsp70 in cerebral ganglion is thus consistent with the attenuation expected for a vertical parasite whose evolutionary fate greatly depends on not being very harmful for its transmission vehicle [Camacho, 2005]. In the gonads, however, parasitic B-chromosomes do interfere with chromosome segregation, and this can produce correlated effects on some cellular parameters (e.g. nucleolar size and Hsp70 levels).

Our present results did not allow us to determine which proteins of the Hsp70 family the B-chromosome affected (Hsp70, Hsc70 or both) as more specific antibodies are not commercially available. The fact that a high level was detected in the absence of heat shock suggests that the antibody at least detected a constitutively expressed Hsp70 family member. This could suggest that it is, at least primarily, a constitutively expressed Hsc70 protein that was affected by B-chromosomes.

Finally, we would like to note that the association of B_{24} chromosomes with lower levels of Hsp70/Hsc70 resulted from correlative evidence and thus it should be taken with caution. In any case, this intriguing associa-

tion deserves deeper analyses for inferring a causal relationship between these B-chromosomes and Hsp70 levels, controlling other possible variables such as environmental and genetic factors not specifically addressed in the present experiments.

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