Comparative analyses of the transcriptomes of adult *Dictyocaulus filaria* and *D. viviparus* elucidate molecules involved in parasite-host interactions

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ABSTRACT

Parasitic nematodes cause diseases of major economic importance in animals. Key representatives are species of *Dictyocaulus* (= lungworms), which cause bronchitis (= dictyocaulosis, commonly known as “husk”) and have a major adverse impact on the health of livestock animals. In spite of their economic importance, very little is known about the immunomolecular biology of these parasites. Here, we established and used a bioinformatic workflow to compare, both qualitatively and quantitatively, the transcriptomes of the adult stages of the lungworms *Dictyocaulus filaria* and *D. viviparus* in the absence of reference genomes. We investigated differential transcription between *Dictyocaulus filaria* and *D. viviparus*. Our analyses identified a select group of molecules, including transthyretin-like proteins, a lipase and fatty-acid and/or retinol-binding protein, β-galactoside-binding lectin, glutathione peroxidase and glutathione S-transferase, cathepsin B and type 2-like cystatins, inferred to be enriched in adult *D. filaria* and linked to parasite-host interactions. We then studied, in depth, homologs of type 2-like cystatins from the two species of *Dictyocaulus* and 24 other nematodes representing seven distinct taxonomic orders, with a particular focus on their proposed role in immunomodulation and/or metabolism. Taken together, the present study provides new insights into nematode-host interactions. The findings lay the foundation for future experimental studies, and could have significant implications for designing new interventions against lungworms and other parasitic nematodes. The future characterization of the genomes of *Dictyocaulus* spp. should underpin these endeavors.

*Keywords:*  
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1. Introduction

Parasitic nematodes cause diseases of major economic importance in animals. Particularly significant nematodes are members of the order Strongylida, including the Ancylostomoidea, Strongyoidea, Trichostrongyloidea and Metastrongyloidea (Anderson, 2000). The latter superfamily includes pathogens of livestock, such as cattle and small ruminants (sheep and goats) (Panuska, 2006). Important representatives are species of *Dictyocaulus* (= lungworms); these parasitic nematodes live in the bronchi and bronchioles, and cause bronchitis (i.e., dictyocaulosis, commonly known as ‘husk’) particularly in young animals (Panuska, 2006; Holzhauer et al., 2011).

*Dictyocaulus* species of ruminants have direct life cycles (Anderson, 2000; Panuska, 2006). Adults live in the bronchi and trachea. Embryonated eggs are coughed up, swallowed and hatch in the small intestine. First-stage larvae (L1s) are excreted in faeces into the environment. Under suitable environmental conditions, L1s moult to the second- (L2s) and then third-stage larvae (L3s). The rate of development of the larvae to the L3 stage depends on temperature and humidity, but can be achieved in a week. Infective L3s actively move from faeces to herbage and are ingested by the grazing animal; they can also be disseminated via the sporangia of particular fungi (Panuska, 2006). Following ingestion, L3s exsheath in the small intestine, penetrate the intestinal wall and enter the mesenteric lymph nodes. Here, larvae develop, moult and then migrate via the thoracic duct, anterior vena cava, heart and pulmonary arteries to the lungs (Panuska, 2006). They penetrate the walls of the alveoli, and enter the airways. Here, the larvae become sexually mature, dioecious adults approximately four weeks following infection with L3s, after which the females produce embryonated eggs, or can also undergo arrested development (hypobiosis).

The life cycles of *Dictyocaulus* species are remarkably similar; yet, there are biological differences, particularly with regard to host preference (Panuska, 2006). *Dictyocaulus viviparus* infects cattle, other bovids and cervids, whereas *Dictyocaulus filaria* infects sheep and goats (the latter being more susceptible) (Panuska, 2006), and immunity to homologous reinfection is strong (reviewed by Panuska, 2006; Foster and Eisheikha, 2012). Interestingly, *D. filaria* can establish in the lungs of calves - disease has been reported to develop, but patent infection does not establish, although some degree of immunity against challenge infection with infective L3s of *D. viviparus* has been shown (Parfitt and Sinclair, 1967; Panuska, 2006).

Although there is clear evidence that cattle elicit mixed Th1/Th2 responses, with elevated (Th2-dependent) IgE and eosinophil levels, against *D. viviparus* (reviewed by Foster and Eisheikha, 2012), no detailed information is available on the immunobiology of *D. filaria*. Nonetheless, it is known that *D. filaria* can infect calves (Parfitt and Sinclair, 1967) but *D. viviparus* does not infect sheep, which suggests a difference in host permissiveness, such that *D. filaria* might induce an immune response that is distinct from that induced by *D. viviparus*. Molecular studies of other parasitic nematodes (reviewed by Cantacessi et al., 2009; Hewitson et al., 2009; Klotz et al., 2011) have indicated that key groups of molecules, such as SCP/Tpx-1/Ag5/PR-1/Sc7 (SCP/TAPS) proteins, transthyretin-like proteins and type 2-like cystatins, are intimately involved in the parasite-host interplay. However, information is lacking for lungworms. Clearly, improving our understanding of the differences/similarities between *Dictyocaulus* species at the molecular level, through comparative transcriptomic analyses, could elucidate their immunobiology and the
pathogenesis of disease, and might also assist in guiding future interventions against them.

Advanced genomic and transcriptomic sequencing technologies (e.g., RNA-seq; Illumina) enable detailed molecular analyses and comparisons (Allen et al., 2011; Cantacessi et al., 2012; Li et al., 2012; Liu et al., 2012; Heizer et al., 2013; Mangiola et al., 2013). Thus far, using RNA-seq, transcriptomic analyses of life stages and/or sexes of *D. viviparus* have been conducted (Cantacessi et al., 2011a; Strube et al., 2012), but there have been no comparative analyses between closely related species, such as *D. viviparus* and *D. filaria*. Indeed, nothing is known about *D. filaria* at the molecular level. RNA-seq has become a key approach to compare, in a quantitative manner, the transcription of orthologous genes of closely related species (Busby et al., 2011; Liu et al., 2011), and offers a major advantage over microarrays in that analyses can be performed directly without the need for designing probes for arrays. In addition, RNA-seq allows the comparison of the transcription of orthologous genes without significant complications of biases or saturation in hybridization to probes, typical of microarray experiments (Busby et al., 2011). Moreover, RNA-seq has been shown to have a higher capacity than microarray for the detection of variation in transcript abundance (Cloonan et al., 2008). Here, we established a bioinformatic workflow for the comparative analysis of RNA-seq data sets of *D. filaria* and *D. viviparus* adults in the absence of reference genomes for these species. In this comparative biological investigation, an emphasis was placed on exploring molecules inferred to be involved in parasite-host interactions.

2. Materials and methods

2.1. Production and procurement of parasite material, RNA isolation and sequencing

Adult specimens of *D. filaria* (n = 20; mixed sexes) were collected from the trachea and bronchi of an infected sheep in Victoria, Australia, following a routine autopsy; adults of *D. viviparus* (n = 20; mixed sexes) were collected from the trachea of an infected cow from Hannover, Germany (permit AZ 33-42502-06/1160; ethics commission of the Lower Saxony State Office for Consumer Protection and Food Safety). The worms were washed extensively in phosphate-buffered saline (PBS) and frozen at -70 °C. For each species, total RNA was extracted, purified and quantified spectrophotometrically (NanoDrop ND-1000 UV-VIS v.3.2.1). RNA libraries were prepared and paired-end sequenced using Illumina technology (Bentley et al., 2008) as described previously (Cantacessi et al., 2011b).

2.2. Curation of RNA-seq data, and assembly of transcriptomes

The RNA-seq data sets for *D. filaria* and *D. viviparus* were filtered for PHRED quality (< 30), and sequencing adapters removed using the program Trimmomatic (Lohse et al., 2012). The redundancy among reads was reduced using the program khmer (https://khmer.readthedocs.org), in order to obtain a read coverage of ≤ 20. Each non-redundant data set was assembled into contigs using the program Oases v.0.1.18 (Schulz et al., 2012) using a combination of k-mer lengths (i.e. 19-51 for *D. filaria*; 19-69 for *D. viviparus*) and read-coverage cut-offs (i.e. 5-20 for *D. filaria*; 3-20 for *D. viviparus*). Using this approach, 240 and 425 assemblies were produced for *D. filaria* and *D. viviparus*, respectively. For each assembly, five parameters were
determined: (i) sequence redundancy - calculated as the number of contigs with significant similarity to those in the same assembly (BLASTn, E-value cut-off: ≤ 10^{-6}) (Altschul et al., 1997); (ii) average contig length; (iii) number of open reading frames (ORFs) of ≥ 100 nucleotides, encoded in each contig; (iv) portion of the paired-end raw read data set that mapped to the assembled transcriptome using the program BWA (Li and Durbin, 2009) - employing a mismatch probability threshold of 0.05, and a minimum fraction gap opening of 3; the program flagstat (included in the SAMTools package) was used to undertake statistical analyses (Li et al., 2009); and (v) total number of contigs. The transcriptomes assembled for adult *D. filaria* and *D. viviparus* with the most similar parameters (i-v) were selected for direct, comparative analyses.

Sequences that did not share homology (E-value cut-off: ≤ 10^{-05}) to those of other nematodes, *Ovis aries* (sheep for *D. filaria*), *Bos taurus* (cattle for *D. viviparus*), bacteria, viruses and/or fungi, were identified and removed from these two transcriptome data sets by performing an homology search at the amino acid level against protein sequences in the NCBI non-redundant database (www.ncbi.nlm.nih.gov) using the program BLASTx (Altschul et al., 1997). Furthermore viral-like retrotransposons were also identified and removed from the sequence data according to: (i) results from InterProScan (Zdobnov and Apweiler, 2001), using selected keywords, and (ii) an homology search against the database RepBase (Buisine et al., 2008) using the BLASTx algorithm (E-value cut-off: ≤ 10^{-05}). Mitochondrial DNA and ribosomal RNA sequences were detected and removed using BLASTx and BLASTn algorithms (Altschul et al., 1997) (E-value cut-off: ≤ 10^{-05}) employing sequence data sets for nematodes, available in NCBI. All remaining sequences of ≥ 150 bases were subjected to protein predictions using an established workflow system (Mangiola et al., 2013). An additional redundancy was removed by clustering using CD-HIT (Fu et al., 2012) using identity thresholds of 0.95 (*D. filaria*) and 0.90 (*D. viviparus*). The estimated completeness of each transcriptome was assessed for the presence of conserved genes shared among metazoan organisms using the program CEGMA (Parra et al., 2007).

2.3. Functional annotation of transcriptomes

Each curated, non-redundant transcriptome was functionally annotated using an established workflow (Mangiola et al., 2013). In brief, homologs were identified (at the amino acid level; E-value cut-off of ≤ 10^{-15}) in the following databases: SwissProt (Magrane and Consortium, 2011), WormBase (*Caenorhabditis elegans*) (Yook et al., 2012), MEROPS (peptidase and peptidase inhibitors) (Rawlings, 2009), Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto, 2000) and SPD (secreted proteins) (Chen et al., 2005). Conserved domains and Gene Ontology (GO) (Dimmer et al., 2012) annotations were identified by InterProScan (cf. subsection 2.2). Signal peptide and transmembrane domains were predicted for individual protein sequences using the program Phobius (Kall et al., 2004). Homologs with roles in parasite-host interactions were predicted (BLASTp; E-value cut-off: ≤ 10^{-15}) using a curated, in-house database established from published data (Hartman et al., 2001; Zhan et al., 2002; Basavaraju et al., 2003; van Rossum et al., 2004; Gregory and Maizels, 2008; Ranjit et al., 2008; Bath et al., 2009; Cuellar et al., 2009; Hewitson et al., 2009; Klotz et al., 2011). Annotations from KEGG BRITE and the in-house parasite-host interaction database were merged, and functional categories of the two transcriptomes were defined. The portion of inferred proteins of *D. filaria* genes
with homologs in *D. viviparus* or *C. elegans* was defined using the BLASTp algorithm (E-value cut-off: ≤ 10\(^{-15}\)).

### 2.4. Differential transcription, and comparative analyses

To calculate the numbers of genes differentially transcribed between *D. filaria* and *D. viviparus* adults, 1:1 orthologs were identified using a specifically constructed workflow (Fig. 1): First, orthologs between *D. filaria* and *D. viviparus* were defined using the program InParanoid (Ostlund et al., 2010), employing stringent criteria. Specifically, a match area (i.e. percentage of longest amino acid sequence alignment) of 50% was used identify homology (using the substitution matrix BLOSUM80), and a confidence score of 10\(^{-3}\) was used identify paralogs; *C. elegans* was used as an outgroup. *D. filaria* and *D. viviparus* homologs were discarded if at least one of them had a higher similarity to a *C. elegans* homolog. Paralogs predicted were quarantined from the analyses. Second, differentially transcribed 1:1 orthologs of *D. filaria* and *D. viviparus* were identified. For each of the two *Dictyocaulus* species, RNA-seq data were mapped against their own transcriptome using BWA (Li and Durbin, 2009), permitting up to 5% nucleotide mismatches and 3 nucleotide gaps per aligned read. Third, the number of mapped RNA-seq reads per transcript was calculated using the idxstats tool in the SAMtools software package (Li et al., 2009), and the number of mapped reads per transcript was adjusted to be proportional to the number of contigs assembled for each species. Fourth, for each pair of 1:1 orthologs, mapped reads were normalized for variation in transcript length by dividing the number of mapped reads by the length of the transcripts (in nucleotides) to which they mapped (Li et al., 2010). Fifth, individual mapped reads were normalized for scaling artefacts using the trimmed mean of M-values (TMM) method (Robinson and Smyth, 2007; Dillies et al., 2012). The difference in transcription between 1:1 orthologs was expressed as a log\(_2\) fold-change (FC) in gene transcription; an FC of at least four (i.e. log\(_2\)(4)=2) was recorded as significant. Transcription levels of the ‘housekeeping’ genes encoding β-tubulin, protein disulfide isomerase-1 (pdi-1), elongation factor 1-α (ef1-α) and glyceraldehyde 3-phosphate dehydrogenase (gapdh) were used to assess the method of data normalization (Strube et al., 2008).

Proteins encoded by orthologous genes differentially transcribed between *D. filaria* and *D. viviparus* adults were categorized by homology to proteins in the KEGG BRITE and parasite-host interactions databases (cf. subsection 2.3). For each species, the numbers of highly transcribed genes of individual categories were calculated, and categories with the greatest differences in numbers between the two species were compared. In addition, full-length sequences of type 2-like cystatin homologs, identified (BLASTp or tBLASTn, E-value ≤ 10\(^{-65}\)) in the transcriptomes of *D. filaria* and *D. viviparus*, and in the transcriptomes and/or genomes of other, key nematodes (Table 3) were compared. Amino acid sequences were selected as homologs if: (i) were predicted (Zdobnov and Apweiler, 2001) to encode one or more cystatin I25 inhibitor domains (InterPro proteinase inhibitor I25, cystatin-like domains IPR000010, IPR018073 and IPR020381); (ii) contained a predicted signal peptide domain; (iii) had four or more conserved motifs necessary for the inhibition of papain-like (MEROPS C01 family) and/or asparaginyl endopeptidase-like peptidases (MEROPS C13 family) (Gregory and Maizels, 2008) (see Table 3); and (iv) encoded a single proteinase inhibitor I25 domain (Abrahamson et al., 2003). All homologous sequences were then aligned using MAFFT (Katoh and Standley, 2013), and the alignment was adjusted manually. Then, a phylogenetic analysis of these sequence
data was conducted using a Bayesian inference (BI) method (MrBayes Ronquist et al., 2012), employing the ‘Whelan and Goldman’ amino acid model (Whelan and Goldman, 2001) and using the final 75% of 2.3 million Markov chain Monte Carlo iterations (Metropolis et al., 1953; Hastings, 1970; Geyer, 1992) to construct a 50% majority rule tree; nodal support was expressed as a posterior probability (pp).

3. Results

3.1. Characterization and comparison of the transcriptomes of adult D. filaria and D. viviparus

Optimum transcriptomes of D. filaria (k-mer value: 25; read coverage cut-off: 10) and D. viviparus (k-mer value: 51; read coverage cut-off: 8) were assembled (Table 1) and selected for direct comparative analyses. Totals of 11,859,459 and 15,334,902 paired-end mapped reads were selected for further analyses. The transcriptomes of adult D. filaria and D. viviparus were inferred to encode totals of 14,523 and 18,203 proteins, respectively (Table 1), including > 80 % of 248 core eukaryotic proteins (based on CEGMA). In total, 8,611 (59.3%) and 9,649 (53.0%) of all proteins predicted were annotated (E-value cut-off of ≤ 10^{-15}) for D. filaria and D. viviparus, respectively (Table 1), with most (8,324 for D. filaria, and 9,647 for D. viviparus) having homology to non-hypothetical genes or protein sequences present in the NCBI non-redundant and SwissProt databases. For D. filaria, a total of 4,338 predicted genes could be classified into 38 unique protein KEGG classes, and 2,758 into 285 unique KEGG pathways. For this species, 7,237 conserved domains were identified, allowing 6,188 genes to be assigned 1,417 unique GO terms. For D. viviparus, a total of 5,017 predicted genes could be classified into 44 unique protein KEGG classes, and 3,120 into 303 unique KEGG pathways. For this species, 8,153 conserved domains were identified, allowing 6,725 genes to be assigned 1,377 unique GO terms.

A comparison showed that 9,152 (63.0%) of the 14,523 sequences of D. filaria had homologs in D. viviparus; 7,969 (54.9%) had homologs in C. elegans, of which 949 were not detected in D. viviparus. Of 4,423 (30.5%) D. filaria sequences, with no homologs detected in either C. elegans or D. viviparus, 310 (7%) had homologs (E-value cut-off: ≤ 10^{-15}) in other nematodes, including Ascaris suum, Necator americanus, Oesophagostomum dentatum, Trichostrongylus colubriformis and Trichuris suis, and 44 (1%) with homology to non-nematode protein sequences in public databases. The proteins predicted from the transcriptomes of adult D. filaria and D. viviparus were assigned to 14 functional categories (see Fig. 2A). Despite the similarity in number of proteins in each category between these two species, key differences related mainly to “membrane transport” (Fig. 2A), including major facilitator superfamily (MFS) transporters (45 vs. 23), the phosphotransferase system (0 vs. 11) and the ATP-binding cassette (38 vs. 28).

3.2. Differential transcription between D. filaria and D. viviparus

The comparison between the two transcriptomes identified 5,075 putative orthologs (1:1) between adult D. filaria and D. viviparus, of which 697 were differentially transcribed. The four house-keeping genes used here were constitutively transcribed (Fig. 3) within the limits of significance defined. D. filaria transcripts (n =
486; Fig. 2B) inferred to be enriched related mainly to host interactions (with a ratio between the number of genes highly transcribed in *D. viviparous* and those in *D. filaria* of < 0.11; 0 vs. 19), protein folding/degradation (0.11; 1 vs. 9), mRNA translation (0.18; 14 vs. 76), ubiquitin/proteasome system (0.25; 6 vs. 24), cell mobility (0.25; 2 vs. 8), and peptidases (0.29; 2 vs. 7). *D. viviparous* transcripts inferred to be enriched represented mainly kinases (with a ratio between number of genes highly transcribed in *D. filaria* and *D. viviparous* of 0.45; 5 vs. 11) and membrane transport (0.5; 2 vs. 4) (Fig. 2B). The most pronounced differences between *D. filaria* and *D. viviparous* related to genes inferred to be involved in parasite-host interactions, such as transthrein-like (TTL) proteins (n = 4), lipase and fatty-acid and/or retinol-binding proteins (3), β-galactoside-binding lectins (2), peroxidases (2) and glutathione S-transferase (2), cathepsin B (1) and type 2-like cystatins (1). The latter protein was of particular interest, because there is a substantial body of knowledge surrounding its dual role in immunomodulation and metabolism in parasitic nematodes (Hartmann et al., 1997; Dainichi et al., 2001; Manoury et al., 2001; Schonemeyer et al., 2001; Pfaff et al., 2002); immunomodulation appears to relate to (relatively) conserved sequence motifs (Hartmann and Lucius, 2003; Gregory and Maizels, 2008; Klotz et al., 2011). For this reason, we explored, in detail, the relationship of this protein to homologs from a range of other nematodes.

### 3.3. Analysis of the type 2-like cystatins in *D. filaria* and *D. viviparous* and selected nematodes

The type 2-like cystatins predicted for *D. filaria* and *D. viviparous* were compared with homologs in other nematodes (Table 3), accessible from public databases (Elsworth et al., 2011; Martin et al., 2012; Yook et al., 2012; Mangiola et al., 2013). Assisted by the identification of type-2 cystatin domains, 43 full-length homologs were identified in 24 other species of nematodes (Table 3). Overall, based on alignment, the amino acid sequences had similar features (Table 3; Fig. 4) but there were some differences among them.

The signal peptide was present in most sequences, except for protein *Na-CPI/a* (*N. americanus*) (Fig. 4). The three ‘functional’ motifs involved in the binding to the cathepsins B, L and S (C1 papain-like peptidases) (Bode et al., 1988; Alvarez-Fernandez et al., 1999) were relatively conserved among sequences (Fig. 4). However, there were some variations: (i) the conserved glycine residue at the N-terminus of the mature peptide was absent from the proteins *Bm-CPI-1* and *Bm-CPI-3* (*Brugia malayi*); (ii) the central cystatin-specific flexible loop glutamine-X-valine-X-glycine (QXVXG) was absent from *Bm-CPI-3* (*B. malayi*); (iii) the proline-tryptophan (PW) hairpin loop-pair at the C-terminus of the mature peptide was absent from *Av-CPI* (*Acanthocheilonema viteae*); (iv) the disulfide bond, essential for positioning the N-terminal glycine to the right spatial conformation (Bode et al., 1988), was absent from protein *Sr-CPI* (*S. ratti*). Interestingly, a total of four sequences for *As-CPI/b* (*Ascaris suum*), *Tsp-CPI/a*, *Tsp-CPI/b* (*Trichinella spiralis*) and *TsU-CPI/a* (*Trichuris suis*) (Table 3) were predicted to form a second disulfide bond at the C-terminus of the protein (Fig. 4), similar to the cystatin of chicken (*Gallus gallus*) (Bode et al., 1988) and mammals, such as cattle (*Bos taurus*), sheep (*Ovis aries*) and humans (*Homo sapiens*) (Gregory and Maizels, 2008; Klotz et al., 2011). The C13 legumain-like asparaginyl endopeptidase (AEP)-binding loop motif was present in some sequences but absent from others, which likely relates to the evolution of type 2-like cystatins in nematodes (Fig. 5).
To explore the evolutionary relationships of type 2-like cystatins of *D. filaria* and *D. viviparux* with respect to the 43 homologs from other nematode species, an alignment of amino acid sequences was made (Fig. 4), taking into account key motifs (cf. Alvarez-Fernandez et al., 1999). The Bayesian inference (BI) analysis, characterized by an average standard deviation of split frequencies of 0.012 and an average potential scale reduction factor (PSRF; excluding NA and >10.0) of 1.004 (cf. Ronquist et al., 2012), showed three main clusters of type 2-like cystatin sequences (A-C) (Fig. 5). Cluster A comprised sequences of nematodes of the orders Strongylida and Rhabditida, including the superfamilies Ancylostomatoidea, Metastrogyloidea, Strongyloidea and Trichostrongyloidea (pp = 0.87), whereas the well-separated clusters B and C both represented nematodes of the superfamily Filarioidea (pp = 1.00 and 0.99, respectively). In addition to the genes that clustered, sequences representing the Strongyloidea related to branch a, whereas others representing superfamilies Ascaridoidea, Strongyloidoidea, Trichuroidea and Trichinelloidea were dispersed on branch c; for the majority of them, nodal support (pp values of < 0.70) was insufficient to infer clusters for these sequences. In the aligned amino acid sequence region encoding an AEP-like motif, four and eight of 19 sequences within cluster A contained SNA and SNE motifs, respectively, and two contained an SND/SNS motif (Table 3). In contrast, six of eight sequences in cluster B contained SND/SNS motifs. None of the five sequences in cluster C had an SNE, SNA or SND/SNS motif. Although the codon usage for the AEP motif was relatively conserved within a particular nematode order, it was variable for the SND/SNS motifs (Table 3). For a small number of sequences in each cluster, one or more motifs linked to the binding of cysteine proteases or signal peptides were lacking.

4. Discussion

Here, we employed a bioinformatic workflow to compare, both qualitatively and quantitatively, the transcriptomes of the adult stages of *D. filaria* and *D. viviparux*, in the absence of reference genomes. Following the sub-selection of annotated 1:1 orthologs, we investigated differential transcription between these two closely related species. This analysis enabled us to focus on exploring a select group of molecules in *D. filaria* inferred to be enriched and involved in establishing and maintaining the host-parasite relationship.

4.1. Similarities between *D. filaria* and *D. viviparux* adults at the molecular level

More than half of predicted proteins conceptually translated from adult transcriptome of *D. filaria* (*n* = 14,523) and *D. viviparux* (*n* = 18,203) were homologous (*n* = 9,152; BLASTp, E-value ≤ $10^{-15}$), reflecting the similarity in biology of the two species. Only 7% of the ‘orphan’ genes in *D. filaria* could be inferred to encode functional proteins by homology comparisons with sequences in public databases. As expected, the molecular similarity between *D. filaria* and *D. viviparux* was supported by comparable numbers of encoded proteins in individual functional categories (cf. Fig. 2A). Nonetheless, in spite of the similarities, there were some differences between the two species. For instance, in *D. viviparux*, the larger number of transcribed genes encoding MFS transporters and ATP-binding molecules within the category ‘membrane transport’ appears to be linked to the efflux of drugs (De Rossi et al., 2002), suggesting that this parasite might have a greater propensity to
evade the effect of drugs. To gain an improved understanding of molecular differences between the two lungworms, we quantitatively assessed differential transcription in protein encoding genes.

4.2. A small subset of differentially transcribed orthologs relates to parasite-host interactions

The number of genes inferred to be transcriptionally enriched was greater in *D. filaria* (*n* = 486) than in *D. viviparus* (*n* = 211) (Fig. 3). This finding is also supported by an enrichment in *D. filaria* of the functional categories ‘mRNA translation’ and ‘protein folding/degradation’ (Fig. 2), and of genes involved in the oxidative phosphorylation pathway (Falk et al., 2008; Cox, 2013). Even considering the ratio in the numbers of genes highly transcribed between *D. filaria* and *D. viviparus*, the enrichment of the functional category ‘parasite-host interactions’ (*sensu lato*) in *D. filaria* was considerable (cf. Table 2).

Within the category ‘parasite-host interactions’, one group of enriched transcripts in *D. filaria* represented a peroxidase and a glutathione S-transferase (GST). In some worms, peroxidases are reported to represent a component of the excretory/secretory (ES) products and appear to protect parasite lipids from reactive oxygen species (ROS) produced by phagocytes (Henkle-Dührsen and Kampkötter, 2001; Melendez et al., 2007; Chiumiento and Bruschi, 2009); and, GSTs represent major phase-II detoxification enzymes (Jaffe and Lambert, 1986; Kampkotter et al., 2003; Yadav et al., 2010), which are reported to neutralize cytotoxic products arising from ROS to protect nematodes, such as filaria, against host immune responses (Jaffe and Lambert, 1986; Brophy and Pritchard, 1994; Kampkotter et al., 2003; Yadav et al., 2010).

Another group of transcripts inferred to be enriched in *D. filaria* likely represents proteins involved in immunomodulation; such molecules include beta-galactoside-binding lectins (= galectins), known inhibitors of metalloproteases, SCP/Tpx-1/Ag5/PR-1/Sc7 (SCP/TAPS) and secreted proteins rich in cysteine (SPARC) (Table 2). Specifically, galectins are known to be abundant in some other nematodes, such as *Haemonchus contortus* (see Greenhalgh et al., 2000), and can interfere with antigen uptake and presentation, cell adhesion, apoptosis and T cell polarization in the host (Hewitson et al., 2009). Some secreted metallopeptidase inhibitors identified here have orthologs in, for example, *A. caninum* (Zhan et al., 2002) and *A. ceylanicum* (Mitreva et al., 2005), and can also modify host immune responses (Cuellar et al., 2009). For example, *Ac-TMP-1* of *A. caninum* has been reported to modify dendritic cell function and promote the production of CD4 and CD8 suppressor T cells, which produce anti-inflammatory cytokines (e.g., IL-10) (Cuellar et al., 2009). In *D. filaria*, SCP/TAPS proteins might also be involved in regulating or altering some immune responses and/or can play a role in host invasion (Cantacessi and Gasser, 2011). This statement is supported somewhat by the proposal that the SCP/TAPS protein *Na-ASP-2* of the hookworm *N. americanus* is an antagonistic ligand of complement receptor 3 (CR3) (Asojo et al., 2005; Bower et al., 2008). Because of their known role in preventing cell adhesion, SPARC have been proposed to alter host immune responses by interfering with lymphocyte migration (Brekken and Sage, 2001).

In addition, some genes highly transcribed in adult *D. filaria* encoded proteins with a predicted role in immunomodulation and/or metabolism. These proteins include fatty acid- and retinol-binding proteins, TTL proteins, cathepsins B and
cystatins. Fatty acid- and retinol-binding proteins identified in *D. filaria* were predicted to have secretory signals, and have been identified previously in ES proteins from some parasites, such as *A. caninum* (Zhan et al., 2003). Besides their known role in nutrient acquisition (Mei et al., 1997; Kennedy, 2000; McDermott et al., 2002), these proteins are proposed to play an active role in the establishment or maintenance of infection. The ability of fatty acid- and retinol-binding proteins to sequester retinol – which is involved in the synthesis of collagen, tissue repair (Bulger and Helton, 1998) and IgA production (Nikawa et al., 1999) – suggests that these proteins might regulate immune responses in the host animal (McSorley et al., 2013). In addition, TTL proteins were encoded and identified as secretary molecules, and have been characterized previously in ES products of the related strongylid nematodes *Ostertagia ostertagi* and *H. contortus* (Hewitson et al., 2009), and of distantly related nematodes, including *B. malayi* and *D. immitis* (Geary et al., 2012). Hewitson et al. (Hewitson et al., 2009) hypothesized that some TTL proteins also bind retinoids and, thus, might be involved in immunoregulatory processes in *D. filaria*.

Transcripts encoding cathepsin B proteins were predicted as enriched in the transcriptome of adult *D. filaria*. These cysteine peptidases are an important component of the degradome of nematodes, including haematophagous worms (Williamson et al., 2003; Cantacessi et al., 2010a; Cantacessi et al., 2010b; Knox, 2011; Mangiola et al., 2013; Schwarz et al., 2013). Cathepsin-like cysteine peptidases are enzymes involved in the digestion of blood, and are also involved in other key biological processes, including the establishment and maintenance of infection in the host (Tort et al., 1999; Williamson et al., 2003; McKerrow et al., 2006). As most cathepsins play a general role in intracellular protein metabolism, they are usually tightly regulated and expressed as inactive zymogens, which contain cysteine peptidase inhibitor domains and protect cells from proteolytic damage (Dickinson, 2009). In addition to cathepsin B, a *D. filaria* type 2-like cystatin was enriched in the transcriptome. Type 2-like cystatins are an important group of endogenous proteins that inhibit cysteine peptidases, including members of the papain- (C01) and AEP-like (C13) peptidases. In parasites, excreted/secreted cystatins are also potent inhibitors of host peptidases, and can modulate the host immune response via the inhibition of host cathepsins and AEPs that are required for antigen processing/presentation and/or the regulation of pattern recognition receptor signaling (see Vray et al., 2002; Klotz et al., 2011). Secreted cystatins of some parasitic nematodes can also down-regulate inflammation by employing multiple immunological pathways in the host, for example, by reducing Th2-related inflammation via the induction of IL-10 cytokine production in macrophages (Schnoeller et al., 2008). Interestingly, a number of the host immune-modulatory characteristics of nematode secreted type 2-like cystatins appear to have evolved independently from those that inhibit peptidases (Klotz et al., 2011). Exploring the levels of conservation in protein domain sequences revealed various groups of type 2-like cystatins among 26 nematode species (Figs. 4 and 5).

4.3. Type-2-like cystatins of *Dictyocaulus spp.* are proposed to play an important immunomodulatory role

Cystatins were of particular interest because of the differential transcription between *D. filaria* and *D. viviparusb*, and because of their role in immunomodulation and/or metabolism in parasitic nematodes (Hartmann et al., 1997; Dainichi et al., 2001; Manoury et al., 2001; Schonemeyer et al., 2001; Pfaff et al., 2002). The type 2-like cystatins predicted for *Dictyocaulus* and orthologs of related strongylid
nematodes seem to have co-evolved with cystatins from distantly related parasitic nematodes (cf. Table 3; Fig. 5). However, the phylogenetic relationships of all type 2-like cystatins of nematodes are not consistent with the evolution of the nematodes themselves. For parasitic nematodes, the role of cystatins as endogenous and/or host-derived papain-like cysteine peptidase inhibitors appears to be relatively conserved, with various nematode species, including D. filaria and D. viviparus, retaining at least one type 2-like cystatin with the three conserved domains that interact with papain-like cysteine peptidases (Bode et al., 1988). In contrast, the ability of nematode cystatins to inhibit host AEP-like cysteine peptidases is not a characteristic shared by all nematode cystatins studied to date, rather a trait acquired by some parasites to enhance their capacity to evade immune responses (Manoury et al., 2001; Murray et al., 2005). The presence of a relatively conserved SND/SNS domain in cystatins is not restricted to a particular nematode order, but is dispersed among nematode groups (Fig. 4). Interestingly, an alignment of the type 2-like cystatin sequences of key parasitic nematodes (Fig. 4) reveals an amino acid substitution in this domain in strongylid and rhabditid nematodes compared with other representatives. Furthermore, an alignment of the AEP-motif coding domain suggests that a functional AEP binding motif (encoding an SND/SNS) evolved independently more than once, being lost from a common ancestor of the Strongylida/Rhabditida and then re-emerging, possibly via convergent evolution, as an isoform of the type 2-like cystatins of H. contortus, N. americanus and Pristionchus pacificus. This is consistent with the hypothesis that parasitism evolved more than once in the Phylum Nematoda (Blaxter et al., 1998), and suggests that the AEP-motif plays an important role in nematode-mammalian host interactions. At this point, the ability of type 2-like cystatins of D. filaria and D. viviparus to inhibit host AEP is uncertain. Findings from a functional study of the free-living nematode C. elegans suggest that a cystatin encoding an AEP inhibitory site with an alternative polar/hydrophilic amino acid residue at the third position (SNN) does not inhibit mammalian AEP (Murray et al., 2005). Nonetheless, a negatively charged, polar amino acid residue at the third position of the conserved AEP inhibitory site (SNE) (Table 3; Fig. 4) suggests that a broader range of nematode cystatins may inhibit host AEP and function via the disruption of antigen processing/presentation (Murray et al., 2005). Certainly, in other studies, substituting glutamic acid (E) for aspartic acid (D) did not result in a loss of the conserved structure or of function (Vik and Antonio, 1994; Ellis et al., 1995). Current evidence (Table 3; Fig. 4) suggests that conserved domains appear to have been lost upon cystatin gene duplication, which might relate to a loss of cysteine peptidase inhibition of respective cystatins. A striking example is B. malayi, whose genome encodes three type 2-like cystatins, two of which are developmentally regulated and do not retain the conserved domains required for peptidase inhibition. A loss of conserved domains linked to cysteine peptidase inhibition was also observed for other nematodes in which more than one copy of the cystatin gene was encoded in genomic/transcriptomic data sets (Table 3; Fig. 4).

Experimental evidence for various parasitic nematodes (Klotz et al., 2011), including strongylids, suggests that cystatins expressed in the adult stages of D. filaria and D. viviparus might play one or multiple roles in modulating host immunity, independent of inhibition of host cysteine peptidases. For instance, cystatins of some parasitic nematodes retain a conserved mechanism to modulate cytokine production (Hartmann et al., 2002) and/or nitric oxide (NO) synthesis in antigen-presenting cells in the host animal (reviewed by Klotz et al., 2005), but are not linked to a common immune pathway. While cystatins of selected filarial nematodes, for example,
induced NO production in IFN-gamma-primed macrophages in a TNF-alpha and IL-10-dependent manner (Hartmann et al., 1997; Schnoeller et al., 2008), cystatins of other nematodes can stimulate NO production in an IL10-independent manner (Garraud et al., 1995). Clearly, based on current opinion (Klotz et al., 2011), particular structures of cystatins secreted by parasitic nematodes are likely to be critical in maintaining an effective interplay with the host animal. Interestingly, Hartmann et al. (Hartmann et al., 2002) demonstrated also that the N-terminal region of cystatins of some filarial nematodes is essential for cysteine peptidase inhibition, but not for the induction of NO in host macrophages. Future study, focused on the conserved structures within the C-terminal region of these proteins, is needed to assess the functionality of conserved (secondary or tertiary) domains/motifs. This work could be done by cloning an array of genes encoding secreted cystatins from non-filarial nematodes, expressing these proteins and testing them on host antigen-presenting cells to elucidate their immunomodulatory capacities.

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