TITLE: Proteomic analysis of the somatic and surface compartments from *Dirofilaria immitis* adult worms

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ABSTRACT

Dirofilaria immitis (hearthworm) is a filarial roundworm transmitted by mosquitoes to different vertebrate hosts (dogs, cats and humans, among others), causing dirofilariosis. The adult worms reside in the pulmonary arteries affecting vessels and tissues and resulting in different pathological manifestations. Worms migrate to the heart and surrounding major vessels in heavy infections. Dirofilariosis can result in serious damage to affected hosts. In the last few years, a re-emergence of the disease driven by the climate change has been pointed out. Very recently, the knowledge at molecular level of this parasite has been extended by the published studies on its genome and transcriptome. Nevertheless, studies on the expression of defined protein sets in different parasite compartments and the corresponding role of those proteins in the host-parasite relationship have been relatively scarce to date. These include the description of the adult worm secretome, and some of the proteins eliciting humoral immune responses and those related with plasminogen binding in secreted and surface extracts of the parasite. Here, we investigate by proteomics the somatic and surface compartments of the *D. immitis* adult worm, adding new information on protein expression and localization that would facilitate a deeper understanding of the host-parasite relationships in dirofilariosis.

KEYWORDS: *Dirofilaria immitis*; proteomics; somatic; surface.

1. Introduction

Dirofilariosis is a vector-borne disease in temperate and tropical areas worldwide caused by several *Dirofilaria* species (Simón et al., 2009). *Dirofilaria immitis* is responsible of cardiopulmonary dirofilariosis in canine and feline animals and accidentally humans. *D. immitis* adult worms can survive for several years (>7) in the lung arteries and right ventricle of immunocompetent hosts, while releasing microfilariae that circulate in the peripheral blood vessels. These can be taken by mosquito vectors when they feed on infected individuals and transmitted to the next vertebrate host (McCall et al., 2008).

Cardiopulmonary dirofilariosis is usually a chronic disease. It progressively affects lung arteries, the lung parenchyma and the hearth in the last stage of the disease (Venco, 2007). The pathogenic mechanisms are related with both inflammatory and non-inflammatory responses (Kaiser et al., 1989). Additionally, acute seizures overlapping with the chronic phase of the disease can occur, due to massive death of adult worms either spontaneously or after an adulticide treatment, occasionally resulting in the death of the affected animal. This massive death of worms is linked with the triggering of inflammatory responses and occurrence of thrombi that can result in the death of affected animals (Simón et al., 2012).

The complex host-parasite relationships of dirofilariasis resulting in a long-lasting survival of the parasite, the diverse pathogenic mechanisms displayed by the parasite and the control of pathological consequences that adult worms exert over the host, as well as the relatively complex metabolic machinery of the parasite are not well known at molecular level to date. Information about the protein composition of *D. immitis* is still scarce when compared to the available proteomic information in other filarial nematodes (Simón et al., 2012). Many of the related studies in *D. immitis* have focused on single, specific proteins, resulting in a very low number of *D. immitis* proteins present in databanks (e.g., 143 entries

in the GenBank Protein database using "*Dirofilaria immitis*" as key words). Only few studies have looked at sets of proteins, among them the recent study on the identification of those proteins present in the secretome of the adult parasite (Geary et al., 2012) and also those performed by our group. These included proteins identified by two-dimensional electrophoresis and mass spectrometry in *D. immitis* adult worms somatic extracts that were reactive against sera from infected dogs, cats and human patients suffering dirofilariasis (Oleaga et al., 2009; González-Miguel et al., 2010a, b). These studies allowed the identification of immunoreactive proteins of the parasite involved in parasite metabolism, plasminogen binding, detoxification, up-regulation of anti-inflammatory (Th2) responses and others.

Regarding antigen characterization, excreted/secreted antigens (E/S) of *D. immitis* adult worms, these have shown to stimulate a Th2 anti-inflammatory response driven by prostaglandin E2 and accompanied by a decrease of permeability in blood vessels (Morchón et al., 2010). Later, it has been demonstrated that the *D. immitis* E/S and surface proteins also activate the fibrinolytic system of the host promoting thrombi elimination, and 10 and 11 plasminogen-binding proteins, respectively, were identified in those two worm compartments (González-Miguel et al., 2012, 2013). Somatic antigens showed on the contrary pro-inflammatory properties, mainly driven by antigens from the symbiotic bacteria *Wolbachia*. The inflammatory phenomenon is then exacerbated at the vascular endothelium level when adult worms die and somatic antigens are released *in situ* (Morchón et al., 2010), although the participation of specific molecules of the adult worm in the triggering of inflammatory responses has not been demonstrated to date.

Lately, the study of the genome of *D. immitis* has added new valuable information on the potential set of proteins that could be expressed by the parasite (Godel et al., 2012). The transcriptome of *D. immitis* adult worms has also bee recently released (Fu et al., 2012).

Nonetheless, the identification and characterization of the protein expression patterns in specific parasite compartments and stages is still a priority. This information could aid to focus on defined interventions against dirofilariosis based in the definition of new drug and immune targets in the parasite. Especially important in this context are those molecules exposed by the parasite and used by *D. immitis* to modulate its relationships with the vertebrate host.

Here we present our contribution to expand the knowledge about protein expression of *D. immitis* adult worms in specific parasite compartments, and the potential relationship of some of the identified proteins with parasite survival and pathogenic mechanisms during dirofilariasis. For this, we analyze two different compartments of the worms - somatic and surface- using a proteomic approach for the identification and comparison of the most abundant proteins in both parasite's compartments. Additionally, we perform the immunolocalization of two of the identified proteins –GAPDH and P22U- in adult worms.

2. Material and methods

2.1. Protein extracts of *D. immitis* adult worms.

Somatic and surface antigens were obtained from *D. immitis* adult worms as previously described (Morchón et al., 2008; Hernandez-González et al., 2010). Briefly, 10 male and female worms were obtained from a naturally infected dog and extensively washed with PBS. For somatic antigens, 4 male and female worms were sonicated in 5 cycles of 70 kHz (30 seg each) at 4°C and centrifuged at 10,000 *g* for 20 min and the supernatant was collected. To obtain the surface antigens, 5 worms were subjected to trypsin digestion by incubation with 1 ng/µl of sequencing grade trypsin (Sigma) in PBS at 37ºC for 45 min. The supernatant containing the released peptides was then subjected to DTT and iodoacetamide treatment as follows: supernatants were reduced with 10 mM dithiothreitol (DTT) in 50 mM

NH_4HCO_3 in a microwave oven for 3 min at 560 W. The reaction was stopped with 10% trifluoroacetic acid. The remaining adult worm was used for immunolocalization studies (see 2.3.).

A cocktail of protease inhibitors (1mM EDTA, 1mM N-ethylmaleimide, 0.1 µM pepstatin A, 1mM PMSF and 0.1 mM N-tosylamide-L-phenylalanine chloromethyl ketone) was added to all samples and were lyophilized and stored at -20°C until analysis.

2.2. Liquid chromatography and tandem mass spectrometry (LC–MS/MS).

Fifty µg of each of the above-mentioned samples were resuspended in 100µl of Urea 2M in 50 mM ABC and 60µl of each sample were loaded onto a trap column (PepMap C18, 300µm×5mm, LC Packings, Amsterdam The Netherlands) and desalted with 0.1% TFA at 30µl/min during 10 min. Previously, all samples were digested with 500 ug of trypsin at 37ºC. The peptides were then loaded onto an analytical column (PepMap C18 3µ 100 A, 75µm×15 cm, LC Packings) equilibrated in 5% acetonitrile and 0.1% formic acid. Elution was carried out with a linear 5–65% gradient of solvent B (95% acetonitrile, 0.1% formic acid) in 120 min at a flow rate of 300nl/min. The eluted peptides were analyzed with a nanoESI-Q-TOF mass spectrometer (QSTAR-XL, Applied Biosystems) in an information dependent acquisition mode (IDA).

Protein identification was performed using Mascot v2.2 (Matrix Science) search engines. Mascot were used to process peak list generated directly from QSTAR wiff files with Sciex Analyst import filter options using the default parameters and used to search NCBInr protein database (20090602; 8430240 sequences; 2898477468 residues). Searches were also performed on the recently released set of ESTs of *D. immitis* (assembled unigenes longer than 300 bp) available from Transcriptome Shotgun Assembly Sequence Database (TSA) at NCBI with the following accession numbers: JR895929–JR916738, and on the *D.*

immitis whole genome tryptic peptides dataset available at http://nematodes.org/downloads/959nematodegenomes/blast/db/Dirofilaria_immitis_v1.3_20110901.fna. Additional searches were performed against the 1.1 Mb genome of *Wolbachia* isolated from *Brugia malayi* (wBm; Foster et al., 2005). Taxonomy search was done in Metazoa (Animals) sequences available at http://www.sanger.ac.uk (1175176 sequences). The search parameters were set to tryptic specificity, no cys-alkylation, restricted taxonomy to metazoa (animals), three missed cleavage and a tolerance in the mass measurement of 100ppm in MS mode and 0.5 Da for MS/MS ions. Met oxidation and Asn/Gln deamination were set as variable modifications. To avoid using the same spectral evidence in more than one protein, the identified proteins are grouped based on MS/MS spectra by Mascot. Thus, proteins sharing MS/MS spectra are grouped, regardless of the peptide sequence assigned. The protein within each group that can explain more spectral data with confidence is shown as the primary protein of the group. Only the proteins of the group for which there is individual evidence (unique peptides with enough confidence) are also listed, usually toward the end of the protein list. Only primary proteins are shown in the results. The MS/MS spectra of the proteins identified with a single protein were inspected manually. Individual ions scores >69 indicate identity or extensive homology which is equivalent to a protein confidence threshold greater than 95%, was considered significant ($p<0.05$).

For the proteins identified, the molecular function and biological process were assigned according to the AmiGO and the UniProtKB databases (http://amigo.geneontology.org and http://www.uniprot.org). Finally, the relative abundance of the predicted proteins in the trypsin digestion was assessed using the Exponentially Modified Protein Abundance Index – emPAI – calculated by Mascot and thus applying the default parameters and statistics used by Mascot. For redundant identifications the emPAI value from the higher score hit was considered. The relative abundance in percentage for the

identified proteins in each compartment was calculated assuming that 100% is equal to the sum of all the emPAI values in each compartment.

2.3. Immunolocalization of the GAPDH and P22U proteins in *D. immitis* adult worms.

One *D. immitis* adult worm was fixed in 10% formaldehyde and embedded in paraffin. Following, 5-µm-thick sections were placed on slides and incubated for 1 hour at 37ºC with 1% bovine serum albumin (BSA) in PBS (blocking solution). Sections were then incubated for 1 hour at 37ºC either with a negative rabbit serum or a polyclonal rabbit serum against the recombinant GAPDH protein from *Schistosoma bovis* (GenBank accesión number GI:186462282) obtained by immunization of rabbits three times with 50 µg of the *S. bovis* recombinant GAPDH as described elsewhere (Hernández-González et al., 2012) plus 100 µg saponin in PBS, or a polyclonal rabbit serum against the recombinant P22U protein from *D. immitis* (Frank et al., 1999), diluted 1:50 in blocking solution. Sections were washed four times with 0.05% Tween 20 in PBS and once with PBS, and incubated with an anti-rabbit IgG marked with Alexa Fluor 594 and with phalloidin Alexa Fluor 488 (contrast staining binding actin) at 1:50 in blocking solution for 1 hour at 37ºC. The luminescent reaction was studied and recorded with a confocal microscope.

3. Results

3.1. Identified proteins.

The proteomic analysis performed in this work allowed for the identification of 108 proteins in the somatic extract and 16 in the surface digestion of *D. immitis* adult worms, from which 11 were shared by both extracts. The percentage (mean) of the peptide mass peaks that could not be assigned in the searched databases was 46% for somatic extracts and 60% for surface extracts. The false discovery rate was 12.2% and 15.09% for somatic and

surface extracts, respectively. Table 1 shows the relevant information of the identified polypeptides, including their homology, score and their relative abundance or emPAI value. Of the identified proteins, 13 proteins were represented by 2 to 8 isoforms (data not shown). None of those proteins were from *Wolbachia*.

As shown in Figure 1, 11 proteins –major sperm protein, polyprotein antigen, GAPDH, P22U, tropomyosin, small heat shock protein 12.6, paramyosin, MFP3, heat shock protein 70, troponin T and intermediate filament protein - are present in the two extracts studied here, being the relative abundance of the major sperm protein, polyprotein antigen, GAPDH, P22U, HSP12.6, paramyosin, MFP3 and HSP70 higher in the surface compartment than in the somatic extract (Fig. 1).

Only twelve of the 108 proteins in the somatic extract (fructose-bisphosphate aldolase, DiNCF, pepsin inhibitor Dit33, galectin, P22U, small heat shock protein p27, glutathione peroxidase Di29, intermediate filament protein, calreticulin precursor, peroxiredoxin, superoxide dismutase and polyprotein antigen) and two in the surface extract (polyprotein antigen and P22U) were identified as homologous to *D. immitis* proteins present in the databanks that were used to perform the searches. The remaining proteins were identified by homology with sequences from other organisms, mainly from the filarial nematodes *Brugia malayi (23)/pahangi* (2), *Loa loa* (24), *Onchocerca volvulus* (17)*/O. gibsoni* (1), *Wuchereria bancrofti* (13) and other nematodes: *Caenorhabditis elegans* (12)*/C. briggsae* (2) and *Ascaris suum* (10)*/A. lumbricoides* (2) (Table 1). Some other proteins that were identified over more taxonomically distant invertebrate species were discarded because scores were very low, probably indicating unreliable homologies (data not shown), with the exception of the high-affinity octopamine transporter, identified on the homologous protein from *Lumbricus terrestris* (Table 1). In the surface extract, half of the identifications were done on the sequences from the lymph vessel residing parasite *B. malayi* (Table 1).

The majority of the identified proteins in both compartments are associated with molecular binding processes, being the catalytic activity group best represented in somatic extract, while the structural molecules were more abundant in the surface extract than in the somatic proteins (Fig. 2). Regarding biological processes assigned by homology, the identified proteins could be grouped into 9 families: structural-motility, energy-metabolism-redox processes, stress response, immune response, proteolysis, transcription-translation, signalling, other functions and unknown. The representation of each family for each parasite compartment is shown in Figure 3, indicating the relative percentage for each family. Those molecules related with energy, metabolism and redox processes are the most abundant in the somatic extract, while structural-motility proteins are dominant in the surface digestion products (Fig. 3). In the "proteolysis" group, main differences were found between somatic and surface extracts: while 3 out of 5 molecules in this group were identified as protease inhibitors in the somatic extract, a proteolytic papain-like enzyme was the only representative of this group in the surface extract and was not identified in the somatic proteins.

3.2. The GAPDH and P22U proteins mainly localize in the cuticle of *D. immitis* adult worms.

Two of the proteins shared by the two parasite compartments analyzed here were localized in transverse sections of *D. immitis* adult worms after incubation with specific anti-sera using a confocal microscope. As Figure 4 shows, both proteins were predominantly present in the cuticle, although the P22U showed to be localized within the cuticle, while the anti-GAPDH serum sharply stained the outermost surface of the worm. Anti-GAPDH reactivity was also found inside the worm, although less abundantly than at the surface of

233 the parasite. No reactivity was found in worm sections incubated with the preimmune
234 negative serum (Fig. 4).
235
236 **4. Discussion**.
237 The expansion of animal dirofilariasis to temperate and cold climate areas and the
238 rising in the number of human cases poses this disease as a globally emerging problem
239 (Simón et al., 2012). The proper application and use of preventive tools and treatment
240 alternatives better than those available to date are required (Fu et al., 2012). Nonetheless, the
241 identification of new targets in the parasite have been precluded due to the limited
242 information about *D. immitis* protein expression and, more importantly, the scarce
243 knowledge about *D. immitis* proteins interacting with the host and about the related
244 mechanisms of interaction.
245 The present study, together with that performed by Geary et al. (2012) on the
246 secretome of *D. immitis*, partially fills this gap for adult worms. Here, we have used the
247 LC/MS/MS technique to identify 108 and 16 proteins of the parasite in specific
248 compartments, some related with the pathology triggered after the sudden death of adult
249 worms –usually after treatment-, specifically the somatic components, and some others
250 associated with the host-parasite relationship during the chronic infection and putatively
251 found at the host-parasite interface (surface proteins). The recent release of a transcriptomic
252 database of the parasite (Fu et al., 2012) and the available data on the *D. immitis* genome
253 have facilitated the identification of proteins in this work, although due to the used approach
254 only the most abundant proteins in both parasite's compartments studied here have been
255 identified.
256 Somatic proteins represent those exported to the outside of the parasite, including
257 excreted/secreted and surface components, as well as those which are only exposed to the

host after parasite's death and destruction. When adult worms die spontaneously or by adulticide treatments, a sudden release of somatic antigens and *Wolbachia* molecules to the circulation occurs, triggering the most serious pathological effects of dirofilariosis. These are mainly related with inflammatory processes that have been investigated on vascular endothelial cells *in vitro* (Morchón et al., 2008; Simón et al., 2008) and in infected animals (Oleaga et al., 2009; González-Miguel et al., 2010a, 2010b) by proteomic techniques. A number of allergens found here in the somatic extract could contribute as well to the inflammatory reaction after parasites death.

Here, the ten proteins with the highest relative abundance found in the somatic extract are tropomyosin, major sperm protein, P22U, pepsin inhibitor Dit33, an ubiquitin family member, the small heat shock protein 12.6, myosin regulatory light chain 1, DiNCF, triose phosphate isomerase and acyl CoA binding protein. The DiNCF (IL8-like) molecule could contribute to the proinflammatory reaction upon release from death parasites, due to the potential of the IL8-like molecules to trigger inflammation. Remarkably, DiNCF has not been found in the secretome of *D. immitis* or at the surface in the present work, thus it could be usually not exposed to the host and only released after parasite's damage.

The fourth most abundant protein in the somatic extract is the pepsin inhibitor Dit33, belonging to a family that has been also identified in the secretome of *D. immitis* (Av33; Willenbücher et al., 1993). Two other peptidase inhibitors (serpin and cysteine protease inhibitor) were also found in the somatic extract, being absent in the secretome and in the surface extract. No protease inhibitor was found at the surface. Inside the proteolytic family, the papain-like protease was found to be potentially associated to the surface of the worm. This protease could be used to digest host antibodies and other blood components, and certainly to regulate host immune responses, since it is well known that parasite cysteine

proteases are unusually immunogenic (Sajid and McKerrow, 2002) and potent allergens (e.g., Rodríguez-Maíllo et al., 2007), situating those proteases at the host-parasite interface.

Of the ten most abundant proteins in the somatic extract, the tropomyosin, major sperm protein, P22U and small heat shock protein 12.6 were also found in the surface extract during our study. None of those four proteins have been found by Geary et al. (2012) in the secretome of the parasite, although the P22U has been described as a component of the excretory/secretory products of *D. immitis* by other authors (Frank et al., 1999; González-Miguel et al., 2012) and characterized as a plasminogen-binding protein (González-Miguel et al., 2012), thus potentially interacting with the host. Its presence at the surface of *D. immitis* is confirmed here by immunolocalization, suggesting the re-association of this molecule to the parasite surface after being excreted.

The presence of the other three above-mentioned molecules at the host-parasite interface could also be inferred from former publications that have identified them as protective antigens (Sereda et al., 2008; Gnanasekar et al., 2008; Dakshinamoorthy et al., 2012) or as diagnostic antigens (Park et al., 2008) for other filarial nematodes. It is important mentioning that the majority of the proteins identified at the surface of worms in the present work have been described as well as diagnostic or vaccine candidate antigens in dirofilariasis and other nematode infections -polyprotein antigen (Poole et al., 1992, 1996; Tekuza et al., 2002a, b; Vercauteren et al., 2004), paramyosin (Zhang et al., 2011), intermediate filament protein (Cho-Ngwa et al., 2011), HSP70 (Ravi et al., 2004), and the papain family cysteine protease (Rodriguez-Mahillo et al., 2007).

Of those shared with the somatic extract, the major sperm protein, the polyprotein antigen and the P22U showed to rank among the 5 most abundant proteins in the surface extract, together with the the papain-like cysteine proteinase and the GAPDH. The presence of this glycolic enzyme in the surface of *D. immitis* adult worms, confirmed by

307 immunolocalization in the present work, could be related with the newly described function
308 of GAPDH in this and other helminth parasites as a plasminogen binding molecule at the
309 host-parasite interface (Erttmann et al., 2005; Ramajo-Hernández et al., 2007; González-
310 Miguel et al., 2012, 2013).

311 The remaining molecules identified here as potentially associated with the surface
312 structures of *D. immitis* adult worms, either at the cuticle or at the intestinal tract, were not
313 detected in the secretome of the parasite, with the exception of the HSP70. Some of them
314 have been characterized as immunodominant antigens in other filarial nematodes (HSP70;
315 Ravi et al., 2004) or as potential immunomodulators (JNK-associated leucine-zipper protein;
316 Wang et al., 2013), although their presence at the host-parasite interface and related
317 functions in dirofilariosis should be further investigated.

318 *D. immitis* contains an endosymbiont, *Wolbachia*, which is essential for the survival
319 and reproduction of the parasite. It has also been postulated that *Wolbachia*-derived products
320 may impact upon the host immune system (rev. in Simón et al., 2012). We therefore,
321 specifically searched with our spectra derived from somatic and surface extracts of *D.*
322 *immitis* for matches to the genome of *Wolbachia* wBm, but found none. Similarly, previous
323 proteomic studies on *D. immitis* failed to identify endosymbiont proteins in adult worm
324 extracts (González-Miguel et al., 2010a, b; 2012; 2013; Geary et al., 2012). This could be
325 attributed to a low representation of *Wolbachia* proteins in the extracts of *D. immitis*. It is, of
326 course, very likely that dying parasites would release *Wolbachia* proteins, and the relative
327 influence of parasite and endosymbiont products on host immunity *in vivo* remains to be
328 determined.

329 In summary, the present work has allowed the identification of 108 and 16 proteins
330 expressed in the somatic and the surface compartments of *D. immitis* adult worms. This
331 represents an extension of those parasite compartments already characterized by other

authors (secretome), and opens the way for further studies on the functionality and the participation of the identified proteins in the host-parasite relationships of dirofilariasis.

Conflict of interests

No conflict of interests is declared.

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Table

Table 1. Proteins of *D. immitis* somatic and surface adult worm extracts identified by LC-MS/MS.

Figure captions

Figure 1. Somatic and surface proteins identified in *Dirofilaria immitis* adult worms by LC-MS/MS. 108 and 16 proteins were identified in somatic (SO) and surface (SU) extracts, respectively. From those, 11 were found in both compartments. The comparison of the relative abundance of the 11 shared proteins in somatic and surface extracts is shown. Grey bars, somatic proteins; dotted bars, surface proteins. MSP, major sperm protein; PPA, polyprotein antigen; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; TROPM, tropomyosin; HSP12.6, small heat shock protein 12.6; PARAM, paramyosin; HSP70, heat shock protein 70; TROPO, troponin T; IFP, intermediate filament protein.

Figure 2. Comparison of the relative abundance of the proteins indentified by LC-MS/MS in somatic (grey bars) and surface (Dotted bars) extracts of *D. immitis* adult worms, classified by their molecular function (GO).

Figure 3. Comparison of the relative abundance of the nine family groups of proteins (GO) detected in *D. immitis* somatic (A) and surface (B) adult worm extracts.

Figure 4. Immunolocalization of the GAPDH (second file) and P22U (third file) proteins in *D. immitis* adult worms (transversal sections). The confocal microscope images (4×) are shown under normal light (C)) and green (A) or red fluorescence (B) after incubation with phalloidin plus an anti-GAPDH rabbit serum (second file), an anti-P22U rabbit serum (third

file) or the corresponding naïve serum (first file). Specific reactivity of actin is shown in green and specific anti-GAPDH and P22U reactivity is shown in red.

Table 1 - R2

PROTEINS OF *D. immitis* SOMATIC AND SURFACE ADULT WORM EXTRACTS IDENTIFIED BY LC-MS/MS					
Protein name	**Database ID nb.**	**Species**	**Score**	**NP**	**emPAI**
SOMATIC PROTEINS					
Mesocentin	ADY39862	*Ascaris suum*	3987	32	0.12
Myosin regulatory light chain 1	XM_003138853	*Loa loa*	2311	12	1.31
Aminopeptidase ES-62 precursor	AF077194	*Acanthocheilonema viteae*	1769	38	0.91
Polyprotein antigen	DIU52967	*Dirofilaria immitis*	1714	10	1.05
Apolipophorin	ADY39826	*Ascaris suum*	1651	35	0.42
Thrombospondin	AAB99830	*Haemonchus contortus*	1616	46	0.09
Fructose-bisphosphate aldolase	JQ780094	*Dirofilaria immitis*	1245	22	0.70
Tropomyosin	gi|154466686	*Ascaris lumbricoides*	1207	29	4.56
Aspartic protease	OVU81605	*Onchocerca volvulus*	1120	66	0.30
Dosage compensation protein dpy-30	EJW84023	*Wuchereria bancrofti*	893	51	0.64
MFP3	XM_003137007	*Loa loa*	855	27	0.92
Glycogen phosphorylase	XM_003141550	*Loa loa*	842	11	0.47
Triosephosphate isomerase	XM_003145134	*Loa loa*	702	22	1.20
DiNCF	gi|2160474	*Dirofilaria immitis*	679	18	1.26
Heat shock protein 70	gi|7673686	*Wuchereria bancrofti*	639	10	0.58
Calponin protein 3	XM_003148786	*Loa loa*	614	23	0.68
Pepsin inhibitor Dit33	gi|31339942	*Dirofilaria immitis*	609	21	2.29
ATP-dependent DNA helicase II	XM_001899708	*Brugia malayi*	497	22	0.27
Actin	gi|6626	*Caenorhabditis elegans*	473	12	0.84
Tumor D52 family protein	XM_003143389	*Loa loa*	438	7	0.37
Adenine phosphoribosyltransferase	XM_001901344	*Brugia malayi*	425	11	0.61
FKBP-type peptidyl-prolyl cis-trans isomerase-59	EJW82003	*Wuchereria bancrofti*	410	11	0.15
Alpha tubulin	DQ010542	*Onchocerca volvulus*	397	9	0.11
TTR-51 protein	XM_003145789	*Loa loa*	360	17	1.09
Chaperonin HSP-60	EJD75295	*Loa loa*	354	10	0.90
Calponin (Ov9M)	gi|1352090	*Onchocerca volvulus*	352	8	0.58
Galectin	gi|7159326	*Dirofilaria immitis*	349	8	0.80

P22U	gi|3253097	*Dirofilaria immitis*	337	47	2.78
Cytoplasmic intermediate filament protein	XP_001902854	*Brugia malayi*	333	10	0.29
Major sperm protein	gi|118137388	*Ascaris suum*	324	9	3.45
Immunodominant hypodermal antigen OV17	OV17_ONCVO	*Onchocerca volvulus*	309	21	0.61
14-3-3 family member (ftt-2)	gi|17568359	*Caenorhabditis elegans*	304	7	0.40
Small heat shock protein 12.6	gi|170591664	*Brugia malayi*	301	6	1.55
Octapeptide-repeat protein T2	XM_001900570	*Brugia malayi*	300	6	0.24
Heat shock protein 90	gi|3096951	*Brugia pahangi*	296	7	0.26
Adenylate kinase isoenzyme 1	XP_001894222	*Brugia malayi*	294	6	0.26
Annexin A5	gb|ADY46116	*Ascaris suum*	285	11	0.15
Major antigen	EJD74046	*Loa loa*	271	8	0.04
Beta-galactoside-binding lectin	gi|433317	*Onchocerca volvulus*	269	7	0.35
Small heat shock protein p27	gi|1206025	*Dirofilaria immitis*	268	7	1.07
DS DNA-binding domain containing protein	XP_001902452	*Brugia malayi*	268	5	0.33
Serpin protein 6	XP_001900434	*Brugia malayi*	261	6	0.11
GAPDH	gi|1945477	*Onchocerca volvulus*	259	12	0.30
OV25 heat shock protein	gi|9777	*Onchocerca volvulus*	255	8	0.88
OV-16 antigen precursor	AAA29411	*Onchocerca volvulus*	251	11	0.47
Glycosyl hydrolase family 31 protein	EJW88543	*Wuchereria bancrofti*	249	11	0.14
Hypothetical protein	XP_003147689	*Loa loa*	247	5	0.18
Galectin	XP_003139211	*Loa loa*	245	8	0.34
Troponin-c	ABO84939	*Brugia pahangi*	228	11	0.21
Phosphoglycerate kinase 1	gi|17508823	*Caenorhabditis elegans*	221	4	0.34
Acyl CoA binding protein	XM_001895645	*Brugia malayi*	215	8	1.20
Glutathione peroxidase Di29	gi|1708061	*Dirofilaria immitis*	213	6	0.64
Enolase	gi|32440997	*Onchocerca volvulus*	206	3	0.22
Ubiquitin family member	gi|25151716	*Caenorhabditis elegans*	202	4	1.80
Thioredoxin	XP_001900803	*Brugia malayi*	196	14	1.10
Heat shock factor binding protein 1	XP_003145843	*Loa loa*	189	3	0.14

MCC	EJD75504	*Loa loa*	186	3	0.04
Troponin	gi|6065738	*Anisakis simplex*	183	5	0.65
ML domain-containing protein	XP_003136496	*Loa loa*	182	6	0.32
Muscle positioning family member	gi|17569083	*Caenorhabditis elegans*	181	4	0.23
Transthyretin-like family protein	XP_001892758	*Brugia malayi*	180	8	0.36
Transthyretin-like protein 46	ADY47514	*Ascaris suum*	172	4	0.29
Phosphofructokinase	XP_003136359	*Loa loa*	169	4	0.10
Oxidoreductase	EJW80846	*Wuchereria bancrofti*	169	3	0.16
Cysteine protease inhibitor	XP_003147913	*Loa loa*	168	7	0.68
Polyprotein	XP_001901258	*Brugia malayi*	168	2	0.08
SPARC family protein	EJW80102	*Wuchereria bancrofti*	165	7	0.18
Heat shock protein 10	EJW83482	*Wuchereria bancrofti*	167	4	0.24
Hypothetical protein	XM_003144363	*Loa loa*	162	4	0.27
Hypothetical protein CBG17351	gi|157768372	*Caenorhabditis briggsae*	162	4	0.25
Intermediate filament protein	gi|7159290	*Dirofilaria immitis*	159	4	0.11
HD domain-containing protein	EJW87016	*Wuchereria bancrofti*	157	8	0.11
Disorganized muscle protein 1	ADY45671	*Ascaris suum*	153	6	0.23
Putative fatty acid retinoid binding protein 2	ACT55269	*Onchocerca volvulus*	138	2	0.09
Hypothetical protein	XP_003145485	*Loa loa*	135	2	0.10
Hypothetical protein T22F3.3	gi|17564550	*Caenorhabditis elegans*	132	4	0.10
Calreticulin precursor	gi|4115903	*Dirofilaria immitis*	128	3	0.15
Small heat shock protein	gi|1518125	*Brugia malayi*	125	4	0.19
Translation elongation factor aEF-2	XP_003141031	*Loa loa*	123	3	0.22
Peroxiredoxin (thioredoxin peroxidase)	gi|2352262	*Dirofilaria immitis*	122	3	0.32
MFP2 sperm cell motility protein	XP_003144076	*Loa loa*	121	6	0.29
TB2/DP1 family protein	EJW82952	*Wuchereria bancrofti*	119	3	0.29
Glucose phosphate isomerase	XP_001900986	*Brugia malayi*	119	3	0.08
DJ-1 family protein	XP_003142071	*Loa loa*	117	2	0.09
Fumarase	gi|31580769	*Ascaris suum*	116	3	0.01

60S ribosomal protein L5	EJW84695	*Wuchereria bancrofti*	115	2	0.08
ATP synthase subunit beta	XP_003143628	*Loa loa*	114	3	0.08
Mitochondrial prohibitin complex protein 2	EJW83593	*Wuchereria bancrofti*	113	2	0.08
Antigen maltose binding protein	gi|558046	*Onchocerca volvulus*	112	2	0.53
Cell Division Cycle related family member	gi|17532375	*Caenorhabditis elegans*	103	3	0.04
Translationally controlled tumor protein	gi|2501147	*Brugia malayi*	100	2	0.35
S1 protein/Ov20	gi|1019801	*Onchocerca volvulus*	90	2	0.20
DnaK protein	EJW87493	*Wuchereria bancrofti*	90	2	0.06
Cyclophilin Ovcyp-2	AAC47233	*Onchocerca volvulus*	89	6	0.39
Hydrolase	EJW81476	*Wuchereria bancrofti*	87	6	0.07
Paramyosin	gi|126256672	*Trichinella spiralis*	85	1	0.03
CK1/WORM6 protein kinase	EJW80549	*Wuchereria bancrofti*	80	4	0.15
Microfilariae surface-associated protein	gi|45602845	*Onchocerca volvulus*	76	1	0.19
Profilin	EFO23797	*Loa loa*	74	2	0.24
Glutathione S-transferase 1	ADY45818	*Ascaris suum*	70	2	0.11
Lactate dehydrogenase	gi|17535107	*Caenorhabditis elegans*	69	1	0.09
Superoxide dismutase	gi|2209364	*Dirofilaria immitis*	67	1	0.21
Hypothetical protein CBG08063	gi|157766821	*Caenorhabditis briggsae*	65	1	0.26
As37 immunoglobulin-like family protein	gi|22036079	*Ascaris suum*	63	1	0.09
Ezrin/Radixin/Moesin family member	gi|17505420	*Caenorhabditis elegans*	63	1	0.05
SMC	gi|56758564	*Schistosoma japonicum*	60	2	0.07
Titin	gi|72000919	*Caenorhabditis elegans*	54	3	0.00
Ov87 (galectin)	gi|4100353	*Onchocerca volvulus*	53	1	0.09
Antigen	gi|170590552	*Brugia malayi*	52	2	-
Myosin heavy chain	gi|3941223	*Schistosoma japonicum*	51	3	-
Chromodomain protein family member	gi|17569817	*Caenorhabditis elegans*	50	1	0.02
SURFACE PROTEINS					
Polyprotein antigen	gi|1663728	*Dirofilaria immitis*	191	7	0.20
GAPDH	gi|1945477	*Onchocerca volvulus*	114	4	0.19

Paramyosin	gi|915306	*Onchocerca gibsoni*	80	2	0.06
Intermediate filament protein	gi|170596673	*Brugia malayi*	79	2	-
Tropomyosin	gi|42559553	*Ascaris lumbricoides*	79	5	0.10
Major sperm protein	gi|118137388	*Ascaris suum*	76	2	0.24
P22U	gi|3253097	*Dirofilaria immitis*	69	3	0.14
High-affinity octopamine transporter	gi|110816318	*Lumbricus terrestris*	63	1	0.04
Papain family cysteine protease	gi|170595047	*Brugia malayi*	59	1	0.11
NSF	gi|133901658	*Caenorhabditis elegans*	53	6	-
Troponin T	ADY43415.1	*Ascaris suum*	51	2	0.04
JNK-associated leucine-zipper protein	XM_001899700.1	*Brugia malayi*	47	2	0.02
MFP3	XM_003137007.1	*Loa loa*	45	2	0.05
Small heat shock protein 12.6	XM_001900555.1	*Brugia malayi*	37	2	0.07
Conserved hypothetical protein	XP_001901762.1	*Brugia malayi*	30	2	0.03
Heat shock 70 kDa protein	XM_001901744.1	*Brugia malayi*	27	10	0.04
RhoGEF domain containing protein	XM_001896860.1	*Brugia malayi*	24	2	0.04
DnaJ C terminal region family protein	XM_001900397.1	*Brugia malayi*	23	2	0.05

Table footnotes	
NP	Number of matching peptides
MFP3	PP2A targeted MSP fiber protein 3
DiNCF	*Dirofilaria immitis* neutrophil chemotactic factor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
MCC	Macroglobulin complement component family protein
ML	MD-2-related lipid-recognition domain
SPARC	Secreted protein acidic and rich in cysteine
MFP2	PP2A targeted MSP fiber protein 2
SMC	Structural maintenance of chromosomes
NSF	N-ethylmaleimide sensitive secretion factor

Figure 1

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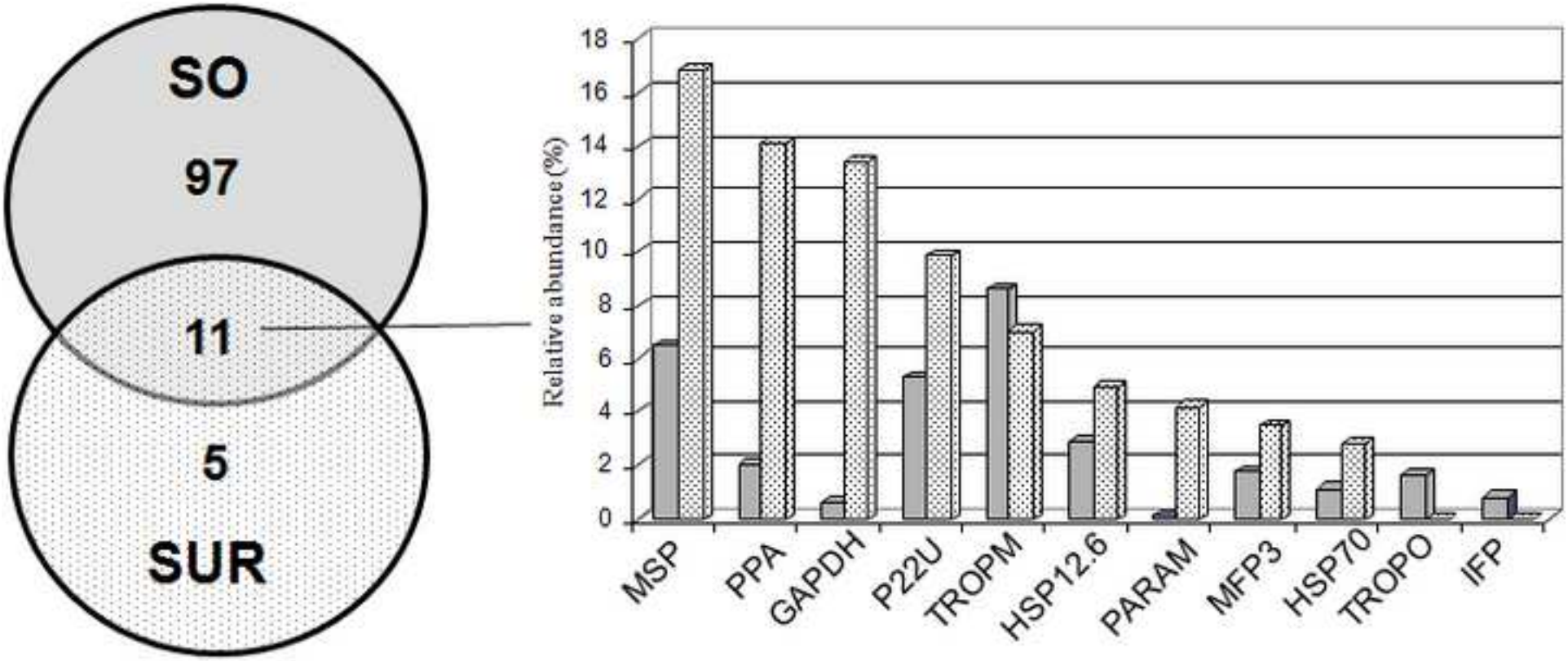

Figure 2

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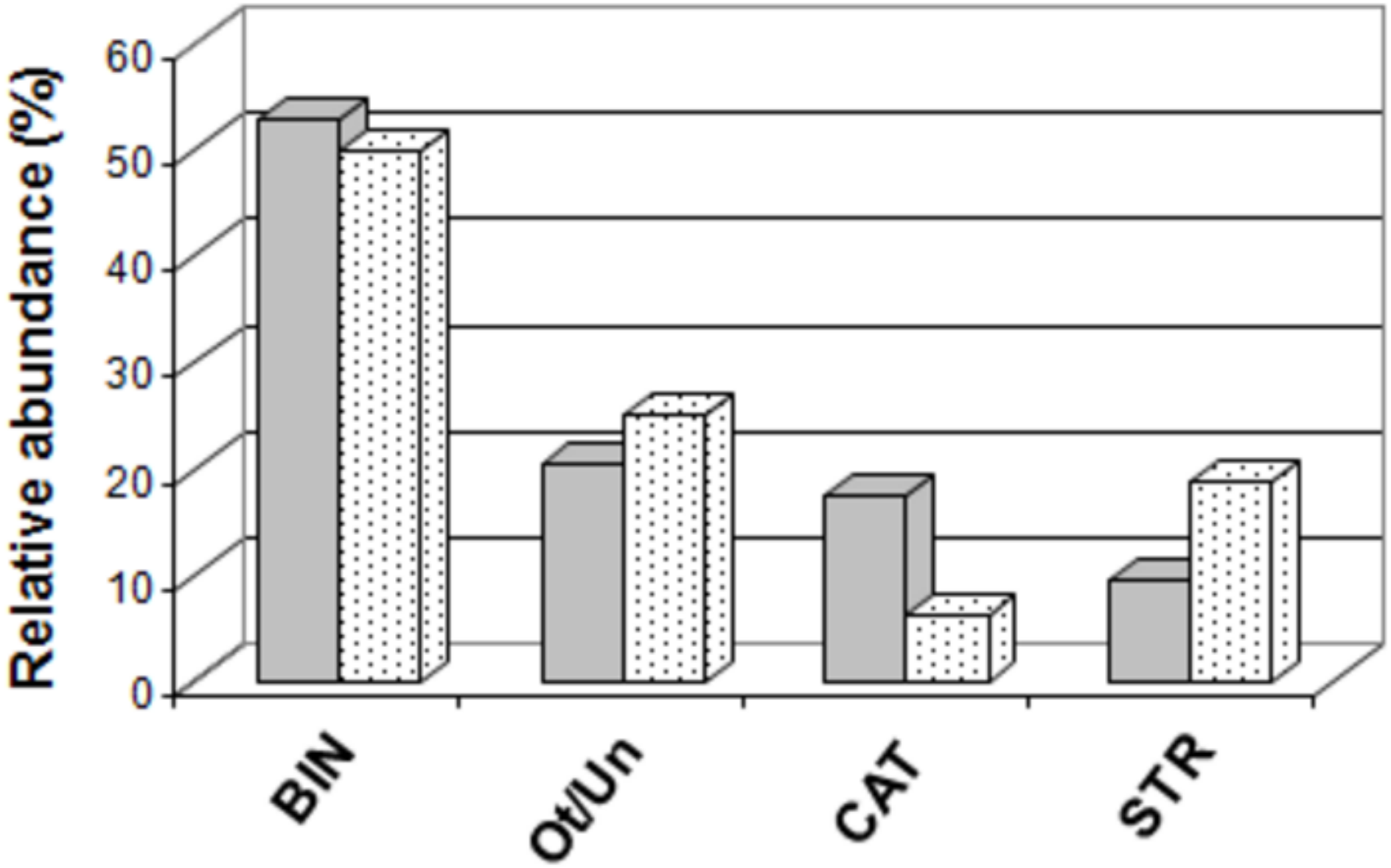

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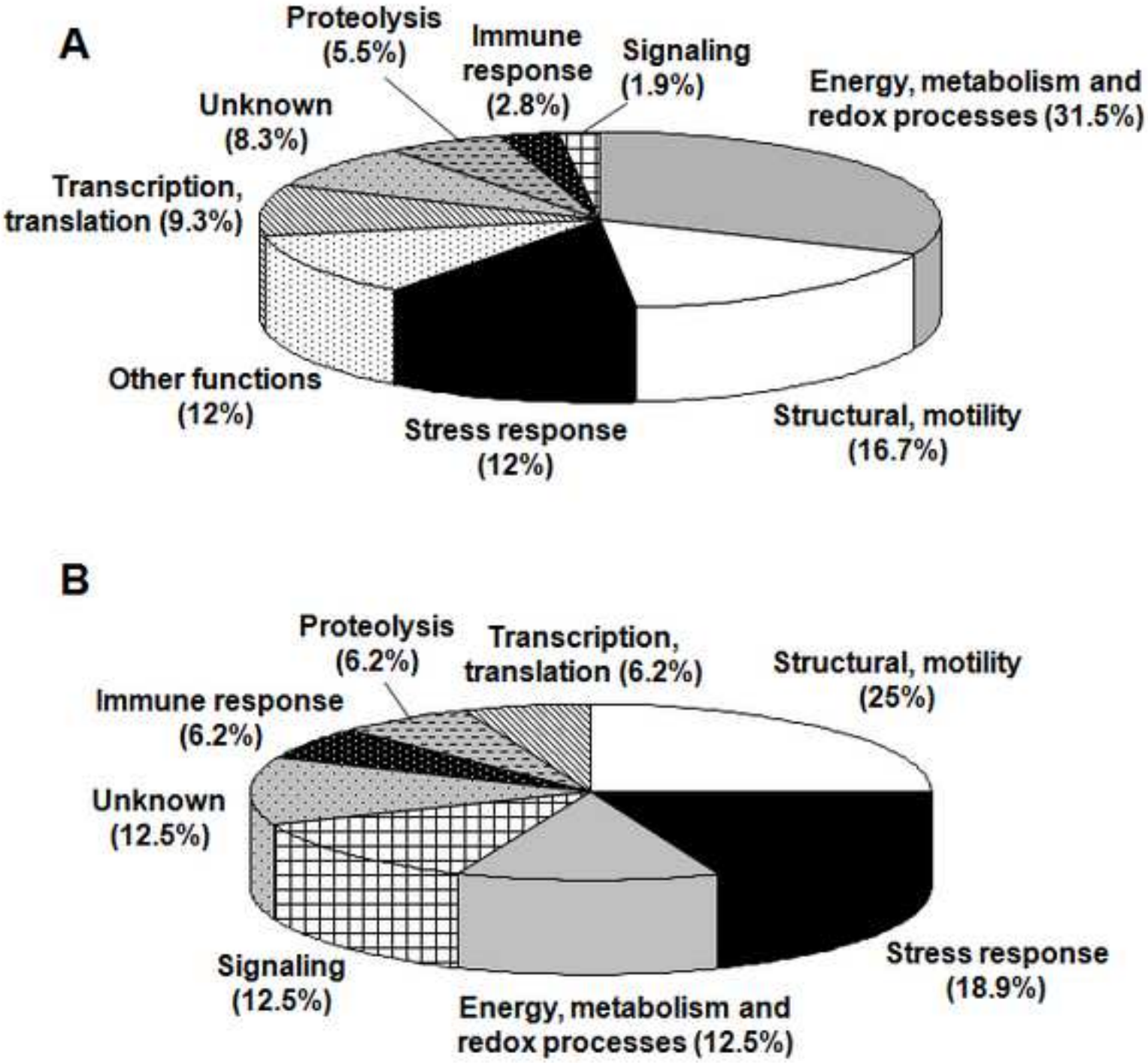

Figure 4

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