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Project: Is there a relationship between thermal adaptation and chromosomal polymorphism in *Drosophila subobscura*? A proteinomic approach

INTRODUCTION

Drosophila subobscura is a species that presents a rich chromosomal inversion polymorphism with many of these inversions showing latitudinal clines (Krimbas, 1993). Moreover, in the colonized areas by this species, similar clines to the ones found in the Old World were soon detected, an indication of their adaptive nature (Balanyà et al, 2006).

According to their correlation with latitude, the inversions can be classified as "warm-adapted" or "cold-adapted" (Menozzi & Krimbas, 1992). As inversions inhibit the recombination inside them, we would expect the genetic content of each inversion to be different. In this study we aim to determine if there is an association between gene arrangements and heat shock protein (HSP) levels in lines differing in their arrangement for the O chromosome and with a homogeneous background for the other chromosomes. Particularly, we will test whether flies carrying "cold-adapted" chromosomal arrangements present higher levels of Hsp70 after a heat shock. The experimental design used, involving both homokaryotypes and heterokaryotypes, will allow us to test for the effects of chromosomal inversion and heterozygosity and analyze the expression profile of HSP after a heat shock.

The aim of the short visit to Dr. Volker Loeschcke's lab was to test whether their protocols worked in *D. subobscura* as a preliminary test for the experiment on thermal tolerance and adaptation that we have designed. We chose this research group because of its extensive experience with HSP quantification in different organisms.

MATERIAL AND METHODS

We used homokaryotypic lines for two different arrangements from a Barcelona population. The flies were sampled in October 2007, and were crossed with the strain chcu (cherry-curled) during several generations in order to homogenize the genetic background of the different lines (with the exception of the O chromosome). These lines were subsequently made homokaryotypic for the O chromosome using the balancer strain Va/Ba (Varicose/Bare). As this was a test, we only used lines carrying either O_{ST} or O_{3+4} (Fig. 1). Both arrangements present latitudinal clines (Balanyà et al., 2003): O_{ST} is a "cold-adapted" inversion while O_{3+4} is "warm adapted".

Fig. 1: Diagram of the arrangements studied. The boxes indicate the region that it is inverted in relation with the standard arrangement.

The *Hsp70* gene is located in the 94A cytological band of the O chromosome, included inside the O_{3+4} arrangement and close to one of the breakpoints of inversions O_3 and O_4.

Crosses

Six independently obtained lines from each arrangement were used to obtain outbred homokaryotypic and heterokaryotypic individuals (Table 1). But the crosses did not yield sufficient progeny to test for karyotype and recovery time effects, so a pooled sample with the offspring for each type of cross was used to determine the variation in the quantity of HSP70 at different times after a heat shock.

Cross type (includes reciprocal cross)

O_{ST}/O_{ST}	O_{3+4}/O_{3+4}	O_{ST}/O_{3+4}
ST(1) x ST(2)	3+4 (1) x 3+4 (2)	ST(1) x 3+4 (1)
ST(2) x ST(3)	3+4 (2) x 3+4 (3)	ST(2) x 3+4 (2)
ST(3) x ST(4)	3+4 (3) x 3+4 (4)	ST(3) x 3+4 (3)
ST(4)x ST(5)	3+4 (4) x 3+4 (5)	ST(4) x 3+4 (4)
ST(5) x ST(6)	3+4 (5) x 3+4 (6)	ST(5) x 3+4 (5)
ST(6) x ST(1)	3+4 (6) x 3+4 (1)	ST(6) x 3+4 (6)

Table 1: Schematic representation of the crosses carried out.

With the pooled offspring of the crosses 5 vials of 10 flies per cross and sex were obtained and used to do the heat shock. Both sexes were treated separately because previous studies showed that males and females have different thermal behavior (Rego et al, 2009). Four vials of each cross and sex were used to test for heat hardening and the remaining one was kept untreated as a control.

Heat hardening exposure

To induce the heat shock response, batches of ten flies from each sex and cross were transferred to empty glass vials with moistened stoppers and exposed to the heat hardening treatment (30°C during 1h in a water-bath). After treatment, the flies were kept at 18°C in vials containing fresh food and allowed to recover during different time periods (2h, 4h, 6h and 24h). After each period, a sample of ten individuals was frozen in liquid nitrogen and maintained at -80°C until HSP quantification. One control group of both males and females (which did not undergo stress) was also frozen in order to quantify the basal level of HSP70.

All the work described so far was carried out at Universitat de Barcelona and Universitat Autònoma de Barcelona (Spain).

Immunochemical analysis

BCA assay

The determination of HSP70 expression levels was carried out for all male samples and only the controls for females due to time constraints. Ten flies per treatment were homogenized and the total protein concentration in the supernatant was determined by means of a BCA assay. The bicinchoninic acid assay is a biochemical assay to determine the total level of protein in a solution. The total protein concentration is exhibited by a colour change of the sample solution from green to purple proportional to protein concentration, which can then be measured using colorimetric techniques. The amount of protein present in a solution can be quantified by measuring the absorption spectrum and comparing it that of a protein solution with known concentrations. In our case, a standard of albumin serum of known concentrations (from 0 to 2.0 mg/ml) was used.

Elisa

Enzyme-linked immunosorbent assay is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample. The Hsp70 expression was assayed using two different antibodies: a monoclonal inducible HSP70 antibody (7.FB; Velazquez et al, 1983) and the monoclonal HSP70 antibody 5A5 (Abcam, UK). To ensure replicates per sample, only 18 samples were processed (the males for each cross and treatment and the control females for each cross) because it is the number that better fits the plate.

Western Blot

The western blot (or protein immunoblot) is an analytical technique used to detect specific proteins in a given sample of tissue homogenate or extract. It uses gel electrophoresis to separate denatured proteins by the length of the polypeptide. The proteins are then transferred to a membrane (typically nitrocellulose), where they are probed using antibodies specific to the target protein.

For the western blot only eight samples were analyzed because of time constraints. The samples analyzed were the controls for all the crosses and sexes and the males after 2h of recovering time. The antibody used was the monoclonal HSP70 antibody 5A5 (Abcam, UK).

RESULTS

BCA assay

The O_{ST} homokaryotypes seem to present the lowest protein concentration, while the heterokaryotypes present the highest (Table 2).

	OST/OST		O3+4/O3+4		OST/O3+4	
	Calc'd Conc	Std Dev	Calc'd Conc	Std Dev	Calc'd Conc	Std Dev
♂Control	0.454	0.093	0.624	0.065	0.712	0.073
♂2h	0.431	0.112	0.259	0.058	0.694	0.087
♂4h	0.505	0.054	0.662	0.065	0.507	0.115
♂6h	0.413	0.057	0.561	0.077	0.572	0.152
♂24h	0.282	0.048	0.636	0.111	-	-
♀ Control	0.655	0.034	0.937	0.085	1.204	0.109
♀ 24h	0.268	0.050	-	-	-	-

Table 2: Total amount of protein (mg/ml) per sample calculated by BCA assay. The concentration has been calculated from the mean absorbance values of four replicates.

However, due to pipeting problems, the final volume could not have been the same in all the wells and this could have affected the lecture of the protein concentration of the samples. This can be deduced from the standard curve, which although it fits quite well, could be much better (Fig. 2).

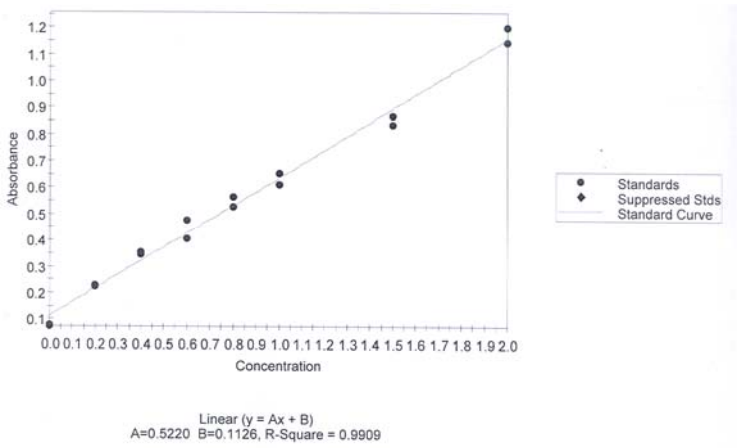

Fig. 2: Absorbance curve for the standard used. It can be observed that the measurements do not always fit the known concentration loaded in the plate. In the figure no suppressed standards appear.

Elisa

The Elisa worked with both antibodies tested: 5A5 and 7.FB (Fig.3). In both cases, there was no correlation between the blank data and the sample data, showing that the different values for the samples are not an artifact of the test.

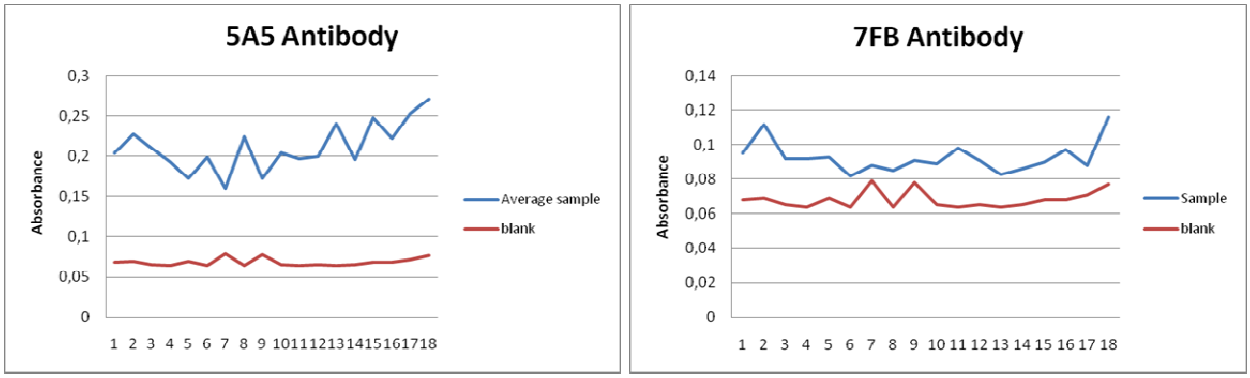

Fig. 3: In these graphs the value for the blank data per each value of the sample data (numbers in the X axis) is plotted. It can be observed that there is no relationship between them

When the shape of the absorbance curve of 5A5 is analyzed (Fig. 4), it can be observed that the peak of maximum absorbance of HSP70 is 2h after the heat shock for the heterokaryotypes. However, for the homokaryotypic O_{3+4} males, this value is the lowest of the treatment. This low value could be probably due to a mistake related to the problems during the BCA assay.

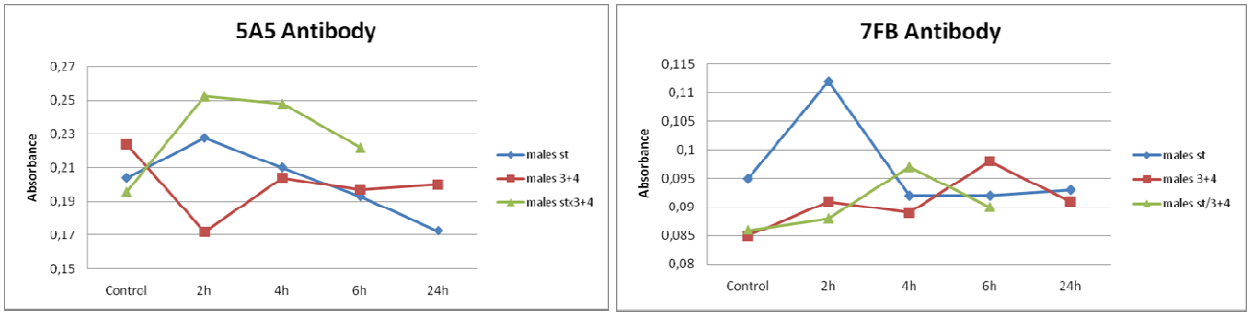

Fig. 4: Absorbance levels of HSP70 at different recovering times after heat shock for both antibodies used. The control group was not treated.

As it seemed that the 7.FB had a very weak signal (Fig. 4), it was also tested if the protein concentration at which the Elisa was carried out affected the results. A new plate with one sample (the heterokaryotypic control female) in decreasing concentrations was run, from 50 µg protein/ml (2X) to 0 µg protein/ml (0X). In this case both antibodies worked fine again (Fig. 5). It can be observed that the absorbance obtained for the same sample was very different when comparing between plates. As no sample of known concentration was loaded in any of the plates, the values of absorbance cannot be compared between them. Moreover, the use of different batches of primary antibody (which might have different activities) in each plate could have introduced additional differences.

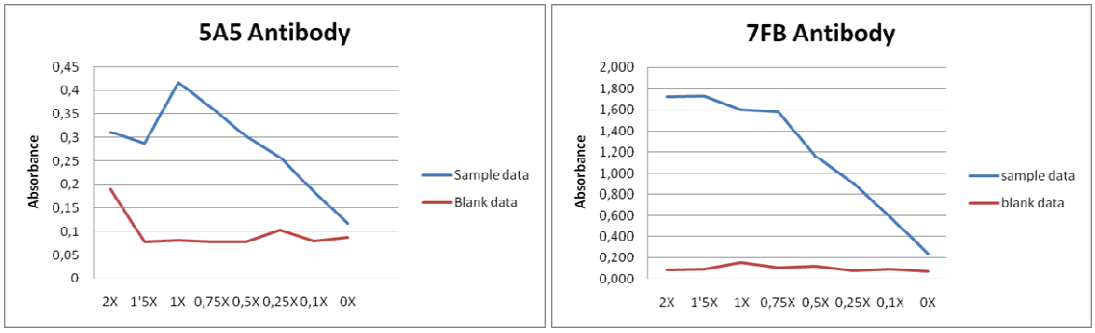

Fig. 5: Curves of dilutions for both antibodies.

From these graphs it can be said that the plates would work well with a concentration of 1X (25 µg protein/ml).

The different results could have arisen from the initial dilution in the first plate which most probably was submitted pipeting problems. We are more confident with the results in the second plate.

There was no time to process all the female samples, and only the control females were processed. Thus, the differences between sexes could only be analyzed for the controls (Fig. 6). It can be observed that heterokaryotypic females present higher values of absorbance.

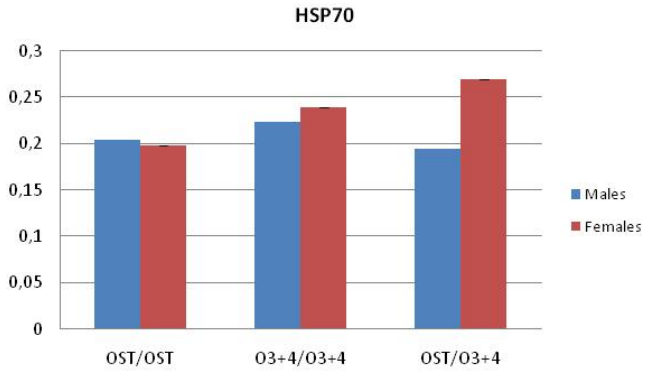

Fig. 6: Comparison between males and females for the control group.

Western Blot

Unfortunately neither of the two western blots gave any result. The gels in which we run the electrophoresis for the western blots were very old and maybe they did not work properly. Other things that could have failed were either the transfer to the membrane or the incubation with the antibodies. When we repeated the western blot, the camera required to read the results failed and when it was ready again, the membrane were probably too old to keep the signal.

DISCUSSION AND FUTURE WORK

From the results we can infer that the highest values of Hsp70 production can be observed 2h after the heat shock. Some studies done in other *Drosophila* species have shown that a response in the HSP production can be detected after only one hour of recovery (Sørensen et al., 1999; Sarup et al., 2006). Other studies conclude that the maximum peak of HSP70 is reached after two hours of the heat shock (Dahlgaard et al., 1998; Sørensen et al., 2001).

The response to the temperature chosen give good results but as the measurements for Hsp70 seem to be low, maybe we should try with a higher temperature. In the work of Moltó et al. (1992), they analyzed the heat shock response at the cytological level and they observed that the optimum temperature to induce the puffing pattern of the HSP's in *D. subobscura* larvae was 34°C. Furthermore, in *D. buzzatii* has been shown that with treatments differing only in 2 degrees (37°C and 39°C), the higher temperature produced a higher response (Sørensen et al., 2001; Sarup et al., 2006).

Regarding the differences between sexes, our results of a higher response in females agree with those observed in other *Drosophila* species (Dahlgaard et al. 1998, Sørensen et al., 2001). In the case of *Drosophila buzzatii* (Sarup et al., 2006), the expression of Hsp70 in females is twice than in males. These results, together with differences in thermal tolerance observed in males and females of *Drosophila subobscura* (Rego et al., 2009), confirm the necessity of working with males and females separately.

With all these results now we are preparing the samples for the experiment of HSP quantification. In the future, we want to analyze three chromosomal arrangements: $O_{ST,}$ O_{3+4}, O_{3+4+8} (Fig. 7). This last arrangement has been added because it is increasing in frequency in all populations sampled recently and is expanding northwards (Balanyà et al., 2004). Furthermore, Hsp70 is located close to the breakpoints of inversions O_3, O_4 and O_8, and different alleles can easily be in linkage disequilibrium with the three arrangements.

Fig. 7: Diagram of the three arrangements studied. The boxes indicate the region that it is inverted in relation with the standard arrangement.

The experiment will involve five lines per arrangement which will be used to obtain homokaryotypic (outbred) and heterokaryotypic individuals for each combination (Table 3).

Cross type (includes reciprocal cross)

O_{ST}/O_{ST}	O_{3+4}/O_{3+4}	O_{3+4+8}/O_{3+4+8}	O_{ST}/O_{3+4}	O_{ST}/O_{3+4+8}	O_{3+4+8}/O_{3+4}
ST(1) x ST(2)	3+4 (1) x 3+4 (2)	3+4+8 (1) x 3+4+8 (2)	ST(1) x 3+4 (1)	ST(1) x 3+4+8 (1)	3+4+8 (1) x 3+4 (1)
ST(2) x ST(3)	3+4 (2) x 3+4 (3)	3+4+8 (2) x 3+4+8 (3)	ST(2) x 3+4 (2)	ST(2) x 3+4+8 (2)	3+4+8 (2) x 3+4 (2)
ST(3) x ST(4)	3+4 (3) x 3+4 (4)	3+4+8 (3) x 3+4+8 (4)	ST(3) x 3+4 (3)	ST(3) x 3+4+8 (3)	3+4+8 (3) x 3+4 (3)
ST(4) x ST(5)	3+4 (4) x 3+4 (5)	3+4+8 (4) x 3+4+8 (5)	ST(4) x 3+4 (4)	ST(4) x 3+4+8 (4)	3+4+8 (4) x 3+4 (4)
ST(5) x ST(6)	3+4 (5) x 3+4 (6)	3+4+8 (5) x 3+4+8 (6)	ST(5) x 3+4 (5)	ST(5) x 3+4+8 (5)	3+4+8 (5) x 3+4 (5)

Table 3: Scheme with all the crosses carried out. The number in the parenthesis refers to the number of the line that is used.

The aim is to analyze the Hsp70 production of the offspring of these crosses after a heat-shock trough an ELISA and with mRNA production. The samples will be prepared as described in Material and Methods. We will increase the heat hardening temperature to 34°C. We will also keep six tubes per sample (hsp70 quantification, mRNA quantification × 3 replicates) at -80°C until its utilization. The quantification of Hsp70 with ELISA will be carried out as described previously and we plan to quantify mRNA with RT-PCR.

The results obtained are going to be published and included as a chapter of my doctoral thesis with title *Identification of the adaptative genetic variation in* Drosophila subobscura *through the study of latitudinal clines in molecular markers of O chromosome*.

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