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Towards a new conceptual approach to 'parasitoproteomics'

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Many parasitologists are betting heavily on proteomic studies to explain biochemical host–parasite interactions and, thus, to contribute to disease control. However, many 'parasitoproteomic' studies are performed with powerful techniques but without a conceptual approach to determine whether the host genomic responses during a parasite infection represent a nonspecific response that might be induced by any parasite or any other stress. In this article, a new conceptual approach, based on evolutionary concepts of immune responses of a host to a parasite, is suggested for parasitologists to study the host proteome reaction after parasite invasion. Also, this new conceptual approach can be used to study other host–parasite interactions such as behavioral manipulation.

The study of host–parasite interactions

Host–parasite interactions have been studied for many centuries using many disciplines (including agroecology, microbiology, evolutionary ecology, evolutionary medicine, biochemistry, medicine and veterinary medicine, immunology, molecular biology and proteomics), leading to a proliferation of research avenues that become ever more diverse. Proteomic applications to parasitic agents are in their infancy but have already led to new insights about molecular pathogenesis and microorganism identification [1–3]. The study of all proteins encoded by the genome of parasites and hosts using proteomics, or 'parasitoproteomics' [i.e. the study of the reaction of the host and parasite genomes through the expression of the host and parasite proteomes (genome-operating systems) during their complex biochemical cross-talk], is being used to investigate global protein synthesis and gene expression. A key issue is whether the host genomic responses during parasite infection are nonspecific responses that might be induced by any parasite or any other stress.

Proteomic tools

Infectious and parasitic agents remain a major cause of morbidity and mortality in humans and domestic livestock, especially in developing countries [4–6]. Molecular biologists remain confident that complete sequencing of the genome of host–parasite systems will enable the total understanding of the molecular mechanisms implied in most of the infectious and parasitic diseases and will contribute to finding new drugs for treating them [4,7]. The completion of the genomic sequences of many organisms (hosts or parasites) is the greatest triumph of molecular reductionism since the discovery of the DNA double helix in 1953 [4]. However, the use of molecular reductionism is becoming limited and holistic approaches, including theories and techniques, are desperately needed in the postgenomic era. In the field of infectious and parasitic diseases, there is an urgent need for global approaches that can efficiently, precisely and integratively study structural and functional genomics, and proteomics of microbial infections [8,9].

The structure, function, abundance and even the number of proteins in an organism cannot yet be predicted from the DNA sequence alone [10–12]. Also, posttranslational modifications such as phosphorylation and glycosylation are often extremely important for the function of many proteins, although most of these modifications cannot yet be predicted from genomic or mRNA sequences [11,12]. The cells of an organism are reactive systems in which information flows not only from genes to proteins but also in the reverse direction [6]. Some authors suggest that the proteome is the genome-operating system by which the cells of an organism react to environmental signals [12].

Proteomics offers an excellent way to examine the host genome in action, through the evaluation of the host proteome during the host–parasite interaction process. However, little proteomic information is available regarding the biochemical and physiological interactions in many host–parasite systems. Using the first generation of proteomic tools – 2D gel electrophoresis (2DE) and mass spectrometry (MS) – host proteome responses such as posttranslational modifications of host proteins (phosphorylation, glycosylation, acetylation and methylation) in reaction to parasite invasion can be detected and identified [13–15].

Although 2DE offers a high-quality approach for studying the host and/or parasite proteomes, several proteomic tools have been developed that will complement this

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Table 1. Comparison of different proteomic tools[a]

Proteomics tools	Separation	Quantification	Identification	Advantages	Disadvantages
2DE	Electrophoresis: IEF PAGE	Densitometry of stains	MS (PMF)	Well-established method Powerful for detecting protein modifications Low cost	Might provide biased quantification according to the stains used. Each stain has a specific dynamic range and a specific sensitivity
2DIGE	Electrophoresis: IEF PAGE	Densitometry of Cy3- and Cy5-labeled proteins normalized to Cy2	MS (PMF)	Good quality for quantification of multiple samples Total number of gels for an experiment reduced compared with traditional 2DE Powerful detection of protein modifications	Requires expensive dedicated instrumentation and labeling reagents Risk of nonlinear dynamic range of fluorescent dyes, as observed recently in differential levels of mRNA (transcriptome)
MudPIT	LC–LC of peptides	None	MS–MS	Excellent approach if no quantification is desired Much higher sensitivity than 2DE techniques (much larger coverage of the proteome for biomarker discovery)	No quantification Requires high level of MS skill Complicated data compilation
ICAT™	LC of peptides	Through use of heavy and light tags	MS–MS	Designed for quantification Theoretically, provides higher coverage of proteome Simultaneity of protein identification	Requires high level of MS skill Complicated data compilation At each experiment, only two treatments compared
SELDI-TOF MS	Binding of proteins based on their chemical and physical characteristics	Comparison of MS peaks	Difficult, requires serial of sample or coupling to second MS instrument	Ability to bind a range of proteins to different molecular surfaces without the requirement for antibody production Easiest MS instrumentation	Problems with reproducibility and repeatability Difficult to identify proteins
Protein arrays	Antibody-based chips (binding to affinity reagent)	Densitometry of binding	Binding to particular reagent	High throughput	Specificity of antigen or antibody binding Queries about quantitative accuracy

[a]Abbreviations: LC–LC, tandem liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PMF, peptide-mass fingerprint.

approach (Table 1). The estimated cost of acquiring the proteomic tools excludes the price of a mass spectrometer (at least US$300 000) (Table 1). 2D fluorescence difference gel electrophoresis (2DIGE) uses direct labeling of proteins with cyanine dyes before isoelectric focusing (IEF). This method relies on cyanine dyes that react with lysine groups on proteins. Differentially treated samples can be labeled separately with different dyes and then run on the same gel, eliminating inconsistencies associated with 2DE and providing a quantitative fluorescence-based measurement for relative differences in protein abundance. The dyes (Cy3, Cy5 and Cy2) are comparable in sensitivity to silver-staining methods and are compatible with MS. One tandem MS (MS–MS) method that is particularly suited to proteome determination, but not quantification, is multidimensional protein identification technology (MudPIT) [16,17]. Using MudPIT technology, all of the proteins in a sample are digested and loaded onto liquid chromatography (LC) columns. After the peptides are fractionated, they are fed into an MS–MS instrument for protein identification. This method can identify thousands of proteins and can detect membrane proteins. MudPIT is similar in concept to shotgun sequencing of DNA. Recently, a study demonstrated that MudPIT can be used in conjunction with stable-isotope labeling to provide quantitative measurement [11]. An exciting development by Abersold and colleagues is the isotope-coded affinity tag (ICAT™) method, which can be used to label proteins before separation [12]. Similar to DIGE, ICAT™ is used to label peptides so that two differentially treated samples can be combined and analyzed simultaneously in an MS. The ICAT™ method has the potential to detect more-lowly expressed proteins and is more likely than 2DE to detect hydrophobic and large proteins. Surface-enhanced laser desorption–ionization time-of-flight (SELDI-TOF) MS is a proteomic tool that attempts to overcome the requirement for purification and separation of proteins before MS analysis. SELDI-TOF uses a variety of selective chips on which complex biomaterials (e.g. body fluids and cell extracts) can be spotted. This proteomic tool, also known by its trade name ProteinChip™, is hindered by the difficulty identifying proteins of interest [18]. Protein arrays (antibody-based chips) have been developed to capture and separate known proteins selectively. The basic approach is extremely similar to that of microarrays [19,20]. The advantage of this kind of proteomic tool is the high-throughput nature of the technology, making it applicable to routine testing. However, many limitations cannot be ignored. A high-quality antibody is needed for each protein of interest and each modification of that protein. The generation of antibodies remains a laborious task that is almost as much art as it is science. Finally, it should be remembered that sequence and structure knowledge is needed for any protein that is to be analyzed by protein

microarrays, to generate the affinity reagent, thus limiting this approach to known protein sequences and modifications [21].

Limitations of the current approach to parasitoproteomics

Studies of parasitoproteomics have, hitherto, involved parasite proteome expression during infection by a given parasite [22–25], the reaction of the host proteome following invasion by a parasite species [26–29] or the injection of immune elicitors [2]. Some elegant studies of the differential expression of the proteome of insect hosts during their biochemical interactions with parasites [2,30–33] concluded that insects could react rapidly to infection by a given parasite, bacteria or fungi by producing a variety of immune-induced molecules, including antibacterial and/or antifungal peptides or polypeptides [34]. Many studies of parasite-responsive proteins of hosts dealt with a limited framework (current philosophical approach): studying the differential expression of the proteome of the host during the infection process by a specific parasite [8,27].

This approach to parasitoproteomics makes it possible to identify proteins of interest for a given host–parasite system. For example, Wattam and Christensen [26] associated some polypeptides with the genome response of *Aedes aegypti* (Diptera, Culicidae) to the invasion of the filarial worm *Brugia malayi* (Spiruria, Filariidae). This pioneering study provided important new information about the response of *Ae. aegypti* to invasion by a specific filarial worm species. However, it is not possible to determine whether the response detected in *Ae. aegypti* is specific to *B. malayi* or whether it can be observed for other worm species. A major limitation of the current philosophical approach is the absence of comparison with other stresses imposed on the host. Indeed, response of the host to a parasite infection will cause the differential expression of protein spots [induction, suppression (absence), lower expression or overexpression of common proteins shared with the control treatment], some of which will be specific to parasite infection. Others, however, might be involved in general stress responses. We suggest that this refers to another process that deserves to be distinguished from specific host–parasite interactions.

Some studies have shown the limitations of the current approach to parasitoproteomics by showing that, in the host–parasite interaction, many immune mechanisms are used (e.g. constitutive, induced and specific) [2,3,22,23]. By using two treatments – the injection of lipopolysaccharides (LPSs) and a sterile injury – Vierstraete *et al.* [2] could differentiate proteome modifications induced by immunity from those induced by a physical stress. Moura and Visvesvara [22] studied protein expression of two microsporidian isolates from humans, *Encephalitozoon intestinalis* (CDC V:307) and *Brachiola algerae* (CDC V:404), during their multiplication within monkey kidney (E6) cells. They found specific proteins for each microsporidian species that could be potential markers for both diagnosis and drug targets. Levy *et al.* [3] studied the immune response of *Drosophila* to bacterial (*Micrococcus luteus* and *Escherichia coli*) and fungal (*Beauveria bassiana*) infections. Their proteomic data revealed that 70 of the 160 detected protein spots were differentially expressed at least fivefold after a fungal or a bacterial challenge. Furthermore, the majority of these protein spots was specifically regulated by one pathogen, whereas only a small number of protein spots corresponded to proteins altered in all cases of infection. In summary, the current approach to parasitoproteomics encourages neither an increase in the knowledge of host proteome responses to different parasite species nor the creation of a proteomic database with a holistic view of host–parasite interactions.

Towards a new conceptual approach

Some studies have shown common features in the innate response of plants, insects and mammals [35,36]. Disease-resistance genes (R genes) mediate the plant defense response. They are abundant and confer resistance to many microorganisms, nematodes and/or insects. The R gene family of plants shows homology to the *Drosophila* receptor Toll and the mammalian interleukin-1 receptor. In addition, plants, invertebrates and vertebrates produce a family of peptides called defensins that is pathogen inducible. Some peptides and/or proteins used by phytophagous or animal parasites to modify the genome expression of their host share many homologous or analogous structures [35,37]. For instance, phytoparasites such as the nematode *Meloidogyne* sp. secrete substances in their hosts to make a giant cell that is used as a feeding site. A similar system has been observed for the zooparasite *Trichinella spiralis* (Stichosomida, Trichinellidae) [38]. In addition, the injection of a peptide from nematode secretion to either plant protoplasts or human cells enhances cell division [38]. The mechanism is well known but protein induction is also considered possible. Many data concerning the host–parasite interaction are now available from genomic and proteomic projects but no study has been planned with a holistic aim of increasing the knowledge about immune responses of hosts or about biochemical cross-talk between hosts and parasites.

From an evolutionary ecology point of view, host immune responses to a particular parasite can be plotted in a chart according to the immune mechanisms used (constitutive versus induced) and the degree of specificity (Figure 1). The first axis of the defense chart refers to the immune mechanisms used by the host in the two extreme cases: (i) a constitutive immune mechanism used by the host to impair rapidly the invasion of a parasite; and (ii) an induced immune mechanism that has the advantage of avoiding a costly defense system but the disadvantage of the parasite possibly escaping host control [39]. The second axis of the defense chart refers to the degree of specificity of the host immune response. Whichever immune mechanism is used and whatever the degree of specificity, the host genome ensures the adequate operation of the immune response through the proteome. Each immune mechanism involves many proteins. The genome response through the genome-operating system can be generalized in a 2D chart for all host–parasite interactions studied. The first axis of the genome-response chart refers to mechanisms of the genome-operating system used by an organism with

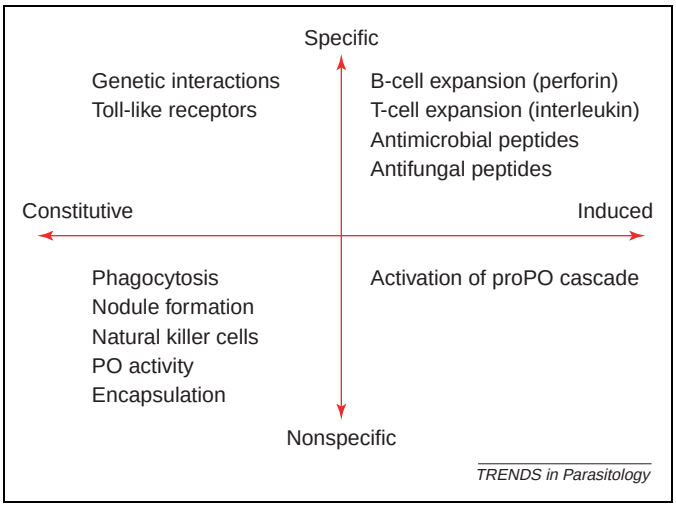

Figure 1. Defense chart, according to Schmid-Hempel and Ebert [39], summarizing the different immune mechanisms that can be used by the host genome during its biochemical interactions with a parasite. Abbreviation: PO, phenoloxidase.

constitutive and induced mechanisms. The second axis refers to the degree of specificity of the genome response.

A new conceptual approach would enable the specific immune responses of the host to be distinguished from the nonspecific immune responses to parasite attacks. In addition, the host proteome responses induced by immunity could be distinguished from the proteome responses induced by other stresses such as physical stress. The suggested conceptual approach is based on three premises: (i) in any given study, the differential expression of a host proteome (cells or tissues) should be examined across different parasite types (i.e. genotypes, isolates, strains or species) and in response to other stresses (e.g. hypoxia) for certain preselected times (e.g. the crucial moments of the process of infection by a parasite); (ii) according to the proteomic tools chosen, the results of the experiment should be analyzed with appropriate statistical tools to find all of the candidate proteins; and (iii) the proteomic results (identified proteins) should be categorized according to the genome-response chart (e.g. the defense chart for immunity and the manipulative chart for manipulative strategy).

Choosing the appropriate proteomic tools is a key step. Each technique shows advantages and disadvantages, and requires years of experience. In certain cases, several proteomic tools can be used in complementarity. For example, a study demonstrated that 2DE is the most accessible and efficient proteomic tool for a laboratory and that the ICAT™ method complements it. The ICAT™ method provided better results with proteins of high molecular weight, whereas 2DE detected more-hydrophobic proteins and proteins with small molecular weight [40]. Nonetheless, no proteomic tool can detect the total differential proteome expression. Many researchers are limited by access to proteomic tools, particularly because of cost (Table 1), and collaboration among research teams could increase accessibility.

However, all researchers in parasitoproteomics working with the conceptual approach suggested here and with powerful proteomic tools will be able to categorize the host and/or parasite genome reaction for any parasite at any given time. This conceptual approach will be hypothesis-generating for parasitoproteomics, will contribute to increasing the knowledge of host immune mechanisms, will help to discover new drugs and vaccines against parasites and will open the way to creating protein databases based on the defense chart. In addition, this strategy will provide the possibility of identifying more rapidly the efficient immune mechanisms used by a host against the different families of parasites.

Figure 2 provides an example of a parasitoproteomics study based on the new conceptual approach. This study follows the differential expression of the host proteome using three types of treatment: (i) control (e.g. a noninfected host such as *Ae. aegypti* larvae); (ii) mechanical treatment

Box 1. Analysis of data obtained with 2DE and 2DIGE

Traditionally in parasitology, proteomic tools are used to reveal the differential expression of a given proteome (cells or tissues of a given host or parasite species) between different treatments, with the aim of finding and identifying proteins linked to a biological phenomenon. With our new conceptual approach, a second aim is to categorize the identified proteins according to the chart of host and parasite genome responses and to create a proteomic database about host–parasite interactions. At least three replicated pictures are conserved per treatment. Replicated gels for a treatment are compared with 2DE software to build a synthetic gel for each treatment. The normalized volume data are generally used for the common protein spots (groups). By definition (Equation I):

$$\%Vol = \frac{Vol}{\sum_{s-1}^{n} Vol_s} \times 100 \qquad \text{(I)}$$

where Vol_S is the volume of spot S in a gel containing n spots.

After the control, treatment is used to compare the different treatments, with the aim of finding the differential expression of proteome between treatments. The analysis of 2D data is divided into two steps. First, the gels are classified with the help of heuristic cluster analysis to identify treatments (gels) showing similar patterns of proteome expression [44]. Because it is difficult to homologize loci among treatments using 2DE, the generally employed genetic distance methods cannot be used. Instead, we use the association coefficient for the heuristic classification (Equation II):

$$F = 2nxy \div (nx + ny) \qquad \text{(II)}$$

where nx and ny are the number of protein spots scored in species x and y, respectively, and where nxy is the total number of protein spots shared by both species x and y. The proteinic distance between treatments is $1-F$ [45,46]. The proteinic distance and the common protein spots ($\%Vol$) are used to perform a heuristic analysis to classify gels (treatments). Second, qualitative (presence or absence) and semi-quantitative analyses of only common protein spots between treatments are done to help identify proteins linked to different biological events. Qualitative analysis, which involves determination of the induced and suppressed protein spots (not detectable), can help to identify proteome responses. Semi-quantitative analysis (heuristic analysis [47], principal-component analysis [43] and adaptation of the Eisen method [48]) based on the relative abundance of the common protein spots enables the identification of common protein spots differentially expressed between treatments.

For 2DIGE proteomic data, the DeCyder Differential Analysis Software (http://www.apczech.cz/pdf/DF-DeCyder-2D.pdf) has been developed specifically as a key element of the Ettan DIGE system (http://www1.amershambiosciences.com/APTRIX/upp00919.nsf/Content/Proteomics+DIGE). This proprietary software considerably increases throughput by accurately addressing measurement of protein differences with statistical confidence, and enables the export of data in XML files for complementary analysis using the same steps and statistical tools as 2DE analysis (cluster, principal-component analysis and Eisen analysis).

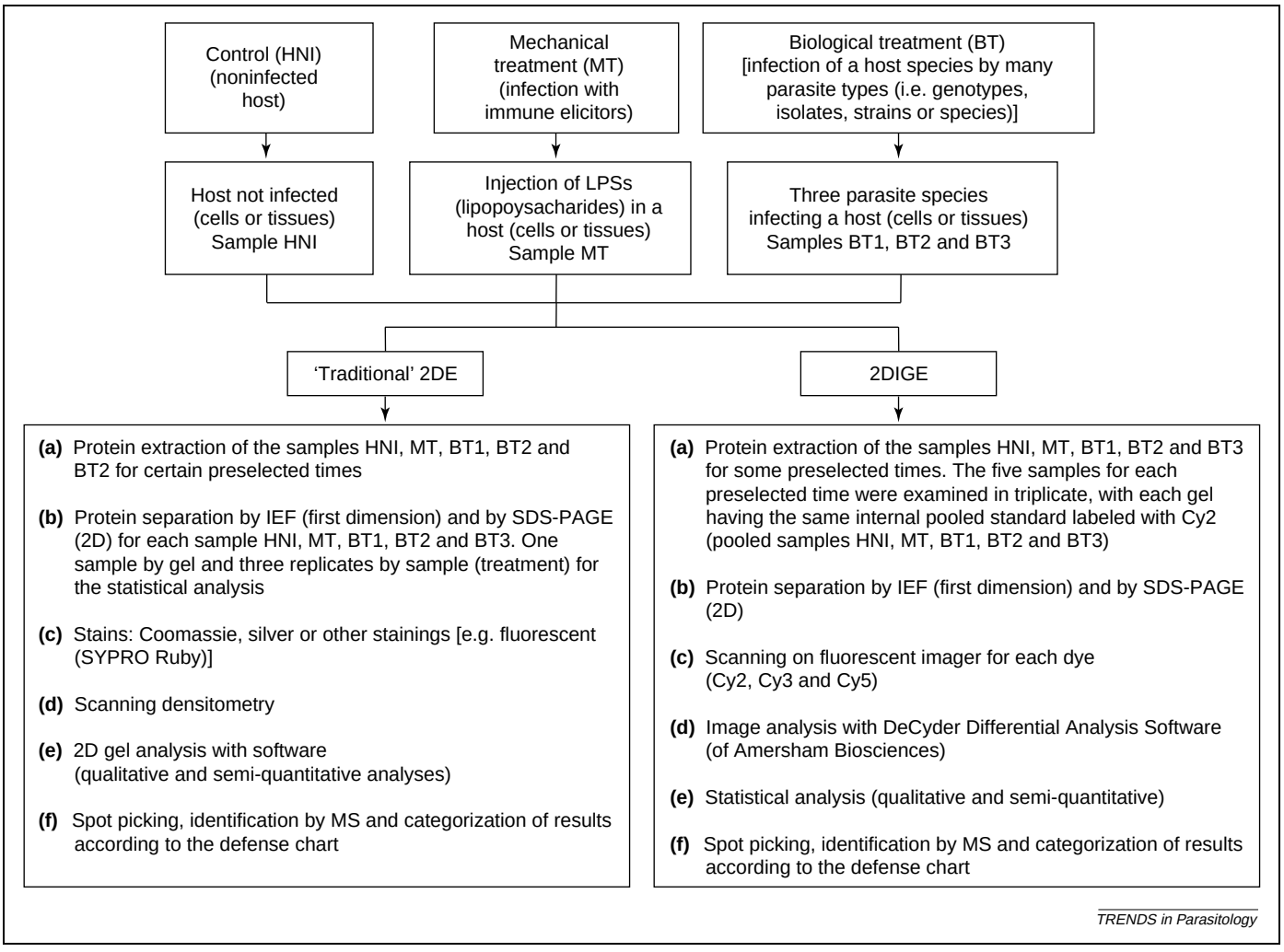

Figure 2. An example of a parasitoproteomics study based on the new conceptual approach with classical 2DE techniques (visible and fluorescent stains) or with 2DIGE (incorporation of Cy3, Cy5 and Cy2 dyes).

(injection of immune elicitors such as LPSs in *Ae. aegypti* larvae); and (iii) infection of a host species by a given number of parasite species (e.g. *Ae. aegypti* larvae infected by different microsporidian species). Software with different statistical tools is continuously developed to interpret proteomic data obtained in proteomics [41–43]. For 2DE and 2DIGE, this study enables both qualitative (presence or absence) and semi-quantitative analysis to reveal the differential host proteome expression, with the aim of categorizing the protein spots linked to the immune host response according to the defense chart. There is a strong likelihood that many proteins classified as being 'absent' are, in fact, present below the detection limit of the methods used. Nevertheless, to prevent statistical biases, analysis of the relative volume of the protein spots must be done after excluding specific ones [44–46]. Box 1 explains in detail the different steps for analyzing proteomic results obtained with 2DE and 2DIGE. The statistical method suggested for analyzing the protein spots not only compares the difference among treatments but also enables the host–parasite interaction in its entirety to be considered by analyzing each treatment (gel) as a variable. In this way, the global impact of the parasite activity on the expression of host proteome can be observed, in addition to the impact of parasite types.

The use of the conceptual approach is not limited to the study of host immune mechanisms. For instance, one strategy of parasite transmission that is particularly intriguing among parasitic organisms is the host manipulation that occurs when a parasite enhances its own transmission by altering host behaviors [49]. Despite widespread belief, there is little proof that parasites change host behavior by secreting substances that function directly on the central nervous system (CNS) of the host [50]. Less is known about the biochemical and physiological interactions between the manipulated host and its parasite. The study of the manipulation strategy of the parasite, using proteomic tools, seems to be a good opportunity to create new data and to reveal for the first time new products (proteins) implicated in the alteration of host behavior. Figure 3 suggests a way to use the conceptual approach for studying the manipulative strategy. In this case, a new chart is proposed: the manipulative chart. The first axis of the manipulative chart refers to manipulative mechanisms used by the parasite in two extremes cases: (i) a constitutive manipulative strategy used by the

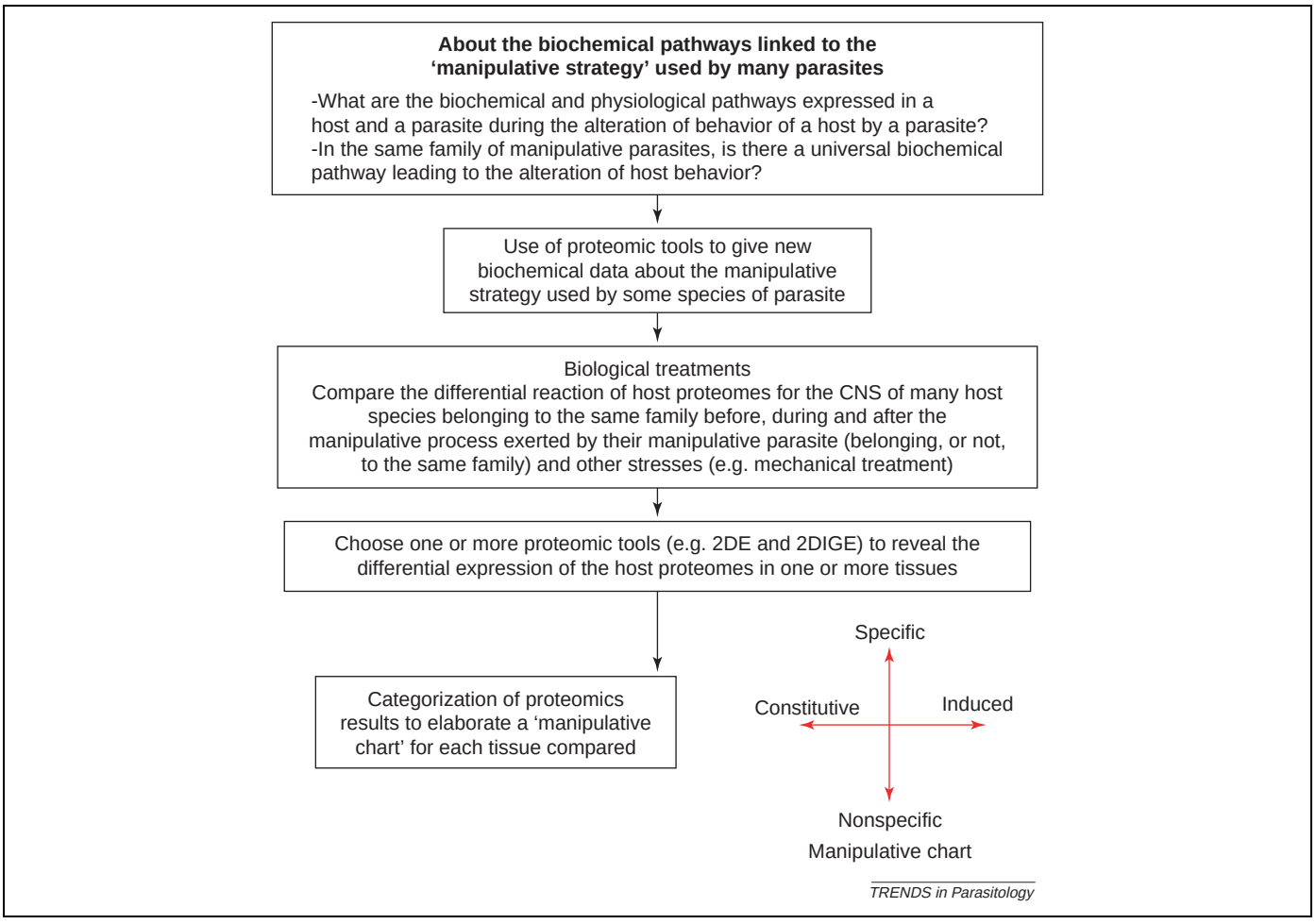

Figure 3. Application of the new conceptual approach to the study of proteins whose function is implied in behavioral manipulation of a host by a parasite.

parasite to manipulate the host [parasites continuously release products (e.g. proteins), causing directly and/or indirectly the continuous secretion of product in the host CNS]; and (ii) an induced manipulative strategy to avoid a costly permanent manipulative capability [parasites release products (e.g. proteins) at a specific moment, causing directly or indirectly the punctual production of products in the host CNS that lead to abnormal host behavior]. The second axis of the manipulative chart refers to the degree of specificity of the biochemical manipulative strategy. Each manipulative strategy involves proteins that can be categorized in the manipulative chart. This type of study will increase the knowledge of manipulative strategies and open the way to creating protein databases based on a manipulative chart. The conceptual approach can also be adapted to study the differential expression of host and parasite proteome when parasites manipulate host apoptosis. Whatever the type of study of host–parasite interactions, the conceptual approach provides many advantages, even though some traditionally technical difficulties persist (such as reproducibility, and quantity of proteins needed for the identification and extraction of hydrophobic proteins).

Concluding remarks

The new conceptual approach suggested for parasito-proteomics will help to increase the knowledge of immune responses to different parasite species, in addition to the creation of a proteomic database with a holistic view of host–parasite interactions, based on evolutionary concepts of host immune responses to a parasite. This new methodological approach offers a new way not only to discover drugs and vaccines but also to study host–parasite interactions, such as characterizing proteins whose function is implied in the behavioral manipulation of host in many taxa. In addition, it will open the way to reconstructing the molecular phylogeny of proteins such as those involved in the host immune response and to determining their level of conservation during evolution.

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