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International Journal for Parasitology 35 (2005) 1385–1397
www.elsevier.com/locate/ijpara

Proteome of *Aedes aegypti* larvae in response to infection by the intracellular parasite *Vavraia culicis*

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Received 17 February 2005; received in revised form 3 May 2005; accepted 15 May 2005

Abstract

We report on the modification of the *Aedes aegypti* larval proteome following infection by the microsporidian parasite *Vavraia culicis*. Mosquito larvae were sampled at 5 and 15 days of age to compare the effects of infection when the parasite was in two different developmental stages. Modifications of the host proteome due to the stress of infection were distinguished from those of a more general nature by treatments involving hypoxia. We found that the major reaction to stress was the suppression of particular protein spots. Older (15 days) larvae reacted more strongly to infection by *V. culicis* (46% of the total number of spots affected; 17% for 5 days larvae), while the strongest reaction of younger (5 days) larvae was to hypoxia for pH range 5–8 and to combined effects of infection and hypoxia for pH range 3–6. MALDI-TOF results indicate that proteins induced or suppressed by infection are involved directly or indirectly in defense against microorganisms. Finally, our MALDI-TOF results suggest that *A. aegypti* larvae try to control or clear *V. culicis* infection and also that *V. culicis* probably impairs the immune defense of this host via arginases-NOS competition.

Keywords: Proteomics; Proteome; *Aedes aegypti*; Microsporidia; *Vavraia culicis*; Nitric oxide synthase

1. Introduction

Insects must resist attack from various pathogens of a microbial or eukaryotic origin. Their defences start with physical barriers to prevent infectious agents from gaining entry into the body cavity. These include the thick waxy cuticles of exoskeleton and the peritrophic matrix lining the digestive tract. Invaders managing to breach these defenses often arrive in the host's hemocoel where they are exposed to both humoral and cellular components of the host's innate immune system. These include a wealth of molecules involved in recognizing and signaling the presence of foreign or non-self material. These responses set in motion cellular defense mechanisms, including phagocytosis and encaspulation, as well as causing cells, such as those in the fat body, to rapidly produce and release a battery of anti-microbial and anti-fungal peptides into the hemocoel. Additional responses include those to protect the host, for example, by repairing damaged tissues or buffering against free radicals produced during intensive bouts of metabolic activity.

Much of our knowledge on the immune system of invertebrates has been derived from studies involving the genetics of *Drosophila melanogaster*. This has been through its comparison with other organisms or by experimental approaches making use of molecular and genetic tools developed using this species as a model organism. However, data from other organisms are making an increasing contribution to this field. Mosquitoes in particular feature in this diversification following the sequencing of the *Anopheles gambiae* genome (Holt et al., 2002). For example, diversity in the recognition and effector molecules of the *Drosophila* and *Anopheles* immune systems is indicative of adaptive evolutionary change associated with their divergent ecologies and contrasting exposure to

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doi:10.1016/j.ijpara.2005.05.015

pathogens (Zdobnov et al., 2002). Furthermore, direct experimental evidence of how mosquito genes function in response to pathogens is also becoming available with the application of techniques such as double-stranded RNA knockdown (Blandin et al., 2004).

Despite these advances in the genetic domain relatively less is known about the mechanisms initiating these responses, the proteins or molecules directly affecting parasite development, or how parasites evade or try to manipulate them. Protein-based approaches promise to make a useful contribution in this area (Engström et al., 2004; Biron et al., 2005a). Recent improvements in the techniques of 2-DE (two-dimensional gel electrophoresis) and mass spectrometry, in combination with accumulating database resources, have made it possible to characterize the entire protein complement (or proteome) expressed by individual cells, tissues, or whole organisms. These techniques have already been applied to *D. melanogaster* in characterizing how the profile of hemolymph proteins changes within the minutes or hours following wounding, challenge by immune-stimulatory molecules, or infection by microorganisms (e.g. Levy et al., 2004; Vierstraete et al., 2004a,b). In these studies, researchers have been able to identify increases or decreases in the presence of particular proteins and in many cases have been able to assign them a functional role, e.g. as antimicrobials or responding to oxidative stress.

In this study we investigated the proteome of the yellow fever mosquito *Aedes aegypti* (L.) in response to infection by the microsporidian parasite *Vavraia culicis* Weiser. The microsporidia are amitochondrial eukaryotes currently thought to be closely related to the fungi, if not directly descended from them (Keeling et al., 2000). Their host range spans most of the animal kingdom, including man, but they have mainly been described from invertebrate hosts (Becnel and Andreadis, 1999). In particular, they are among the most common pathogens infecting natural populations of mosquitoes (Castillo, 1980). *Vavraia culicis* itself has been reported from natural populations of *Aedes*, *Anopheles* and *Culex* mosquitoes (Weiser, 1980), and has been subject to investigation in a variety of laboratory studies (Bano, 1958; Reynolds, 1970; Kelly et al., 1981; Agnew et al., 1999, 2004; Bedhomme et al., 2004). Host larvae become infected when they ingest the parasites' spores from their aquatic environment. Germination causes a tube coiled within the spore to be everted with force. It is thought this tube crosses the peritrophic matrix and penetrates epithelial cells lining the digestive tract. Contents of the spore pass down the tube and directly into the host cell's cytoplasm, from which point the parasite's development can begin. New spores are formed approximately 8–10 days p.i. They can germinate in situ and directly infect other host cells. The accumulation of spores within a cell may also cause its rupture, in which case spores can be liberated back into the intestine or into the host's hemocoel from where other cells or tissues can be infected, e.g. the fat body. Infections can either be chronic, increasing host morbidity, or cause host mortality, depending on the particular conditions involved (Agnew et al., 2004; Bedhomme et al., 2004). Little is known about the immune responses invertebrate hosts deploy against microsporidian infections. Melanized spores are occasionally reported in the hemocoel of insects with mature infections (Weiser, 1976). However, such spores must have been produced in the host concerned, thus the infection must already have been established and the parasite able to develop through to spore production.

Our study differs from the proteomic studies cited above in two important aspects. First, unlike many of the pathogens used to date, the development of the microsporidia is obligatorily intracellular; they thus pose a different type of challenge for the host immune response as compared to pathogens circulating in the hemocoel. Second, we investigated changes in the host's proteome at 5 and 15 days p.i. These intervals are much longer than those used above and correspond with different stages of infection where the parasite's development is predominantly directed towards proliferation (5 days) or spore production (15 days). Both developmental stages are likely to have different metabolic requirements and impose different stresses upon the host (Agnew et al., 2003).

We were particularly interested in changes of the host proteome that were specifically in response to *V. culicis* infection, as opposed to those in response to stress per se. To help distinguish between these possibilities, infected and uninfected larvae were subjected to hypoxia as a source of general stress by denying them access to atmospheric oxygen in the 5 h prior to being sampled. We analyzed how these treatment combinations quantitatively affected the numbers of protein spots expressed by the host proteome. We subsequently focused on those showing a qualitatively different pattern of expression, being either specifically induced or suppressed in association with infection.

2. Materials and methods

2.1. Host and parasite material

The strain of *A. aegypti* used in this study was collected by colleagues at the Pasteur Institute in Dakar, Senegal and had been maintained in the laboratory for at least 10 generations in outbred conditions (>500 breeding females) in an insect room maintained at 25 (±3)°C, >50% relative humidity and a 12 h:12 h light:dark photoperiod. The isolate of *V. culicis* used was provided by Dr J.J. Becnel of the USDA Gainesville, USA and originally isolated from *Aedes albopictus* (Fukuda et al., 1997).

2.2. Experimental protocol

Our experiment involved four treatment conditions that combined the stressful effects of infection and hypoxia.

These treatments were: C (uninfected); I (infected); H (uninfected and hypoxia) and I+H (infection and hypoxia).

Mosquito eggs were simultaneously hatched under reduced atmospheric pressure and within 8 h of hatching, 16 groups of 60 *A. aegypti* larvae were transferred to Petri dishes (diameter 55 mm) containing 10 ml of de-ionized water and 0.06 mg/individual of fish food (Tetramin MicroFood). Four Petri dishes were randomly assigned to each of the four treatments. Larvae of the I and I+H treatments were exposed to 20,000 *V. culicis* spores/larva. Following Agnew et al. (2001), after 24 h, larvae of the four treatments were rinsed in de-ionized water and transferred to their own individual *Drosophila* vial (20×90 mm) containing 5 ml of de-ionized water and 0.8 mg of fish food; with this amount of food mosquitoes remain in the larval stage for at least 15 days (unpublished data).

Larvae were sampled at two different intervals, either 5 or 15 days. In the H and the I+H treatments, a layer of vegetable oil (1.5 ml) was added to tubes 5 h before sampling the larvae. This prevented them having access to atmospheric oxygen during this period without causing their death. Following sampling larvae were stored at −20 °C until further use.

2.3. Two-dimensional electrophoresis (2-DE)

For each treatment, two groups of 15 larvae were prepared from the 5 and 15 day sampling periods by dissecting them at the third abdominal segment on an ice bath under sterilized conditions. The anterior section, from head to third abdominal segment, was used for the 2-DE analysis; this contained fat body tissue which is known to produce a battery of protective proteins against invading organisms (Fehlbaum et al., 1994). Samples were transferred to 1.5 ml micro-centrifuge tubes and stored at −20 °C. For the two treatments involving infection (I and I+H), the posterior section (third abdominal segment to respiratory siphon) of 15 days larvae were checked for *V. culicis* spores. Only those larvae confirmed as being infected by the presence of spores were retained for further analysis (55.0 and 51.7% of larvae from the I and I+H treatments, respectively). This procedure could not be used for larvae sampled at 5 days as it is before spore production has begun. Consequently, all larvae exposed to infection and sampled at 5 days were retained for analyses.

Water-soluble proteins were recovered according to the method of Biron et al. (2005b). The concentration of each protein sample was estimated by measuring the shift of extinction of Coomasie Blue G-250 at 595 nm (Bradford, 1976) and standardized at 2 μg/μl by the addition of the required volume of the homogenizing solution. Protein samples were stored at −70 °C until electrophoresis. The two dimensional gels were done following Biron et al. (2005b). At least four IPG strips of pH 5–8 were run for treatment. Gels were stained using tetrathionate–silver nitrate (Oakley et al., 1980; Rabilloud et al., 1994).

2.4. Computer analyses

The replicated 2D gels of each treatment were compared using the MELANIE software (GeneBio, Geneva, Switzerland). The best 2D gels obtained for each treatment at 5 and 15 days were used to build a 2D synthetic gel for each treatment and sample date. The selection criteria of the 2D gels were the absence of significant distortion, a low background and good staining. For each treatment (C (uninfected); I (infected); H (uninfected and hypoxia) and I+H (infection and hypoxia)) common protein spots observed on all least three replica gels of the four were retained to build 2D synthetic gels. The isoelectric point (p*I*) and molecular weight (M_w) scales of the 2D gels were determined with a protein standards kit from Bio-Rad (2D SDS-PAGE Standards).

2.5. Identification of induced or suppressed proteins of infected larvae

The basic information that can be obtained from a 2D gel is the approximate pI and Mw (molecular mass in Da or kDa). These data can then be used by search software such as TAGIDENT (http://www.expasy.org/tools/tagident.html) to generate a list of proteins whose molecular mass and p*I* are close to the given values (Barret et al., 2005).

The main selection criteria of protein spots selected for Peptide Mass Fingerprints (PMF) analyses in our study was based on a search done with TAGIDENT for induced and suppressed proteins in SWISS-PROT and TREMBL protein databases. Parameters were set, as recommended on EXPASY Proteomics server (http://www.expasy.org/), for delta-p*I* (±2.0) and for delta-M_w (20%).

Once initial analyses suggested protein spots of interest, new gels were run and silver stained following Shevchenko et al. (1996). Candidate proteins were identified and peptide digestion and MALDI-TOF analysis were done following Biron et al. (2005b). Protein identification was obtained by conducting a database search of the peptide masses generated from MALDI analysis. Identification of proteins was performed using PEPTIDENT (http://www.expasy.org/tools/PepIdent.html), PROTEIN PROSPECTOR MS-FIT (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm) and ALDENTE (http://www.expasy.org/tools/aldente/) software. Monoisotopic peak lists were imported into PEPTIDENT, PROTEIN PROSPECTOR MS-Fit and ALDENTE software with the following search parameters: INSECTA, OTHER INSECTA and All ENTRIES in the species field, p*I* ±2.0, M_w ±30%, one missed cleavage, tryptic digestion, carbamidomethylation as a cysteine modification and oxidation of methionine. For the species fields, we did a parsimony search by taking into consideration the possible molecular cross-talk during host-parasite interactions (Salzet et al., 2000). Search tolerance was set at 100 ppm with a MH^+ charge state. Proteins with the highest score, the higher significant 'Probability value' ($P<0.05$,

i.e. the probability that the observed match is a random event), a minimum of missed cleavages, a minimum of Delta ppm between the molecular mass of the experimental peptides and the corresponding theoretical peptides, a theoretical pI/M_w close to the experimental pI/M_w and more than 20% coverage were retained (Wilkins and Williams, 1997; Mathesius et al., 2002; Habermann et al., 2004; Ostrowski et al., 2004; Barret et al., 2005). Matching peptides with missed cleavages were considered as relevant only when there were two consecutive basic residues or when arginine and lysine residues were followed by a proline or acidic residues inside the peptide amino acid sequence (Garin et al., 2001; Bécamel et al., 2002).

3. Results

3.1. Proteome expression of A. aegypti larvae following aggression by V. culicis and/or by temporary hypoxia

The total number of spots observed for each treatment, pH and larval range is reported in Table 1. For our four treatment conditions, we can classify different protein spots in 15 categories according to the treatment(s) in which each spot was observed. These categories can be grouped based on their biological interpretation: protein spots not present in the control gels but present in another treatment were induced by the treatment; those present in the control but absent in another treatment were suppressed (break-even point of detection) by the treatment; those present under all conditions or always expressed (Table 1). Spots can be further classified according to whether their expression was modified by a specific stress, alone or in combination with the other: for example, the expression of spots in categories 2, 6, 9, and 13 was either induced or suppressed by infection. Fig. 1 shows the proteome response of *A. aegypti* larvae to infection by *V. culicis* at pH 3–6 and 5–8 for 5 day larvae, and at pH 5–8 for 15 day larvae, respectively.

Fig. 2 gives the percentage of spots whose expression was affected by each treatment relative to the total number of spots whose expression was affected by any treatment for each combination of age and pH range. Results suggest that 15 day larvae reacted most strongly to infection with 46% of the total number of spots affected compared with 17% for the 5 day larvae. Hypoxia had the major impact on 5 day larvae with 34% of the number of spots affected. These observations were supported by a heuristic cluster analysis (Appel et al., 1988; data not shown).

3.2. Temporal expression of the A. aegypti larval proteome

Comparisons of the larval proteome at 5 and 15 days were only possible for the pH range 5–8. Expression of the host larval proteome was different for these two ages. Fig. 3A gives the number of common protein spots,

Table 1
Number of protein spots induced, suppressed and always expressed for each treatment at 5 and 15 days

Interpretation	Category	Treatment where spot occurred				5 day larvae		15 day larvae
						pH range 3–6	pH range 5–8	pH range 5–8
		C	H	I	I+H	Number of spots	Number of spots	Number of spots
Induced by hypoxia only	1		X			9	1	16
Induced by infection only	2			X		2	12	21
Induced by both only	3				X	8	4	5
Induced by single stresses only	4		X	X		0	1	1
Induced by hypoxia	5		X		X	1	3	5
Induced by infection	6			X	X	2	0	4
Induced by any stress	7		X	X	X	5	0	4
Total induced (%)						27 (7.4)	21 (5.0)	56 (6.3)
Suppressed by stress	8	X				3	18	31
Suppressed by infection	9	X	X			19	30	56
Suppressed by hypoxia	10	X		X		9	92	7
Suppressed by single stresses	11	X			X	6	2	5
Suppressed by combined stresses	12	X	X	X		78	90	48
Suppressed by infection only	13	X	X		X	6	3	26
Suppressed by hypoxia only	14	X		X	X	16	17	6
Total suppressed (%)						137(37.4)	252 (60.1)	179 (20.0)
Always expressed	15	X	X	X	X	202	146	659
Spots present in control (%)		X				339 (92.6)	398 (95.0)	838 (93.7)
Total number of spots						366	419	894

Numbers in parentheses give the percentage relative to the total number of spots. C: control, H: hypoxia, I: infection by *Vavraia culicis*, I+H: infection and hypoxia. The category 'spots present in control' is the sum of categories 8–15. (Total=protein spots present in Control plus Induced.)

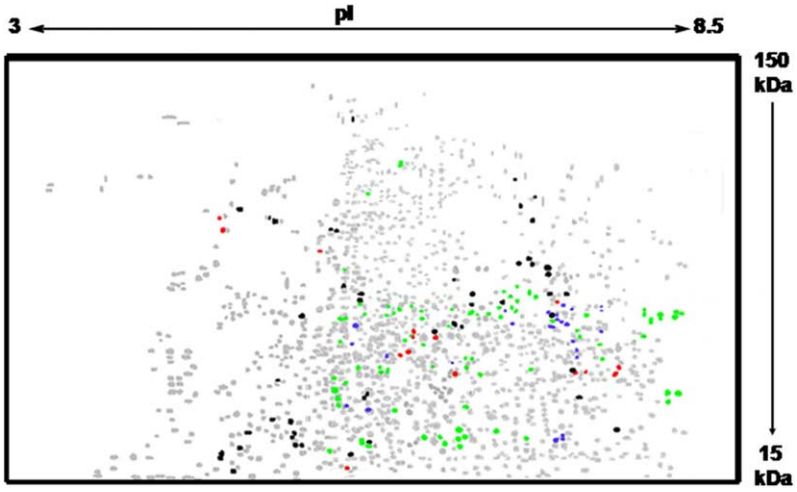

Fig. 1. Two-dimensional synthetic gel image of *Aedes aegypti* larvae showing proteome response to *Vavraia. culicis*: 5-day-old larvae [induced by infection (Inf; Inf + HI): ●, Suppressed by infection: ●]; 15 days old larvae [Induced by infection (Inf; Inf + HI): ●, Suppressed by infection: ●]; Other proteins for 5 and 15 days old larvae: ●.

i.e. observed both at 5 and 15 days, and temporally specific protein spots, i.e. observed in only one of the two ages, for each treatment.

The consideration of the temporal dimension allows us to categorize protein spots differently: spots can be specific to a given treatment and age, i.e. they are only observed at a given age under specific conditions (see Fig. 3B). Interestingly, some protein spots can be specific to a given treatment at one age, and shared with other treatments of a different age (see Fig. 3B). This situation may arise when a treatment accelerates or suppresses the accumulation of the protein(s) corresponding to the spot. For example, infection increases the accumulation of two spots (specific at 5 days, shared at 15 days) and suppresses the accumulation of two other spots (shared at 5 days and specific at 15 days). Finally some spots are specific to a given treatment and observed only under that treatment at both time points we considered.

3.3. Identification of proteins induced and suppressed in infected larvae

Table 2 gives the putative identification of many *A. aegypti* protein spots induced or suppressed in treatments involving *V. culicis*. Many of them are of known function and are linked directly or indirectly to defense against invading microorganisms, e.g. NF-κB, Gasp [Precursor], Odorant binding proteins (OBPs), gluthatione S-transferase (GSTs), serine/threonine phosphatase, ornithine amino-transferase (OAT), ornithine decarboxylase antizyme (ODC-AZ) and nitric-oxide synthase (NOS). No induction or suppression was observed for TEP1 belonging

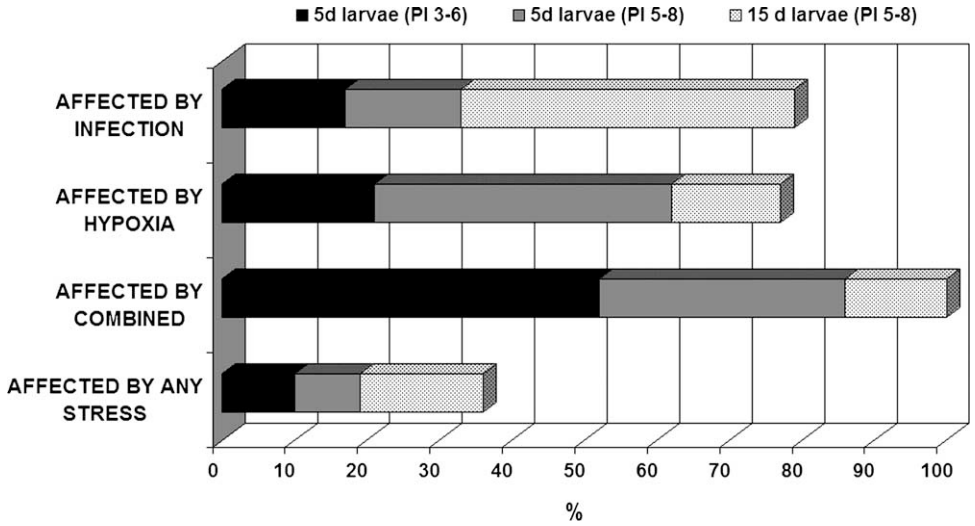

Fig. 2. Percentage of protein spots whose expression was affected by each treatment for 5 and 15 days larvae for different p*I* gel ranges.

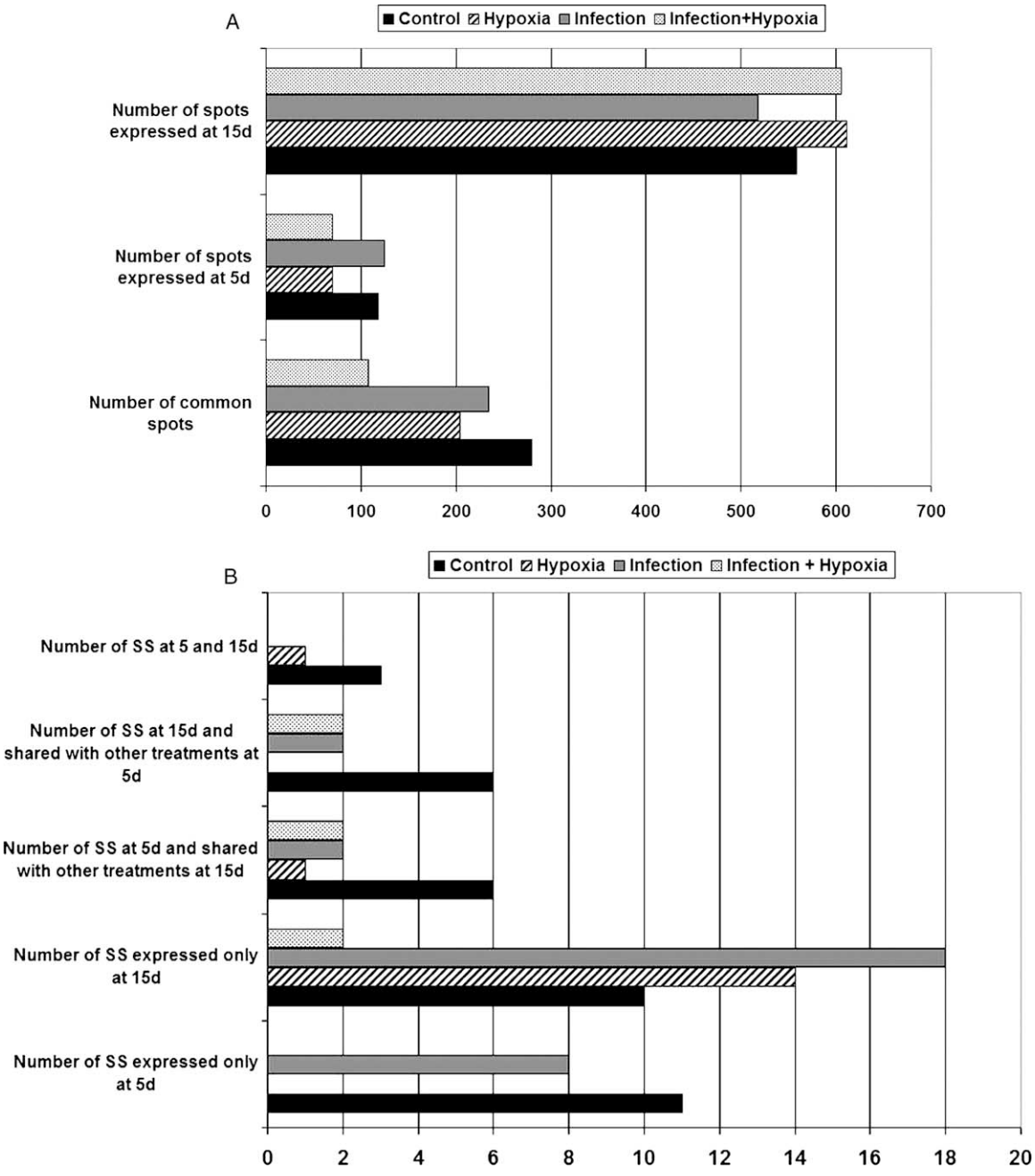

Fig. 3. (A) Number of common and temporal specific spots observed at 5 and 15 days resulting from a differential expression of larval proteome. (B) Temporal comparison between the 2D synthetic gels for each treatment (Specific Spots (SS)).

to alpha-2-macroglobulin (Accession Code: PF00207 in PFAM database (http://www.sanger.ac.uk/cgi-bin/Pfam/)), an endopeptidase inhibitor activity protein, known as a conserved immune protein in the Animal Kingdom (Blandin and Levashina, 2004). Some of the proteins identified in protein databases currently have no known function. For five digested protein spots (not mentioned in Table 2), PMF were obtained but it was impossible to identify and to link these proteins to any known family in protein databases (5 day larvae pH 3–6: (1) p*I* 4.8 ± 37.3 kDa; 5 day larvae pH 5–8: (2) p*I* 5.2 ± 31.4 kDa, (3) p*I*: 5.3 ± 32.8 kDa, (4) p*I* 7.6 ± 24.5 kDa; 15 day larvae (5) p*I* 6.1 ± 28.6 kDa).

4. Discussion

The use of 2-DE gel electrophoresis and mass spectrometry proved to be a sensitive method for detecting changes in the expression of the *A. aegypti* proteome. We found considerable differences in protein expression among treatments and between the two sampling dates considered.

Table 2
Identification of *Aedes aegypti* proteins with mass spectrometry for induced (I) and suppressed (S) proteins by *Vavraia culicis*

Protein spot	Protein name (find in SWISS-PROT (#) and TREMBL(*))	Accession number	Family of protein according to Pfam database of Sanger Institute	Treatment	(I) or (S)	p$I \pm M_w$ (Exp.; Theo.)	No. of peptides matched (sequence coverage (%))	*P* value obtained with ALDENTE software	Known function according to SWISS-PROT, TREMBL and Pfam databases
A	Ryanodine receptor 44F (fragment) (#)	Q24498	RYDR_ITPR; 2	5 day larvae pH range 3–6	S	4.6 ± 34.3; 5.4 ± 58.1	6 (19.0)	2.2×10^{-3}	Intracellular calcium channel that is required for proper muscle function during embryonic development and may be essential for excitation-contraction coupling in larval body wall muscles. The ryanodine protein is essential for larval development
B	Ornithine aminotransferase (Fragment) (#)	P49724	Aminotransferase class-III	5 day larvae pH range 3–6	S	4.6 ± 25.2; Unknown ± 47.3	7 (17.2)	2.7×10^{-4}	Aminotransferases share certain mechanistic features with other pyridoxalphosphate-dependent enzymes, such as the covalent binding of the pyridoxalphosphate group to a lysine residue. CATALYTIC ACTIVITY: L-ornithine + a 2-oxo acid = L-glutamate 5-CC semialdehyde + an L-amino acid
C	Lin1-like protein (#)	Q9VKV5	GYF; 1	5 day larvae pH range 3–6	I	4.4 ± 56.6; 4.8 ± 36.8	5 (16.0)	2.3×10^{-4}	Binding experiments and mutational analyses have demonstrated the critical importance of the GYF tripeptide in ligand binding. A GYF domain is also found in several other eukaryotic proteins of unknown function. It has been proposed that the GYF domain found in these proteins could also be involved in proline-rich sequence recognition
D	Hsp90 co-chaperone Cdc37 (#)	Q24276	CDC37; 1	5 day larvae pH range 3–6	I	4.4 ± 51.0; 5.0 ± 45.2	10 (21.0)	2.7×10^{-5}	Co-chaperone that binds to numerous kinases and promotes their interaction with the Hsp90 complex, resulting in stabilization and promotion of their activity
E	Flotillin-1 (#)	O61491	Flotillin; 1	5 day larvae pH range 5–8	S	5.6 ± 32.0; 5.6 ± 47.1	10 (20.4)	1.7×10^{-5}	The molecular function of flotillins remains uncertain. They are probably involved in organising the structure of caveolae and lipid rafts, and other detergent resistant membrane domains. They may also be involved in signal transduction
F	NF-κB essential modulator (#)	Q9GYV5	zf-C2H2; 1	5 day larvae pH range 5–8	S	5.5 ± 35.8; 5.5 ± 43.9	6 (23.5)	2.1×10^{-3}	Essential signaling component in transmitting the lipopolysaccharide (LPS) signal leading to cact degradation, which is required for NF-kappa-B (Rel) activation. Required for antibacterial immune response
G	ENSANGP00000002018-1 [Fragment] (*)	Q7PV61	GRAM; 1	5 day larvae pH range 5–8	I	5.3 ± 31.6; Undefined ± 24.3	15 (17.9)	2.8×10^{-6}	The GRAM domain is found in glucosyltransferases, myotubularins and other putative membrane-associated proteins

(*continued on next page*)

Table 2 (*continued*)

Protein spot	Protein name (find in SWISS-PROT (#) and TREMBL(*))	Accession number	Family of protein according to Pfam database of Sanger Institute	Treatment	(I) or (S)	p$I \pm M_w$ (Exp.; Theo.)	No. of peptides matched (sequence coverage (%))	*P* value obtained with ALDENTE software	Known function according to SWISS-PROT, TREMBL and Pfam databases
H	RE16440p (*)	Q8SZ54	Not determined	5 day larvae pH range 5–8	I	7.1 ± 29.8; 7.8 ± 27.9	5 (20.2)	7.8×10^{-7}	Transcription initiation from Pol II promoter
I	Similar to Drosophila melanogaster RpS3 [Fragment] (*)	Q6XI37	Ribosomal_S3_C; 1	5 day larvae pH range 5–8	I	7.2 ± 29.9 Undefined ± 23.9	5 (22.8)	1.0×10^{-3}	Protein biosynthesis
J	Similar to Drosophila melanogaster RpL15 [Fragment] (*)	Q6XIM6	Ribosomal_L15e; 1	5 day larvae pH range 5–8	I	5.6 ± 29.8; Undefined ± 24.3	7 (21.2)	5.1×10^{-5}	Protein biosynthesis
K	ENSANGP OOOOO-O24097 (*)	Q7PG91	PBP_GOBP; 1	5 day larvae pH range 5–8	I	5.4 ± 32.7; 5.3 ± 36.5	6 (24.3)	5.2×10^{-3}	Molecular function: odorant binding
L	Elongation factor 1 alpha (Fragment) (*)	Q9U9M5	GTP_EFTU; 1	5 day larvae pH range 5–8	I	6.5 ± 33.2; 6.0 ± 30.3	7 (19.0)	2.5×10^{-4}	Elongation factors belong to a family of proteins that promote the GTP-dependent binding of aminoacyl-tRNA to the A site of ribosomes during protein biosynthesis, and catalyse the translocation of the synthesised protein chain from the A to the P site
M	ENSANGP 00000017614 (*)	Q7PRQ6	zf-CCHC; 2	5 day larvae pH range 5–8	I	7.5 ± 33.4; 8.3 ± 22.6	8 (24.0)	9.6×10^{-5}	Molecular function: nucleic acid binding
N	EbiP7561 [Fragment] (#)	Q7QIY4	zf-C2H2; 6	5 day larvae pH range 3–6 pH range 5–8	S	4.9 ± 36.5; 8.5 ± 37.0	7 (24.7)	1×10^{-4}	Zinc ion binding
O	Glutathione S-transferase D6 (DmGST25) (#)	Q9VG94	GST_C; 1	5 day larvae pH range 3–6, pH range 5–8	S	4.9 ± 27.2; 5.0 ± 24.5	7 (24.0)	1.4×10^{-4}	Conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles
P	Gasp [Precursor] (*)	O97377	CBM_14; 3	5 day larvae pH range 3–6, pH range 5–8	I	4.8 ± 21.5; 4.9 ± 24.2	5 (25.4)	9.1×10^{-6}	This domain is called the Peritrophin-A domain and is found in chitin binding proteins particularly peritrophic matrix proteins of insects and animal chitinases
Q	Ornithine decarboxylase antizyme (ODC-AZ) (*)	Q95P51	ODC_AZ; 1	5 day larvae pH range 3–6 pH range 5–8	S	4.9 ± 27.8; 4.9 ± 27.1	10 (28.4)	3.3×10^{-5}	Ornithine decarboxylase inhibitor activity
R	Nitrophorin 4A precursor	Q7YT15	Nitrophorin; 1	15 day larvae pH range 5–8	I	7.1 ± 21.5; 6.8 ± 22.5	6 (21.3)	4.8×10^{-3}	Biological process: regulation of hemolymph pressure

S	CG8097 [Fragment] (*)	Q71D42	tRNA-synt_1d_C; 1	15 day larvae pH range 5–8	I	5.0±24.2; Undefined±28.1	10 (33.1)	2.5×10^{-4}	Biological process: protein biosynthesis
T	Tc1-like transposase (Fragment) (*)	O96917	Transposase_5; 1	15 day larvae pH range 5–8	I	4.8±24.3; Undefined±30.4	7 (29.2)	2.1×10^{-5}	Transpose proteins are necessary for efficient DNA transposition. Transpose activity
U	ENSANGP0000001148-8 (*)	Q7PXK7	Acetyltransf_1; 1	15 day larvae pH range 5–8	S	5.2±23.8; 5.4±27.2	7 (21.1)	2.4×10^{-5}	Molecular function: *N*-acetyltransferase activity
V	Glutathione S-transferase 1–5 (#)	Q93112	GST_N; 1	15 day larvae pH range 5–8	S	4.7±26.7; 5.8±23.9	6 (19.9)	1.1×10^{-5}	Conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles
W	GH08336p (CG31207-PA) (*)	Q8MSJ6	DUF233; 1	15 day larvae pH range 5–8	S	6.4±32.3; 5.7±30.2	5 (21.2)	5.4×10^{-5}	Molecular function: odorant binding
X	ENSANGP0000002042-9 [Fragment] (*)	Q7QEI8	APH; 1	15 day larvae pH range 5–8	S	5.5±32.6; 5.9±33.2	6 (24.7)	7.9×10^{-5}	This family consists of bacterial antibiotic resistance proteins, which confer resistance to various aminoglycosides they include:-aminoglycoside 3′-phosphotransferase or kanamycin kinase/neomycin-kanamycin phosphotransferase and streptomycin 3″-kinase or streptomycin 3″-phosphotransferase
Y	Probable transaldolase (#)	Q9W1G0	Transaldolase; 1	15 day larvae pH range 5–8	S	4.7±30.4; 5.9±35.5	10 (23.4)	4.4×10^{-5}	Transaldolase is important for the balance of metabolites in the pentose-phosphate pathway. Transaldolase is an enzyme of about 34 kDa whose sequence has been well conserved throughout evolution
Z	General odorant binding protein, GOBP2-sexi (*)	Q9GRH5	PBP_GOBP; 1	15 day larvae pH range 5–8	S	5.9±21.5; 5.3±17.0	8 (31.3)	2.5×10^{-5}	Molecular function: odorant binding
A1	ENSANGP0000002184-8 (*)	Q7PNS5	Acyl_transf_1; 1.	15 day larvae pH range 5–8	S	5.2±27.4; Undefined; Undefined	6 (50.1)	5.9×10^{-3}	Biological process: metabolism
B1	Nitric-oxide synthase (NOS) (Fragment) (#)	O61608	NO_synthase; 1	15 day larvae pH range 5–8	S	5.1±27.3; 7.0±141.6	7 (25.5)	2.3×10^{-4}	Produces nitric oxide (NO) which is a messenger molecule with diverse functions throughout the body. Nitric oxide limits plasmodium development in the midgut
C1	Glutathione S-transferase (GSTs) (*)	O77473	GST_C; 1	15 day larvae pH range 5–8	I	4.8±24.4; 5.2±24.4	9 (19.4)	8.4×10^{-4}	Conjugation of reduced glutathione to a variety of targets. Also included in the alignment, but are not GSTs: *S-crystallins from squid. Similarity to GST previously noted. HSP family of stress-related proteins, including auxin-regulated proteins in plants and stringent starvation proteins in *E. coli*

(*continued on next page*)

Table 2 (*continued*)

Protein spot	Protein name (find in SWISS-PROT (#) and TREMBL(*))	Accession number	Family of protein according to Pfam database of Sanger Institute	Treatment	(I) or (S)	pI±M_w (Exp.; Theo.)	No. of peptides matched (sequence coverage (%))	P value obtained with ALDENTE software	Known function according to SWISS-PROT, TREMBL and Pfam databases
D1	Serine/threonine phosphatase alpha-2 isoform (#)	P12982	Metallophos; 1	15 day larvae pH range 5–8	I	5.2±29.7; 5.4±34.5	7 (25.5)	7.4×10^{-4}	Protein phosphorylation plays a central role in the regulation of cell functions causing the activation or inhibition of many enzymes involved in various biochemical pathways. This protein is essential for the regulation of mitotic chromosomal segregation as well as regulation of chromatin condensation during interphase
Reference protein spot	Actin (#)	P07836	Actin; 1	5 day larvae pH range 3–6, pH range 5–8, 15 day larvae pH range 5–8	–	5.0±41.5; 5.3±41.9	15 (35.4)	4.0×10^{-6}	Actins are highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells. Multiple isoforms are involved in various cellular functions such as cytoskeleton structure, cell mobility, chromosome movement and muscle contraction

The number of protein spots detected in this study was considerably greater than for previous proteomic studies involving *D. melanogaster* (see above). Some of these spots will be explained by the longer period of host-parasite interaction in our experiment allowing more differences to accumulate with time (see also Levy et al., 2004). However, much will be due to our samples containing a greater diversity of material than studies specifically involving hemolymph proteins. It seems unlikely a greater number of spots was detected because mosquitoes inherently produce more proteins than fruit flies. It is also unlikely that protein spots in infected treatments were of parasite origin. Most host cells will not have been infected, thus proteins of parasite origin would have been greatly diluted in homogenized host material and below the threshold of 10^4 copies/cell required for proteins to be detected (Rabilloud, 2002). Spores would not have been damaged during mechanical grinding of tissues and the proteins forming their walls are not water-soluble. Furthermore, PEPTIDENT, PROTEIN PROSPECTOR MS-FIT and ALDENTE results for proteins induced or suppressed in infected treatments identified many as being involved in host immune responses to microbial organisms (Table 2).

We decided to analyze protein expression in terms of the presence or absence of spots, rather than in terms of how intensely they were expressed. This was not only because of the number of spots identified in this study but also because we lacked objective criteria for determining the scale at which changes in expression become functionally significant.

The number of proteins expressed by control larvae increased substantially between the 5 and 15 days sampling periods, e.g. in the pH range 5–8, 5 day larvae expressed a total of 398 proteins while 15 day larvae expressed 838 (Table 1). Much of this difference is probably related to stage-specific development of the larvae (Fig. 3A); ~30% (118/398) of the proteins expressed by 5 day larvae were specific to that age, whereas the value for 15 day larvae was ~67% (558/838). By 15 days, larvae will have begun development of their reproductive structures and are in an intensive period of metabolic activity leading up to metamorphosis (Timmermann and Briegel, 1999).

The overall effect of stressful treatments (H, I, I+H) was to reduce the total number of protein spots expressed by 5 and 15 day larvae, whatever the pH range considered. This pattern arose because they caused more protein spots to be suppressed than induced, e.g. in the pH range 5–8 for 5 day larvae, a total of 252 spots were suppressed as opposed to the 21 induced (Table 1). Furthermore, the pattern of suppression was greatest in the treatment where larvae were subjected to infection and hypoxia (I+H). Of the 252 suppressed proteins above, only 22 were exclusively absent from gels in which either infection (I) or hypoxia (H) were applied separately (sum of categories 11, 13, 14), while 90 were suppressed in gels of the I+H treatment (category 12). This pattern also applied to 5 day larvae in the pH range 3–6

and for 15 day larvae (pH 5–8). Thus, the effect of each stressful treatment on the suppression of protein spots was amplified when acting in combination with one another.

The suppression of protein spots could be explained by a reduction in protein synthesis or by an increase in the rate at which proteins were metabolized. If protein synthesis was reduced, this could be because the stressors had a directly adverse effect on protein production or because larvae were actively responding by shutting down their synthetic machinery. The latter has been suggested as a mechanism to conserve ATP and reduce the rate of nectrotic processes in response to stresses such as hypoxia (Hochachka and Lutz, 2001). Reduced production may also function as a way to deprive resources from pathogens in a manner akin to sequestering iron away from bacteria.

More proteins were differentially expressed by 5 day than 15 day larvae, 437 (56%) vs. 235 (26%), respectively (Fig. 2). The combined effects of infection and hypoxia had the greatest effect in 5 day larvae, particularly for the pH range 3–6, and were associated with suppressed protein expression; much of this was due to hypoxia with infection having a lesser role. In contrast, although relatively fewer proteins were involved in the differential expression of larvae at 15 days, the pattern was dominated by those involving infection by *V. culicis*. Indeed, 46% of proteins differentially expressed were due to infection, with 15 and 22% being attributed to the effects of hypoxia and the combined effects of infection and hypoxia, respectively. It was notable that approximately one-quarter of this 46% was due to the induced expression of proteins (Fig. 2).

Among the proteins differentially expressed by 5 day larvae infected with *V. culicis* was the induction of a peritrophic matrix protein (protein spot P, Table 2). This suggests larvae had detected the presence of a pathogen and were responding to reinforce this physical barrier and reduce the chances of further infection. The identification of such proteins is of interest as the ability to induce their production is seen as a potential target for human intervention in the fight against vector-borne disease (Levashina, 2004). We note this protein was being produced 4 days after larvae had been in an environment containing spores. This indicates larvae were actively investing in protection against infection that extended beyond a short-term response. Deploying resources in this manner is expected to be at the detriment of other activities of the host such as growth and development.

Another protein differentially expressed in 5 day larvae was identified as an essential component implied in the NF-κB (Rel) activation used by insects as an antibacterial immune response (spot F, Table 2) (Shin et al., 2003). The suppression of spot 'F' in infected treatments indicating the protein was being mobilized in the activation of the cascade (Shin et al., 2003).

The induced expression of odorant binding proteins (OBP) in 5 and 15 day larvae (spots K, W and Z, Table 2) was notable, especially as proteins with this function have been reported in the hemolymph of *Drosophila* following infection. Some OBPs are thought to play a role in recognizing or neutralizing viral and bacterial infections, although Levy et al. (2004) found one to be particularly over-expressed in response to a fungal infection. This latter result is of special interest given the phylogentic proximity of microsporidia and fungi.

For larvae sampled at 15 days, relatively fewer proteins showed differential expression but almost half of those that did were in response to *V. culicis*. Of the proteins responding to infection and identified in Table 2, many have roles in maintaining physiological or metabolic functions. Hosts are likely to have been experiencing greater demands on their resources than 5 day larvae. For example, infected cells often show reduced amounts of stored lipids and carbohydrates (Weiser, 1976). Hosts will also be faced with an increasing demand on their protein resources as spore production begins. In addition to this drain of resources, hosts will also need to cope with increasing tissue damage as accumulating spore numbers cause their cells to rupture. However, not all changes in protein expression were necessarily due to the host as some of the proteins involved in cell maintenance may have actively been manipulated by the parasite (e.g. spot D1, Table 2). For example, Scanlon et al. (2000) have suggested microsporidia actively arrest mitosis and division of host cells to maintain the integrity of their cellular environment.

Other than the immune proteins mentioned above, we found differential expression for six protein spots involved in the L-arginine and L-ornithine metabolic pathways; ornithine aminotransferase (OAT), gluthatione S-transferase D6 (GSTs D6), ornithine decarboxylase antizyme (ODC-AZ), gluthation S-transferase 1–5 (GSTs 1–5), nitric-oxide synthase (NOS), gluthatione S-transferase (GSTs) (respectively, proteins B, O, Q, V, B1 and C1, Table 2). These changes in expression may result from arginase-NOS competition for the common substrate L-arginine. Arginases are a primordial enzyme family which is highly conserved across kingdoms. They may be involved in the synthesis of polyamines, amino acids, and neurotransmitters and compete with nitric-oxide (NO) synthase in vertebrate macrophages (Vincendeau et al., 2003). Parasites, such as, trypanosomes escape host NO production by activating host arginase production. This leads to a depletion of L-arginine which results in lower levels of cytotoxic NO and higher production of polyamines necessary for parasite growth (Vincendeau et al., 2003). This mechanism of parasite escape from host NO has not yet been demonstrated in insects (Samson, 2000; Foley and O'Farrell, 2003) but since the innate immune system is an ancient line of defense shared by all metazoans, Arginase-NOS competition is probably not limited to mammals. It is thus possible that *V. culicis* impairs the immune defense of its host through Arginase-NOS competition.

The insect immune system produces a variety of peptides (e.g. defensins, cercropins and transferins) to impair the

process of infection by particular parasites. Many constitutive proteins with higher molecular weights (20–200 kDa) are also implicated directly in processes of insect immune responses; protease inhibitor, serine protease, serine/threonine, phosphatase, actin, thioester-containing protein (TEP), NF-κB, Gasp [precursor], phosphatidylethalolamine binding protein (PEPB), alcohol dehydrogenase, glutathione-S-transférase (GST), etc. (see Khush and Lemaître, 2000; Oduol et al., 2000; Vierstraete et al., 2004a,b). Many of the known immune constitutive proteins cited above were observed and modified in their expression during the infection by *V. culicis* (see Table 2).

A priori, antimicrobial peptides do not provide an efficient response to microsporidian infections. It is unclear whether their induction in our experiments corresponds to a response by mosquitoes to secondary infections from other microorganisms, such as gut bacteria, or because the mosquitoes detected the presence of infection and tried to react with whatever means at their disposal. Whatever the case, our results demonstrate that a microsporidian infection elicited responses from the immune system of their insect host. The activation of the immune system, through the costs it may incur (Moret and Schmid-Hempel, 2000), may be at least partly responsible for changes in mosquito life history traits observed for this host–parasite relationship (Agnew et al., 1999; Agnew et al., 2004; Bedhomme et al., 2004).

The results on proteins involved in the L-arginine and L-ornithine metabolic pathways indicate that this parasite may be manipulating the immune system of its invertebrate host to its own benefit in a manner similar to that proposed for parasites of vertebrate hosts (Vincendeau et al., 2003). It is thus possible that *V. culicis* impairs the immune defense of its host through the Arginases-NOS competition. Experiments measuring and manipulating levels of NO, NOS, Arginase, L-arginine and polyamines are necessary and should allow confirmation or rejection of these observations.

Acknowledgements

We thank Nathalie Galeotti and Emmanuelle Demey-Thomas from the proteomics platform of Montpellier Genopole® for their help concerning the PMF data, Hercules Moura from National Center for Environmental Health Centers for Disease Control and Prevention of USA and Stéphanie Bedhomme from GEMI UMR CNRS/IRD 2724 Montpellier France for their precious comments on this manuscript. This work was supported by an ATIP CNRS grant to Y. Michalakis.

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