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Do distantly related parasites rely on the same proximate factors to alter the behaviour of their hosts?

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Phylogenetically unrelated parasites often increase the chances of their transmission by inducing similar phenotypic changes in their hosts. However, it is not known whether these convergent strategies rely on the same biochemical precursors. In this paper, we explored such aspects by studying two gammarid species (*Gammarus insensibilis* and *Gammarus pulex*; Crustacea: Amphipoda: Gammaridae) serving as intermediate hosts in the life cycle of two distantly related parasites: the trematode, *Microphallus papillorobustus* and the acanthocephalan, *Polymorphus minutus*. Both these parasite species are known to manipulate the behaviour of their amphipod hosts, bringing them towards the water surface, where they are preferentially eaten by aquatic birds (definitive hosts). By studying and comparing the brains of infected *G. insensibilis* and *G. pulex* with proteomics tools, we have elucidated some of the proximate causes involved in the parasite-induced alterations of host behaviour for each system. Protein identifications suggest that altered physiological compartments in hosts can be similar (e.g. immunoneural connexions) or different (e.g. vision process), and hence specific to the host–parasite association considered. Moreover, proteins required to alter the same physiological compartment can be specific or conversely common in both systems, illustrating in the latter case a molecular convergence in the proximate mechanisms of manipulation.

Keywords: acanthocephalan; gammarid; manipulative parasite; molecular convergence; proteomics; trematode

1. INTRODUCTION

Parasites are capable of altering a wide range of phenotypic traits in their host, which favour the continuation of their life cycle (Poulin 1998; Combes 2001; Moore 2002; Thomas *et al.* 2005). Behavioural changes have been particularly well documented in a variety of host–parasite systems, especially those involving trophically transmitted parasites (Lafferty 1999; Moore 2002). Recently, there has been a growing interest in understanding the origin of similar behavioural changes induced by different parasite species (Moore & Gotelli 1996; Poulin 1998; Thomas & Poulin 1998). Indeed, many parasite species evolve under similar selective pressures for the completion of their life cycle, exploiting the same host species in the same sequence or different host species, but in a similar context. When similar behavioural changes are induced by phylogenetically unrelated parasites experiencing similar selective pressures, convergence is a reasonable explanation, since the same manipulation of host behaviour has arisen independently in different parasite lineages. Whether these behavioural changes rely on similar proximate precursors, however, remains poorly understood, mainly because the mechanisms underlying ethological changes in parasitized hosts are by no means well characterized (see Thomas *et al.* (2005) for a recent review).

The present study focuses on two manipulative parasites that are phylogenetically unrelated: the first one, *Microphallus papillorobustus* (Rankin 1940), is a trematode (Platyhelminthes: Trematoda: Microphallidae) whereas the second one, *Polymorphus minutus* (Goeze 1782), is an acanthocephalan worm (Acanthocephala: Polymorphidae). *Microphallus papillorobustus* has a complex life cycle including snails from the genus *Hydrobia* as first intermediate hosts, gammaridean amphipods (mainly the salt marsh gammarid *Gammarus insensibilis*; Stock 1966) as second intermediate hosts and various sea- and shorebirds as definitive hosts. The life cycle of *P. minutus* displays broad ecological similarities with *M. papillorobustus*, since it also involves a crustacean,

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2869

the freshwater gammarid, *Gammarus pulex* (Linnaeus 1758), as intermediate host and aquatic birds (mainly ducks) as definitive hosts. Unlike metacercariae of *M. papillorobustus* that are always encysted in the brain of *G. insensibilis* (Helluy 1983), cystacanths of *P. minutus* are located in the body cavity of *G. pulex*. Interestingly, both parasites have been shown to manipulate the behaviour of their host, making them more likely to be eaten by predatory definite hosts foraging at the water surface. Detailed studies showed that metacercariae of *M. papillorobustus* induce a positive phototaxis, a negative geotaxis and aberrant evasive behaviour (Helluy 1981). In an experimental setting, infected *G. insensibilis* were, on an average, twice as likely as uninfected ones to be preyed upon by aquatic birds (Helluy 1984). Cystacanths of *P. minutus* do not induce positive phototaxis as in the previous system, but a negative geotaxis and aberrant evasive behaviour are clearly observed in parasitized *G. pulex* (Cézilly *et al.* 2000). Neither of the parasitic larvae induce behavioural alterations from the start of the infection. It seems that the behavioural responses are changed only after several days when the cysts are infective to the definitive hosts (Bethel & Holmes 1974; Helluy 1981). Therefore, *M. papillorobustus* and *P. minutus* have independently evolved the capacity to modulate specific behaviours of their intermediate hosts with precise timing and in very subtle ways, in order to bring them near the surface and to increase their risk of avian predation.

The aim of the present study is to elucidate and compare some of the proximate cause(s) of the behavioural manipulations exerted by *M. papillorobustus* and *P. minutus* in their gammarid hosts. Proteomics has been recently introduced as a promising approach for investigating many aspects of host–parasite interactions (Barrett *et al.* 2000; Ashton *et al.* 2001; Biron *et al.* 2005*a,b*), including manipulative processes (Biron *et al.* 2005*a–c*). Permitting the study of the host genome in action during the expression of the altered behaviour, proteomics *a priori* offers a relevant tool to explore the proximate mechanisms responsible for host manipulation. Here, we performed such an approach by analysing the differential expression of the host brain proteomes of parasitized and uninfected *G. insensibilis* and *G. pulex*. Moreover, given the important ecological differences between animals living at the surface or near the bottom of a body of water (e.g. light, current, temperature, food quality, quantity and density), we may perhaps expect in both systems other proteic spots than those directly linked to the manipulation, which display a particular proteic spot expression between infected (surface) and uninfected (bottom) gammarids (Lopez *et al.* 2001, 2002). In an attempt to control this potentially confounding effect, we also considered uninfected male gammarids experimentally placed for (in a metallic cage 1 m^2) 20 h at the water surface of the lagoon (*G. insensibilis*) and the river (*G. pulex*) in our study. Thus, our experiment involved three categories of individuals for each amphipod species: uninfected- ($U_{insensibilis}$, U_{pulex}), infected- ($I_{insensibilis}$, I_{pulex}) and control- individuals (i.e. uninfected gammarids kept 20 h at the water surface; $C_{insensibilis}$, C_{pulex}). As far as we are aware, this is the first work to explore the hypothesis of molecular convergence in parasite manipulative behavioural processes.

2. MATERIAL AND METHODS

(a) *Sampling*

Since experimental infections of gammarids by the trematode and the acanthocephalan are still problematic, we performed this study with naturally infected specimens. Large samples of infected and uninfected *G. insensibilis* were randomly collected following the methodology described by Thomas *et al.* (1995) in the brackish lagoon of Thau (southern France, 43°25′ N, 3°35′ E) during July 2004. Infected individuals were identified in the field through the aberrant surface behaviour induced by the parasite. In the same way, infected and uninfected *G. pulex* were collected in a natural river, La Bèze (Noiron-sur-Bèze, eastern France, 47°26′ N, 5°18′ E) during July 2004. Infected individuals were identified through their modified behaviour and the orange colour of the acanthocephalan visible through their cuticle (Cézilly *et al.* 2000). To limit the possible effects of multiple infection or host sex-specific factors on the proteomics expressions, only *G. insensibilis* males infected with one to three parasites and mono-infected *G. pulex* males were used for the proteomics analysis.

In infected *G. insensibilis*, the anterior part of the head was carefully dissected in order to remove the one to three metacercariae of *M. papillorobustus*. To verify the uninfected status of *G. insensibilis* collected at the bottom of aquatic systems, we performed a similar dissection on the head. At the same time, such a procedure ensured that heads of infected and uninfected individuals had been prepared with the same method. We also meticulously dissected the body cavity of all *G. insensibilis* in order to verify that no other trematode species were present. Heads of *G. pulex* were also removed and body cavities dissected in order to confirm the presence of only one *Polymorphus* cystacanth in infected individuals, and that no other parasites were present. Heads of *G. insensibilis* and of *G. pulex* were then dried on absorbent paper before being frozen individually in 1.5 ml Eppendorf tube in liquid nitrogen, and after a few hours, were transferred to a $-80\,^{\circ}\text{C}$ freezer for long-term storage prior to electrophoresis testing.

(b) *Two-dimensional electrophoresis*

Proteins were extracted from two samples of 35 heads in each *G. insensibilis* category and 20 heads in each *G. pulex* category. The heads were washed in Tris solution (10 mM, pH 7.4) and crushed with a piston (Potter) in 186.5 µl of extraction buffer solution (lysis solution) consisting of 15 M urea, 10 mM Tris–HCl pH 7.4, 2.5% (w/v) β-mercaptoethanol, 11% solution A (100 µl β-mercaptoethanol, 100 µl biolyte 3–10, H_2O for 400 µl). The sample was centrifuged (10 000*g* for 10 min at 4 °C) to separate proteic phase from the lipidic phase. The concentration of each protein sample was estimated by measuring the shift in the extinction of Coomassie blue G-250 at a wavelength of 595 nm (Bradford 1976) and standardized at $2\,\mu\text{g}\,\mu\text{l}^{-1}$ by the addition of the required volume of the homogenizing solution. The protein samples were stored at $-70\,^{\circ}\text{C}$ prior to electrophoresis. The two-dimensional gels were prepared and run as detailed by Biron *et al.* (2005*c*). At least three immobilized pH gradient (IPG) strips (Immobiline, Dry-Strip gels; BioRad, USA) of pH 3.0–6.0 were run per category. The gels were stained using tetrathionate-silver nitrate (Oakley *et al.* 1980; Rabilloud *et al.* 1994).

Table 1. Number of common protein spots (above diagonal) and proteome distances (below diagonal), between categories of each amphipod species and between both species.

	U_{pulex}	C_{pulex}	I_{pulex}	$U_{insensibilis}$	$C_{insensibilis}$	$I_{insensibilis}$
U_{pulex}	—	710	679	303	328	289
C_{pulex}	0.064	—	718	301	333	288
I_{pulex}	0.103	0.051	—	299	331	288
$U_{insensibilis}$	0.513	0.516	0.518	—	400	359
$C_{insensibilis}$	0.496	0.488	0.49	0.222	—	390
$I_{insensibilis}$	0.510	0.511	0.51	0.208	0.190	—

(c) Computer analyses

At least three well-replicated two-dimensional electrophoresis (2DE) gels were preserved and used for computer analysis of the various *G. insensibilis* and *G. pulex* categories described earlier. Replicated gels for the same treatment were compared using IMAGEMASTER 2D Platinum Software v. 5.0 (Amersham Biosciences, UK; Genebio, Switzerland). The best gels obtained for each category were then used to build a two-dimensional master gel for both *G. insensibilis* and *G. pulex*, respectively. The I_p and M_w scales of 2DE gels were determined using a protein standard kit from BioRad (USA). IMAGEMASTER 2D Platinum was used to compare the proteomics results obtained for both *G. insensibilis* and *G. pulex*. Crowed protein spot areas and areas containing high-molecular weight protein spots were not well defined and thus discarded from analysis.

Protein patterns obtained for both gammarid species were compared by cluster analysis of coordinates and optical density of protein spots (presence or absence) by using the IMAGEMASTER software. This analysis is especially useful in cases where position assignment was uncertain and it ensured that only homologous protein spots (with the same coordinates X and Y) were matched between treatments. We used the generally employed genetic distance for the analysis of 2DE results: the Nei & Li coefficient for the heuristic classification: $F = 2nxy/(nx + ny)$ where nx and ny are the number of protein spots scored in population x and y, respectively, and nxy is the total number of protein spots shared by both populations x and y (Nei & Li 1979). The proteome divergence was computed as a 'proteome distance' measure according to the value of $1 - F$ (Spicer 1988; Thomas & Singh 1992; Tastet *et al.* 1999, 2000; Biron *et al.* 2005*a*). The proteome distance was used to perform a heuristic analysis to classify gels of amphipods earlier analysed using the STATISTICA v.5.0 software (Statsoft, Inc., Tulsa, OK, USA).

(d) Protein identification by MALDI-TOF mass spectrometry

Once initial analyses had revealed protein spots of potential interest, new gels were run and silver stained following the procedure of Shevchenko *et al.* (1996). Thereafter, and following the identification of candidate proteins, these were digested to yield peptides, which in turn were analysed using MALDI-TOF mass spectrometric methods to determine their molecular weight and sequence, as earlier performed by us in previous studies (see Biron *et al.* 2005*c* for details). Spectra peak of all candidate proteins are given in the electronic supplementary materials S1 (*G. insensibilis*) and S2 (*G. pulex*), we also provide the list of known contaminant ions (electronic supplementary material S3). Protein
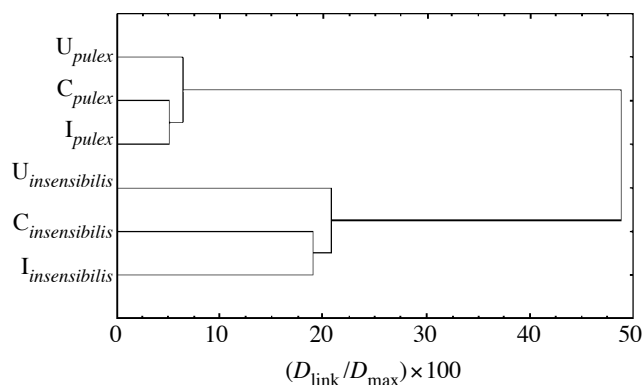

Figure 1. Classification of two-dimensional gels according to proteome distance of the three categories of *Gammarus insensibilis* and *Gammarus pulex*.

identification was obtained by conducting a database search of the peptide masses generated from MALDI analysis. Identification of proteins was performed using ALDENTE (http://www.expasy.org/tools/aldente/) software, available online. Monoisotopic peak lists were imported into ALDENTE software with the following search parameters: other Metazoa, Insecta and other Insecta in the species field, $I_p \pm$ 2.0, $M_w \pm 30\%$, one missed cleavage, tryptic digestion, carbamidomethylation as a cysteine modification and oxidation of methionine. Gels of infected gammarids are mainly made by proteins from amphipod's brain and could also contain proteins secreted by parasites; this method does not permit us to elucidate proteins' origin. Thus, for the species fields, we performed a parsimonious search by taking into consideration the possible molecular 'crosstalk' during host–parasite interactions (Salzet *et al.* 2000). Search tolerance was set at 100 p.p.m. with a MH+ charge state. The proteins were retained with the highest score, the higher significant 'probability value' ($p < 0.01$, i.e. the probability that the observed match is a random event), a minimum of missed cleavages, a minimum of delta parts per million between the molecular mass of the experimental peptides and the corresponding theoretical peptides, a theoretical I_p/M_w close to the experimental I_p/M_w and more than 20% coverage were retained (Wilkins & Williams 1997; Mathesius *et al.* 2002; Habermann *et al.* 2004; Ostrowski *et al.* 2004; Barrett *et al.* 2005).

3. RESULTS

(a) Classification of 2DE gels

Table 1 shows the number of common protein spots resolved for both amphipod species, as well as the proteome distances between *G. insensibilis* and *G. pulex* categories. The qualitative data (presence/absence of protein spots) were analysed with a phenetic study by calculating a 'proteome distance' (see §2) broadly analogous to Nei's genetic distance (Nei & Li 1979). The dendrogram constructed (figure 1) shows that proteome distances between the species are greater than the distances observed between categories of the same species. For both the species, the category consisting of uninfected gammarids ($U_{insensibilis}$, U_{pulex}) is separated from two other ones (infected ($I_{insensibilis}$, I_{pulex}) and uninfected that lived 20 h on the surface ($C_{insensibilis}$, C_{pulex})).
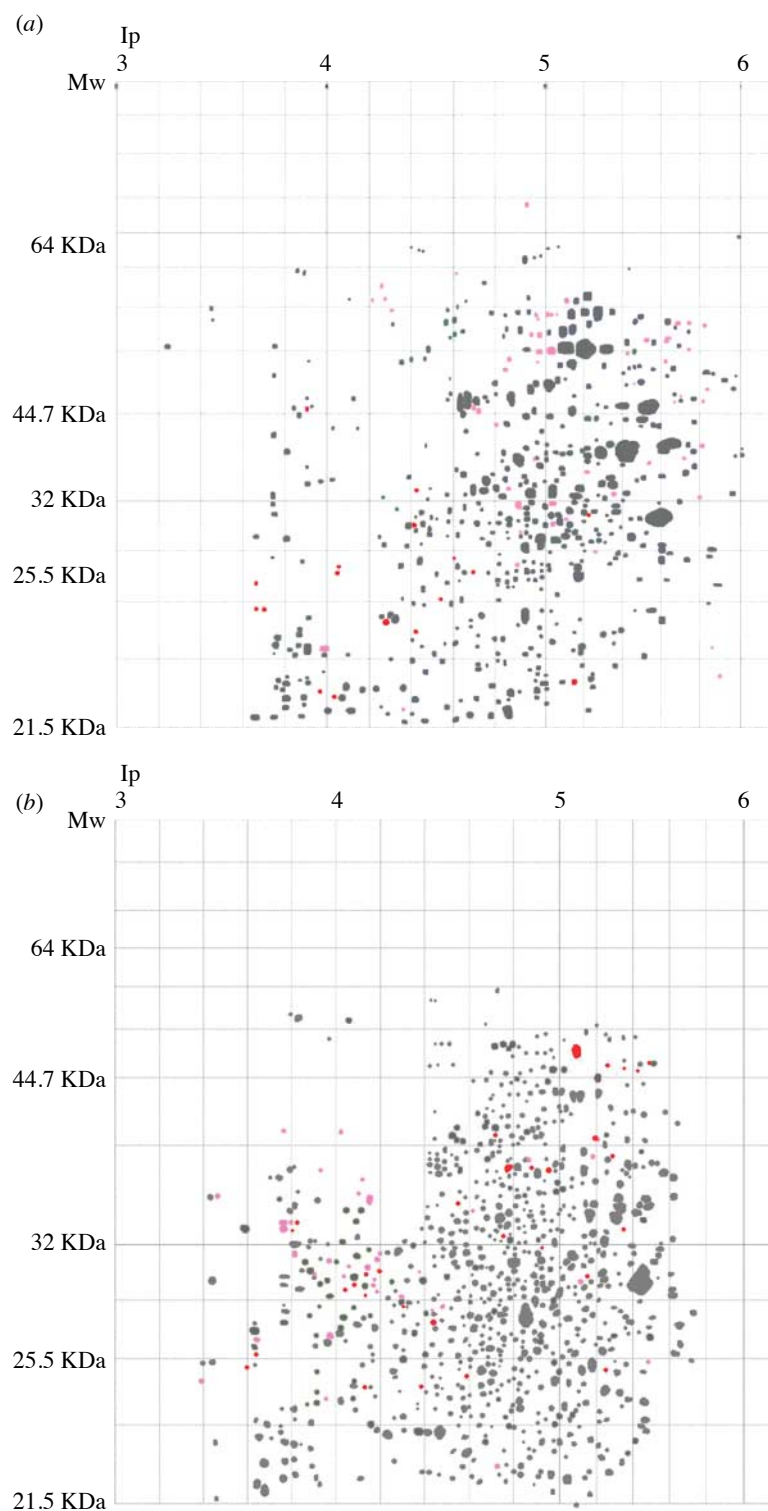
Figure 2. Two-dimensional synthetic gels (pH range 3–6) showing the differential brain proteome expression between the three categories of (*a*) *Gammarus insensibilis* and (*b*) *Gammarus pulex* to disentangle the protein spots potentially linked to the manipulative process. Colours: grey, other protein spots; pink, manipulation effect (suppression); red, manipulation effect (induction).

(b) *Analysis of 2DE gels*

Figure 2 reveals the differential *G. insensibilis* (figure 2*a*) and *G. pulex* (figure 2*b*) brain proteome expression during the alteration of the host behaviour and highlights protein spots that are specific to a subset of categories. Figure 3 gives the proportions of common or specific and induced or suppressed (not detectable (break-even point of detection)) proteins of the different categories for both systems (see electronic supplementary material S4, for

more details). For instance, we considered that a protein spot was likely to be linked to the manipulative process when its presence/absence was specifically observed in $I_{insensibilis}$ and I_{pulex} gels. Thus, for *G. insensibilis*, 345 (62.05%) protein spots are shared between the three categories, whereas 72 protein spots are potentially linked to the manipulative process, i.e. 3.06% for $I_{insensibilis}$ gels and 9.9% for both $U_{insensibilis}$ and $C_{insensibilis}$ gels (figure 3). Moreover, concerning *G. pulex*, 673 protein spots (80.3%)
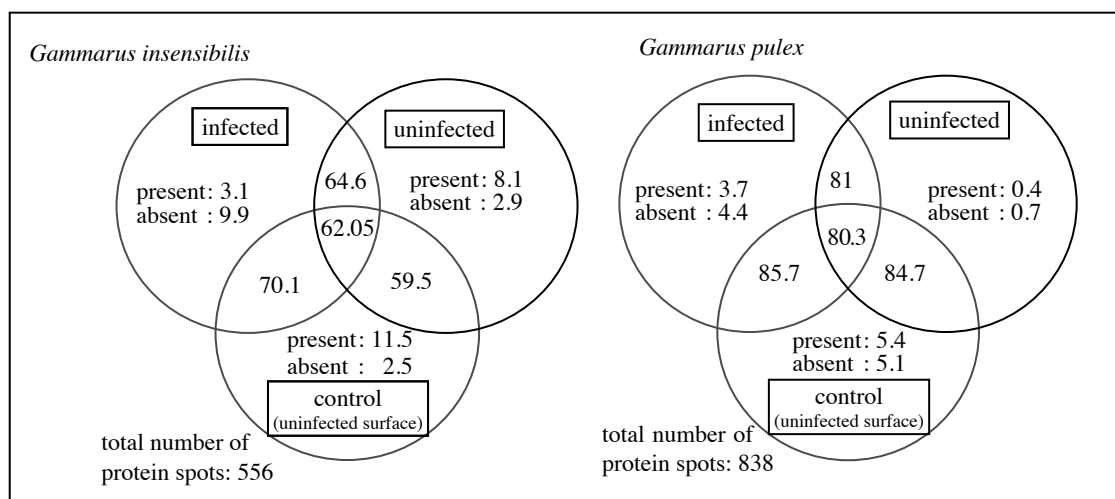
Figure 3. Venn diagram illustrating the overlap in proportion between the sets of proteins of the three amphipod categories for both species *Gammarus insensibilis* and *Gammarus pulex*.

are common to three categories of brain, while 68 protein spots are linked to the manipulative process, i.e. 8.1% were specific to I_{pulex} gels and 4.4% for both U_{pulex} and C_{pulex} gels (figure 3).

(c) Comparison of peptide mass fingerprint between both amphipod species

Analyses were focused on the study of protein spots differentially expressed during the manipulative process in both systems (figure 2*a*,*b* and electronic supplementary material S4). Thus, good peptide mass fingerprint (PMF) was obtained for 35 protein spots in the system *G. insensibilis–M. papillorobustus* (17 specific to $I_{insensibilis}$ and 18 shared by $U_{insensibilis}$ and $C_{insensibilis}$ categories) and 32 protein spots in the system *G. pulex–P. minutus* (15 specific to I_{pulex} category and 17 to U_{pulex} and C_{pulex} categories; electronic supplementary materials S5 and S6).

(d) Identification of candidate proteins

To refine our studies on the molecular mechanisms implied in the modification of host behaviour and molecular convergence, we attempted to identify the candidate protein spots in available online protein databases. Since actin is highly conserved (Sheterline *et al.* 1996) in animal kingdom, it is a good positive control to evaluate the MALDI-TOF protocol employed here. Protein spots identified as actin-2, according to their I_p and M_w, have been excised and PMF obtained for both species (electronic supplementary materials S5 and S6). The results of search in protein databases confirmed that spots excised belonged to the family of actin proteins (electronic supplementary materials S7 and S8). We then performed the same search for the other proteic spots. The results of search in protein databases always suggest that candidate proteins are from the host's genome. The results are summarized in table 2. This table gives the identified protein families for which a differential expression was observed during the expression of the aberrant behaviour by the two amphipod species. In addition, for each family of proteins identified, the accession number in the Pfam database (http://www.sanger.ac.uk/Software/Pfam/) and its function are given in the electronic supplementary materials S7 and S8. Some proteins expressed

differentially (presence/absence) in the central nervous system (CNS) of manipulated and non-manipulated gammarids belong to protein families (*G. insensibilis*: CUB; 1, PBP_GOBP; 1, Pyridoxal_deC; 1, ATP-gua_Ptrans, SGS; 1; *G. pulex*: ATP-gua_Ptrans, haemocyanin, tropomyosin) acting directly and/or indirectly during the CNS development; they represent 37.5 and 23% of the characterized proteins in the brain of infected *G. pulex* and *G. insensibilis*, respectively (figure 4). We also observed differential expression of proteins (*G. insensibilis*: ATP-gua_Ptrans; *G. pulex*: ATP-gua_Ptrans, MAM and Sushi) involved in the immune system; immunity defences are thus required in both systems and involved 23 (*G. insensibilis*) and 37.5% (*G. pulex*) of candidate proteins (figure 4). In addition, two proteins specifically expressed in the brain of manipulated *G. insensibilis* are involved in the vision process (CRAL_TRIO; 1, efhand; 3; 15.5% of candidate proteins, figure 4). Since they are involved in many molecular functions, the other identified proteins did not provide definite information concerning mechanisms of parasite manipulation and/or host proteome reaction. Therefore, as such, we were unable to refine their true biological role using our systems. Even though we obtained very good PMF for the majority of the protein spots, it was impossible to identify nine protein spots specifically expressed in infected *G. insensibilis* CNS and 18 protein spots that were specifically absent (electronic supplementary material S5). Concerning the CNS proteome of *G. pulex*, 34 protein spots specifically absent and 26 protein spots specifically present in infected individuals are unknown in protein databases (electronic supplementary material S6).

4. DISCUSSION

Understanding how parasites alter host behaviour is important in attempting to elucidate the evolution of parasitic manipulation (Poulin 1995; Thomas *et al.* 2005). Most research on the processes underlying behavioural changes in parasitized hosts have been limited to the quantification of relatively few molecules considered in advance as potential candidates. Conversely, the proteomics approach, as used here, makes no assumption of identifying the molecules involved, and for this reason, it

Table 2. Identification of *Gammarus insensibilis* and *Gammarus pulex* protein spots with PMF (see electronic supplementary materials S7 and S8 for more details).

protein spots not detected in the proteome of infected gammarids		protein spots specifically detected in the proteome of infected gammarids	
Gammarus insensibilis, protein name (protein spot name; protein family)	*Gammarus pulex*, protein name (protein spot name; protein family)	*Gammarus insensibilis*, protein name (protein spot name; protein family)	*Gammarus pulex*, protein name (protein spot name; protein family)
CUB-domain containing protein (I; CUB; 1)	allergen Pen m 2 (X; ATP-gua_Ptrans)	AT18354p (fragment) (B; Carb_anhydrase; 1)	arginine kinase (EC 2.7.3.3) (S; ATP-gua_Ptrans)
GH09161p (fragment) (J; family undefined)	ENSANGP00000015032 (fragment) (Y; eIF-5a et KOW)	CG3226-PA (C; SGS; 1)	ENSANGP00000015837 (T; Ras family)
CG9486-PA (K; Acetyltransf_1; 1)	ENSANGP00000028533 (Z; TFIIE; 1)	ENSANG00000017842 (D; CRAL_TRIO; 1)	CG3212 (fragment) (U; MAM. et Sushi)
aromatic-l-amino acid decarboxylase (L; Pyridoxal_deC; 1)		ENSANGP00000004678 (fragment) (E; p450; 1)	ENSANGP00000024231 (V; Tropomyosin)
GA15598-PA (M; family undefined)			
CG5050-PA (N; family undefined)		pheromone-binding protein 1 (F; PBP_GOBP; 1)	prophenoloxidase (W; haemocyanin)
centromeric histone Cid (O; Histone; 1)		Odorant-binding protein 3-1 (G; PBP_GOBP; 1)	
arginine kinase (P; ATP-gua_Ptrans; 1)		neurocalcin homologue (H; efhand; 3)	

appears to be a powerful tool to discover potentially new mechanisms (Biron *et al.* 2005*a*).

Our results initially indicated that the proteomics approach is sensitive enough to detect proteome differences between two related species, since intraspecific differences were smaller than interspecific ones. There was also differential expression between infected and uninfected amphipod brain proteomes within each species. Interestingly, the phenetic analysis revealed the same typology for clusters in both species, with individuals living at the water surface (i.e. uninfected control and infected individuals) being closer than uninfected ones. Thus, it is clear that both parasitic status and environmental conditions have a significant influence on protein expression.

In this study, PMF, which is still widely used and employed in a number of recent parasite studies (Jefferies *et al.* 2000; Chemale *et al.* 2003; Bernal *et al.* 2004; Curwen *et al.* 2004), allowed the characterization of interesting proteins. Among the candidate proteins (i.e. those displaying a particular pattern of expression in the brain of manipulated gammarids), several are implicated in the functioning of the CNS. For instance, we observed differential expression of a protein belonging to the tropomyosin family, which could interact with the development and the plasticity of the nervous system in infected individuals (Stamm *et al.* 1993). In infected *G. insensibilis*, there was a higher expression of a protein involved in the synthesis of serotonin (Aromatic-l-amino acid decarboxylase). This finding agrees with previous work, which suggest a major role of serotonin in the processes linked to behavioural alterations in parasitized animals (Maynard *et al.* 1996; Terenina *et al.* 1997; Overli *et al.* 2001), and especially in gammarids (Helluy & Holmes 1990; Helluy & Thomas 2003). In most cases, alteration of serotonergic labels is related to alteration
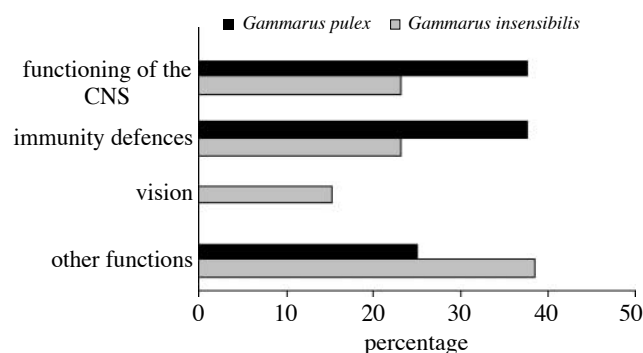

Figure 4. Percentage of proteins with similar function among characterized manipulative proteins for both species *Gammarus insensibilis* and *Gammarus pulex*.

of phototactism. It is interesting in this context to note that the differential expression of aromatic-l-amino acid decarboxylase only concerns the gammarid species, which displays phototactism alterations (i.e. *G. insensibilis*). Finally, it is relevant that for infected *G. insensibilis*, a differential expression of two proteins implied in the vision process was seen (CRAL_TRIO; 1 and efhand; 3). Because these two proteins are differentially expressed only in infected *G. insensibilis*, and not in uninfected controls from the surface, these particular proteins are more likely to be involved as *causes* rather than as *consequences* of the positive phototaxis.

The present results also indicated a differential expression of proteins involved in the immune system. For instance, two protein families in *G. pulex* (MAM and Sushi and ATP-gua_Ptrans) and two protein families in *G. insensibilis* (ATP-gua_Ptrans and PBP_GOBP) were more expressed in the brains of infected individuals. These results suggest the activation of an immune response in the amphipod brain against the parasite. Interestingly, among the proteins differentially expressed by infected

G. insensibilis, there are odorant and pheromone-binding proteins (PBP_GOBP). It is now widely recognized that PBPs are required for the detection of pheromones during odour-oriented navigation by insects (Leal *et al.* 2005). However, odorant-binding protein (OBP) family members have also been found in non-sensory tissues where they act as transporters for other types of hydrophobic molecules. Recently, expression of two OBP proteins was shown to be induced by viral and anti-bacterial infections (Levy *et al.* 2004; Biron *et al.* 2005*d*). Further experiments are necessary in our case to determine the causes and the consequences of the differential expression of OBPs in the brain of infected *G. insensibilis*.

Since it has been shown that immune responses may secondarily affect host nervous system functions and hence behaviour, it is increasingly suggested that parasites could exploit host defence reactions in order to manipulate host behaviour (Adamo 2002; Moore 2002; Helluy & Thomas 2003; Thomas *et al.* 2005). Biogenic amines, which are involved in the functioning of both immune and nervous systems, are frequently found in high concentration in the brain of infected hosts (Overli *et al.* 2001; Helluy & Thomas 2003). In the present study, we found that arginine kinase is differentially expressed in the brain of infected *G. insensibilis* and *G. pulex* compared to uninfected individuals. This phosphotransferase is known to be one of the regulating factors in nitric oxide (NO) synthesis (Mori & Gotoh 2000). NO is liberated during immunological reactions, but it also has a role of neuromediator coordinating numerous neuronal activities. Considering that the production of behaviourally effective neuroactive compounds should be energetically costly for parasites (Poulin 1994), it might be more efficient to induce the host to make them interact with immunity processes. Parasites could then modulate NO-synthesis since bioamines are required for parasite growth and differentiation (Giordanengo *et al.* 2002; Vincendeau *et al.* 2003; Biron *et al.* 2005*d*). An altered expression of the same protein in both systems may be considered as a case of molecular convergence in the proximate processes of the altered behaviour.

Although comparisons between specific protein spots of infected *G. insensibilis* and *G. pulex* reveal that the greater number of these proteins is specific to the host species considered, the proteins involved frequently have similar functions. From comparison with other systems involving behavioural manipulation (e.g. Orthoptera parasitized with hairworms), it seems that the alteration of the CNS is a common means used by parasites to modify behaviour (Holmes & Zohar 1990; Biron *et al.* 2005*b*). Interestingly, as in manipulated *G. insensibilis*, a differential expression of proteins from the family (CRAL_TRIO) has been found in the brain of the wood cricket *Nemobius sylvestris* (Bosc 1792) (Orthoptera: Gryllidae: Nemobiinae) infected by the manipulative nematomorph, *Paragordius tricuspidatus* (Dufour 1828) (Nemotomorpha: Gordioida: Chordodidae; Biron *et al.* in press). Thus, two parasites phylogenetically very distant apparently rely on the same molecular mechanism to alter vision in their arthropod hosts.

In conclusion, this proteomics study on the biochemical pathways altered by manipulative parasites has, for the first time, allowed us to tackle questions of physiological and molecular convergence in the mechanism(s) causing the alteration of arthropod host behaviour. It seems that altered physiological compartments of the host can be similar (e.g. immunoneural connexions) or different (e.g. visual process) and thus apparently specific to the host–parasite association. Moreover, proteins required to alter the same physiological compartment can be specific or interestingly, common in both systems and may illustrate a molecular convergence in the proximate mechanisms of manipulation. Although this study is the first to use global proteomics approaches to investigate parasitic worm manipulation of amphipods, it relies on certain assumptions that must be mentioned. For instance, if the parasite modulation is proactive, and not a cascade of non-specific toxic events, we could expect the production of membrane-bound receptors/messengers to a parasite modulator to be important. In such a case, the global approach we used could miss out key host/parasite proteins (low abundance, low mass or insoluble). Similarly, because our sampling was performed at a specific time and also in only one location, further studies would be necessary to confirm the generality of our findings. Finally, further analyses (peptide sequencing, study of protein's structure, functions and interactions) would also be necessary to fully understand the key roles of the proteins detected here in the manipulative processes.

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