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RESEARCH ARTICLE

Gene expression and allergenic potential of *Pseudoterranova bulbosa* L3 from different infection sites in North Atlantic cod (*Gadus morhua*)

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Abstract

The recent advances in molecular methods and data processing have facilitated research on anisakid nematodes. While most research efforts were made regarding the genus *Anisakis*, since this genus is held responsible for the majority of reported clinical signs, there is still a demand for data on the genus *Pseudoterranova*. Several case studies of severe invasive anisakidosis affecting various organs caused by species of the *P. decipiens* complex have been described. To better understand the way these parasites might infest their fish host, we examined whether parasite location within the fish host affects gene expression. A de novo assembly of the transcriptome of *Pseudoterranova bulbosa*, isolated from North Atlantic cod, was analysed for patterns of differential gene expression between samples taken from liver and viscera. We additionally searched for homologs to known nematode allergens, to give a first estimate of the potential allergenicity of *P. bulbosa*. There was a subtle difference in the gene expression of samples taken from liver and viscera. Seventy genes were differentially expressed, 32 genes were upregulated in parasites isolated from liver and 38 genes were upregulated in parasites from viscera. Homologs of five nematode allergens were identified among the genes expressed by *P. bulbosa*. Our transcriptome of *P. bulbosa* will be a valuable resource for further meta-analyses and resequencing projects.

KEYWORDS
Anisakidae, differentially expressed genes, human pathogenic Nematoda, seafood allergy, transcriptome

1 | INTRODUCTION

The occurrence of zoonotic nematodes of the family Anisakidae in food fish has gained awareness during the 1990s (Bouree et al., 1995; Hochberg & Hamer, 2010), and not only provoked a high research interest but also the establishment of food safety measures. While past studies on human-pathogenic anisakids mainly focused on molecular species identification (Bullini et al., 1997; D'Amelio et al., 2000; Mattiucci et al., 1998, 2001, 2002, 2005, 2009, 2014, 2016; Nascetti et al., 1986, 1993; Paggi et al., 1991, 2000; Paggi & Bullini, 1994; Santoro et al., 2010; Timi et al., 2014; Umehara et al., 2008; Zhu et al., 2002) , the recent advances of sequencing methods and computing power have made molecular studies more affordable and have opened up new perspectives for the research

on parasites, including genomic, proteomic and transcriptomic studies (Baird et al., 2016; Bušelić et al., 2018; Cavallero et al., 2018, 2020; Consortium International Helminth Genomes, 2017; D'Amelio et al., 2020; Fæste et al., 2014; Kim et al., 2018; Kochanowski et al., 2019, 2020; Nam et al., 2020).

Anisakidosis is a collective term for different clinical pictures caused by marine Nematoda from the family Anisakidae (Hochberg & Hamer, 2010). It includes signs caused by the tissue invasion behaviour of the worms causing lesions in the invaded tissues and allergic signs caused by parasitic antigens present in the family Anisakidae (Hochberg & Hamer, 2010). Most cases of Anisakidosis are attributed to the eponymous genus *Anisakis*, but morphological or molecular identification methods are rarely applied (Rahmati et al., 2020). Clinical signs have been linked to *A. simplex s.s.*, *A. pegreffii*, *Contracaecum* and *Pseudoterranova* spp. infections (Buchmann & Mehrdana, 2016; Rahmati et al., 2020; Shamsi & Sheorey, 2018). Medical case studies of *Pseudoterranova* infections show their pathogenic potential and relevance (Arcos et al., 2014; Arizono et al., 2011; Brunet et al., 2017; Cavallero et al., 2016; Llorens et al., 2018; Menghi et al., 2020; Mercado et al., 1997, 2001; Mitsuboshi et al., 2017; Murata et al., 2018; Nordholm et al., 2020; Pinel et al., 1996; Ramanan et al., 2013; Sánchez-Alonso et al., 2020; Suzuki et al., 2021; Timi et al., 2014; Torres et al., 2007; Weitzel et al., 2015).

The migrating behaviour of Anisakidae within the fish host leads to infections of muscle tissue and has therefore become a major concern for seafood safety. The phenomenon of migrating from common infection sites within the body cavity (e.g., liver and viscera) into muscle tissue has mostly been observed after the host's death, in positive relation to storage temperature and duration (Cattan & Carvajal, 1984; Cipriani et al., 2016; Šimat et al., 2015; Smith & Wootten, 1975). Processing and gutting fish promptly after catching has proven to be an effective prevention of heavy infestation of fish fillets with anisakid nematodes (Klapper et al., 2015). Food safety institutions impose visual inspections and requirements for storage (deep freezing at −20°C for 7 days, freezing at −35°C until solid and subsequently store either at −35°C for 15 h or at −20°C for 24 h) and preparation (cooking for at least 1 min at 60°C) to prevent transmission of live larvae to humans when consuming fishery products (FDA, 2021).

The understanding of the pathogenicity of anisakid nematodes was facilitated by the published genome of *Anisakis simplex s.s.* (Consortium International Helminth Genomes, 2017) and transcriptomes of *A. simplex s.s.* (Baird et al., 2016), *A. pegreffii* (Nam et al., 2020) and their hybrids (Cavallero et al., 2018), which enabled resequencing projects and meta-analyses. However, there is still a demand for data on other pathogenic marine nematodes relevant for food-safety.

The drivers for parasite migration and distribution within their host are still under debate. Hypotheses discussed in the literature are physical proximity of organs or tissues adjacent to the stomach (Quiazon et al., 2011; Strømnes & Andersen, 1998; Sukhdeo & Sukhdeo, 1994; Young, 1972), nutrient uptake (Strømnes & Andersen, 2003), evasion of the host's immune system (Buchmann, 2012) and beneficial properties, e.g., lipid tissue (Strømnes, 2014). Our study set out to assess whether and how parasite larval gene expression differs between infection sites within their intermediate host. We observed a distribution of *Pseudoterranova bulbosa* in different microhabitats, liver and viscera, within the body cavity of Atlantic Cod (*Gadus morhua*) caught off Greenland. Since aggregations of anisakid larvae have often been observed in the liver of fish hosts (Klapper et al., 2018; Muñoz-Caro et al., 2022; Nadolna & Podolska, 2014; Najda et al., 2018; Severin et al., 2020), we hypothesized that this effect could become visible in a differential gene expression analysis, giving insight into the activities (e.g., feeding on blood or tissue in the liver) of anisakid L3 within their paratenic fish host.

Trace amounts of allergens can represent a health hazard to sensitized patients. Currently, 19 nematode allergens are known (WHO/IUIS Allergen Nomenclature Database; Pomés et al., 2018), which are potentially cross-reactive with other allergens and have known homologs in other nematodes and invertebrates. Therefore, we screened the *P. bulbosa* transcriptome to assess whether homologs of these allergens were expressed.

2 | MATERIAL AND METHODS

In this study, we analysed the transcriptomes of *Pseudoterranova bulbosa* samples taken from different infection sites (liver and viscera) in their intermediate fish host, *Gadus morhua*. The transcriptomes were screened for the expression of anisakid allergens to study their potential release into fishery products for human consumption.

2.1 | Sampling

Parasite samples ($n = 54$) were taken during the 341st expedition of the German fishery research vessel Walther Herwig III in October 2019 during a bottom-fish survey off Greenland (Figure 1; Table S1; Table S2). Live *Pseudoterranova* sp. were isolated from twelve Atlantic cod (*Gadus morhua*) individuals, with coincident infections of liver and viscera. Host tissue and cyst tissue were removed in a petri dish with autoclaved 0.9% saline solution, using tweezers. The parasite individuals were washed in fresh saline solution. A sample for molecular barcoding was taken at the apical end of the worm, measuring approximately 1/5 of the parasite's length. Tweezers and scissors were cleaned between each sample. Barcoding samples were stored in individual reaction tubes with 99% ethanol. To preserve RNA and inhibit RNase activity, samples were treated with RNAlater (Thermo Fisher Scientific) or TRIreagent (Sigma-Aldrich) (Table S1) according to the manufacturer's protocol and stored at −20°C during the expedition and subsequently at −80°C. The choice of RNA stabilizing media was arbitrary.

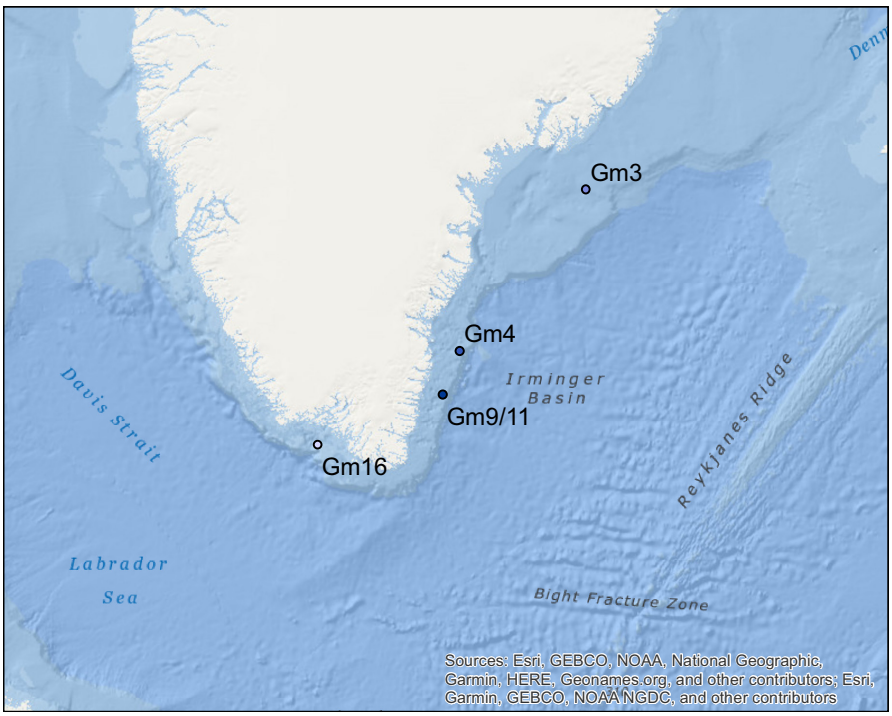

FIGURE 1 Sampling sites of *P. bulbosa* from their intermediate host *Gadus morhua* off Greenland coast. Sampling point and host identifier (Gm3, Gm4, Gm9, Gm11 and Gm16). Figure created with Esri ArcGIS

2.2 | Sample selection

Parasite species were identified with DNA-barcoding of the mitochondrial cytochrome oxidase subunit I (COI) marker (COIf 5′-CTACTACTAAGAATTTGCGT-3', COIr 5′-AATCCAAATACTTACGAGGA-3′, Arizono et al., 2011). The parasites were genotyped using the internal transcribed spacer (ITS+; NC5 5′-GTAGGTGAACCTGCGGAAGGATCATT-3′, NC2 5′-TTAGTTTCTTTTCCTCCGCT-3′; Zhu et al., 1998). Anisakid nematode larvae have a relatively long residence time within their fish host (Smith, 1984). Genotyping was used to select parasite samples from the same population. To reduce the genetic variation of 'host reaction' parasite gene expression, which could influence gene expression patterns, only sample pairs of both infection sites from the same host individual, with the same genotype (according to the ITS+marker) were selected for RNA sequencing. This selection resulted in 14 samples from five host individuals (Table S1, Figure S1).

2.3 | RNA isolation and sequencing

To extract mRNA from the samples a TRIreagent phenole-chloroform extraction for tissue was performed according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, USA) with 95 µl TRIreagent used as lysis buffer. The purity of the RNA isolates was assessed through optical density measuring using a spectrophotometer (Nanodrop, Thermo Fisher Scientific, Waltham, USA). RNA contents were quantified with the Qubit fluorometric quantitation assay (Thermo Fisher Scientific, Waltham, USA).

Library preparation and sequencing of seven sample pairs from both infection sites (liver, viscera) in the same host fish was carried out by the external service provider Novogene UK Company Limited (Cambridge, United Kingdom). The library was prepared with the NEB Next® Ultra™ RNA Library Preparation Kit using a 250–300 bp insert. Sequencing of 150 bp paired end reads was carried out on the Illumina NovaSeq 6000 platform (Illumina Inc., San Diego, USA) for each of the individual 14 samples. The aimed sequencing yield was 8 Gb which we obtained for 13 samples (Table S1, Figure S1).

Data are available under study accession number PRJEB42010.

2.4 | Transcriptome assembly, mapping and annotation

The quality of raw reads for each sample was assessed with FastQC version 0.11.9 (Andrews, 2019). As the overall quality was very high, all further steps were performed with untrimmed reads. To identify potential contamination as such, and putative fish transcripts stemming from food uptake, a taxonomic classification was performed using the tool Kraken 2 version 2.1.0 (Wood et al., 2019). The aim of filtering was a dataset including all unclassified reads combined with nematode reads, and a read count for fish reads. As a reference for the most likely contamination, transcripts of cod (Table S3) and a standard library for protozoa and humans were used. A positive control for nematode sequences was designed by including Ascaridoid nematode transcripts (Table S3) accessed through WormBase ParaSite (Howe et al., 2016, 2017). The R-package pavian version 1.0 (Breitwieser & Salzberg, 2020) was used to get an overview of the Kraken 2 output. To filter all reads classified as Nematoda, we

used kraken-tools version 0.1-alpha (Lu et al., 2020) and extracted reads with the respective taxon-ID.

The software Trinity version 2.11.0 was used to assemble the de novo transcriptome of *Pseudoterranova bulbosa* (Grabherr et al., 2011; Haas et al., 2013) including all filtered reads of the 13 samples and using standard settings. To assure that filtering the raw data did not limit the quality of the reference transcriptome, we created and compared the assembly based on the filtered transcript to an assembly based on raw reads. The filtered and raw transcripts were translated into the predicted protein code with TransDecoder version 5.5.0 (Haas, 2018), which accounts for both read directions and all three different reading frames.

The Benchmarking Universal Single-Copy Ortholog (BUSCO, http://busco.ezlab.org) version 4.1.4 was used as a tool for the quality control of the assembly (Simão et al., 2015), to assess the completeness and number of conserved genes in the NCBI nematoda_odb10 reference database (downloaded 27. Nov. 2019). HISAT2 version 2.2.1 (Kim et al., 2019) was used to map the raw reads to the transcriptome. The software Kallisto version 0.46.1 was used to quantify the read counts of the filtered assembly (Bray et al., 2016). Output data were processed into a matrix of isoform counts using a pearl script provided with Trinity. Isoforms were chosen to account for different possible splice variants of genes.

Annotation of the filtered transcriptome was performed using BLASTX version 2.12.0 using the invertebrate protein database, downloaded from NCBI (10 Nov. 2020, ftp://ftp.ncbi.nlm.nih.gov/refseq/release/invertebrate/*protein.faa.gz; Altschul et al., 1990; Gish & States, 1993). Protein classification was predicted using InterProScan version 5.47–82.0 (Jones et al., 2014).

2.5 | Differential gene expression and enrichment analysis

Even though 14 samples were sequenced we finally decided to choose one liver and one viscera sample from four individual fish ($n = 4+4 = 8$) to obtain a balanced sample design for further analyses. We used a random sample pair (R10 and R14) from Gm3, which had three replicate sample pairs, and excluded four samples from this host individual. Sample R28 from Gm9 was removed from the dataset, because it was paired with sample R29, for which RNA-sequencing was unsuccessful (Table S1, Figure S1). DESeq2 version 3.1.2 (Love et al., 2014) was used to analyse differential gene expression in the eight nematode samples using a paired design with 'site' as the main factor but accounting for 'host'.

A PCA was run with variance stabilized count data to identify potential outliers and visualize effects of infection sites (R-package ggplot2, Wickham, 2011). Differential gene expression analysis was performed and filtered with standard settings of the DESeq2 results function and adjusted p-value < .05 (FDR) cutoff (Love et al., 2014). The results were processed into a volcano plot and a heatmap of differentially expressed genes (DEGs) with indication of infection site and host-ID (R-packages pheatmap, RColorBrewer, viridisLite).

A functional enrichment analysis was performed with the R-package topGO, for DEGs upregulated in liver or viscera using the 'weight01' algorithm (Alexa & Rahnenfuhrer, 2022). Significant results were filtered using a $p < .05$ level (Fisher statistic). Gene ontology terms of enriched DEGs were summarized in REVIGO and exported as tree maps using the R-scripts for plotting (Supek et al., 2011).

2.6 | Allergen screen

In order to identify the allergenic potential of *Pseudoterranova bulbosa*, nematode allergen protein sequences listed in the WHO/IUIS Allergen Nomenclature database (Pomés et al., 2018) were collated from NCBI-Genbank (accessions are stated in the results section). These allergens were used as reference data for a BLASTX search of the assembled transcriptome. Sequences with an identity >80% and a match length ≥reference length were considered as potential homologs and putative allergens.

3 | RESULTS

All sampled specimens were assigned to the species *Pseudoterranova bulbosa* (NCBI Reference Sequence: NC_031643.1) using molecular barcoding of the CO1 marker (Data S1). The samples chosen for RNA-seq belonged to the same ITS-genotype (Data S2).

3.1 | Taxonomic classification and filtering of reads

The data used in the transcriptome assembly consisted of reads assigned as Nematoda (from our reference database of 28 spp.; Table S3) and unclassified reads, which included between 88.7 and 97.34% (mean 96.11 ± 2.42%, 5.86% variance). Unclassified reads were included into the assembly because we assume they also belong to nematodes.

Reads assigned to Protozoa (mean 0.73 ± 0.07%, 0.005% variance), *Homo sapiens* (mean 1.49 ± 0.2%, 0.04% variance) and the fish host (*Gadus morhua*, Atlantic cod) were excluded from the dataset. Contamination with cod reads was below 1% in all samples except R12 where 8.22% of reads were assigned to *G. morhua*. Detailed taxonomic filtering results are provided in Table S4.

3.2 | Transcriptome

The assembly of the taxonomically filtered data consisted of 25,405 genes from 65,146 transcripts with 40.54% GC content (Table S5). While the unfiltered assembly included a higher number of bases/reads, which resulted in a higher total number of transcripts and genes, contig N50 and contig length were higher in the filtered assembly, based on all transcripts as well as the longest isoform per

gene. The analysis using BUSCO revealed that taxonomic filtering improved the contents of expected single-copy conserved genes (Table S6), and we thus continued with the assembly based on filtered reads in all downstream analyses.

Peptide prediction with BLASTX resulted in 92.3% annotation (7.7% unknown/missing/new/unique) and 61.1% of the transcripts were functionally annotated with InterProScan (38.9% unknown/missing/new/unique).

Metabolic processes (9.93%, P), binding (19.86%, F) and catalytic activity (31.21%, F) were the most frequent annotation categories.

3.3 | Differential gene expression and functional enrichment

Seventy differentially expressed genes (DEGs) were detected, 32 significantly upregulated in samples from liver and 38 significantly upregulated in specimens from viscera (Table S7, Figure 2). The PCA grouped three sample pairs according to their respective hosts (R19 & R22, R35 & R4, R44 & R46), while the sample pair from Gm3 (R10 & R14) showed a bigger variance. The principal components explained 17 and 20% of the variation and indicated a stronger 'host' than 'site' effect (Figure S2).

Most BLAST hits of the DEGs were obtained in other Nematoda (e.g., *Loa loa* and *Brugia malayi*), and many corresponded to hypothetical proteins, which have not been described further (Table S7). Gene Ontology (GO) terms assigned to the differentially expressed genes in nematode larvae infecting cod liver included metabolic and catabolic processes, protein degradation (proteasome complex GO:0000502), cellular organization (GO:0045104), RNA processing and regulation (mediator complex, GO:0016592). Genes upregulated in larvae isolated from viscera genes were involved in metabolic (organic acid, GO0006082) and catabolic processes (aldehydes, GO:0046185, macromolecules GO:0009057), the processing of small nuclear RNAs (integrator complex, GO:0032039, protein import into the nucleus) and cellular detoxification (Table S8, Figure 3).

3.4 | Putative allergens

The allergen screening of the *Pseudoterranova bulbosa* transcripts identified five putative allergens: Ani s 2, Ani s 3, Ani s 7, Ani s 13 and Asc s 13 (Table 1, additional information included in Table S9).

The highest match identity was discovered for tropomyosin, with 98.94–91.54% identity and 3–24 mismatches, depending on query isoform. Tropomyosin has been identified as an allergen in vertebrates (Teleostei) and invertebrates (Acari, Insecta, Crustacea, Bivalvia, Gastropoda, Cephalopoda). The WHO database includes 37 homolog air- and food-borne allergens. Five homologs of paramyosin from other invertebrates (Acari and Gastropoda) are recognized air- or food-borne allergens (allergen.org). Glutathione-S-transferase (GST) from three taxonomic kingdoms (Plantae, Fungi, Animalia) acts as an allergen, with ten recognized homologs. Haemoglobin from *Anisakis simplex* is a homolog to *Chironomus thummi thummi* (Insecta) (Pomés et al., 2018). The function of Ani s 7 has not been discovered, yet.

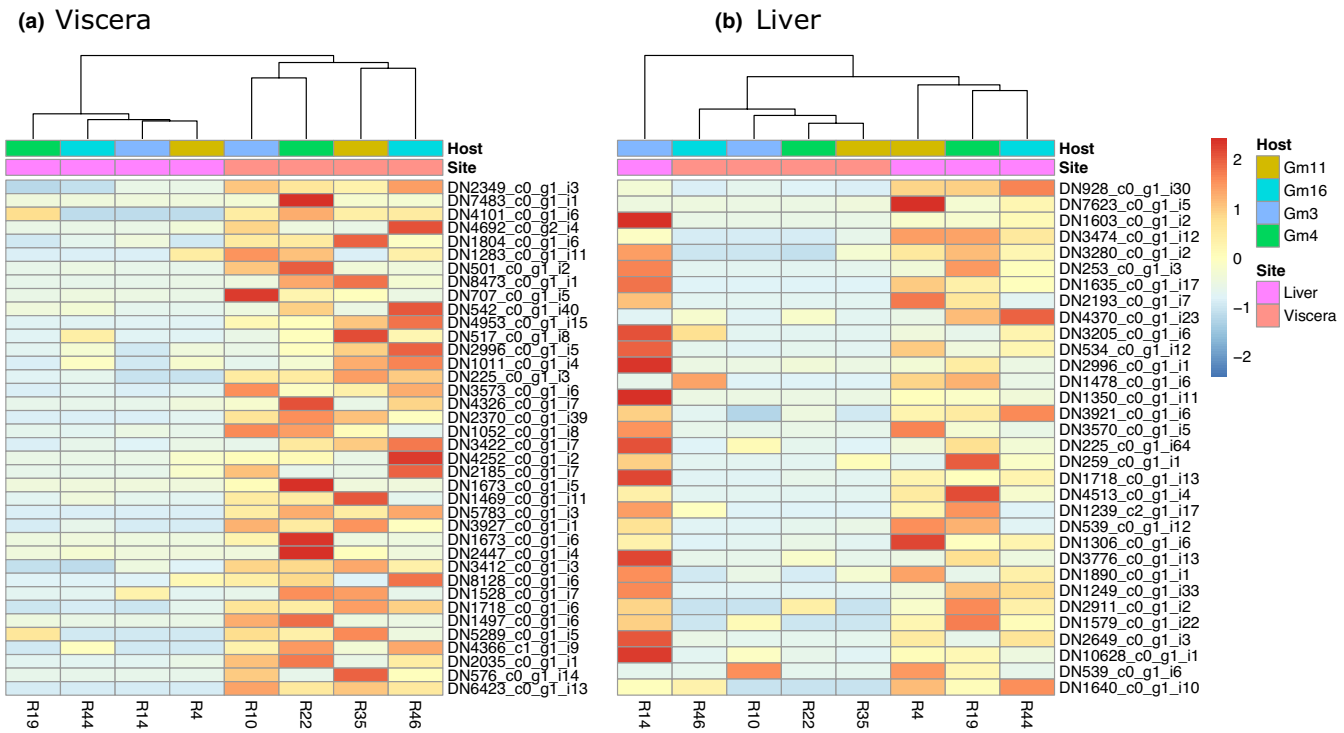

FIGURE 2 Heatmap of differentially expressed genes (rows) of the different samples (columns) in (a) viscera and (b) liver with indication of host-ID (row no. 1) and infection site (row no. 2). Warm colours imply a gene that was significantly upregulated in a sample

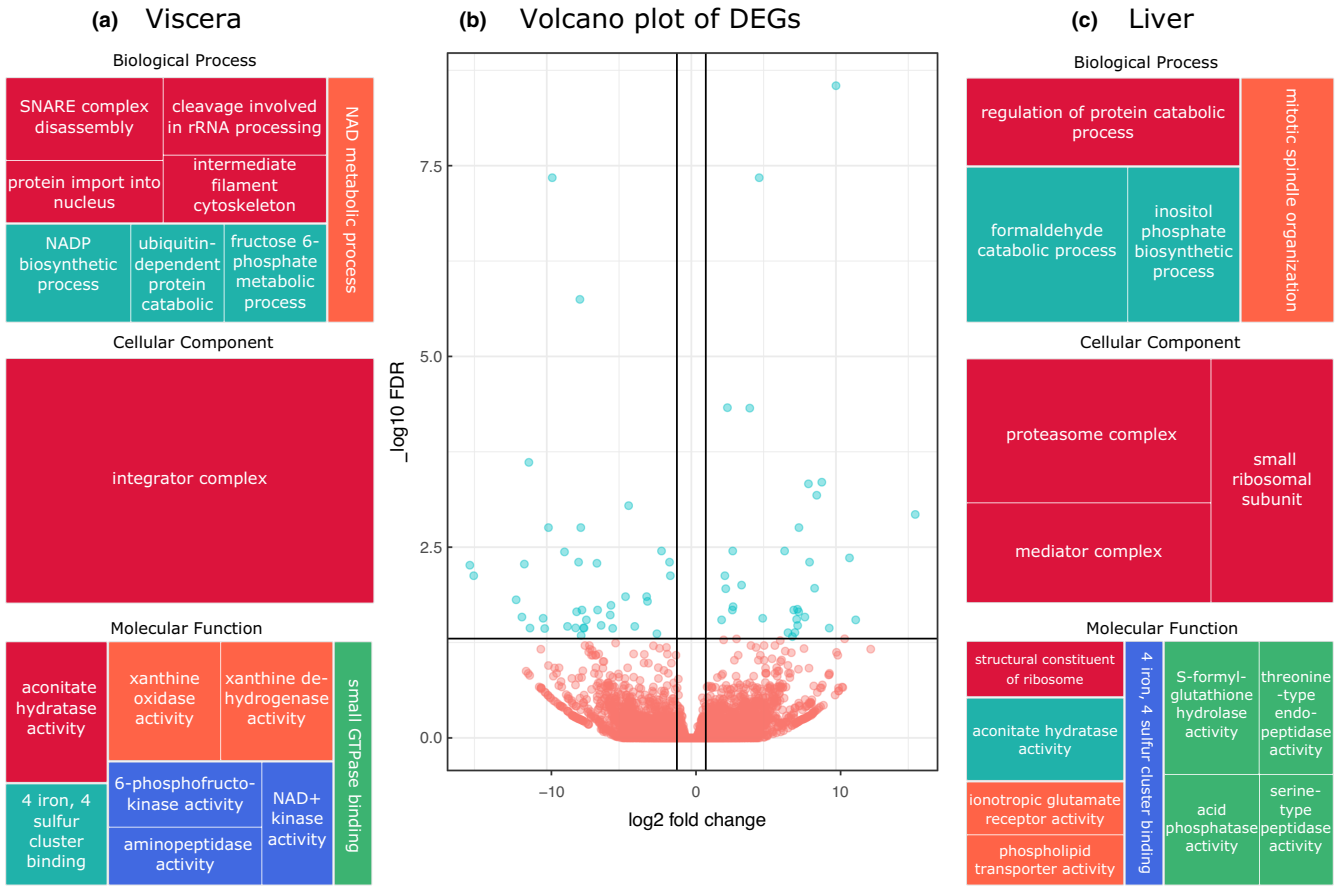

FIGURE 3 Treemaps of significantly over-expressed GO terms in *P. bulbosa* from (a) viscera and (c) liver and (b) volcano plot of differentially expressed genes, negative log2 fold change corresponding to genes upregulated in viscera samples and positive log2 fold change corresponding to genes upregulated in liver samples. Treemap colours are arbitrary in (a) versus. (b). Volcano plot colour: blue = above FDR cut-off, red = below FDR cut-off

4 | DISCUSSION

We assembled and annotated the first transcriptome of the zoonotic anisakid genus *Pseudoterranova*. The differential gene expression analysis between samples from liver and viscera of *P. bulbosa* in *Gadus morhua* revealed only slight gene expression differences between infection sites. We here discuss our results in the light of published (experimental) research on this life cycle stage and its tissue invasion behaviour and presumed preferences.

As a starting point for the evaluation of the allergenic potential of *P. bulbosa*, we discuss the homologs of nematode allergens expressed by *P. bulbosa* in its fish host.

4.1 | Differential gene expression between infection sites

Anisakid larvae have been reported from different infection sites within their teleost hosts (Gay et al., 2018; Najda et al., 2018). The movement patterns of the larvae within the host after their ingestion have been attributed to the temperature difference between mammalian definitive hosts and teleost paratenic hosts (Smith & Wootten, 1975). Hake gutted and examined after different time periods and kept at environmental temperature or on ice did not show significant variation/increase of nematode larvae in muscle (Cattan & Carvajal, 1984). Cipriani et al. (2016) reported that high storage temperatures (14°C) led *Anisakis pegreffii* to leave the fish host body, while temperatures between 2 and 7°C promoted a migration into muscle tissue. The motility and mobility of anisakid larvae strongly correlated with temperature in an experimental study by Guan et al. (2021). Šimat et al. (2015) were able to establish a strong connection between 'post mortem' migration of *Anisakis* sp. and the concentration of biogenic amines and dependent change of pH-value during decomposition of the host rather than temperature. Muscle infections with anisakids also occur in fish frozen or examined immediately after catching, suggesting there are other factors than temperature influencing migration in live hosts.

The distribution of *Pseudoterranova* stage three larvae (L3) infecting Atlantic cod (*Gadus morhua*) within the host's body cavity is primarily focused on liver and liver parenchyma and scarcer in viscera (own observation, Klapper et al., 2018; Mehrdana et al., 2014; Nadolna & Podolska, 2014). If the larval distribution is not coincidental, it can either be explained by the migrating distances defined by the host's anatomy or by the specific preference of liver.

TABLE 1 Putative allergens of *P. bulbosa* expressed in L3 from Atlantic cod. Count avg. = count average, Ident. =identity, Mism. = mismatch, Gap = alignment gap, identity cut-off = 80%. Asterisks indicate transcripts with a count average >1000

Query	Reference	Count avg.	Ident.	Length	Mism.	Gap	Annotation	Allergen
DN6446_c0_g1_i26 *	CAB93501.1	7518.46	98.944	284	3	0	Tropomyosin	Ani s 3
DN6446_c0_g1_i33	CAB93501.1	107.08	98.8	250	3	0	Tropomyosin	Ani s 3
DN7685_c0_g1_i1 *	AAF72796.1	3003.85	97.583	869	21	0	Paramyosin	Ani s 2
DN7685_c0_g1_i2 *	AAF72796.1	12,679.08	97.583	869	21	0	Paramyosin	Ani s 2
DN7685_c0_g1_i4	AAF72796.1	340.77	97.583	869	21	0	Paramyosin	Ani s 2
DN6446_c0_g1_i16 *	CAB93501.1	1894.38	91.549	284	24	0	Tropomyosin	Ani s 3
DN5493_c0_g1_i3	AAF72796.1	155.46	84.977	872	124	2	Paramyosin	Ani s 2
DN5493_c0_g1_i2	AAF72796.1	327.00	84.977	872	124	2	Paramyosin	Ani s 2
DN5493_c0_g1_i4 *	AAF72796.1	28,865.54	84.977	872	124	2	Paramyosin	Ani s 2
DN15669_c0_g1_i5 *	AFY98826.1	7159.69	84.848	297	45	0	Haemoglobin	Ani s 13
DN15669_c0_g1_i8	AFY98826.1	6.00	84.848	297	45	0	Haemoglobin	Ani s 13
DN15669_c0_g1_i2 *	AFY98826.1	7052.62	84.848	297	45	0	Haemoglobin	Ani s 13
DN15669_c0_g1_i7	AFY98826.1	11.38	84.848	297	45	0	Haemoglobin	Ani s 13
DN409_c0_g1_i4	ABL77410.1	25.92	84.593	675	102	1	–	Ani s 7
DN409_c0_g1_i233	ABL77410.1	53.69	84.593	675	102	1	–	Ani s 7
DN409_c0_g1_i79	ABL77410.1	12.23	84.593	675	102	1	–	Ani s 7
DN409_c0_g1_i23	ABL77410.1	19.62	84.546	893	136	1	–	Ani s 7
DN409_c0_g1_i10	ABL77410.1	1.69	84.546	893	136	1	–	Ani s 7
DN409_c0_g1_i129	ABL77410.1	74.00	84.489	1096	168	1	–	Ani s 7
DN409_c0_g1_i56	ABL77410.1	17.77	84.489	1096	168	1	–	Ani s 7
DN409_c0_g1_i169	ABL77410.1	33.00	84.389	442	69	0	–	Ani s 7
DN8128_c0_g1_i4 *	CAA53218.1	1837.69	83.193	119	20	0	Glutathione S-transferase	Asc s 13
DN409_c0_g1_i185	ABL77410.1	48.38	81.514	1136	168	2	–	Ani s 7
DN409_c0_g1_i75	ABL77410.1	7.38	81.514	1136	168	2	–	Ani s 7
DN409_c0_g1_i60	ABL77410.1	0.00	81.514	1136	168	2	–	Ani s 7

Anisakid larvae infect cod through the ingestion of infected invertebrate hosts or the ingestion of a paratenic fish host. The parasites leave the alimentary tract by penetrating the stomach or intestine wall and infecting the body cavity. Young (1972) suspected that a high infection intensity of the liver was a result of the short distance between the fish's stomach and the surrounding lobes of the liver (Young, 1972). Following studies assumed more complex underlying mechanisms, which have been conserved throughout the evolutionary adaptation of the parasite to its paratenic host (Sukhdeo & Sukhdeo, 1994). Here, the host was considered as a habitat with predictable anatomical and chemical structure, where certain stimuli provoke a fixed behavioural pattern of the parasite. Experimental studies showed that the larval response differed when infecting accidental hosts, e.g., freshwater species which they were not adapted to. In these studies, infection was irregular (remaining in the digestive tract) or the parasites did not survive (Quiazon et al., 2011; Sukhdeo & Sukhdeo, 1994). But differences in the distribution of larvae between organ and muscle tissues have also been reported within their adequate intermediate hosts (Strømnes & Andersen, 1998). Fat content of the infected tissue was identified to be positively correlated with the number of anisakid larvae (Strømnes & Andersen, 1998, 2003). Anisakids have been found to sometimes cause severe diseases in other accidental hosts, such as granulomatosis and necrosis in sea turtles (*Caretta caretta*) (Santoro et al., 2010) but were non-pathogenic in others (Shamsi et al., 2017). An experimental study by Bušelić et al. (2018) examined Wistar rats infected with *Anisakis pegreffii* L3 and showed that 30% of nematode larvae penetrated the stomach mucosa or abdominal muscle tissue, causing proinflammatory activity and induced the expression of cuticula degrading enzymes in the host. Stomach tissue reacted with ribosomal stress, while a T-helper 17-type inflammatory response was found in muscle tissue. No consistent behaviour of infective larvae pointing to aimed tissue migration was observed during infection experiments in rats (Bušelić et al., 2018), suggesting, that it could also be arbitrary in human patients.

Our DGE-analysis shows that gene expression patterns of *P. bulbosa* L3 from cod only differed in seventy genes between parasites infecting liver or viscera. Concerning the life-history of *P. bulbosa*, we conclude that during the L3 stage infecting paratenic fish hosts, similar biological processes take place regardless of the infection site. One way to interpret our data is in support of an arbitrary distribution of L3 *P. bulbosa* in cod, which could be a result of the physical proximity

between the host's stomach and liver (Young, 1972). In this case, most larvae remain in the adjacent liver after penetrating the stomach, while a smaller proportion of larvae leave the alimentary tract during the gut passage, thereupon residing in viscera tissue. However, the distribution of anisakid larvae in other fish species challenges this hypothesis (Strømnes & Andersen, 1998) and it does not provide explanatory approaches for the invasion of fish muscle tissue.

It is under debate whether anisakid larvae feed while infecting their fish host. Larval growth has been reported from fish hosts (Strømnes & Andersen, 2003), which would require nutrient uptake. During our sampling of live larvae immediately after capture of the fish, observations were made that could indicate that individual parasites may have fed on host blood. The consumption of cod tissue or blood prior to encapsulation would be supported by the cod reads discovered in the filtering step of this study (Table S4), but they could also be a result of contamination. If true, however, feeding does not occur exclusively in parasites infecting liver, as no differentially expressed genes with a direct link to digestion could be identified. Granted, that conditions in liver are beneficial to the parasite larvae, this benefit was not clearly reflected by their gene expression patterns in our study. Still there are findings pointing to better larval growth and more beneficial conditions, such as lipid content, at certain infection sites (Strømnes & Andersen, 2003). Respective experimental evidence on a transcriptomic level could be achieved by a setup similar to Strømnes (2014), who examined the migration of *Anisakis* on agar with a lipid gradient, accompanied by RNA sampling. Since our study did not include the apical part of the worm into the RNA samples, we recommend to future research of the gene expression of L3 from fish hosts in this body part. Genetic material for genotyping could also be acquired from residual DNA during RNA extraction, before DNAse treatment.

Another possible explanation for the subtle DEG-pattern of *P. bulbosa* in our study could be explained by a dauer-phase of the larvae in both examined microhabitats (Hand et al., 2016). To prevent the release of antigens, hosts encapsulate invasive parasites instead of digesting them (Buchmann, 2012). The sheath covering the parasite is composed of host tissue (Buchmann, 2012), which could be the source of the cod reads found in some of our samples. The parasites undergo a diapause which is regulated by small RNAs and chromatin modifications (Hand et al., 2016). The expression of transcription regulating genes (integrator complex) in our analysis could be connected to this stage. Since the capsule is composed of host tissue, incomplete removal is one possible reason for the high number of cod reads in two of the samples (Table S4).

4.2 | Putative allergens

An important factor of anisakid pathogenicity is allergenic potential. We compared WHO-recognized Nematode allergens to proteins from *P. bulbosa*. Among five putative allergens, the protein tropomyosin showed a high similarity to Ani s 3 from *Anisakis* spp. which has already been detected in *P. decipiens* (*s.s.*). Homologs of four nematode allergens were expressed in all samples. This finding suggests an allergenic potential of *P. bulbosa* which should be a subject of future research.

The putative allergens detected in this study were homologs of tropomyosin, paramyosin, glutathione-S-transferase (GTS), haemoglobin and Ani s 7. Since homology between biomolecules does not directly infer cross-reactive IgE binding, candidate allergens need to be validated by serological studies to verify their activity (Guarneri et al., 2007). Nevertheless, the identification of homologues is another line of evidence for the proposed allergenic properties of *P. bulbosa*.

Tropomyosin is essential to muscle function and cell division (cytoskeleton) in all eukaryotes (Sereda et al., 2008). Nematode tropomyosin is located in the muscle and under the basal layer of the cuticle (Asnoussi et al., 2017). Parasite tropomyosin can regulate host immune molecules through the simultaneous expression of tetraspanin (Monteiro et al., 2010). While vertebrate tropomyosins are non-allergenic to humans due to a closer homology (Sereda et al., 2008), invertebrate tropomyosin and paramyosin are considered pan-allergens (Pérez-Pérez et al., 2000). Anisakid tropomyosin is suspected to be heat-resistant which is relevant for food-safety considerations (Guarneri et al., 2007). Tropomyosin has been debated to be involved in fish allergy (González-Fernández et al., 2018; Keshavarz et al., 2020). Cross-reaction to invertebrate tropomyosins has also been described between house dust mite and nematodes (Acevedo et al., 2009; Bernardini et al., 2005). However, ascribed cross-reaction cannot always be validated through immunoblotting, despite the similarity of the epitopes, e.g., *Anisakis pegrefii* tropomyosin and polyclonal antibodies from crustacean tropomyosin (Asnoussi et al., 2017). Still, cross-reactivity of anisakid tropomyosin and paramyosin with frequent arthropod allergens led to diagnostic problems of allergic anisakidosis (Guarneri et al., 2007), which required a search for a specific diagnostic allergen (Acevedo et al., 2009).

The enzyme glutathione-S-transferase (GST) serves as a catalyst of detoxification processes in eukaryotes (Eaton & Bammler, 1999). Parasite GST is involved in the suppression of the host's inflammatory response to the infection (Mehrdana & Buchmann, 2017; Pritchard, 1995), i.e., by neutralizing oxygen radicals (Mehrdana & Buchmann, 2017; Nielsen & Buchmann, 1997). Several homologs of arthropod GST act as respiratory allergens, causing reactions to cockroaches and mites (Mueller et al., 2015). Acevedo et al. (2013) described allergenic GST from *Ascaris lumbricoides* which was serum cross-reactive with tropical house dust mites. It is a recognized allergen of anisakids and has been proposed by Fæste et al. (2014) and considered a putative allergen of *Pseudoterranova decipiens* (Kochanowski et al., 2020). Cross-reactivity between GTS homologues of invertebrates from different taxonomic groups has been suspected but not confirmed as yet (Mueller et al., 2015).

The identification of the allergenic agent Ani s 7 provided a new candidate for a reliable serum-indicator of acute *Anisakis* infections

(Acevedo et al., 2013; Anadón et al., 2009). Sera acquired through infection experiments of rats with *Anisakis simplex* and *Pseudoterranova decipiens* showed that Ani s 7 was genus specific to *Anisakis*. Because *Pseudoterranova* infections did not result in the sensitization to Ani s 7, antibodies to Ani s 7 were considered an indicator of infections with live larvae from the genus *Anisakis* (Anadón et al., 2009). Later, the role of Ani s 7 as standard indicator for the identification of *Anisakis* infections has been debated to be cross-reactive in serodiagnosis, possibly due to glycosylation (González-Fernández et al., 2017; Lorenzo et al., 2000). Based on these findings, the *Pseudoterranova bulbosa* homolog of Ani s 7 identified in this study has probably lower similarity or different surface structure and may not have the allergenic properties described for *Anisakis*.

The haemoglobins of nematodes are large molecules with a high structural diversity and multiple subunits and domains (Weber, 1980; Weber & Vinogradov, 2001). They are characterized by a high O_2 binding affinity and low dissociation (Coletta et al., 1986; Gibson et al., 1965, 1993; Weber & Vinogradov, 2001), playing role in the neutralization of free radicals and therefore mitigating a host's response to parasite infections (Madala et al., 2008; Nieuwenhuizen et al., 2013). Some invertebrate parasites' haemoglobin has allergenic potential within their vertebrate hosts, i.e., as the main allergenic agent of chironomids (Baur et al., 1986; Mazur et al., 1987, 1990). *Pseudoterranova* spp. haemoglobin accounts for more than 30% of the protein within the nematode's pseudocoel (Dixon et al., 1993). Grey seals (*Halichoerus grypus*), the definitive host of *Pseudoterranova decipiens* (*s.s.*) and *P. krabbei*, produce antibodies against parasite haemoglobin (Dixon et al., 1991; Frenkel et al., 1992; Weber & Vinogradov, 2001). The sensitization of the host only takes place during the exposure with a live parasite (Nieuwenhuizen et al., 2013). Studies by González-Fernández et al. (2015, 2017) suggested that haemoglobin could be used to increase diagnostic sensitivity for the identification of the source of chronic urticaria caused by *Anisakis* infections, because it does not cross-react with *Ascaris*, a parasite genus from the same clade. Our results indicate that a specificity to genus levels within the family Anisakidae should be examined further.

Chen et al. (2015) hypothesized that heat stress could play a role in the allergenicity of anisakids, as a side effect of their reaction to the definitive host's body temperature and may be beneficial in the competition with other gastro-intestinal parasites of their definitive hosts. Temperature seemed to influence the expression of antigenic proteins, when simulating cold stress and body temperatures of intermediate and definitive hosts. Low storage temperatures could reduce the expression of excretory-secretory products (Palomba et al., 2019). However, fast freezing rates of 0.2–0.3°C/min at −10°C seemed to be sufficient to inactivate the nematodes and preserve the quality of the seafood, but rapid freezing kinetics also increased the expression of the allergen Ani s 4. Thus, freezing kinetics may have a bigger effect on allergenicity of anisakids than differences between species (Sánchez-Alonso et al., 2020).

Examining allergenic potential based on transcriptomic instead of genomic data is advantageous, because it reveals the allergens which are acutely expressed under certain conditions. Our data therefore give information on the trace allergen homologs potentially present in fish after the manual removal of *P. bulbosa* larvae. Genomic data would enable to screen for all potential allergen homologs, but like transcriptomic data, they can only be used as an indication for future serological studies.

5 | CONCLUSION

This study provides an annotated de novo transcriptome of the marine parasite *Pseudoterranova bulbosa*, which contributes to resources on Nematoda with human-pathogenic potential. The data can be used in meta-analyses, as a basis for tissue-specific gene expression analysis and as a starting point for resequencing projects.

Our DGE-analysis revealed only slightly differing expression patterns of *P. bulbosa* L3 from cod liver or viscera. Concerning the life-history of *P. bulbosa*, we conclude that during the L3 stage infecting paratenic fish hosts, similar biological processes take place regardless of infection site. Our findings either support the hypothesis that parasite distribution results from the liver's physical proximity to the stomach or is driven by beneficial properties of liver, such as a high lipid content. Allergen traces from *P. bulbosa* contaminating fishery products are a potential food-hazard.

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CONFLICTS OF INTEREST

The authors declare they do not have competing interests.

AUTHORS' CONTRIBUTIONS

KGA, BF, JK and SK conceived the study and wrote the manuscript. KGA carried out the sampling, bioinformatic analysis and created the figures. BF supported the data analysis. BF and JK commented and revised the manuscript.

CONSENT TO PARTICIPATE

Not applicable.

CONSENT FOR PUBLICATION

All authors gave final approval for publication and agree to be held accountable for the work performed therein.

DATA AVAILABILITY STATEMENT

Transcriptomic raw data generated for this study are available at the European Nucleotide Archive database (ENA) under Project number: PRJEB42010. Sequences used for DNA-barcoding are included in the supplemental information (Data S1 and S2).

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