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Proteomics of immune-challenged *Drosophila melanogaster* larvae hemolymph

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Abstract

In the last decade, the fruit fly *Drosophila melanogaster* has emerged as a promising invertebrate model for the investigation of innate immunity, in part because of its well characterised genetics. The information provided by the innumerous reports on *Drosophila*'s immune response indicates that a large number of genes, in addition to the well-known antimicrobial peptide genes, are both up- and down-regulated upon immune challenge. Nevertheless, their contribution to fighting off infection has not been seriously addressed. With the application of recent advances in proteomics, the effects of an immune challenge in the overall modification of *Drosophila* 2-DE protein patterns were investigated. The aim of this study was to investigate hemolymph proteins differentially expressed between control and immunised larvae sets, which could be related solely to the *Drosophila* immune response. The list of immune-related protein spots included heat shock proteins and other proteins with chaperone properties, serine proteases, phenol oxidase, and *Drosophila* antioxidant system components, which accounted for 21% of the total of 70 identified proteins, metabolic enzymes implicated in pathways such as cellular respiration, fatty-acid oxidation, protein biosynthesis, and structural proteins.

Keywords: *Drosophila*; Hemolymph larvae; Immune response; MALDI-TOF/TOF

Life on Earth has radiated to fill virtually every conceivable habitat and niche. In each of these, organisms must interact with their environment and deal with some degree of variations in its parameters [1]. To some extent, environmental variations are accommodated with ease but, beyond certain limits, stress is imposed. This kind of situation stimulates the organism to undergo metabolic adjustments that will allow the counteraction of the stress related negative effects. Studies on the interactions between organisms, their organs and/or cells, and the changes occurring in the environmental parameters cover an enormous part of Modern Biology and Clinical studies within Medicine [1]. This is easily understandable as life is a continuous interplay between environmental stimuli and cellular responses.

In the past few years, the subject of innate immunity has received renewed attention, mainly through studies involving the model system *Drosophila*. Not only the ease of genetic and molecular analysis, combined with a complete genomic sequence, but also the knowledge that molecular mechanisms controlling specific biological processes are conserved between *Drosophila* and mammals makes this organism an essential tool for deciphering the biochemical pathways and other components of innate immunity. As a result, the investigations carried out have generated a significant amount of information focused on the *Drosophila*

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immune response ([2–4], all reviewed in [5]). Like other insects, *Drosophila* has developed several structural barriers and a multifaceted innate immune system comprising a variety of synergistic mechanisms. To combat microbial infections, *Drosophila* has developed a large range of strategies based on cellular and humoral reactions. This includes phagocytosis [6,7] by macrophage-like blood cells, activation of proteolytic cascades [8] leading to localised melanisation and coagulation, and synthesis of a battery of potent antimicrobial peptides by the fat body [9]. Recent studies focusing on the study of the regulation of antimicrobial peptides, through genetic and molecular tools, have led to the detailed characterisation of two distinct intracellular signalling pathways that regulate antimicrobial activity in *Drosophila*. These pathways are the Toll and immune deficiency (Imd) pathways, which are activated by different pathogens. The Toll pathway mainly involves resistance against fungal and Gram-positive bacteria, whereas the Imd pathway primarily directs the defence reactions against Gram-negative bacteria and controls resistance to these microorganisms [2]. Furthermore, other molecules have been implicated in *Drosophila*'s immune response, such as: peptidoglycan recognition proteins (PGRPs) [10], Gram-negative bacteria-binding proteins (GNBPs) [11], and lectins [3] implicated in pathogen recognition; thiolester proteins (TEPs) [12] and lysosomal enzymes involved in phagocytosis; phenol oxidase (PO) and phenol oxidase activating enzymes (PO-AEs), fibrinogen-like proteins, and reactive oxygen species (ROS), involved in melanisation and coagulation reactions; and proteins involved in iron metabolism [13] like ferritins and transferrins.

Although much has been learned about *Drosophila* immunity through genetic studies, a number of issues still remain to be clearly understood in *Drosophila* multifaceted immune response. In particular, the identities of several hundred genes up-regulated upon immune challenge, in addition to the antimicrobial peptide genes, that contribute to fighting off infection have not yet been addressed. However, several groups have reported a poor correlation between mRNA level and protein abundance within the cell [14,15], indicating that, although generating a large amount of data on immune-related genes, transcriptome analysis through micro-array technology turns out to be an indirect approach, pointing to a clear interest in directly monitoring protein levels. Besides, it is also acknowledged that mRNA-based approaches measure message abundances and not the actual proteins, i.e., the real mediators of biological events, and cannot be used for fluids (such as hemolymph). Therefore in this report, with the aim of investigating the protein profile in the context of the systemic immune response of *Drosophila*, a proteomic approach was carried out by combining two-dimensional gel electrophoresis, mass spectrometry (MS), and data bank searches. This proteomic approach allows the qualitative and semi-quantitative comparison of proteins present in the hemolymph of *Drosophila* before and after immunisation, followed by the identification of proteins differentially regulated by the immune challenge.

Recently, two separate studies focusing on *Drosophila* innate immune response have been reported [16,17]. Both studies were based on a proteomic analysis approach, where the aim was to profile the proteins presented in the hemolymph of non-infected versus infected *Drosophila*. In the report by Vierstraete et al. [16], a total of 22 proteins were found to be differentially expressed, mostly up-regulated, upon immune challenge. Those identifications gather proteins implicated in protein and carbohydrate metabolism (larval serum protein 2, enolase, and alcohol dehydrogenase), iron metabolism (ferritin), detoxification (glutathione *S*-transferase, peroxiredoxin), and other proteins such as TEP2 (complement-like protein), phosphatidylethanolamine-binding protein, regucalcin, actin, and a fatty acid-binding protein.

In the report by Levy et al. [17], the proteins found to be associated with *Drosophila* immune response included only up-regulated proteins such as prophenol oxidase activating enzymes and necrosis enzymes (protease and serpin families, respectively), a GNBP-like protein (pathogen recognition family), TEP4 (complement-like protein), an odorant-binding protein, transferrin and ferritin (implicated in iron metabolism), and a phosphatidylethanolamine-binding protein.

Materials and methods

Animals. Drosophila melanogaster were kept in 600 mL bottles and fed with agar medium prepared from 100 g yeast, 100 g sucrose, 12 g agar, and 5 mL propionic acid per litre of water. The growth conditions, including a controlled temperature of 21 °C, were established as described elsewhere [18].

Immunisation procedure. Sets of 40 *D. melanogaster* third-instar larvae were immunised through miming a natural infection procedure by feeding on a conditioned medium. This conditioned medium was prepared in a similar way from that of normal growth conditions, plus the addition of a microorganisms' membrane suspension (Biopental OM, OM Portuguesa). Each millilitre of this suspension contained bacterial lysates from 400 million of all the following bacteria, *Diplococcus pneumoniae*, *Neisseria catarrhalis*, and *Staphylococcus aureus*, and bacterial lisates from 200 million of all the following bacteria, *Klebsiella pneumoniae*, *Haemophilus influenza*, and *Streptococcus pyogenes*. A 1:10 proportion of microorganism's membrane suspension: feeding medium was used in every immunisation experimental procedure. Larvae were placed one by one in this medium and maintained in these conditions for a period of 24 h. For the control set, larvae were also placed one by one but in normal growth medium. Subsequently, the initial set of immunised larvae was divided into two according to the recovery times of 6 and 24 h, respectively. During the recovery times, immunised larvae were replaced into normal growth conditions, similar to the control larvae set.

Preparation of protein samples. Preparation of protein samples was carried out according to Braun et al. [19]. Briefly, the hemolymph of about 40 *D. melanogaster* third-instar larvae was collected by previously anaesthetising the animals using ether embedded cotton for about three minutes, and then pricking and gently squeezing to collect hemolymph, expelled as a droplet upon pressure. Larvae hemolymph was suspended in 400 μL of a lysis solution (8 M urea, 2 M thiourea, 1% Chaps, 13 mM DTT, and 4 μL of a protease inhibitor cocktail (Sigma, P2714)), followed by a centrifugation step of 15 min at 14,000*g* and 4 °C. The total protein content of the supernatant was determined according to Lowry et al. [20].

2-D gel electrophoresis. 2-DE was performed in a horizontal apparatus (IPGphor and Hoefer 600 SE from Amersham–Pharmacia Biotech, Sweden). Briefly, for analytical gels, 120 μg of protein was applied onto IPG strips (13 cm, linear pH 3–10) with a rehydration buffer containing urea, thiourea, Chaps, and DTT according to Rabilloud [21]. After isoelectric focusing, the strip was applied on a SDS–PAGE gel (12 by 14 cm, 12.5%). The SDS–PAGE gel was then silver stained [22]. For tryptic digestion, 700 μg of protein was applied, and the SDS–PAGE gel was stained using colloidal Coomassie blue.

For image analysis, each gel image was acquired using the GS-710 calibrated imaging densitometer (Bio-Rad) and analysed with the PDQuest v7.1 software (Bio-Rad). The software allowed background subtraction, automatic spot detection, as well as automatic gel matching, and subsequential comparative analysis of normalised spot optical densities (ODs). Automatic spot detection in each gel was verified by visual inspection in order to obtain an image pattern as similar as possible with the original gel.

Tryptic digest analysis. The tryptic digestion protocol was performed according to Detweiller et al. [23]. Briefly, after excision with a pipette tip from the gel, the protein spots were transferred to an Investigator ProGest automated digester (Genomic Solutions, Ann Arbor, MI, USA) rack. Then, the spots were washed twice with 25 mM ammonium bicarbonate/in 50% ACN and dried with nitrogen flow. Twenty five microliters of sequence grade modified porcine trypsin (Promega V5111) (10 μg/mL in 50 mM ammonium bicarbonate) was added to the dried residue and samples were incubated overnight at 37 °C. After digestion, tryptic peptides were lyophilised and re-suspended in 10 μL of a 50% acetonitrile/0.1% formic acid solution. The samples were mixed (1:1) with a saturated matrix solution of α-cyano-4-hydroxycinnamic acid prepared in 50% acetonitrile/0.1% formic acid. Peptide mass spectra were obtained on a MALDI-TOF/TOF mass spectrometer (4700 Proteomics Analyzer, Applied Biosystems, Foster City, CA, USA) in the positive ion reflectron mode. For each sample spot, a data dependent acquisition method was created to select the two most intense peaks, excluding those from the matrix, due to trypsin autolysis or acrylamide peaks, for subsequent MS/MS data acquisition.

Database search. Spectra were processed and analysed by the Global Protein Server Workstation (Applied Biosystems, Foster City, CA, USA), which uses internal Mascot (Matrix Science, UK) software for searching the peptide mass fingerprints and MS/MS data. Searches were performed against the NCBI non-redundant protein database.

To ensure a reliable identification, the results from both the MS and MS/MS spectra were used in the database search. Protein identification was accepted when the score reported by the Mascot search routine was higher than 90 and, whenever possible, confirmed with MW/p*I* values. In case of a gene product as protein spot identification, a BLAST search (www.ncbi.nlm.nih.gov/BLAST/) was done based on the obtained sequence. The result was accepted when the protein family was the same for the best scores. Further confirmation of protein identifications was obtained using Protein Prospector (www.prospector.ucsf.edu, from the University of California at San Francisco) and/or Prowl (www.prowl.rockfeller.com, Rockefeller University at New York Universities) software.

Data analysis. Qualitative comparison analysis was performed using silver stained 2D gel images from both control and immunised *Drosophila* larvae gel samples, while spot protein identification and quantification were performed using Coomassie colloidal stained 2D gel images. After spot detection, comparative analyses were performed by matching the gels with a control gel as reference (called "master"), which also required visual inspection because the software relies basically on spot position and checking false matchings turns out to be absolutely essential. In order to analyse quantitative changes between control and immunised *Drosophila* larvae gel samples, spot intensities were normalised using the PD-Quest software.

Finally, matchset results were exported to excel datasheet where a statistical analysis via Student's *t* test ($p < 0.05$) was realised. With the aim of assuring that protein expression level variations were directly related to the immunological stress inflicted, only variations that presented a confidence interval less than 10% between 2D gel sample replicates (three replicate gels per sample) were considered significant.

Results and discussion

To investigate the effects of immune challenge on protein expression, *Drosophila* 2-DE protein separations of control and immunised larvae were obtained according to the methodology described, using three replicate gels per sample. In concurrence with our previous report [24], an example of a 2-DE protein pattern is shown in Fig. 1 for reversible silver stained 2-DE map.

Initially and prior to analysis by mass spectrometry, the reversible silver staining procedure was used for detection of the 2-DE separation, due to its higher sen-

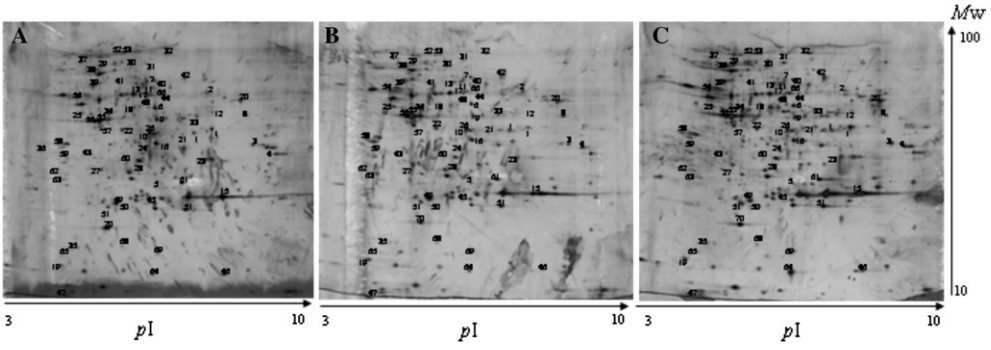

Fig. 1. Two-dimensional gel map of *D. melanogaster* hemolymph larvae obtained in pH range between 3 and 10, and 12.5% of SDS–PAGE and reversible silver staining. (A) The pattern of control experiments, while (B,C) the pattern of immunisation experiments with 6 and 24 h of recovery, respectively. Numbers correspond to identified spots with statistically differential expression levels.

sitivity as well as reasonable trustworthiness in semi-quantifying protein maps. The software detected 336 spots in the control gels, 315 spots in the 6 h recovery gels, and 320 spots in the 24 h recovery gels. The software analysis also revealed that, during the recovery period, both qualitative and quantitative changes occurred. For example, after 6 h recovery, 17 spots were absent from the control gels. None of these spots were identified, and most of them were not visible in the colloidal Coomassie stained gel. Twelve of the 14 spots that appear in the 24 h recovery gels, compared to the 6 h recovery gels, are in common with the group of the 17 spots. Finally, after 24 h recovery 26 spots are still absent compared to the control gels.

Quantitative changes, occurring during the recovery period, were determined by comparison between control and immunised *Drosophila* hemolymph proteins using colloidal Coomassie stained protein patterns (three replicate gels per sample). A total of 131 protein spots were found to be altered during the recovery time. Both the silver stained and Coomassie stained gels generated similar 2-DE protein patterns [24], although according to Smales et al. [25], the reproducibility of quantitative data generated using silver staining is dependent on individual protein spot intensities (which in turn are correlated with the coefficients of variation) and is protein specific. So, for expression analysis studies, confident comparison of individual spot intensities is only feasible, provided the coefficients of variation for those individual spots are known. This is especially important when the spot corresponds to a low abundance protein [25,26]. This limitation can be overcome by using alternative staining procedures such as SYPRO Ruby and Coomassie, which produce a more highly linear measure of relative protein abundance in a gel map than does silver staining [25]. Thus, the quantitative comparison between control and immunised *Drosophila* larvae protein patterns was performed using colloidal Coomassie stained protein patterns for each gel and sample. Although lower sensitivity was a disadvantage, Coomassie staining allowed the detection of an average of 289 spots and was compatible with MS identification. In addition, this staining procedure allowed spot intensity normalization by correlating the spot volume from a marker band of known equal protein quantity chosen in each gel. Hence, quantitative comparison was performed between control and immunised *Drosophila* larvae hemolymph 2-DE colloidal patterns and the calculated variability factors for both recovery times are shown in Table 1. The 24 h recovery values were calculated via the 6 h recovery values in an attempt to verify what was occurring during the recovery period, as well as via the control values. With the purpose of attributing spot volume (intensity) differences between samples as significant changes in protein expression, i.e., not related to gel-to-gel variations, statistical analysis via Student's t test ($p < 0.05$) was performed on the three replicates per sample [25]. The spots that presented a confidence interval larger than 10% between the replicates were not included in the variability factors' estimations between samples, as they were not considered to be reliable.

Hemolymph proteins induced after 6 h of recovery

The up-regulated proteins, sorted into their functional classes, represented enzymes implicated in carbohydrate metabolism, such as glycolysis, pentose phosphate, and Krebs cycle, enzymes implicated in the fatty-acid β-oxidation pathway, and enzymes implicated in protein degradation (proteolysis), in addition to stress response related proteins.

Metabolism

Carbohydrate metabolism. Nearly all the identified glycolytic enzymes presented an increase in their relative abundance after 6 h of recovery. Among the identified enzymes involved in this pathway are fructose-biphosphate aldolase, glyceraldehyde 3-phosphate dehydrogenases I and II, phosphoglycerate mutase, enolase, and dihydrolipoamide dehydrogenase. Enzymes for instance, isocitrate dehydrogenase, fumarase, and malate dehydrogenase, directly implicated in the Krebs cycle, also showed a similar behaviour.

Although not clearly indicated in the literature, these results suggested that, even though a reorganisation of metabolism network was needed, at the cellular level, the organism was using these pathways for one of their major purposes: ATP production. The increase in ATP synthesis, shown by the up-regulation of the subunit enzyme ATP synthase F1 complex α-subunit and ATP synthase α-chain, and the decline of phosphoenolpyruvate carboxykinase gluconeogenesis enzyme also indicated that ATP production was more obligatory to cells than did the use of these pathways for generating building blocks to fatty acid or aminoacid biosynthesis, for instance. At the cellular level, ATP depletion results in proteotoxic stress that can lead to dysfunction, destabilisation, and aggregation of many cellular proteins including enzymes, ion pumps, and constituents of cytoskeletal and contractile structures [27]. Reports focusing on the main effects of heat shock reveal that decline of intracellular ATP abundance is one of the primary events [28,29]. The assumption that similar consequences would occur under our conditions leads to the hypothesis that the pathways involved in cellular respiration were induced as a result of cellular demand for ATP synthesis and energy production. Thus, cells seemed to readjust their metabolic activities according to their energetic requirements and, if necessary, at the cost of their biosynthetic capabilities.

Table 1
List of the statistically significant quantitative changes in the identified *Drosophila* hemolymph proteins after immune challenge for both recovery times (6 and 24 h)

	Identified proteins	Spot no.	Swiss-Prot Accession No.	Recovery time		
				6 h	24 h	
					In relation to 6 h values	In relation to control values
Metabolism						
Carbohydrate metabolism	Fructose-biphosphate aldolase	1	P07764	20 ± 0.3%▲	nv	18 ± 0.4%▲
	Transketolase	2	Q95519	21 ± 1.8%▲	nv	15 ± 0.2%▲
	Glyceraldehyde 3-phosphate dehydrogenase I	3	P07486	nv	37 ± 1.4%▼	40 ± 0.5%▼
	Glyceraldehyde 3-phosphate dehydrogenase II	4	P07487	13 ± 9.4%▲	67 ± 2.1%▼	63 ± 11.4%▼
	Phosphoglycerate mutase	5	Q9GU73	14 ± 0.1%▲	24 ± 1. 1%▼	14 ± 1.0%▼
	Enolase	6	P15007	nv	14 ± 3.0%▼	13 ± 3.8%▼
	Phosphoenolpyruvate carboxykinase	7	P20007	31 ± 1.1%▼	11 ± 1.2%▲	24 ± 0.1%▼
	Citrate synthase	8	Q9W401	22 ± 2.2%▲	41 ± 5.9%▼	28 ± 8.2%▼
	Isocitrate dehydrogenase 1	9	Q9VSI6	nv	20 ± 1.6%▼	25 ± 2.4%▼
	Isocitrate dehydrogenase 3 (NAD^+) subunit	10	Q8IQW9	36 ± 0.6%▲	61 ± 1.4%▼	47 ± 0.8%▼
	Succinate dehydrogenase (ubiquinone flavoprotein)	11	Q94523	15 ± 0.3%▼	76 ± 1.6%▼	80 ± 1.3%▼
	Fumarase	12	Q9W3X6	nv	10 ± 0.3%▲	19 ± 0.3%▲
	Malate dehydrogenase (malic enzyme)	13	Q9NIW2	16 ± 8.6%▲	79 ± 9.7%▼	74 ± 1.1%▼
	Cytosolic malate dehydrogenase	14	Q94523	52 ± 0.3%▲	23 ± 1.2%▼	17 ± 0.9%▲
	Alcohol dehydrogenase	15	P00334	13 ± 0.8%▼	27 ± 3.9%▲	11 ± 2.9%▲
	Aldehyde reductase	16	Q9VTK9	10 ± 1.5%▼	19 ± 1.0%▼	27 ± 2.5%▼
	Dihydrolipoamide dehydrogenase	17	Q9VVL7	47 ± 1.7%▲	44 ± 1.8%▼	17 ± 0.2%▼
Energy metabolism						
Oxidative phosphorylation	NADH dehydrogenase Fe–S protein	18	Q9V4E0	29 ± 0.9%▼	12 ± 1.2%▼	37 ± 2.1%▼
	Succinate dehydrogenase (ubiquinone flavoprotein)	11	Q94523	15 ± 0.3%▼	76 ± 1.6%▼	80 ± 1.3%▼
ATP synthesis	ATP synthase mitochondrial F1 complex d-subunit	19	Q9W2X6	35 ± 1.0%▲	10 ± 2.2%▼	23 ± 1.1%▲
	ATP synthase α-chain	20	P35381	58 ± 0.2%▲	37 ± 2.3%▼	nv
Lipid metabolism						
Fatty acid β-oxidation	Enoyl-CoA hydratase mitochondrial	21	Q9VXI1	70 ± 1.0%▲	38 ± 1.2%▼	nv
	Acyl-CoA dehydrogenase	22	Q9VVU1	42 ± 5.4%▲	48 ± 2.4%▼	nv
	Hydroxyacyl-CoA dehydrogenase	23	Q9VXI1	67 ± 2.3%▲	46 ± 3.1%▼	nv
Complex lipid metabolism	Alcohol dehydrogenase	15	P00334	13 ± 6.8%▼	27 ± 3.9%▲	11 ± 2.9%▲
	Aldehyde reductase	16	Q9VTK9	10 ± 1.5%▼	19 ± 1.0%▼	27 ± 2.5%▼
	Glycerol-3-phosphate dehydrogenase	24	P13706	29 ± 0.7%▼	261 ± 1.8%▲	nv
Protein metabolism	Aminoacylase	25	Q9VCR0	47 ± 3.8%▲	51 ± 4.2%▼	28 ± 0.4%▼
	Arginine kinase	26	P48610	nv	32 ± 7.5%▼	37 ± 17.3%▼
Proteolysis and peptidolysis	Carboxypeptidase A	27	Q9VL86	60 ± 1.2%▲	31 ± 1.5%▼	20 ± 0.3%▲
	Carboxypeptidase B	28	Q961J8	27 ± 1.2%▲	36 ± 1.0%▼	18 ± 1.2%▼
Biosynthesis	Larval serum protein 1 γ-chain	29	P11997	22 ± 1.1%▼	23 ± 1.8%▲	nv
	Larval serum protein 1 α-chain	30	P11997	17 ± 0.4%▼	23 ± 0.1%▲	nv
	Larval serum protein 2	31	Q24388	41 ± 4.9%▼	117 ± 5.8%▲	29 ± 0.9%▲
	Translational elongation factor 2	32	P13060	31 ± 1.2%▼	39 ± 0.1%▲	nv
	Translational elongation factor 1γ	33	Q9NJH0	27 ± 3.3%▼	21 ± 0.1%▼	42 ± 3.2%▼
	Eukaryotic translational initiation factor 4A	34	Q02748	nv	27 ± 2.5%▼	31 ± 0.2%▼
	Eukaryotic initiation factor 5A	35	Q9GGU68	25 ± 1.5%▼	nv	21 ± 2.3%▼
	Alpha NAC	36	O16813	55 ± 0.6%▼	10 ± 0.1%▼	60 ± 0.7%▼

Table 1 (*continued*)

	Identified proteins	Spot no.	Swiss-Prot Accession No.	Recovery time		
				6 h	24 h	
					In relation to 6 h values	In relation to control values
Stress response						
Heat shock response (and chaperone properties)	Heat shock protein 83	37	P02828	27 ± 0.7%▲	25 ± 2.4%▼	nv
	Heat shock protein 60	39	O02649	nv	16 ± 2.6%▼	nv
	Heat shock protein 70	38	P02825	19 ± 1.8%▲	25 ± 1.5%▲	50 ± 3.4%▲
	Heat shock protein 90 related protein TRAP1	40	Q9V9D1	10 ± 0.5%▲	62 ± 0.1%▲	55 ± 0.4%▼
	Chaperonin 2 β-subunit	41	Q9W392	18 ± 0.5%▲	55 ± 0.1%▼	47 ± 0.6%▼
Immune response	Prophenol oxidase	42	Q9BLD9	28 ± 0.5%▼	29 ± 0.2%▲	nv
	Chymotrypsin-like serine protease	43	Q9VPN8	42 ± 0.3%▲	39 ± 0. 2%▼	nv
	Serine proteinase	44		37 ± 0.2%▲	48 ± 0.1%▼	29 ± 0.1%▼
	Serine protease 1	45	Q9VA66	28 ± 2.1%▲	44 ± 0.9%▼	28 ± 1.3%▼
	PPIase	46	P25007	45 ± 3.2%▲	nv	32 ± 0. 4%▲
Detoxification	Thioredoxin	47	Q9V429	43 ± 0.2%▲	30 ± 2.4%▼	nv
	Thioredoxin reductase	48	P91938	40 ± 1.7% ▲	41 ± 1.9%▼	nv
	Thioredoxin peroxidase 1	49	Q9V3P0	30 ± 2.6% ▲	65 ± 0.2%▼	54 ± 2.4%▼
	Thioredoxin peroxidase 3	50	Q9VEJ0	37 ± 0.5% ▲	50 ± 1.2%▼	31 ± 0.7%▼
	Glutathione transferase	51	Q963F1	42 ± 0.3% ▲	27 ± 0.7%▼	nv
Cellular function						
Structural	Paramyosin	52	P35415	39 ± 0.3% ▲	32 ± 0.4%▼	nv
	Paramyosin, long form	53	P35415	23 ± 0.2%▲	46 ± 0.1%▼	33 ± 0.1%▼
	Tubulin 1 β-chain	54	Q8IME1	44 ± 0.6% ▲	32 ± 0.6%▼	nv
	Actin	55	P18091	16 ± 2.7%▼	nv	17 ± 0. 4%▼
	Actin 57B	56	P53501	28 ± 2.9% ▲	29 ± 5.2%▼	nv
	Actin 87E	57	P18091	50 ± 2.1% ▲	18 ± 1.3%▼	23 ± 0.8%▲
	Tropomyosin 1	58	Q9VF97	27 ± 2.1%▼	nv	32 ± 1.9%▼
	Tropomyosin 2	59	P09491	24 ± 0.3% ▲	39 ± 2.0%▲	nv
Voltage-gated channel	Chloride intracellular channel protein	60	Q9VY78	45 ± 0.1% ▲	57 ± 0.3%▼	17 ± 0.3%▼
Porin protein	VDAC	61	Q94920	36 ± 0.2%▲	30 ± 1.0%▼	nv
Cell communication (signal transduction)	14-3-3 protein epsilon	62	P92177	nv	25 ± 0.6%▼	25 ± 0.1%▼
	14-3-3-like protein (Leonard protein)	63	P29310	49 ± 1.9% ▲	29 ± 9.5%▼	nv
Unclassified	Nucleoside diphosphate kinase (NDP kinase)	64	P08879	50 ± 3.1%▼	93 ± 16.8%▲	nv
	Cyclic AMP-regulated protein-like-protein	65	Q9VWQ7	14 ± 1.7%▼	nv	14 ± 1.6%▼
	Receptor mediated endocytosis protein 1, isoform a	66	Q8T8W3	117 ± 0.4%▲	61 ± 0.1%▼	16 ± 0.5%▼
	Guanine nucleotide-binding protein β-subunit like protein	67	O18640	13 ± 0.1%▲	18 ± 1.9%▼	nv
	Fatty acid-binding protein	68	Q9VGM2	nv	31 ± 0.8%▼	33 ± 1. 7%▼
	Twinstar protein (cofilin/actin depolymerising factor homolog)	69	P45594	18 ± 2.0%▲	72 ± 1.5%▼	62 ± 0. 5%▼
	Phosphatidylethanolamine-binding protein	70	Q9VD01	23 ± 1.4%▲	41 ± 5.5%▼	28 ± 4.0%▼

Protein identity, spot number, and Swiss-Prot accession number are indicated. For each differentially expressed protein the average ratio of normalised spot protein quantity is specified. ▲ means up-regulation whereas ▼ means down-regulation; nv means no significant variability and occurs whenever the variability factor is less than 10% between samples.

In addition, an enzyme involved in the pentose phosphate pathway, transketolase, was also observed to be up-regulated after 6 h of recovery. This pathway is known to be the major source of NADPH as a result of glucose oxidation, a very important element in reductive biosynthesis (for instance, fatty acid and nucleotide biosynthesis, and detoxification reactions).

Lipid metabolism. All identified enzymes implicated in fatty acid β-oxidation (and also NADPH produc-

tion)—enoyl-CoA hydratase, acyl-CoA dehydrogenase, and hydroxyacyl-CoA dehydrogenase—were induced upon immune challenge. In fatty acid β-oxidation pathway, fatty acids are first activated and then transported into mitochondria for degradation where they are broken down into acetyl-CoA, which also results in NADPH production (and $FADH_2$). These results suggest that an increase in NADPH cell requirements is caused by an increase in energy demand by ATP production through cellular respiration, or is caused by an increase in the antioxidant defence reactions involved in maintenance of cellular redox homeostasis. In fact, all the identified proteins involved in adaptive response mechanisms like immune response or detoxification, as well as the heat shock proteins, were also induced after 6 h of recovery.

The higher stimulation indexes observed in Table 1 correspond to the proteins involved in both immune response and detoxification defence mechanisms.

Immune proteins

Other proteins induced in response to the immune challenge included serine proteases, one PPIase (immunophilin) and prophenoloxidase. Serine proteases have been reported as important molecules that play a central role in insect immune response [30,31] by regulating important processes such as hemolymph coagulation, melanisation of pathogen surfaces and antimicrobial peptide synthesis, via proteolytic cleavage activation of specific proteins. Thus, their increase after 6 h recovery was likely related to the *Drosophila* larvae immune response reactions.

In this context, PPIase up-regulation occurs similarly. PPIases belong to the peptidyl-prolyl *cis–trans* isomerase family and are responsible for catalysing the isomerisation of peptide bonds N-terminal to proline residues in polypeptide chains [32], having a role in the folding of newly synthesised proteins. Based on drug specificity, PPIases have been divided into three distinct classes—cyclosporine A binding cyclophilins; FK506-binding proteins (FKBPs); and parvulin-like PPIases—which are also structurally divergent. Even though structurally divergent, cyclophilins, and FKBPs are collectively referred to as immunophilins ([33], cited in [34]). During the past several years, a growing number of immunophilins have been characterised from sources from bacteria to yeast and higher plants ([33,35], reviewed in [34]). Their high level of conservation and ubiquitous distribution among divergent organisms and in almost all subcellular compartments indicate that these proteins participate in important cellular processes. Since then, they have been implicated in facilitating protein folding in vivo (due to its rotamase activity) [32], protein trafficking [36], and can function as chaperones, a possibility supported by reports that demonstrate the heat-shock responsive expression of cyclophilin mRNAs in yeast [37] and in a higher plant [38]. Recently, they have also been associated with oxidative stress response ([39], cited in [40]). Therefore, PPIase up-regulation suggested that it is an immune-associated protein because of its capabilities in protein folding and in oxidative stress, both consequences of immune defence reactions.

Also the enzyme phenol oxidase is directly implicated in insects' innate immune response. In this study, the inactive form of this enzyme, prophenol oxidase, was identified and among the other immune-related proteins it is the only one to be repressed upon immune challenge (and after 6 h of recovery). One of the killing mechanisms associated with the insect immune response is encapsulation (cellular defence reaction). Encapsulation is often accompanied by the deposition of melanin and protein-phenol complexes around the intruder, in a reaction termed melanotic encapsulation, which requires the proteolytic activation of phenol oxidase from the inactive precursor termed prophenol oxidase [41]. Hence, the observed prophenol oxidase decline is probably coupled with an increase in the enzymatically active form that contributes to the melanisation reactions as a cellular defence mechanism.

Detoxification proteins

Proteins with antioxidant properties also show induction upon immunological stress. After 6 h of recovery, proteins such as thioredoxin, thioredoxin reductase, thioredoxin peroxidases 1 and 3, and glutathione transferase, presented stimulation indexes comparable to those of immune-related proteins. It is well known that oxidative stress is concurrent with immune response, including the insects' innate immune response. Cellular defences, such as phagocytosis, melanotic encapsulation, result in cytotoxic reactive oxygen intermediates' (ROI) production, as well as reactive nitrogen intermediates (RNI) and associated enzymes. The signalling pathways involved in the activation and consequent generation of ROI (for instance, O_2^- or H_2O_2) are not completely understood, in part because of the "cross-talk" that exists between the signal transduction messengers. Nevertheless, these reactive oxygen species can damage various components of living cells, such as unsaturated lipids (giving rise to organic peroxides), proteins, and nucleic acids [42]. To counter these deleterious events, cells use several protective systems that either repair the various types of damage (for instance, DNA repair enzymes) or destroy the reactive oxygen species which implies the action of enzymatic systems like the peroxidases. In *Drosophila*, the system responsible for re-establishment of cellular redox homeostasis is the thioredoxin system [43,44]. Furthermore, the results about this antioxidant system suggested that, rather than contributing to ATP production, the consumption of NADPH is mainly directed to the protective role of antioxidant defences.

Heat shock proteins

The heat shock proteins analysed in this study are also induced upon immune challenge, as expected, although with a stimulation index comparatively lower than the stimulation index of the immune- and detoxification-related proteins discussed above. Although Hsps are best known for their inducibility by heat, it has been reported that the presence of non-native proteins within cells is sufficient to induce their expression [45]. The results indicated in Table 1 revealed that, among the identified Hsps, a relatively low increase was associated with the cytoplasmatics Hsp 82 and Hsp 70. after 6 h of recovery. Although it is to be considered that the stress imposed in this study was not severe, since the immunisation procedure did not include pricking larvae, in a report by Krebs and Feder [45] Hsp 70 expression in third-instar *Drosophila* larvae was initially very low under severe heat shock treatment (38.5 °C), but increased many hours afterwards. A similar behaviour is suggested by these results associated with Hsps, mainly for Hsp 70 which at the 24 h of recovery is still being up-regulated. The relatively low change in expression of the mitochondrial Hsps, Hsp 60 and Hsp 90 related protein, suggested that the application of the immunological challenge did not significantly affect them or the normal functioning of mitochondria, as already implied by the up-regulated fatty acid β-oxidation pathway.

Hemolymph proteins repressed after 6 h of recovery

The hemolymph proteins repressed due to immune challenge were mainly proteins involved in biosynthetic processes, such as protein biosynthesis (translational apparatus components), complex lipid metabolism (glycerol metabolism), and precursor's biosynthesis (for generating aminoacids, nucleotides, lipids, purines and pyrimidines, and porphyrins).

All the identified proteins involved in protein biosynthesis, such as larval serum proteins (storage proteins for biosynthesis), translational initiation and elongation factors, were down-regulated after 6 h recovery indicating the occurrence of a global slowdown in protein translation. Similarly, the enzymes glycerol-3-phosphate dehydrogenase, aldehyde reductase, and alcohol dehydrogenase, that are involved in glycerol metabolism, also showed a decline in expression after the immune challenge. These results are in agreement with the observed carbohydrate metabolism results, indicating that those pathways were being directed to the production of ATP and NADPH, and not to the consumption of energy as it is the case of glycerol metabolism.

Taken together, these results indicate that the stress response stimulates the important switch of the biosynthetic normal cellular activity towards cellular protective functions by inducing Hsps, immune-related proteins, and *Drosophila* antioxidant system components.

Hemolymph proteins at 24 h of recovery

After 24 h recovery and comparing with the 6 hour recovery values, nearly all hemolymph proteins up-regulated at 6 hour recovery were still being down-regulated. Basically, the enzymes involved in the carbohydrate, energy, and lipid metabolisms, as well as the proteins involved in the overall stress response, including elements of the immune and antioxidant defence reactions, Hsps, and proteins with proteolytic activity, were down-regulated (indicating a little recuperation from the immunological stress at cellular level). Yet, as expected, some of the proteins implicated in protein biosynthesis were still being induced at 24 h recovery, most likely indicating that, at this point, a little recuperation in anabolism was starting to occur at the cellular level.

Furthermore, at this time, there were still cellular processes that had not returned to their control values. In comparison with the controls, enzymes implicated in the carbohydrate, protein, and lipid metabolisms, and stress-related proteins such as thioredoxin peroxidases, serine proteases, heat shock protein 70, and PPIase, still presented stimulation indexes either above or lower than the controls. Only the proteins implicated in ATP synthesis, fatty acid β-oxidation, the heat shock proteins 83 and 60, prophenol oxidase, and the detoxification proteins, thioredoxin, thioredoxin reductase, and glutathione transferase, appeared to regain their normal functioning.

As a whole, these results illustrate that, after reorganisation of the metabolism and stress adaptive response functioning, i.e., up-regulation of stress-related proteins and concomitant protein degradation with global biosynthesis slowdown, at this time resources were being redirected to normal development. With diminished energy demand after stress recuperation at the cellular level, the regaining of normal development could be interpreted based on the emergence of anabolism relative of catabolism due to metabolic adaptation.

Conclusions

This work described a differential proteomic analysis for the study of the *Drosophila* immune response, an effort to highlight the molecular mechanisms underlying this biological event, as well as to point up the proteins affected during this process.

Transcriptomic and peptidomic approaches previously reported have allowed the analysis of differential regulation as a result of the immune challenge of genes or molecules with low molecular masses. However, in order to analyse the effects on the protein expression profile, a proteomic approach based on 2-DE protein separation with extended pH range (3–10) and high sensitivity protein identification by MALDI-TOF/TOF/MS was implemented. Quantitative comparative analy-

sis of the 2-DE colloidal protein patterns, before and after immunisation, showed the up- or/and down-regulation of 131 protein spots. A total of 71 proteins were positively identified and linked with the *Drosophila* immune response. Amongst the identified proteins, proteins such as serine proteases, actins, and phenol oxidase, have already been linked with *Drosophila* immune response, essentially throughout genomics approach studies. Regarding the recently proteomic approach studies, six proteins were identified in common with the report by Vierstraete et al. [16], which included alcohol dehydrogenase, enolase, glutathione transferase, fatty acid-binding protein, larval serum protein 2, and phosphatidylethanolamine-binding protein. This last protein was also identified as an immune-related protein in the report of Levy et al. [17].

In addition to these known or predicted proteins, previously uncharacterised proteins implicated in *Drosophila* immune system were identified in this study. These proteins included a PPIase (immunophilin), thioredoxin system components, heat shock proteins, and a significant number of metabolic enzymes implicated in pathways such as glycolysis, Krebs cycle, fatty-acid β-oxidation, and protein biosynthesis, gathering so far as 64 other proteins implicated in diverse biochemical events and regulated by the immune challenge.

In conclusion, the results achieved in this work increased the present knowledge of the *Drosophila* immune response, supported essentially on mRNA-based approaches showing genes transcriptionally modified after infection.

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References

[1] K.B. Storey, Environmental Stress and Gene Regulation, BIOS Scientific Publishers Ltd, Oxford, UK, 1999, pp. 10–15.
[2] J.A. Hoffmann, J.M. Reichhart, *Drosophila* innate immunity: an evolutionary perspective, Nat. Immunol. 3 (2002) 121–126.
[3] E. De Gregorio, P.T. Spellman, G.M. Rubin, B. Lemaitre:, Genome-wide analysis of the *Drosophila* immune response by using oligonucleotide microarrays, Proc. Natl. Acad. Sci. USA 98 (2001) 12590–12595.
[4] J. Imler, J.A. Hoffmann, Signalling mechanisms in the antimicrobial host defence of *Drosophila*, Curr. Opin. Microbiol. 3 (2000) 16–22.
[5] J.A. Hoffmann, The immune response of *Drosophila*, Nature 426 (2003) 33–38.
[6] O. Schmidt, U. Theopold, M.R. Strand, Innate immunity and evasion by insect parasitoids, Bioessays 23 (2001) 344–351.
[7] Y. Carton, A.J. Nappi, Immunogenetics aspects of the cellular immune responses of *Drosophila* against parasitoids, Immunogenetics 52 (2001) 157–164.
[8] G. Dimopoulos, Insect immunity and its implication in mosquito–malaria interactions, Cell. Microbiol. 5 (2003) 3–14.
[9] D. Hultmark, *Drosophila* immunity: paths and patterns, Curr. Opin. Immunol. 15 (2003) 12–19.
[10] T. Werner, G. Liu, D. Kang, S. Ekengren, H. Steiner, D. Hultmark, A family of peptidoglycan recognition proteins in the fruit fly *Drosophila melanogaster*, Proc. Natl. Acad. Sci. USA 97 (2000) 13772–13777.
[11] Y.S. Kim, J.H. Ryu, S.J. Han, K.H. Choi, K.B. Nam, I.H. Jang, et al., Gram-negative bacteria-binding protein, a pattern recognition receptor for lipopolysaccharide and beta-1,3-glucan that mediates the signalling for the induction of innate immune genes in *Drosophila melanogaster* cells, J. Biol. Chem. 275 (2000) 32721–32727.
[12] M. Lagueux, E. Perrodou, E.A. Levashina, M. Capovilla, J.A. Hoffmann, Constitutive expression of a complement-like protein in toll and JAK gain-of-function mutants of *Drosophila*, Proc. Natl. Acad. Sci. USA 97 (2000) 11427–11432.
[13] A.J. Nappi, E. Vass, Iron, metalloenzymes and cytotoxic reactions, Cell. Mol. Biol. 46 (2000) 637–647.
[14] S.P. Gygy, Y. Rochon, B.R. Franza, R. Aebersold, Correlation between protein and mRNA abundance in yeast, Mol. Cell. Biol. 19 (1999) 1720–1730.
[15] T. Ideker, V. Thorsson, J.A. Ranish, R. Christmas, J. Buhler, J.K. Eng, R. Bumgarner, D.R. Goodlett, R. Aebersold, L. Hood, Integrated genomic and proteomic analysis of a systematically perturbed metabolic network, Science 292 (2001) 929–934.
[16] E. Vierstraete, P. Verleyen, G. Baggerman, W. D'Hertog, G. Van den Bergh, L. Arckens, A. De Loof, L. Schoofs, A proteomic approach for the analysis of instantly released wound and immune proteins in *Drosophila melanogaster* hemolymph, Proc. Natl. Acad. Sci. USA 101 (2004) 470–475.
[17] F. Levy, P. Bulet, L. Ehret-Sabatier, Proteomic analysis of the systemic immune response of *Drosophila*, Mol. Cell. Proteom. 3 (2004) 156–166.
[18] M.P. Mota, Influência genética e estocástica no fenómeno de envelhecimento, University of Trás-os-Montes & Alto Douro, Portugal, 2003, pp. 52–53.
[19] A. Braun, J.A. Hoffmann, M. Meister, Analysis of the *Drosophila* host defence in *domino* mutant larvae, which are devoid of hemocytes, Proc. Natl. Acad. Sci. USA 95 (1998) 14337–14342.
[20] O.H. Lowry, N. Rosebrough, A. Farr, R. Randall, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[21] T. Rabilloud, Use of thiourea to increase the solubility of membrane proteins in two-dimensional electrophoresis, Electrophoresis 19 (1998) 758–760.
[22] J.X. Yan, R. Wait, T. Berkelman, R.A. Harry, J.A. Westbrook, C.H. Wheeler, M.J. Dunn, A modified silver staining protocol for visualization of proteins compatible with matrix-assisted laser desorption/ionization and electrospray ionization mass spectrometry, Electrophoresis 21 (2000) 3666–3672.
[23] C.D. Detweiler, L.J. Deterding, K.B. Tomer, C.F. Chignell, D. Germolec, R.P. Mason, Free Radic. Biol. Med. 33 (2002) 364–369.
[24] S.M. Guedes, R. Vitorino, K. Tomer, M.R.M. Domingues, A.J. Ferrer-Correia, F. Amado, P. Domingues, *Drosophila melanogaster* larval hemolymph protein mapping, Biochem. Biophys. Res. Commun. 304 (2003) 831–838.
[25] C.M. Smales, J.R. Birch, A.J. Racher, C.T. Marshall, D.C. James, Evaluation of individual protein errors in silver-stained two-dimensional gels, Biochem. Biophys. Res. Commun. 306 (2003) 1050–1055.
[26] M.J. Dutt, K.H. Lee, The scaled volume as an image analysis variable for detecting changes in protein expression levels by silver stain, Electrophoresis 22 (2001) 1627–1632.
[27] A.E. Kabakov, K.R. Budagova, D.S. Latchman, H.H. Kampinga, Stressful preconditioning and Hsp 70 overexpression attenuate proteotoxicity of cellular ATP depletion, Am. J. Physiol. Cell Physiol. 283 (2002) C521–C534.

[28] R.C. Findly, R.J. Gillies, R.G. Shulman, In vivo phosphorus-31 nuclear magnetic resonance reveals lowered ATP during heat shock of Tetrahymena, Science 219 (1983) 1223–1225.
[29] A.M. Lambowitz, G.S. Kobayashi, A. Painter, G. Medoff, Possible relationship of morphogenesis in pathogenic fungus, *Histoplasma capsulatum*, to heat shock response, Nature 303 (1983) 806–808.
[30] M.J. Gorman, S.M. Paskewitz, Serine proteases as mediators of mosquito immune responses, Insect Biochem. Mol. Biol. 31 (2001) 257–262.
[31] A.S. Robertson, D. Belorgey, K.S. Lilley, D.A. Lomas, D. Gubb, T.R. Dafforn, Characterization of the necrotic protein that regulates the Toll-mediated immune response in *Drosophila*, J. Biol. Chem. 278 (2003) 6175–6180.
[32] P.E. Shaw, Peptidyl-prolyl isomerases: a new twist to transcription, EMBO Rep. 3 (2002) 521–526.
[33] S.L. Schreiber, Chemistry and biology of immunophilins and their immunosuppressive ligands, Science 251 (1991) 283–287.
[34] S. Luan, Immunophilins in animals and higher plants, Bot. Bull. Acad. Sin. 39 (1998) 217–223.
[35] D.A. Fruman, S.J. Burakoff, B.E. Bierer, Immunophilins in protein folding and immunosuppression, FASEB J. 8 (1994) 391–400.
[36] M.A. Stamnes, B.H. Shieh, L. Chuman, G.L. Harris, C.S. Zuker, The cyclophilin homolog NinaA is a tissue-specific integral membrane protein required for the proper synthesis of a subset of *Drosophila* rhodopsins, Cell 65 (1991) 219–227.
[37] K. Sykes, M.J. Gething, J. Sambrook, Proline isomerases function during heat shock, Proc. Natl. Acad. Sci. USA 90 (1993) 5853–5857.
[38] S. Luan, M.W. Albers, S.L. Schreiber, Light-regulated, tissue specific immunophilins from a higher plant, Proc. Natl. Acad. Sci. USA 91 (1994) 984–988.
[39] A.N. Santos, S. Korber, G. Kullertz, G. Fischer, B. Fischer, Oxygen stress increases prolyl cis/trans isomerase activity and expression of cyclophilin 18 in rabbit blastocysts, Biol. Reprod. 62 (2000) 1–7.
[40] L. Cavarec, T. Kamphausen, B. Dubourg, I. Callebaut, F. Lemeunier, D. Métivier, et al., Identification and characterization of Moca-cyp, J. Biol. Chem. 277 (2002) 41171–41182.
[41] P. Ligoxygkis, N. Pelte, C. Ji, V. Leclerc, B. Duvic, M. Belvin, H. Jiang, J.A. Hoffmann, J. Reichhart, A serpin mutant links Toll activation to melanization in the host defence of *Drosophila*, EMBO J. 21 (2002) 6330–6337.
[42] T. Rabilloud, M. Heller, F. Gasnier, S. Luche, C. Rey, R. Aebersold, et al., Proteomics analysis of cellular response to oxidative stress, J. Biol. Chem. 31 (2002) 19396–19401.
[43] S.M. Kanzok, A. Fechner, H. Bauer, J.K. Ulschmid, H. Müller, J. Botella-Munoz, S. Schneuwly, H. Schirmer, K. Becker, Substitution of the thioredoxin system for the glutathione reductase in *Drosophila melanogaster*, Science 291 (2001) 643–646.
[44] H. Bauer, S.M. Kanzok, H. Schirmer, Thioredoxin-2 but not thioredoxin-1 is a substrate of thioredoxin peroxidase-1 from *Drosophila melanogaster*, J. Biol. Chem. 277 (2002) 17457–17463.
[45] R.A. Krebs, M.E. Feder, Tissue-specific variation in Hsp70 expression and thermal damage in *Drosophila melanogaster* larvae, J. Exp. Biol. 200 (1997) 2007–2015.