'Suicide' of crickets harbouring hairworms: a proteomics investigation

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Abstract

Despite increasing evidence of host phenotypic manipulation by parasites, the underlying mechanisms causing infected hosts to act in ways that benefit the parasite remain enigmatic in most cases. Here, we used proteomics tools to identify the biochemical alterations that occur in the head of the cricket *Nemobius sylvestris* when it is driven to water by the hairworm *Paragordius tricuspidatus*. We characterized host and parasite proteomes during the expression of the water-seeking behaviour. We found that the parasite produces molecules from the Wnt family that may act directly on the development of the central nervous system (CNS). In the head of manipulated cricket, we found differential expression of proteins specifically linked to neurogenesis, circadian rhythm and neurotransmitter activities. We also detected proteins for which the function(s) are still unknown. This proteomics study on the biochemical pathways altered by hairworms has also allowed us to tackle questions of physiological and molecular convergence in the mechanism(s) causing the alteration of orthoptera behaviour. The two hairworm species produce effective molecules acting directly on the CNS of their orthoptera hosts.

Keywords: extended phenotype, parasite–host systems, parasite manipulation, proteomics.

Introduction

How a parasite or its propagules are transmitted from one host to another is a central topic in parasite ecology. One strategy of parasite transmission that is especially intriguing is host manipulation, which occurs when a parasite enhances its own transmission by altering host behaviour. There are many impressive examples of such phenomena. For instance numerous trophically transmitted parasites have been shown to alter the behaviour of their intermediate hosts in a way that increases their vulnerability to predatory definitive hosts (see Lafferty, 1999; Berdoy *et al*., 2000; Moore, 2002; Poulin, 2002; Thomas *et al*., 2005 for recent reviews). From an evolutionary point of view, these changes are traditionally seen as compelling illustrations of the ‘extended phenotype’ concept (Dawkins, 1982), in which genes in one organism (i.e. the parasite) have phenotypic effects on another organism (i.e. the host).

Examples of behavioural manipulation by parasites are numerous but the mechanisms underlying these ethological changes are by no means well characterized (Helluy & Holmes, 1990; Thompson & Kavaliers, 1994; Beckage, 1997; Adamo, 2002; Thomas *et al*., 2002a; Helluy & Thomas, 2003; Klein, 2003). Parasites can alter host behaviour directly by interacting with the host’s central nervous system (CNS) or muscle. They can also have indirect effects on host behaviour by affecting host tissues other than neurones and muscles, resulting in host-mediated changes in behaviour. When parasitic alteration of behaviour has been examined in detail, the change in host behaviour is usually an indirect effect of the parasite, or a mix of direct and indirect effects of parasites on their hosts’ CNS (Adamo, 2002; Helluy & Thomas, 2003; Beckage & Gelman, 2004).

Proteomics promises to bridge the gap between our understanding of genome sequence and cellular behaviour; it can be viewed as a biological assay or tool for determining gene function (Biron *et al*., 2005a,b). By permitting the study of the host and the parasite in action during the manipulative process, proteomics thus a priori offers an excellent tool to explore the proximate mechanisms responsible for host manipulation and to test the ‘manipulation hypothesis’ (Webster, 2001; Biron *et al*., 2005c). Here we performed such an approach in the association between

the cricket *Nemobius syslvetris* and its manipulative parasite, the hairworm *Paragordius tricuspidatus* (Nematomorpha). From an ecological point of view, hairworms have typical parasitoid life cycles, developing in arthropods, mainly terrestrial species, until they are ready to exit the host (Schmidt-Rhaesa, 2001). Arthropod hosts become infected when they ingest parasitic larvae. During development, the larvae, microscopic at first, grow to become very large worms, whose size exceeds the length of the host by a considerable amount and ready to leave the host. Because parasite mating and early development occur in aquatic environments, adult hairworms must seek water. It has been shown that insects harbouring mature hairworms display in the first part of the night a behaviour originally not present in the host's repertoire: they seek water and jump into it (Thomas *et al*., 2002a) (see http://www.canal.ird.fr/programmes/recherches/grillons_us/index.htm). Finally, in this paper, we explored the hypothesis of molecular convergence for the manipulative process in orthoptera–hairworm systems by comparing our new results with those obtained in our previous study on the *Meconema thalassinum–Spinochordodes tellinii* system (Biron *et al*., 2005c).

Results

Heuristic classification

The differential expression of the *N. sylvetris* proteome was examined in five categories, i.e. day control (CD), night control (CN), before manipulation (BM), during manipulation (DM) and after manipulation (AM). By collecting hairworms from BM, DM and AM crickets, the responses of the *P. tricuspidatus* proteome were analysed during the manipulative process. In the paper, the asterisk is used to designate the hairworm categories, i.e. before (BM*), during (DM*) and after (AM*) manipulation. Table 1 indicates for both host and parasite the number of common protein spots, as well as the proteome distances between categories. The presence/absence data were analysed with a phenetic study by calculating a 'proteome distance'. The heuristic cluster based on proteome distances suggests for the host a dendogram with two groups, one containing the two uninfected categories (day control CD and night control CN) and the other containing the three parasitized categories (before, BM, during, DM and after, AM, manipulation) (Fig. 1a). Furthermore, among parasitized categories, crickets after the manipulation (AM) are isolated from the two other categories. For the three hairworm categories, the heuristic cluster based on proteome distances (Fig. 1b) suggests a dendogram with two groups, one containing 'worm in host' (BM* and AM*) and the other containing 'worm outside host' (AM*).

Analysis of two-dimensional gels

Figure 2 shows the differential *N. syvelstris* (Fig. 2a) and *P. trisucpidatus* (Fig. 2b) proteome expression at different

Table 1. Number of common protein spots (above diagonal) and proteome distances (below diagonal) between *N. sylvestris* categories also for *P. tricuspidatus* categories

	Host proteome reaction				
	CD	CN	BM	DM	AM
CD	–	834	811	803	791
CN	0.01	–	803	809	797
BM	0.03	0.04	–	830	814
DM	0.05	0.04	0.01	–	828
AM	0.06	0.05	0.03	0.02	–

	Parasite proteome reaction		
	BM*	DM*	AM*
BM*	–	656	650
DM*	0.06	–	625
AM*	0.08	0.07	–

Cricket categories: day control (CD), night control (CN), before (BM), during (DM) and after manipulation (AM).
Hairworms categories: before (BM*), during (DM*) and after (AM*) manipulation.

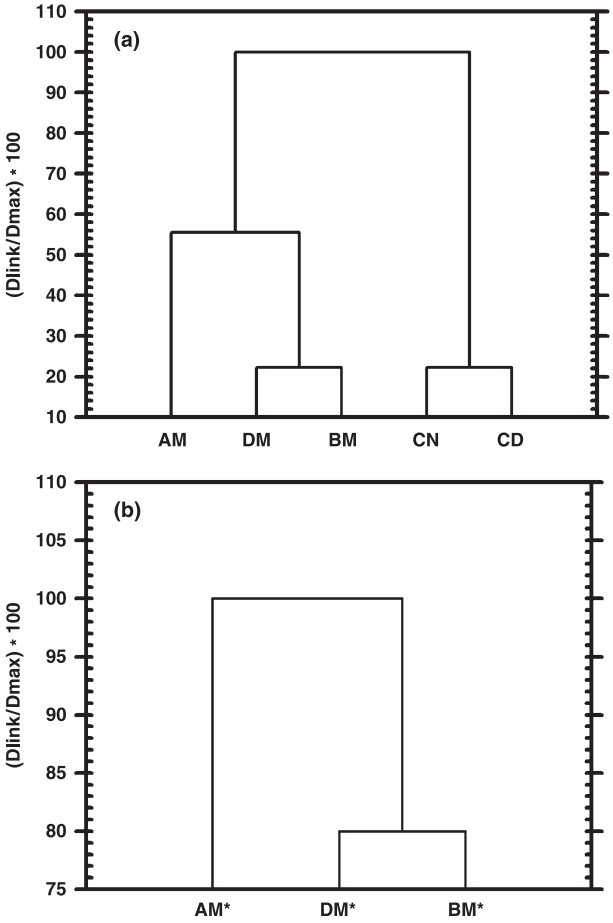

Figure 1. Classification of our 2D gels resulting from an heuristic analysis of the five cricket categories (day control (CD), night control (CN), before (BM), during (DM) and after manipulation (AM)) (a) and also on the three worm categories (before (BM*), during (DM*) and after (AM*) manipulation) (b).

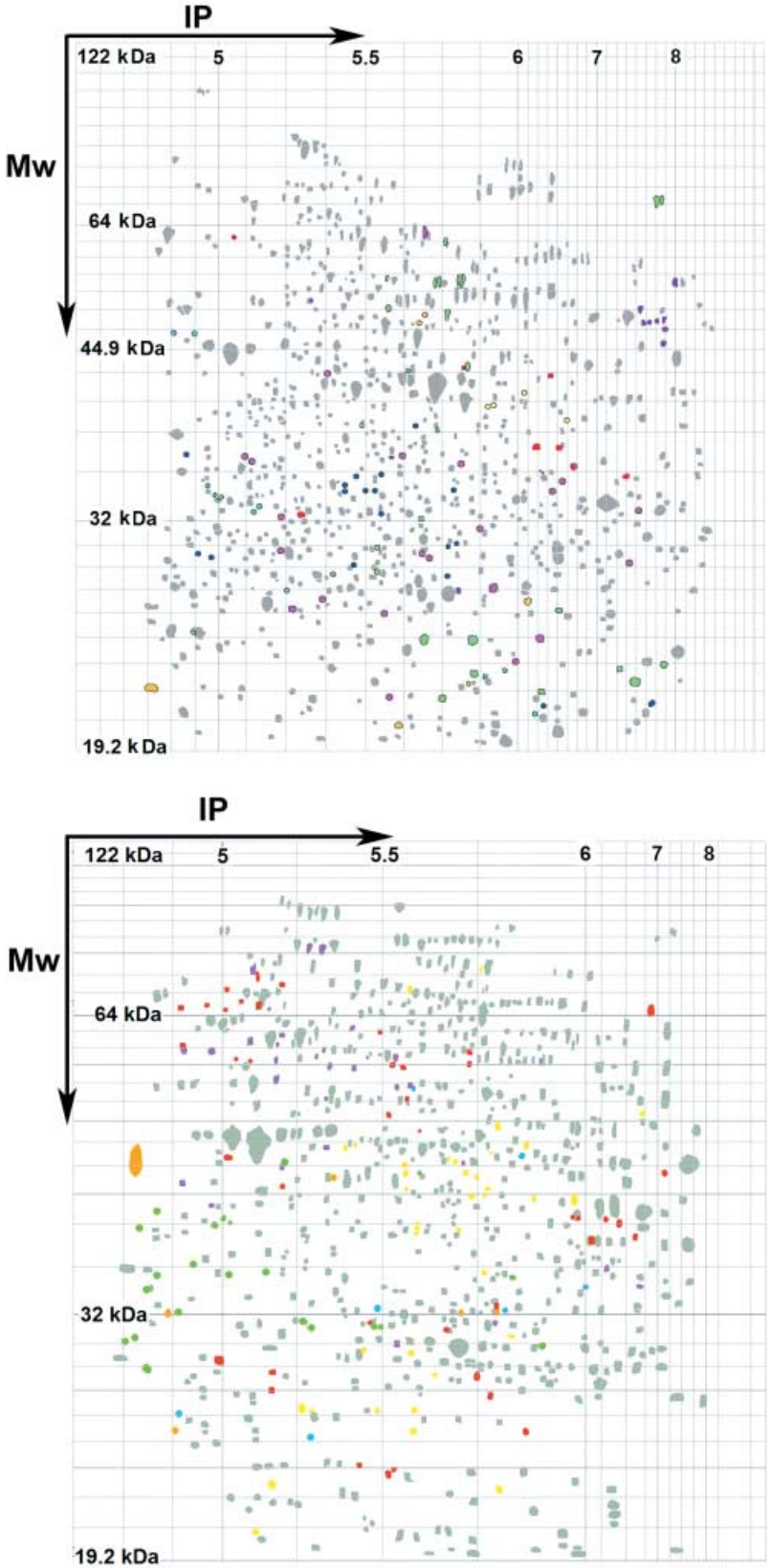

Figure 2. (a) Two-dimensional synthetic gel (pH range 5–8) showing the differential daily expression of the head *N. sylvestris* proteome during its interaction with its manipulative parasite, *P.tricuspidatus*. Common protein spots Day effect (circacdian cycle) Night effect (circacdian cycle) Non-specific stress (induction) Non-specific stress (Suppression) Manipulation effect (Induction) Emergence effect (Induction) Emergence effect (Suppression) (b) Two-dimensional synthetic gel (pH range 5–8) showing the differential daily expression of the proteome of *P. tricuspidatus* following its manipulative action on its host, *N. sylvetris*. Common protein spots Day effect (circacdian cycle) Night effect (circacdian cycle) Non-specific stress Emergence effect (Induction) Manipulation effect (Inducation) Manipulation effect (Suppression).

Table 2. Number of *N. sylvestris* and *P. tricuspidatus* protein spots induced or suppressed (not detected) during their host–parasite interaction. Number in parentheses give the percentage relative to the total number of protein spots (see Fig. 2 for the illustration of each category about the biological interpretation for the cricket and the hairworm proteome responses)

(a) Host proteome reaction

Biological interpretation		Gels where protein spots occurred					No. of protein spots
		CD	CN	BM	DM	AM	
Always expressed (common protein spots)		X	X	X	X	X	791 (87.7)
Circadian cycle	Day	X		X			8 (0.9)
	Night		X		X	X	6 (0.7)
Nonspecific stress	Induction			X	X	X	23 (2.6)
	Suppression			X	X		4 (0.4)
		X	X				31 (3.4)
Manipulation	Induction				X	X	1 (0.1)
					X		8 (0.9)
Emergence effect	Induction					X	18 (2.0)
	Suppression	X	X	X	X		12 (1.3)
Total number of specific protein spots							111 (12.3)
Total number of protein spots							902

(b) Parasite proteome reaction

Biological interpretation		Gels where protein spots occurred			No. of protein spots
		BM*	DM*	AM*	
Always expressed (common protein spots)		X	X	X	619 (81.1)
Circadian cycle	Day	X			41 (5.4)
	Night		X	X	6 (0.8)
Habitat	Inside host (parasite action)	X	X		37 (4.8)
	Outside host (free living)			X	22 (2.9)
Manipulation	Induction		X		7 (0.9)
	Suppression	X		X	31 (4.1)
Total number of specific protein spots					144 (18.9)
Total number of protein spots					763

Cricket categories: day control (CD), night control (CN), before (BM), during (DM) and after manipulation (AM), Hairworms categories: before (BM*), during (DM*) and after (AM*) manipulation.

periods of their interaction and highlights spots that are specific to a subset of categories. Seven hundred and ninety-one protein spots were common to the five cricket categories while 111 were specific to one or another category. Table 2a gives the number of cricket protein spots induced or suppressed (not detectable) in relation to the different factors characterizing the five categories of host. For instance, we considered that a protein spot was likely to be linked to the manipulative process when its presence or its absence (i.e. not detectable) was specifically observed in DM gels, or in both DM and AM gels. Among the 111 specific protein spots observed, 12.6% were related to a circadian effect, 52.3% to nonspecific stress (i.e. common to all parasitized categories), 27.0% to the parasite emergence and 8.1% (i.e. 9) to the manipulation. Six hundred and nineteen protein spots were common to the three *P. tricuspidatus* categories while 144 were specific (18.9% of the total number of protein spots) (see Table 2b). Among these specific proteins, 38 (26.3%) are expressed when the host exhibits the water seeking behaviour.

To study the variations in the expression of common protein spots among cricket and hairworm categories, we performed a PCA analysis. In all cases the first axis, F1, reflects the size (volume) of the proteins, the largest volumes having highest F1 scores and the smallest ones having smallest F1 scores. Other axes are important for separating the gels according to the factors under study, and for detecting those proteins that contribute the most to this separation. For the host, the 'protein size' axis explained 81% of the overall variability across the five categories of crickets. Interestingly, axis 4 separates manipulated crickets (DM) from other categories (see supplementary Fig. S1a). This axis is thus very important as it also permits the identification of protein spots that are over- or underexpressed during the manipulative processes, and thus potentially linked to it. For the hairworm analysis, axis 1 reflects as before the size of the protein spots. This axis also explained 81% of the overall variability across the three categories of hairworms. Axis 2 opposed the hairworms during (DM*) and after (AM*) manipulation (see supplementary Fig. S1b). Analysing the contributions of protein spots to this axis permitted the selection of protein spots characterizing, by their relative volume variation, the manipulative period and the emergence of the hairworms from their hosts. The cluster

analysis, based on Eisen method, allows the detection of semiquantitative differential protein expression during the manipulation process, and thus potentially permit the identification of other candidate protein spots (see supplementary Figs S2 (Host) and S3 (Parasite)). In supplementary Table S1, we give for both the host and the parasite the coordinates of all the candidate proteins linked to the expression of the water seeking behaviour by the host.

Identification of candidate proteins

Table 3 summarizes for the cricket and for *P. tricuspidatus* the identified protein families for which a differential expression was observed during the expression of the water-seeking behaviour by the host. Many of the identified families of protein expressed in the head of the cricket (Actins, ATPase, BIR;2, Clatrin_lg_ch, Wnt) are involved directly and/or indirectly in the development of the CNS. Also, other identified family of cricket proteins are linked to the visual system: CRAL_TRIO and PCI;1. One protein, the '994', was only expressed in the head of manipulated cricket individuals. However, it is impossible to link the '994 protein' to a known family with the PMF (peptide mass fingerprinting). This protein is unknown in all protein databases. The sequence tags obtained with the ESI-Q-TOF for the '994 protein' are given in Table 4. Also, it was impossible to link the '825 protein' (overexpression in DM gels), the '490 protein' (overexpression in DM gels) and the '84 protein' (overexpression in DM gels) to a known family. The sequence tags obtained for these proteins are given in Table 4. For the '722 protein' (overexpression in DM gels) it was possible to link these protein to the Cofilin_ADF; 1 family (see Table 4).

Concerning the hairworm proteome reaction, the PCI;1 involved in the biochemical pathways of the visual system, showed a differential expression in hairworm proteome during the alteration of host behaviour. Also, we observed that the parasite produced host-like proteins. More specifically, an overproduction of two protein fragments from the Wnt family acting directly in the development of the CNS was observed. Maldi-TOF mass spectrometry signals suggest that these two proteins are synthesized by the hairworm but are mimetic to proteins observed in the Order Insecta. For the Wnts found in the hairworm, the first responses (i.e. those with the highest scores of similarity) given by the analysis performed with PeptIdent software with restrictive search parameters as recommended for cross-species identification (see table S4 for the PMF list peaks) were those of insects not those of nematodes. In other words, these had the highest similarity scores. The '751 protein' first matched with Wg (Fragment; American grasshopper) protein (51.7 of sequence coverage). Other searches were done with Mascot and Aldente software without restriction of pI and Mw. The highest score obtained in protein databases with Mascot (score: 101; 46% of sequence coverage; see supplementary Fig. S5A) and Aldente (score 2.61; 42% of sequence coverage; pValue = 1.8e-12) confirmed the protein obtained with PeptIdent interrogations. The '532 protein' first matched with Wnt-4_Drome protein (31% of sequence coverage). As '751 protein', other searches were done with Mascot and Aldente. The highest score obtained with Mascot (Score 139; 30% of sequence coverage; see supplementary Fig. S5B) and Aldente (score: 1.97; 31% of sequence coverage; pValue = 4.3e-10) confirmed the protein obtained with PeptIdent software. The homology between the '532 protein' and the Wnt-4_Drome was confirmed by microsequences (see Table 4 and supplementary Fig. S6 for a 'Clustal alignment').

Comparison of proteomics results obtained for the two orthoptera–hairworm systems during the manipulative process

For *N. sylvestris*, 902 protein spots were detected and 566 for *M. thalassinum* (see Biron *et al.*, 2005c). Figure 3(a) gives the percentage of protein spots differentially expressed (induced or suppressed) during the manipulative process: 1.0% for *N. sylvestris* and 16.7% for *M. thalassinum*. As a first analysis of an eventual molecular convergence between the two orthoptera–hairworm systems, we studied the distribution of Ip and Mw of orthoptera candidate protein spots linked to the manipulative process. The distributions of the manipulative protein spots for the Ip are significantly different between the two orthoptera species (Kolmogorov–Smirnov test: $D = 0.0112$, $P < 0.01$; see supplementary Fig. S7a). However, distributions of the manipulative protein spots for the Mw are not significantly different between the orthoptera species (Kolmogorov–Smirnov test: $D = 0.0362$, $P > 0.100$; see supplementary Fig. S7b). Table 3(a) summarizes for both orthoptera species the identification of manipulative candidate protein spots and the Fig. 3(c) gives the altered physiological compartments.

A total of 763 protein spots were revealed for *P. tricuspidatus* and 729 for *S. tellinii* (see Biron *et al.*, 2005c). Figure 3(b) gives the percentage of protein spots differentially expressed during the manipulative process: 5% for *P. tricuspidatus* and 8.1% for *S. tellinii*. As for the orthoptera, the Ip distributions of the hairworm manipulative proteins are significantly different (Kolmogorov–Smirnov test: $D = 0.0112$, $P < 0.001$; see supplementary Fig. S7c) but the Mw distributions are not significantly different (Kolmogorov–Smirnov test: $D = 0.0362$, $P > 0.100$; supplementary Fig. S7d). Table 3(b) summarizes for both hairworms species the identification of manipulative candidate protein spots and Fig. 3(d) gives the altered physiological compartments.

Discussion

This work clearly showed that proteomics tools are sensitive enough to disentangle proteome alterations linked to factors as various as the circadian cycle, the parasitic

Table 3. Proteins secreted in the orthoptera head and hairworms showing a differential expression during the observation of abnormal behaviour of orthoptera hosts (for more details concerning the identification of proteins see supplementary tables S2 and S3 for the *N. sylvestris–P. tricuspidatus* system and Biron *et al.* (2005c) for the *M. thalassinum–S. tellinii* system)

(a) In orthoptera's head

Protein name (protein spot identity, family of proteins) in:	
Cricket (*N. sylvestris*)	Grasshopper (*M. thalassinum*)
Act2 [nsA, Actin; 1]	Act2 [mtA, Actin; 1]
ENSANGP00000013866 [nsB, Ras; 1]	Alpha-tubulin [mtB, Tubulin; 1]
Mariner transposase 1 [nsC, transposase]	CG31732-PD, isoform D [mtC; Unknown]
Clathrin ligh chain [nsD, Clathrin_lg_ch]	Hunchback protein [mtD; zf-C2H2; 6]
CG8031 protein [nsE, DUF52; 1]	Moesin/ezrin/radixin homolog 1 [mtE; Band_41; 1]
Serine/threonine protein phosphatase alpha-1 isoform [nsF, Calcineurin-like phosphoesterase]	Flotillin-2 [mtF; [Band_7; 1, Flotillin; 1]]
CG32673-PA [nsG, Ras; 1]	CG8863-PA, isoform A [mtG; DnaJ; 1]
ATP synthase beta chain, mitochondrial (Precursor) [nsH, ATPase alpha/beta]	Neural/ectodermal development factor IMP-L2 [Precursor] [mtH; Ig; 2]
CH4 protein [nsI, PCI; 1]	Wingless [Fragment] [mtI; Wnt; 1]
Pol polyprotein (fragment) [nsJ, unknown]	Synaptosome-associated protein SNAP-25-1 [mtJ; [SNAP-25; 1. SNARE; 1]]
Glutathion S-Transferase (fragment) [nsK, GST_N; 1]	Wingless [Fragment] [mtK; wnt; 1]
Inhibitor of apoptosis [nsL, BIR; 2]	Similar to Drosophila melanogaster qm [Fragment] [mtL; Ribosomal_L10e; 1]
Putative GDP-fucose protein O-fucosyltransferase 1 (precursor) [nsM, Glycosyltransferase O-Fuc]	Unknown [mtM; Unknown]
6-Phosphogluconate dehydrogenase, decarboaxylating [nsN, 6-phosphogluconate dehydrogenase]	Unknown [mtN; Unknown]
AgCP5224 (fragment) [nsO, SHMT; 1]	Unknown [mtO; Unknown]
Glycerol-3-phosphate dehydrogenase (fragment) [nsP, NAD_Gly3P_dh; 1]	Unknown [mtP; Unknown]
GH16463p [nsQ, CRAL_TRIO; 1]	Unknown [mtQ; Unknown]
CG5958 [nsR, CRAL_TRIO_C]	Unknown [mtR; Unknown]
GST (fragment) [nsS, GST_N; 1]	
Wingless [fragment] [nsT, Wnt 1]	
Wingless (Fragment) [nsU, wnt-1]	
CG759-PC (nsV, PGAM; 1]	
ORF 2 (fragment) [nsW, transposase]	
CG5896 protein [nsX, Trypsin]	
Clatrin light chain (dClc) [mtY; Clathrin_lg_ch; 1]	
Unknown [nsZ; unknown]	
Unknown [nsA1; unknown]	
Unknown [nsB1, unknown]	
Unknown [nsC1; unknown]	

(b) In hairworms

Protein name (protein spot identity, family of proteins) in:	
P. tricuspidatus (parasite of cricket)	*S. tellinii* (parasite of grasshopper
Act2 [ptA, Actin; 1]	Actin [stA, Actin; 1]
Y45F10B.9 protien [ptB, zf-C3CH4; 1]	Hypothetical protein Y49E10.23a [stB, CARD; 1]
Clone ZZD1079 mRNA sequence////[ptC, Proteasome; 1]	Hypothetical protein C47D12.6b [stC, HGTP_anticodon; 1]
Hypothetical protein T21G5.5 [ptD, KH; 1]	Heat shock protein 60 [stD, Cpn60_TCP1; 1]
C37A5.2 protein [ptE; PIR; 1]	Tyrosine 3-monooxygenase [stE, Biopterin_H; 1]
M04C9.3 protein [ptF, DUF976; 1]	Putative acetylcholine regulator unc-18 [stF, Sec1; 1]
Hypothetical protein E04A4.1 [ptG, F-box; 1]	Intermediate filament protein [Fragment] [stG, Filament; 1]
Beta-tubulin (fragment) [ptH, Tubulin; 1]	Beta-tubulin [stH, [Tubulin; 1; Tubulin_C; 1]]
Y25C1A.13 [ptI, ECH; 1]	Guanine nucleotide-binding protein alpha-16 subunit [stI, G-alpha; 1]
T06D8.8 protein [ptJ, PCI, 1]	Bestrophin 1 [stJ, Bestrophin; 1]
F36D3.1 protein [ptK, Glycoside hydrolase]	Arginine kinase [stK, ATP-gua_Ptrans; 1]
Polycomb protein mes-6 [ptL, WD40; 4]	Hypothetical protein CBG14575 [stL, Unknown]
Hypothetical protein T21G5.5 [ptM, KH_1; 1]	Heat shock protein 60 [stM, Cpn60_TCP1; 1]
Wingless (fragment) [ptN, Wnt; 1]	Wnt5A protein [Fragment] [stN, Wnt; 1]
Wnt4 protein (fragment) [ptO, Wnt; 1]	Hypothetical protein CBG08254 [Fragment] [stO, Unknown]
	DNA binding protein [Fragment] [stP, zf-C2H2; 8]
	Troponin t protein 4, isoform b [stQ, Troponin; 1]
	Probable deoxyhypusine synthase [stR, DS; 1]
	NOA36-like protein [stS, NOA36; 1]
	Wnt-4 protein [Fragment] [stT, wnt; 1]
	Hypothetical protein C54D10.10 [stU, Kunitz_BPTI; 2]
	Binding protein 2 like protein [Fragment] [stV, FKBP_C; 1]
	Hypothetical protein CBG15114 [Fragment] [stW, Unknown]
	Unknown [stX, Unknown]
	Unknown [stY, Unknown]
	Unknown [stZ, Unknown]
	Unknown [stA1, Unknown]

Table 4. Micro-sequences (Sequence Tags) obtained for some cricket and hairworm proteins expressed during the alteration of the *N. sylvestris* behaviour

Identity no. on the synthetic gels (C for cricket); H for hairworm)	Experimental pI_Mw	Theoretical pI_Mw	Sequence tags (by MALDI-TOF/TOF MS (*) ESI-Q-TOF MS/MS (**), by Protein Sequencer (***))	Spectra available in Supplementary figures	Identification (Protein name, AN in SwissProt, Pfam)	Function according protein families database from Sanger Institute
994 (C)	5.57_35530	–	b-[IL][IL].*FNGN (**) b-YA[IL]YDFDYTVQR (**)	Fig. S4A Fig. S4B	Unknown	–
825 (C)	4.74_47566	–	b-QP[IL]PVADT.*AAK (**). *APGAPGVP[IL].* or. *[IL]PVGPAGPA.* (**)	Fig. S4C Fig. S4D	Unknown	–
490 (C)	5.51_31857	–	b-XLVYIVNTPTFR (***)	–	Unknown	–
84 (C)	6.21_53354	–	b-LLFYIWEPADAK (***)	–	Unknown	–
722 (C)	5.47_22209	6.73_17153	b-LFLMSWCPDTAK (***)	–	Cofilin/actin depolymerizing factor homolog, P45594, Cofilin_ADF; 1	The ADF/cofilins are a family of actin-binding proteins expressed in all eukaryotic cells so far examined. Members of this family remodel the actin cytoskeleton. In insecta, this protein encodes a product with actin binding
532 (H)	5.98_39377	Undefined; 56485	b-GYTTQVVK (*) b-QVSSSRMK (*) b-MADFNATATLLRQK (*) b-VTRSFLDLR (***)	Fig. S4E Fig. S4F Fig. S4G –	Wnt4_Drome, P40589, Wnt; 1	involved in border cell migration See Table 3

Note: The syntax used for the description of microsequences is: b- (N → C sequence); [IL] (the amino acid is either I or L); .* (a sequence of one or more unknown amino acids).

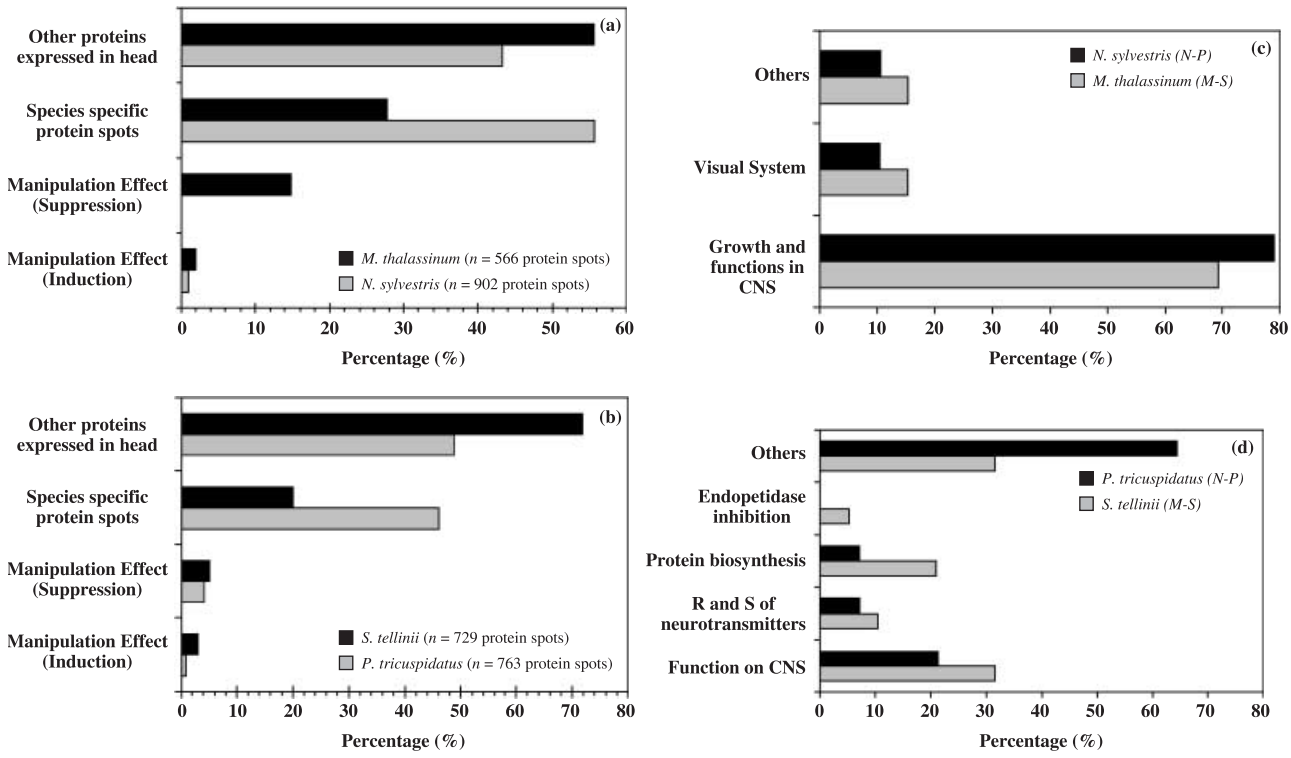

Figure 3. Percentage of orthoptera head protein spots (a) and of hairworm protein spots (b) induced (present) or suppressed (not detected) during the alteration of the host behaviour and proportion of identified proteins linked to a biological process and expressed during the manipulative process in proteomes of the orthoptera hosts (c) and of the two nematomorph parasites (d).

status, parasitic emergence and the manipulative process itself. However, what is the meaning of these differential proteome expressions? Although this study is purely correlational, interesting protein families have been shown to be present at key periods of the alteration of host behaviour. For instance, two protein families (CRAL_TRIO and PCI;1) were more highly expressed in the infected *N. sylvestris* heads with the highest values observed during alteration of the host behaviour. This result indicates an induced change in the manipulated host's visual system. In addition, the 'BIR;2' family is overexpressed in the head of parasitized crickets with the highest values again seen during manipulation. Given the role of BIR;2 in cellular apoptosis inhibition, this result suggests an inhibition of apoptosis in the brains of manipulated crickets and an increased number of cells in *N. sylvestris* brains. We can speculate that an abnormal neuronal production might interfere with normal neural circuitry and where neurogenesis takes place, the analysis of environmental cues by the cricket might be perturbed, leading to aberrant behavioural responses (Klein, 2003; Thomas *et al.*, 2003).

The proteins from the Clathrin_lg_ch family are the major coat-forming proteins that enclose vesicles such as coated pits and form cell surface patches involved in membrane traffic within eukaryotic cells. The proteins from the Clathrin_lg_ch family could suggest an increased neurotransmitter activity but also a higher absorption of macromolecules by endocytosis during the expression of the water-seeking behaviour by the host. The overexpression of the Actin family in the head of infected crickets, especially during manipulation, can be linked to the increased activity of synaptic vesicle coating but also to the overproduction of brain cells. Two proteins from the GST_N; 1 family were overexpressed during the abnormal host behaviour. A study suggests that this family of protein plays a protective role against deleterious effects of oxidative stress (Singh *et al.*, 2001). The proteins from the 'RAS; 1' family play a part in cell growth, cytokine production, vesicle-trafficking and phagocytosis. This protein family was less expressed during manipulation probably because they are heavily used in the increase of neurogenesis in the brain of infected crickets. The most fascinating result is the overexpression in the brain of infected crickets of two proteins from the Wnt family: n°736 and n°1036. More specifically, these two proteins show a higher expression during the induction of the abnormal behaviour and this higher expression is correlated with a higher synthesis in the parasite proteome of two proteins from the Wnt family: n°751 and n°532. The protein spots n°736 from the crickets and n°751 from the hairworms matched with the same Wg (Fragment; American grasshopper) protein. These proteins play an important part in the development of the CNS. In the animal kingdom, the Wnt proteins play several important parts in embryonic development and in adult function of the CNS: synaptogenesis, regulation of apoptosis, axon guidance, formation of neural crest, CNS patterning and cell migration (Patapoutian & Reichardt, 2000; Packard *et al.*, 2003; Zou, 2004). Wnt genes and Wnt proteins of *C. elegans* show the highest homology with those of arthropods. A phylogenetic analysis of the Wnt gene family confirmed the orthology relationships between the arthropods and the nematodes (Prud'homme *et al.*, 2002).

To date, Wnt genes have not been identified in Nematomorphs. However, Nematomorphs are closely related to nematodes. Thus, Wnt genes and Wnt proteins of *P. tricuspidatus* should present a high degree of sequence identity with those of nematodes. However, taking into consideration the possibility of molecular cross-talk between *N. sylvestris* and *P. tricuspidatus*, we performed protein searches within all categories of insect–parasite systems (Salzet *et al.*, 2000). Astonishingly, the nematomorph mass spectrometry results and microsequences suggested a list of Wnt matching proteins only for the order Insecta. These results suggest that the two Wnt proteins synthesized in the parasite proteome during the alteration of the host behaviour shared more similarity with the Insect Wnt proteins than those of Nematoda. Given the orthology relationships between arthropods and nematodes for Wnt proteins, it is very possible that natural selection retained and/or elaborated in the *P. tricuspidatus* genome Wnt genes producing mimetic Insect Wnt proteins capable of altering the host behaviour via the CNS. In this scenario, the mimetic proteins would be injected by the parasite into the host's CNS via the haemolymph, using proteinic transporters to pass through the brain–blood barrier, or by a direct injection by the parasite into the host's CNS. Further analyses would be needed to clarify this point. Although we favour the adaptive manipulation hypothesis we cannot exclude the hypothesis of absorption of Wnt host proteins by the hairworms.

Until now, there is little evidence that zooparasites can change host behaviour by secreting molecules that act directly on the host's CNS. Producing physiologically potent concentrations of molecules (e.g. neuromodulators) may be energetically expensive for many parasites (Adamo, 2002; Beckage, 2002; Thomas *et al.*, 2005). For this reason, it is generally argued that parasites should mainly exploit indirect and less energetically expensive methods to alter host behaviour (Adamo, 2002; Thomas *et al.*, 2005). A direct contact between hairworm species and the brain of their hosts (i.e. the worm touches the host's brain) has sometimes been observed (Hanelt, unpublished data) suggesting the possibility of a direct injection of molecules by the parasite into the host's CNS. Such a phenomenon would allow the parasite to circumvent the protective role of the blood–brain barrier of the *N. sylvestris* CNS. The proteomic results of our study indicate that the hairworm *P. tricuspidatus* may act directly on the CNS of the cricket *N. sylvestris* via the expression of mimetic proteins. Given the very large size of the adult hairworm, it is possibly not too expensive

for such a parasite to produce potent concentrations of mimetic molecules (e.g. Wnt family) acting directly on the CNS of its host to alter its behaviour (Biron *et al*., 2005c).

This proteomics study on the biochemical pathways altered by hairworm parasites has allowed us to tackle questions of physiological and molecular convergence in the mechanism(s) causing the alteration of orthoptera host behaviour. For the orthoptera heads, the qualitative proteome of *M. thalassinum* reacted strongly to the manipulative process by its hairworm with 16.7% of the total protein spots observed compared with only 1% for *N. sylvestris* (see Fig. 3a). The altered physiological compartments for the orthoptera hosts are similar, e.g. visual processes (see Fig. 3c). For the hairworms, 5% of the *P. tricuspidatus* qualitative proteome is linked to the manipulative process compared with 8.1% for *S. tellinii* (see Fig. 3b). The altered physiological compartments are similar for both nematomorph species except for some families of proteins implied in endopeptidase inhibition only expressed in *S. tellinii* (Fig. 3d). Interestingly, our proteomics results suggest that *P. tricuspidatus* inhibits apoptosis in the *N. sylvestris* CNS while *S. tellinii* apparently induces apoptosis in the *M. thalassinum* CNS. Thus, these two different parasite strategies may potentially disrupt CNS functions (Klein, 2003; James & Green, 2004).

In summary, some of the identified proteins in the heads of the two species of infected orthoptera indicate a perturbation of their neurogenesis: an increase of neurogenesis in *N. sylvestris* (cricket) and a decrease of neurogenesis in *M. thalassinum* (grasshopper). Other identified proteins in the heads of the two orthoptera species indicate a modification of their visual processes causing a perturbation of their circadian rhythm. These proteomic changes in the host's head can result from a direct and/or an indirect action by the parasite on the host genome. However, the main results of this proteomic study indicate that the adult hairworms produce host mimetic proteins having a known function on the development of a CNS. Moreover, during the nocturnal manipulation phase, Wnt proteins are overexpressed in the *N. sylvestris* and *M. thalassinum* heads, this differential Wnt protein expression can be linked to a contribution of the mimetic Wnt protein synthesized by the hairworm. The mimetic Wnt proteins suggest a direct action of the hairworms on the host's CNS that can lead directly to an alteration of the host behaviour or indirectly via a host genome response. It will be necessary to confirm that these proteins are the manipulating agents by isolating and injecting them into the orthoptera host CNS. The final step will be to identify the mechanisms by which the hairworms secreted the mimetic proteins into the host's CNS, to make polymerase chain reaction primers and protein chips from these proteomics results (for instance with the protein sequence tags, see Table 3), in order to confirm the expression of these molecules in other orthoptera–hairworm systems and in other arthropod–hairworm systems. These studies will also open the way to find new families of proteins of medical interest more specifically in neurobiology (Kavaliers *et al*., 1999; Klein, 2003).

Experimental procedures

Sampling

N. sylvestris crickets infected by *P. tricuspidatus* were captured nocturnally (22.00–01.00 h) in June and July 2002 around a swimming pool (15 m × 10 m) in Avènes les Bains (southern France, 70 km north of Montpellier) (Thomas *et al*., 2002a). Between this swimming pool and the forest, a concrete area 5 m wide allowed the direct observation and capture of arriving infected crickets (Thomas *et al*., 2002a). To avoid the possible effects of multiple infection, or host and/or parasite sex-specific factors on the proteomics expressions (Thomas *et al*., 2002b), only male crickets infected with only one adult male hairworm were used for the proteomics analysis. We also captured uninfected individuals in the forest around the swimming pool. Our sampling procedure distinguished five categories of crickets (all were nymphs). The first category corresponded to manipulated crickets (DM 'during manipulation'), i.e. infected individuals captured between 22.00 h and 01.00 h near the edge of the swimming pool just before they jumped into water (Thomas *et al*., 2002a). As a control for this category, we also collected uninfected crickets at night in the nearby forest; we called this category CN crickets (night control). Third, in order to obtain crickets harbouring a mature worm without being manipulated, we captured manipulated crickets (i.e. 'DM' category) and kept them until the day after in a terrarium containing wood and leaves from their natural habitat. We dissected these crickets between 13.00 and 15.00 h, i.e. at a period of the day for which no behavioural change is observed in natural conditions (at least for *N. sylvestris*, F. Thomas, unpublished data). As the behavioural change recurs every night, we called this third category 'BM' crickets (before manipulation). As a control for this category, we also collected uninfected crickets and dissected them the following day (13.00–15.00 h); we called this fourth category 'CD' crickets (day control). Finally, we considered crickets that have released their worm. Arriving infected insects were visually tracked until they entered the swimming pool itself. After worm emergence, the cricket was placed in a dry opaque plastic tumbler for 1 h. After this delay, most crickets were vigorous and were dissected. We called this fifth category 'AM' crickets (i.e. after manipulation). Individuals of each category were placed individually in a microcentrifuge tube of 1.5 ml and stored at −80 °C until analysis. By collecting hairworms from BM, DM and AM crickets, we also obtained hairworms during the manipulative process. The asterisk is used to designate the hairworm categories, i.e. before (BM*), during (DM*) and after (AM*) manipulation. Hairworms from cricket categories BM and DM were recuperated by dissecting the crickets' abdomen on a sterile ice bath. All worms were stored at −80 °C. The hairworms of the AM* category were placed after their emergence in a glass of water for 1 h before being stored at −80 °C.

Two-dimensional electrophoresis

For each *N. sylvestris* category, 15 heads were cut into fine pieces on a sterile ice bath. Also, for each *P. tricuspidatus* category, five individuals were cut into fine pieces on a sterile ice bath. All samples were put in microcentrifuge tubes of 1.5 ml at −80 °C. Water-soluble

proteins were extracted as detailed by Biron *et al.* (2005c,d). The concentration of each protein sample was estimated according to Bradford (1976) and standardized at 2 μg/μl by the addition of the required volume of homogenizing solution. Two-dimensional electrophoresis (2-DE) were done as detailed by Biron *et al.* (2005c,d). At least five IPG strips (Immobiline DryStrip gels; Bio-Rad, Hercules, CA, USA) of pH 5–8 were run per treatment. Gels were stained using the tetrathionate-silver nitrate technique of Oakley *et al.* (1980).

The computer analyses were done according Biron *et al.* (2005c). To visualize the global effects of each treatment on the expression of the *N. sylvestris* and *P. tricuspidatus* proteomes, we used an heuristic clustering analysis that allowed classification of gels into two or more groups, along with determination of the characteristic protein spots of each group, i.e. proteins that were differentially expressed (Appel *et al.*, 1988; Biron *et al.*, 2005c). As it is difficult to homologize loci among populations and/or species using 2-DE, so the generally employed genetic distance methods could not be employed. Instead, we used the Nei & Li coefficient (1979) for the heuristic classification. Finally, our methodological approach to identify candidate proteins implied both qualitative (presence/absence) and semiquantitative (heuristic analysis (Appel *et al.*, 1988), principal components analysis (Pun *et al.*, 1988), adaptation of Eisen method (Eisen *et al.*, 1998; Caraux & Pinloche, 2005) analyses of common protein spots between the five cricket categories (BM, DM, AM, CD and CN) and also between the three hairworm categories (BM*, DM*, AM*).

Protein identification by mass spectrometry and by microsequences for N. sylvestris *and* P. btricuspidatus

New gels with the candidate protein spots were silver stained (Shevchenko *et al.*, 1996). Peptide digestion and MALDI-TOF analysis followed Biron *et al.* (2005c), the MALDI-TOF/TOF MS and Sequence Tag (ESI-Q-TOF MS/MS) followed Bécamel *et al.* (2002) and the Micro-sequences followed Tastet *et al.* (2001).

MALDI-TOF mass spectrometry (PMF)

Digest products were completely dehydrated in a vacuum centrifuge and resuspended in 10 μl formic acid (2% v/v), desalted using Zip Tips C18 (Millipore, Bedford, MA), eluted with 10 μl acetonitrile/trifluoroacetic acid (60 : 0.1%) and concentrated to a 2 μl volume. 0.3 μl of analyte solutions were mixed with the same volume of α-cyano-4-hydroxy-*trans*-cinnamic acid (saturated solution is prepared in acetronile/trifluoroacetic acid, 50 : 0.1%, vortexed, sonicated 30 s and microcentrifuged 30 s then a 1/3 dilution of the supernatant is used as the matrix). The mixture is deposited on a 384-well MALDI target using the dry-droplet procedure (Karas & Hillenkamp, 1988) then air dried at room temperature. Analysis was performed using an UltraFlex MALDI-TOF mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany) in a reflectron mode with an accelerating voltage of 20 kV and a delayed extraction of 70 ns. Mass spectra were acquired in an automatic mode using the AutoXecuteTM module of FlexcontrolTM (Bruker-Franzen Analytik).

Spectra were analysed using the FlexAnalysis TM software (Bruker-Franzen Analytik) and calibrated internally with the autoproteolysis peptides of trypsin (m/z 842.51, 1045.56, 2211.10). Peptides were selected in the mass range of 800–4000 Da. Identification of proteins was performed using Mascot, PeptIdent, Aldente ProteinProspector softwares, available online, respectively. A mass deviation of 100 p.p.m. was allowed for database interrogation. Coverage of the full-length protein exceeding 15% and a significant score ($P \leq 0.05$) was considered to be sufficient unless there were some obvious conflicts between the experimental molecular weight or isoelectric point and those of the identified protein (Garin *et al.*, 2001). Matching peptides with missed cleavages were considered as pertinent only when there were two consecutives basic residues or when arginine and lysine residues were followed acidic residues inside the peptide amino acid sequence.

MALDI-TOF/TOF MS (tandem mass spectrometry)

MS/MS spectra were acquired on the same mass spectrometer in a LIFTTM mode, using a 0.5–1% range for the ion mass selector window. Each spectra were recorded manually in a single run, setting laser power and detector gain so that the parent ion fragmentation generated a satisfying number of different fragment ions. Automatic calibration was performed in FlexcontrolTM (Bruker-Franzen Analytik) for the parent ion in the first part of acquisition of MS/MS data then in FlexAnalysis TM software (Bruker-Franzen Analytik) for full spectrum (based on the fragmentation of known peptides), before being analysed using Mascot and ProteinProspector softwares (a mass deviation of 100 p.p.m. was allowed for database interrogation for parent mass and 0.5 Da for fragment ions).

Esi-q-tof ms/ms

Nanoelectrospray mass spectrometry was performed offline on a quadrupole time-of-flight (Q-TOF) mass spectrometer (QSTAR Pulsar-i, Applied Biosystems, Foster City, CA) fitted with a Protana nanospray inlet system (Protana, Odense, Denmark). Spectra were recorded using the Analyst QS software (Applied Biosystems). Parameters were adjusted as follows: ion spray voltage (IS), 900 V; curtain gas (CUR), 25; declustering potential (DP), 45–75 V; focusing potential (FP), 265 V; declustering potential 2 (DP2), 15 V. Peptides fragmentation was performed in the collision cell using nitrogen gas on the doubly or triply charged ions detected, with a collision energy profile optimized individually (30–55 V) and MS/MS spectra were manually interpreted. Before being placed in the source tip holder, capillaries (Protana) were loaded with the samples according to the following procedure: each aliquot from trypsin cleavage was solubilized in 5 μl of 1% formic acid, desalted on Poros 20 R2 (Applied Biosystems) packed in a gel-loader pipette tip and eluted with 1.5 μl 50 : 50 : 1 methanol/water/formic acid. Identification of proteins was performed using Mascot, PeptIdent, Proteinprospector and Aldente softwares, available online, respectively (Wilm & Mann, 1996).

Determination of amino acid sequences

For amino acid sequencing, four 2-DE gels were run and stained with Coomassie Brilliant Blue R-250 (CBB R-250). Three hundred micrograms of soluble proteins were loaded on each gel. After migration, the 2-DE gels were soaked twice in 450 ml of CH_3OH/CH_3COOH/deionized water solution (50 : 10 : 40, v/v/v) for 30 min. The fixing solution was then replaced with 450 ml of staining solution containing 0.003% w/v CBB R-250 in CH_3OH/CH_3COOH/deionized water (45 : 10 : 45, v/v/v). When the staining was sufficient to locate the proteins of interest, the 2-DE gels were washed 10 times with deionized water, in order to remove CH_3OH, CH_3COOH and excess of dye.

The protein spots excised from the CBB R-250 stained gels were rehydrated in 150–200 μl of 0,05 M Tris–HCl, pH 8.6, 0.01% Tween 20 (Pierce, Rockford, IL, USA) and digested for 18 h at 35 °C, with modified Trypsine (Promega Corporation, Madison, WI, USA) at a final concentration of 1 μg/ml. The supernatant was recovered

and the pellet was rinsed with 60% C_2H_3N. The C_2H_3N rinse was added to the supernatant and C_2H_3N was removed in a Speed-Vac. The sample was injected into a DEAE-HPLC column linked to a C18 reverse phase HPLC column and eluted with a 2–70% C_2H_3N, O.1% TFA gradient (Kawazaki & Suzuki, 1990). Peaks recorded at 214 nm were collected manually and frozen (–20 °C) until sequencing. Sequencing was performed on Applied Biosystems 494 Protein sequencer. Protein identification by search of sequence homologies were performed with MS-PATTERN (http://prospector.ucsf.edu/ucsfhtml4.0/mspattern.htm) and BLAST (http://www.ncbi.nlm.nih.gov/BLAST) (BLASTP, PSI- and PHI-BLAST) softwares. We used the NCBI, SwissProt and TrEMBL protein databases.

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