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Long live the host! Proteomic analysis reveals possible strategies for parasitic manipulation of its social host

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Abstract

Parasites with complex life cycles often manipulate the phenotype of their intermediate hosts to increase the probability of transmission to their definitive hosts. Infection with *Anomotaenia brevis*, a cestode that uses *Temnothorax nylanderi* ants as intermediate hosts, leads to a multiple-fold extension of host lifespan and to changes in behaviour, morphology and colouration. The mechanisms behind these changes are unknown, as is whether the increased longevity is achieved through parasite manipulation. Here, we demonstrate that the parasite releases proteins into its host with functions that might explain the observed changes. These parasitic proteins make up a substantial portion of the proteome of the hosts' haemolymph, and thioredoxin peroxidase and superoxide dismutase, two antioxidants, exhibited the highest abundances among them. The largest part of the secreted proteins could not be annotated, indicating they are either novel or severely altered during recent coevolution to function in host manipulation. We also detected shifts in the hosts' proteome with infection, in particular an overabundance of vitellogenin-like A in infected ants, a protein that regulates division of labour in *Temnothorax* ants, which could explain the observed behavioural changes. Our results thus suggest two different strategies that might be employed by this parasite to manipulate its host: secreting proteins with immediate influence on the host's phenotype and altering the host's translational activity. Our findings highlight the intricate molecular interplay required to influence the phenotype of a host and point to potential signalling pathways and genes involved in parasite–host communication.

KEYWORDS
ageing, antioxidants, cestode, intermediate host, lifespan, parasite

1 | INTRODUCTION

The closest molecular interactions and antagonistic dynamics occur between parasites and their hosts, resulting in highly specialised morphologies, physiologies and life cycles of parasites that can be characterised as either direct or indirect (Olsen, 1986). Parasites with a direct life cycle transmit themselves or their progeny directly from one host to the next. In contrast, parasites with an indirect or

'complex' life cycle require at least one intermediate host or vector before they can complete their life cycle in the definitive host (Olsen, 1986). The common denominator of those parasites is that they require a host switch to complete their life cycle. Although difficult to verify experimentally, many of these parasites are thought to actively increase the likelihood of transmission. The malaria parasite *Plasmodium falciparum* alters the odour of their human hosts, possibly to increase their attractiveness towards mosquitoes during the infective stage (de Moraes et al., 2014; Lacroix et al., 2005). The fungus *Ophiocordiceps unilateralis* manipulates *Camponotus leonardi* ants to seek out sites that provide optimal conditions for fungal growth before they die from the infection (Andersen et al., 2009). Those direct changes in host behaviour or odour are examples of adaptive host manipulation by the parasite. Despite the description of numerous case studies in which host manipulation appears to occur, we lack general knowledge of the underlying mechanisms by which parasites alter the phenotype or behaviour of their host. Among others, proposed pathways of manipulation include energy deprivation (Lafferty & Shaw, 2013), the parasite's location within the body of the host (e.g. nervous system or around muscle) and the release of manipulative agents (Martin et al., 2015). The difficulty in determining the exact mechanism by which host phenotype change is achieved arises not only from the distinction between parasite-induced effects encoded in the parasite's genome and the side effects of infection (e.g. host immunity) that happen to favour the parasite but also from the fact that several interconnected signalling pathways are often affected during infection (Kavaliers et al., 1999).

One particularly interesting parasite for which increased transmission due to host manipulation has been postulated (Beros et al., 2015) is the cestode *Anomotaenia brevis*. This parasite has an indirect life cycle, with the ant *Temnothorax nylanderi* as an intermediate host and two species of woodpecker (*Dendrocopos major* and *D. minor*) as definitive hosts (Plateaux, 1972). The infection of the intermediate host happens when foraging ants bring bird faeces with cestode eggs into the nest and feed those to the developing larvae. Within the ant, the cestode eggs hatch into larvae and pass through the gut wall into the haemocoel, where they develop into cysticercoids. When a woodpecker opens acorns or sticks, in which *T. nylanderi* build their nests, and feeds on the infected ants, the cysticercoids develop into adult tapeworms and complete their life cycle (Figure 1).

Infected ants show several traits that distinguish them from their uninfected nestmates, and that might be the result of parasite manipulation to increase the chance of transmission to the final host. They have a yellow, less pigmented and sclerotised cuticle, in contrast to the brown colouration of the healthy workers (Plateaux, 1972; see Figure 1). Depending on the background, their light colouration could increase their visibility to foraging birds, and the reduced sclerotisation could enhance tapeworm escape from the ant carcass and establishment in the gut of avian definite hosts. Interestingly, infected adult workers show a normal melanisation reaction in response to injury (unpublished experiments), suggesting that the parasite may influence melanisation only during the pupal stage of the ant. Infected ants are fed and cared for more frequently but are themselves less active than their uninfected nestmates and almost never leave the nest; indeed, they do not flee from nest sites even when they are opened (Beros et al., 2015; Feldmeyer et al., 2016; Scharf et al., 2012). Woodpeckers search for ant colonies by opening sticks and acorns, so it could be advantageous for the parasite if infected individuals remained in the nest and are well cared for. *Temnothorax* workers are also only two millimetres in size, which makes them hard to find outside of the nest. An inconspicuous trait at first glance is their lifespan, but over a period of 3 years, infected workers showed no difference in survival compared to queens, which can live up to two decades in this species (Plateaux, 1986). In contrast, all uninfected workers, including newly hatched ones, died during this period (Beros et al., 2021). Longer term observations that could provide information on the maximum life expectancy of infected individuals are not yet available, but anecdotal observations demonstrate that infected workers can live up to 7 years (A. Buschinger, pers. comm.). The drastic increase in life expectancy naturally also extends the period during which an infected ant can be preyed upon by the final host, which increases the probability of transmission.

In this study, we have focused on the potential mechanisms underlying the extended lifespan. Melanisation and sclerotisation of the cuticle are established during development, and thus unsuitable targets for proteomic analysis of adult individuals, but we will also discuss shifts in the proteome that may be related to the other

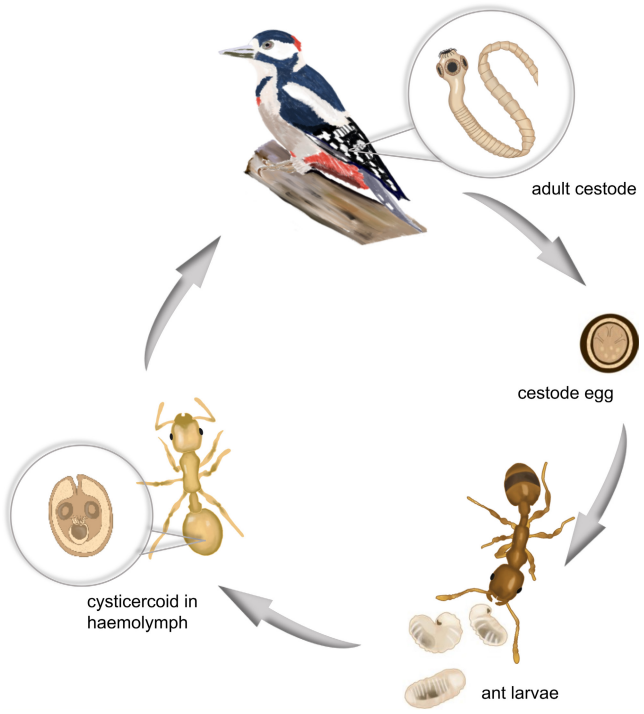

FIGURE 1 Life cycle of *Anomotaenia brevis*. The adult stages in different woodpecker species produce eggs that are excreted by the bird and subsequently fed to growing ant larvae of *Temnothorax nylanderi*. The eggs develop into cysticercoids that are attached to the gut in the haemocoel. The life cycle is completed when infected ants are eaten by the woodpecker.

altered traits of infected workers. Previous studies investigating the factors contributing to the longer lived phenotype of infected ants focused mainly on changes in gene expression after infection (Feldmeyer et al., 2016; Stoldt et al., 2021). Another promising target to identify which molecular pathways are affected by an infection, however, is the proteome. The proteome reflects the biological activity and downstream effects of infection even more directly and includes not only the proteins synthesised by the host itself, but possibly also those released by the parasite. By analysing the proteome of the haemolymph, it is possible to identify circulating substances, whether parasitic or host-derived, that increase the fitness of the parasite.

To be able to detect active secretion of proteins by the cestode, we analysed the haemolymph of infected and uninfected workers of *T. nylanderi* for the presence of secreted proteins of the cestode *A. brevis*. In addition, we compared the abundance of proteins from whole parasite proteomes to those found in the haemolymph, as this would provide further evidence of active delivery of some proteins into the host organism and thus possibly of host manipulation. We also compared workers and queens from infected and uninfected colonies, which gives insight into the consequences of infection for the nestmates of infected ants, which also show an altered phenotype, for example, increased mortality. Finally, a caste comparison was of interest, as queens show a similar low mortality rate as infected workers, a similarity that could have the same or a different molecular basis. Although we worked with very limited material, as *Temnothorax* ants and their colonies are very small and infected individuals are rare, we were able to (i) identify cestode proteins with potential manipulative functions (i.e. the cestode proteins) and (ii) uncover changes in the ant proteome that may be the result of the secreted parasite proteins.

2 | MATERIALS AND METHODS

2.1 | Ant collection and maintenance

Temnothorax nylanderi colonies were collected in the Lenneberg Forest in Mainz (50°00′41.7″ N; 8°10′32.8″ E). In the laboratory, the colonies were moved to a small slide nest covered with red foil to give them a darkened environment, which was placed in a larger Plexiglas nest consisting of three chambers with a plastered floor. They received water and honey and were kept in the laboratory at constant conditions of 20°C and 70% humidity for an acclimation period of 7 days before haemolymph extractions.

2.2 | Haemolymph and cestode sampling

Haemolymph was extracted for proteomic analyses from three different groups of ants: infected worker ants, non-infected worker ants that belong to the same colony (hereafter short: nestmate ants) and non-infected worker ants from healthy colonies (hereafter short: healthy ants). For each extraction, the haemolymph of 10 individual ants was pooled, and we performed four biological replicates. For each colony that was included in the proteome analysis, we also extracted the haemolymph of the (uninfected) queen from the colony, which resulted in the same four replicates for both infected and healthy colonies, but with only one individual each.

For the collection of the haemolymph, the ants were immobilised by placing their heads into a flexible foam plug so that the abdomen was freely accessible. By creating a small perforation with a microscissor between two segments of the abdomen, we gained access to the haemolymph that was collected with a narrowed capillary (Hirschmann, Eberstadt, Germany) to ensure that no cestodes were transferred. The collected clear fluid (1–5 μL per pooled sample) was transferred into a 1.5-mL reaction tube filled with 8 M urea (Sigma Aldrich). All samples were stored at −20°C until mass spectrometry analysis.

Following haemolymph extraction, the gaster of each individual was dissected to confirm the infection status of the individual and to collect the cysticercoid cestodes for proteomic analyses. Cysticercoids reside in the haemolymph in close proximity to the midgut, sometimes loosely attached to it. The cestodes of four infected ants were pooled, carefully taken up with a pipette and transferred to a concave microscope slide, where they were washed with PBS and counted before transferred to PBS and further processing by the IMB core facility for mass spectrometry analysis. We prepared three replicates for the whole cestode analysis.

2.3 | Mass spectrometry analysis

The haemolymph solution was diluted with 150 μL of 50 mM ammonium bicarbonate (ABC) buffer at pH 8.0. The samples (ca. 200 μL) were reduced with 10 mM f.c. dithiotreitol (Sigma Aldrich) for 40 min and subsequently alkylated in the dark with 50 mM f.c. iodoacetamide (Sigma Aldrich) for 40 min. Afterwards, 500 ng of LysC protease (Wako) was added, and after 3 h of incubation, the samples were supplemented with 500 ng of MS-grade trypsin (Serva) and digested overnight. The peptides were loaded onto a C18 StageTip (Rappsilber et al., 2007) and stored until measurement. The eluted peptides were separated on a heated 50-cm reverse-phase capillary (75 μM inner diameter) packed in-house with Reprosil C18 material (Dr. Maisch GmbH). Peptides were eluted along an optimised 90 min gradient from 6% to 40% Mixture B (80% ACN/0.5% formic acid) with an EASY-nLC 1200 system (Thermo Fisher Scientific). The spray voltage was set to 2.2 kV. Measurement was done on an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific) operated with a Top20 data-dependent MS/MS acquisition method per full scan. The full scan had a resolution of 60,000 with a scan window set at 350–1600 m/z. The isolation window was 1.4 m/z. The MS/MS fragment scan was obtained with a resolution of 15,000 after HCD fragmentation. Fragmentation was restricted to peptides with charge states 2–6. All raw files were processed with MaxQuant version 1.6.0.5 (Cox & Mann, 2008), and peptides were matched in a

single search to a combination of the protein database of *T. nylanderi* with 43,252 entries (Stoldt et al., 2021) and the three-frame translated (at least 100 aa ORFs) protein sequences of the cestode *A. brevis* with 173,911 entries (Stoldt et al., 2021). Only peptides that could be matched unambiguously to either species were included in further analyses. MaxQuant was used with pre-set standard settings, but we activated the following options: match between runs, label-free quantitation (LFQ), intensity-based absolute quantification (iBAQ) and deactivated fastLFQ. LFQ quantitation was only performed on unique peptides. Prior to further analysis, contaminants, reverse hits and proteins that were only identified by a modified peptide were removed. The statistical analyses were done with R Studio (v3.6.2).

2.4 | Analyses of host proteins

We employed two separate bioinformatic approaches to analyse the *T. nylanderi* and *A. brevis* proteins. For *T. nylanderi* proteins, abundances of the different groups (healthy, nestmates and infected) were compared in a pairwise fashion using a Welch *t*-test, and the mean difference of $\log_2$ transformed and imputed LFQ intensities between the different conditions was computed. This was repeated for a comparison of queens from healthy colonies and queens from infected colonies.

To test whether infected workers show higher abundances of proteins that are usually only found in queens, we compared proteins that are significantly more abundant in queens (compared to healthy and nestmate workers) to those that are significantly more abundant in infected workers (compared to their nestmates and to healthy workers). Since *T. nylanderi* is usually monogynous, we could only use one queen per nest for the mass spectrometry analysis, as opposed to 10 pooled workers, rendering a direct comparison of iBAQ between our queen and worker samples impossible. Thus, for pairwise comparisons between queens and workers, we first filtered out proteins that were only present in one data set and then calculated the relative protein abundances (iBAQ single protein/iBAQ sum of all proteins) separately for each of the four replicates. The pairwise comparisons were done in the same way as described above. For all differential abundance analyses, we added the same analyses including corrections for multiple testing to Data S1.

In addition, we extracted proteins that were unique to nestmate queens (queens from infected colonies) compared to nestmate workers, and in healthy queens compared to healthy workers. We checked whether those proteins tended to occur in higher abundances in infected workers, both compared to their nestmates and healthy workers, and whether infected workers also express those proteins uniquely.

Putative protein functions were obtained by performing a BLASTp search against the non-redundant invertebrate protein database (accessed 14.09.2021). Proteins were functionally annotated using InterProScan v5.46-81.0. A Gene Ontology (GO) term enrichment analysis of biological processes was conducted with topGO (Alexa & Rahnenführer, 2016) compared to the *T. nylanderi* transcriptome. We used the weight01 algorithm and checked for significance using Fisher statistics with a *p*-value cut-off of $p = .05$. Furthermore, a text mining approach was conducted to assign functional descriptions from reviewed entries in the UniProt database for *Drosophila* to the identified proteins. We then searched those functions for terms that are associated with longevity or ageing, immunity, transport, stress and epigenetics, as we expected those functions to be of particular interest as a target for host manipulation (for a list of search terms, see Table S9 in Data S1) to identify potential candidate genes implicated in infection phenotypes.

During the analysis of the host proteome, we found vitellogenin (Vg) proteins in differential abundances between the groups. Vgs have diversified in ants and are known to play a role in caste differentiation. To identify the type of Vgs, we aligned the protein sequences to the protein sequences of different insect Vgs obtained by Kohlmeier et al. (2018) with MAFFT (v7.487; Madeira et al., 2019) using standard settings. The resulting tree (see Figure S8 in Data S1) was visualised using the iTOL online tool (Letunic & Bork, 2019).

2.5 | Analysis of parasite proteins

A similar approach was employed for the cestode proteins. Here, no differential abundance analysis was conducted since cestode proteins should only be found in infected ants, thus rendering comparative analyses impossible. We therefore conducted all the following analyses with proteins that were (i) found in at least two out of four replicates and (ii) present in all four replicates. With those proteins, as with the ant proteins, we conducted a BLASTp and Interproscan search to obtain functional characterizations and GO terms. We searched reviewed UniProt entries for *C. elegans* to assign functions to the identified proteins. Those lists were then also searched for terms that are associated with longevity or ageing, immunity, transport, stress and epigenetics (Table S9 in Data S1). The functional annotation of cestode proteins, by only using BLASTp, proved to be more difficult than for the ant host since not many cestode genomes have been annotated and the closest relative with an annotated genome is *Echinococcus granulosus*. We thus additionally chose those 15 cestode proteins with the highest abundances in the haemolymph of infected ants and employed a eukaryotic feature-based function prediction with FFPred 3 (Cozzetto et al., 2016). We furthermore identified significantly enriched GO terms with the use of topGO as described above (Alexa & Rahnenführer, 2016) in a comparison to the transcriptome of *A. brevis*.

To identify whether cestode proteins were found in the haemolymph of ants due to secretion, we ran SignalP 5.0 (Nielsen et al., 2019) on all identified cestode proteins to predict the presence of signal peptides, as well as SecretomeP 1.0f (Bendtsen et al., 2004) to identify non-classical (i.e. not signal peptide-triggered) protein secretion. For the SecretomeP analyses, all proteins were considered putatively secreted when they showed a neural network score >0.6, according to Bendtsen et al. (2004). We calculated the share of

proteins with SignalP sites for the whole known *A. brevis* protein set as well as for all proteins present in at least one replicate, in at least two replicates, in at least three replicates and in all four replicates. We compared the share of proteins with SignalP sites among those groups using a χ^2 test.

For the identification of cestode candidate proteins that might contribute to the phenotypic changes observed in infected ants, we used only those proteins that are putatively secreted, were annotated in other organisms, have functions in longevity or immunity and were found in at least two/all four replicates.

As the resulting protein abundances of whole cestode mass spectrometry analysis and haemolymph analysis are not directly comparable, we employed the same approach as for the comparison of ant proteins between queens and workers. Briefly, we filtered out proteins that were only found in one of the measurements of haemolymph and whole cestode samples and calculated the relative abundances of the remaining proteins. Those values were used for a Welch *t*-test. Significantly more abundant proteins in either haemolymph or whole cestode samples were identified based on $\log_{10}$ transformed *p*-values and $\log_2$fold change differences in the relative abundances. We furthermore compared the percentage of annotated proteins between haemolymph and whole cestode data, and between proteins that are predicted to be secreted and non-secreted. We also compared the two data sets regarding the results of automated word category searches for longevity, immunity, transport and epigenetics.

3 | RESULTS

3.1 | Parasite protein identification

We identified 263 proteins from the haemolymph of infected ants that matched the transcriptome of *A. brevis* and that were present in at least two of four biological replicates. Identification of protein functions proved difficult. Only 137 proteins could be functionally identified via a BLAST search against the non-redundant invertebrate database. For the remaining 126 proteins, either no BLAST result was found (112 proteins)—even after adjusting the parameters to be less stringent—, or the proteins from related species were annotated as 'hypothetical protein' (14 proteins).

Next, we investigated whether those parasitic proteins exhibit evidence of active secretion by exhibiting a predicted signal of peptide cleavage (SignalP). While the percentage of putatively secreted proteins versus non-secreted proteins was constant across the number of replicates in which proteins were found (Figure S1 in Data S1), the share of proteins with SignalP sites, indicating their secretion, increased with the number of replicates (Figure 2d). Compared to the percentage of proteins with SignalP sites that were predicted across the complete proteome of *A. brevis* (8.6%), we found a higher-than-expected proportion of those proteins present in the haemolymph with this molecular indicator of secretion (present in at least one replicate (20.3%): $\chi^2=5$, $p=.03$; present in at least two replicates (20.5%): $\chi^2=5$, $p=.03$; present in at least three replicates (28.3%): $\chi^2=12$, $p=.0007$; present in all four replicates (33.7%): $\chi^2=17$, $p=.00003$).

The mean share of cestode proteins in the haemolymph of infected ants amounted to 7% of all identified proteins across the four biological replicates (Figure 2c). Of the 15 most abundant cestode proteins, only two (thioredoxin peroxidase and acylneuraminate cytidylyltransferase) could be annotated (Figure 2a). For the remaining candidates, we procured a prediction for functional GO terms with FFPred 3 (Cozzetto et al., 2016), of which transport, regulation of metabolic process, regulation of nitrogen compound metabolic process and cellular localisation were found for all 15 proteins (Figure 2b).

3.2 | Comparison of whole cestode and haemolymph proteome

More proteins were identified from whole cestode samples (3188) compared to cestode proteins detected in *T. nylanderi* haemolymph (263). Of those proteins, 3035 are uniquely found in whole cestodes, whereas 110 are uniquely found in the haemolymph, possibly due to dynamic range detection by mass spectrometry and/or low abundance in whole cestode samples. The proportion of unknown proteins (i.e. proteins that had no BLASTp hit at an e-value of $1e^{-5}$) was much higher in the haemolymph samples (66.9%) compared to whole cestode samples (16.5%). Of the 51 proteins that showed a higher relative abundance in the haemolymph compared to whole cestodes, only two proteins had an annotation (Figure S2 in Data S1): u1 small nuclear ribonucleoprotein A and *C. briggsae* CBR-HIS-71 protein.

3.3 | Identification of candidate parasite proteins

Based on their presence in all four replicates and a positive prediction of secretion, we identified cestode proteins that could contribute to the phenotype of infection (Table 1; for proteins that occur in at least two replicates, see Table S1). The two main candidates are superoxide dismutase and thioredoxin peroxidase, which both reduce oxidative stress. Markedly, both proteins were among the cestode proteins with the highest abundance in the ant's haemolymph (Figure 2a). We also identified tetraspanin, a protein that is commonly used as a marker for exosomes, extracellular vesicles that enable intercellular communication (Drurey & Maizels, 2021). Notably, tetraspanin is not among the most abundant cestode proteins in this context.

3.4 | Host protein differential abundance analysis

Of all proteins that were found in the ant's haemolymph, 93% could be matched to the protein database of *T. nylanderi* (Stoldt et al., 2021)

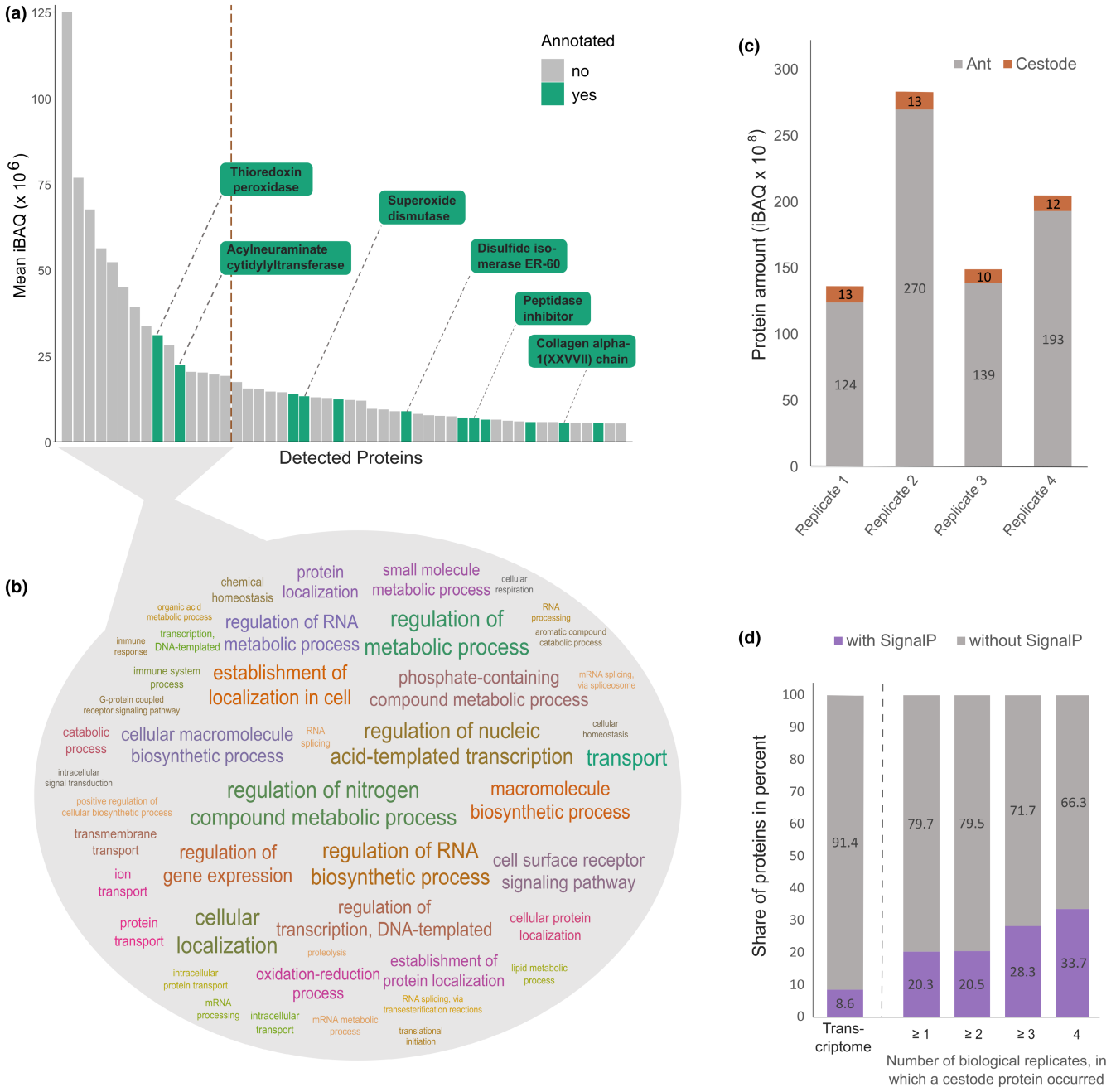

FIGURE 2 (a) Mean sum of the intensity-based absolute quantification (iBAQ) of peptides across the four replicates, depicted for the 50 most abundant *A. brevis* proteins in the haemolymph of infected ants. Proteins for which a BLAST result was found are shown in turquoise. Dashed line indicated cut-off of 15 most abundant proteins for GO prediction. (b) Summary of GO term prediction of the 15 most abundant cestode proteins found in the haemolymph of infected ants with an occurrence >3. (c) Abundance of proteins that are identified as *T. nylanderi* or *A. brevis* within the haemolymph of infected ants. (d) Share of *A. brevis* proteins with or without SignalP sites indicative of secretion. Shown are proteins that are found in at least 1, at least 2, at least 3 and in all biological replicates.

and amount to 1646 proteins. Of those proteins, 57 were significantly more abundant ($p < .05$, Welch *t*-test, and $\log_2$ fold change >1) in infected ants, 21 proteins in comparison to uninfected nestmates (Figure 3a) and 36 in comparison to uninfected workers from uninfected colonies (Figure 3b). In uninfected nestmates, 66 proteins were significantly more commonly detected, 37 compared to infected individuals and 29 compared to healthy individuals (Figure 3c). Healthy individuals from uninfected nests showed an overrepresentation of 20 proteins, including 19 compared to infected ants and one protein compared to uninfected nestmates of infected workers.

A differential abundance analysis yielded several candidate pathways that might contribute to the observed phenotypic changes due to cestode infection (Table 2; for a complete list, see Data S2A), many of which were postulated to extend lifespan in ants and other insects (Currin-Ross et al., 2021; Fang et al., 2014; Słowińska et al., 2019; Su et al., 2021), including sequestosome-1, chymotrypsin-1 and

TABLE 1 Cestode proteins with secretory signals and occurrences across all four replicates that potentially contribute to the parasitised phenotype.

Protein ID	Gene name (BLAST)	Function	Citation/organism
TRINITY_DN236_c0_g2_i3	Peptidyl-prolyl cis-trans isomerase [*Echinococcus granulosus*]	Regulation of histone H3-K36 trimethylation	Ahn et al., 2016 *C. elegans*
TRINITY_DN4073_c0_g2_i2	Thioredoxin peroxidase [*Echinococcus granulosus*]	Oxidative stress reduction	(Wang et al., 2019) *E. granulosus*
TRINITY_DN4612_c0_g2_i3	Calreticulin [*Echinococcus granulosus*]	Oxidative stress reduction	(Lim et al., 2014) *C. elegans*
TRINITY_DN897_c0_g1_i2	Superoxide dismutase [Cu-Zn] [*Echinococcus granulosus*]	Oxidative stress reduction	(Yang et al., 2007) *C. elegans*

chymotrypsin-2, while one protein with additional functions in immunity, ras-related protein Rac1, is postulated to decrease lifespan in *Drosophila* (Slack, 2017). Infected ants showed a higher abundance of proteins that are implicated in GO functions such as cytosolic 10-formyltetrahydrofolate catabolic process, innate immune response or L-serine biosynthetic process (Figure 3d, for a complete list, see Tables S2 and S3 in Data S1).

We also noticed an overabundance of the protein very long-chain fatty acid—CoA ligase bubble gum, which has recently been found to increase in expression with age in termite queens (Séité et al., 2022). Finally, infected individuals, compared to nestmates and healthy individuals, showed a 7- and 18-time higher abundance of Vg-like A (Figures S4 and S5 in Data S1), a protein that elicits brood care behaviour in *Temnothorax longispinosus* (Kohlmeier et al., 2018) and *Diacamma* sp. workers (Miyazaki et al., 2021).

In non-infected nestmates of infected workers, we found a higher abundance of proteins that reduce lifespan in other species, such as calpain-A, ras-related protein Rac1 and beta-1,3-glucan-binding protein (through upregulation of the toll-signalling pathway), as well as peroxiredoxin-6, which has a positive influence on lifespan through detoxification (Quigley et al., 2018). For the results of a GO term enrichment analysis in non-infected nestmates and healthy individuals, see (Tables S4–S7 in Data S1).

For the results of differential abundance analyses following corrections for multiple testing, refer to Data S6 and Tables S10–S12.

3.5 | Comparison of queen and worker proteome

We compared the proteome of queens to the proteome of healthy and infected workers in a differential abundance analysis to identify proteins that long-lived infected workers have in common with long-lived queens. Two proteins were more abundant in queens compared to healthy workers, icarapin and Vg-like-A (Figure S3 in Data S1). Those two proteins were also more abundant in infected workers compared to both healthy workers (Figure S4 in Data S1) and nestmate workers (Figure S5 in Data S1).

We furthermore checked for proteins that are uniquely detected in both healthy queens and infected workers and found fatty acid-binding protein 1, liver-like and facilitated trehalose transporter Tret1-like (Figure S6 in Data S1). The latter protein regulates the level of trehalose within the haemolymph (Elbein et al., 2003). In healthy queens, we found 30 different proteins that were unique to them as compared to healthy workers (Table S8 and Figure S7 in Data S1). Of those, 24 proteins were uniquely shared with infected workers (Table S8 in Data S1), of which four were previously found in the enrichment analysis in infected workers (two uncharacterised proteins, death-associated protein 1 and chymotrypsin-2). While conclusive studies on its function in insects are missing, death-associated protein 1 positively regulates apoptosis in shrimp (Xia et al., 2017), while chymotrypsin-2 was shown to extend lifespan in *Drosophila* (Nguyen et al., 2019).

4 | DISCUSSION

In the course of host–parasite co-evolution, parasites have found many fascinating ways to take advantage of the host resources, evading immune responses and increasing their chances of transmission to the next host. The latter category in particular has been identified as a target for parasite-mediated host manipulation. Cases in which parasites actively alter the behaviour of their hosts to facilitate the encounter between intermediate and final hosts are well known (Fayard et al., 2020; Santiago-Alarcon & Ferreira, 2020; Tong et al., 2021). Not only can potential targeted tissues or metabolic pathways be diverse but also the mechanisms by which parasites influence them. Parasites can cause changes in the host, for example, by their location alone, by energy drain, by inflammation of neural tissue, by influencing the host's epigenome or by protein–protein interactions (Doherty & Matthews, 2022; Lafferty & Shaw, 2013; Martin et al., 2015). By analysing the parasitic proteins present in the host's haemolymph, we gain insight into the latter mechanism. We, however, not only analysed proteins of parasitic origin but also the host's haemolymph proteome and found significant changes when comparing infected to healthy individuals. Those changes could be products of epigenetic manipulation or protein–protein interactions and thus signs of adaptive manipulation. They could, however, also be signals of untargeted but nevertheless beneficial by-products of the infection. In general, active manipulation is particularly difficult to prove because the symptoms of infection and exploitation are complex and their ultimate causes are difficult to separate from untargeted side effects, such

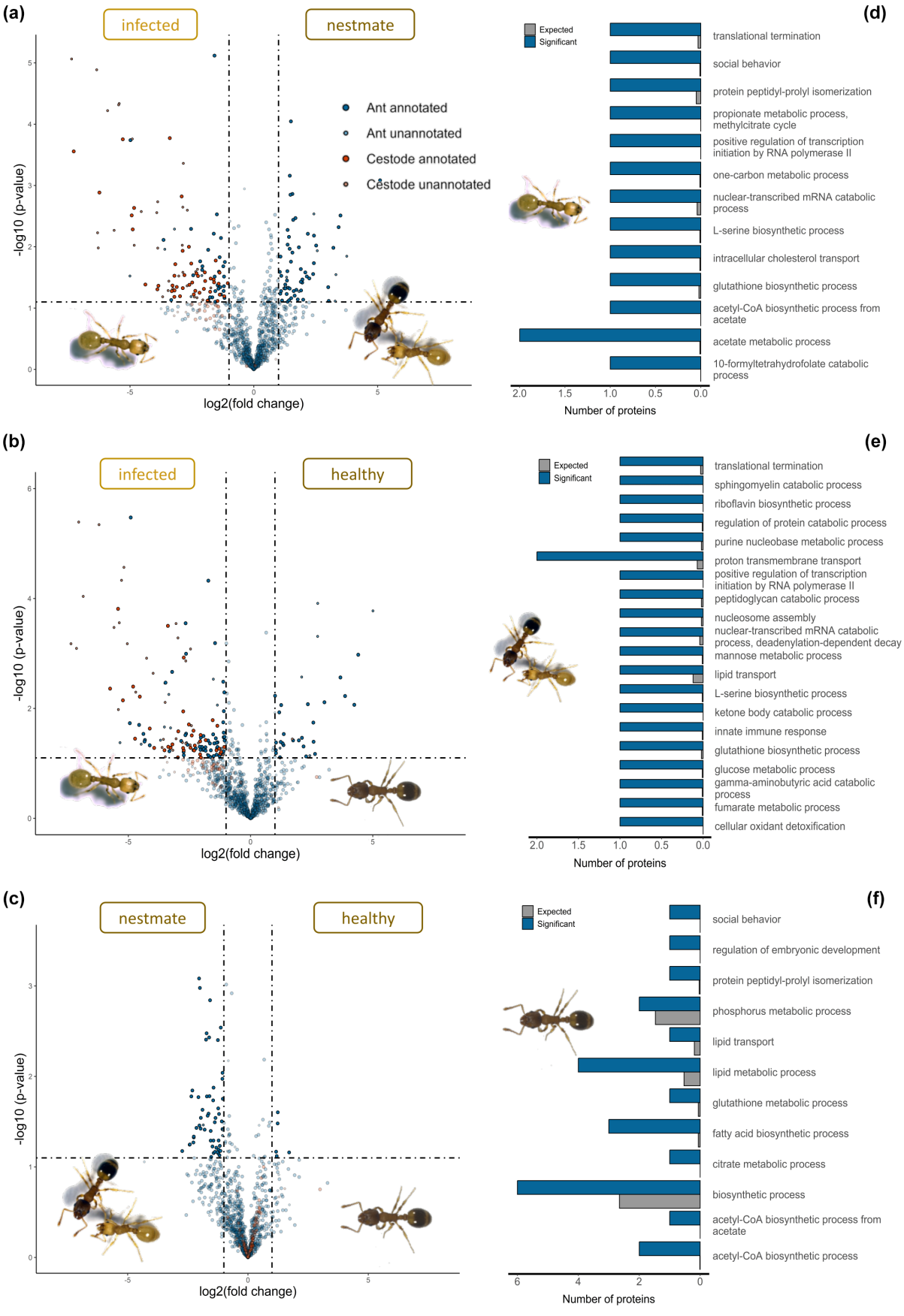

FIGURE 3 (a–c) Volcano plots of pairwise comparisons between infected, nestmates of infected and uninfected ants. Proteins which were identified as *T. nylanderi* proteins are marked in blue and proteins which were identified as *A. brevis* proteins are marked in red. Proteins with significantly higher abundances are depicted with stronger colours. (d–f) Results of a combined GO enrichment analyses encompassing all positively enriched GO terms found in both pairwise comparisons that included (d) infected ants, (e) uninfected nestmates and (f) healthy ants. Shown are the number of proteins that were enriched in the specific GO functions as well as the number of proteins that were expected to be enriched, based on the number of annotated proteins.

TABLE 2 Candidate genes for facilitating the parasitised phenotype, resulting from a differential abundance analysis.

Protein ID	Protein name (BLAST)	Function	Citation/organism
MSTRG.11757.1.p1	Death-associated protein 1 [*Solenopsis invicta*]	Regulator of apoptosis	Xia et al., 2017 *Marsupenaeus japonicus*
MSTRG.8546.2.p1	Chymotrypsin-1 [*Temnothorax curvispinosus*]	Deubiquitination	Nguyen et al., 2019 *D. melanogaster*
MSTRG.8542.1.p1	Chymotrypsin-2 [*Temnothorax curvispinosus*]	Deubiquitination	Nguyen et al., 2019 *D. melanogaster*
MSTRG.7977.1.p1	Chymotrypsin-2 [*Temnothorax curvispinosus*]	Deubiquitination	Nguyen et al., 2019 *D. melanogaster*
MSTRG.8184.1.p1	Sequestosome-1 [*Temnothorax curvispinosus*]	Autophagy, inactivation of TOR	Negroni et al., 2021 *T. rugatulus*
MSTRG.174.1.p1	Vitellogenin-like A [*Temnothorax curvispinosus*]	Division of labour, brood care	Kohlmeier et al., 2018 *T. longispinosus*

as the host's immune response (Heil, 2016). Furthermore, since experimental infections are not yet possible in this system, we are forced to use naturally infected ants. Therefore, we were not able to take into account several confounding factors that could have influenced our results, such as the age and diet of the workers and the duration of the infection. In addition, infected ants might have come into contact with parasite eggs once or several times during their development. Seemingly uninfected ants may have been able to overcome the infection. However, we have never yet found workers exhibiting the infected phenotype that were not infested with cysticercoids. Therefore, if parasite clearance is possible, it likely happens in the larval stage of the ants, which subsequently develop normal melanisation and sclerotisation of their cuticle. We therefore urge caution when interpreting the results and will discuss them accordingly below.

4.1 | Parasite proteins in the host haemolymph

We were particularly interested in determining the molecular basis of the extended lifespan of infected workers. In social insects, the life expectancy of individuals belonging to different castes can vary vastly. Reproductive individuals generally live much longer and show little sign of senescence compared to their worker counterparts. Oxidative stress is often discussed as the main cause of senescence (Finkel & Holbrook, 2000), but the links between oxidative stress, antioxidant processes and lifespan seem to be highly species-specific in social insects (de Verges & Nehring, 2016; Kramer et al., 2021; Lucas & Keller, 2014; Majoe et al., 2021). According to the oxidative stress theory of ageing (Finkel & Holbrook, 2000), long-lived organisms should be characterised by enhanced molecular repair capabilities, lower rates of molecular damage (i.e. lower production of reactive oxygen species or fewer replication defects) and/or higher antioxidant production. Manipulation of any of these mechanisms could alter the ability of individuals to cope with oxidative stress and thus influence the progression of senescence. Indeed, among the most highly secreted (annotated) cestode proteins, we found several proteins with a potential function in oxidation reduction. However, infection adds another dimension to this picture, as oxidation is a common strategy of an insect's immune system in the defence against parasites, especially by using encapsulation and melanisation (reviewed in Zug & Hammerstein, 2015). In turn, parasites release antioxidants to counter those attacks and ensure a successful infection (de Bekker et al., 2015; Mesías et al., 2019; Piacenza et al., 2013). We would nevertheless argue that the most plausible reason for the common release of such antioxidants is to actively prolong the lifespan of the ant host, as we did not find an enrichment of ant proteins with oxidative functions and considering that we only analysed adult ants with an (potentially long) established infection, which takes place during the larval stage, making it unlikely that the release of antioxidants is targeted at countering an attack by the host.

For most of the proteins released by the cestode into the ant's haemolymph, no functional annotations could be found. This stands in striking contrast to the complete proteome of the cestode, where more than 80% of the proteins could be annotated. The closest relative with an annotated genome, *Echinococcus granulosus* (Korhonen et al., 2022), belongs to a different family, the *Taeniidae*; thus, it was to be expected to find proteins that are not present in the other species. However, the profound discrepancy in the share of novel proteins between the secretome and proteome of the parasitic cysticercoid was surprising in its extent and might reflect unique

adaptations specific to this host–parasite interaction. In several other fungal and cestode parasites, researchers indeed discovered many novel proteins that the parasites release during infection (Berger et al., 2021; de Bekker et al., 2015). In this regard, it would be interesting to examine which proteins are released during the different life stages of the ant, as we analysed adult ants with already established infections and the release of proteins could be age-dependent. Functional annotations, especially of the most secreted proteins, could reveal new and specific properties important for infection dynamics or lifespan extension.

One of the most important challenges to overcome for any parasite is the host's immune system. Many protozoan or helminthic parasites choose immune-privileged sites (i.e. sites that are less targeted by immune responses), employ antigenic variation or masking, molecular mimicry, antibody trapping or secrete proteases to evade the immune system (Cortés et al., 2017; Flisser et al., 1986). Dissections of the infected ants so far showed no detectable immune response in the form of encapsulation or melanisation (Stoldt et al., 2021), which indicates successful evasion of the immune system, although the mechanism used is so far unknown. One of the most abundant cestode proteins that we found in the haemolymph of infected ants, acylneuraminate cytidylyltransferase, might provide a possible explanation for this. This protein synthesises CMP-Neu5Ac, the activated form of N-Acetylneuraminic, which constitutes the basic monomer of polysialic acid (Bravo et al., 2001). Polysialic acid, either as a capsule or as components of mucins, is used by different parasites and seems to convey a means to escape the host immune response through molecular mimicry (Ghosh, 2020).

Parasites not only attempt to modify host behaviour or physiology through the release of proteins. Especially helminths are known to use exosomes to facilitate intercellular parasite–host communication (Coakley et al., 2016). Exosomes can contain proteins, but also lipids and nucleic acids such as miRNAs (Coakley et al., 2016; Drurey & Maizels, 2021). Especially the latter are proposed to interfere with host gene expression and thus directly influence the production of proteins (reviewed in Britton et al., 2020). We found tetraspanin, which is a known marker for exosome activity, in all replicates of cestode proteins within the ant haemolymph, suggesting that transfer of materials occurs between the cells of the host and the cestode. In line with this, a gene expression study on the same system found vesicle-mediated transport to be enriched in cestodes from ants with a low parasitic load compared to highly infected individuals (Sistermans et al., 2023).

4.2 | Infection-induced changes of the ant proteome

Infected ants exhibit a mixture of traits characteristic of young workers (morphology, high fat content and metabolic rate; Beros et al., 2021) and queens (increased longevity, reduced activity and high reproductive potential; Beros et al., 2019). Since caste differences in social insects are usually not due to genetic differences but are controlled by differential gene expression, hijacking pre-existing regulatory pathways that make a worker more queen-like might be an elegant strategy from the parasite's point of view. However, as discussed earlier, experimental proof for such mechanisms is nearly impossible, as those changes in phenotype might be by-products of the infection and, as such, though beneficial to the parasite, cannot be regarded as adaptive host manipulation. We nevertheless would like to highlight potential pathways and genes that constitute potential targets of adaptive manipulation according to our findings.

One objective was to find proteins that might facilitate the more queen-like traits of the infected workers, that is, proteins that queens and infected workers have in common compared to healthy workers. A previous transcriptome study on this system found no more overlap in gene expression between queens and infected workers than expected by chance, albeit both overexpress carboxypeptidase B, which in *D. melanogaster* is encoded by the silver gene, and linked to a prolonged lifespan (Carnes et al., 2015; Stoldt et al., 2021). Due to post-transcriptional and post-translational regulation mechanisms, mRNA expression will not directly translate 1:1 to protein abundances (Gunawardana & Niranjan, 2013) and might only show parts of the picture. Proteomic data can thus help to add to our understanding of how an infection translates into an infection phenotype and, furthermore, allow us to sample and analyse processes that are happening in the haemolymph. Indeed, we found two candidates that were more abundant in both groups. One of them is vitellogenin-like A. In social hymenopterans, *Vg* genes have diversified and are especially known for their functions with regard to the division of labour (Corona et al., 2013; Kohlmeier et al., 2018; Morandin et al., 2014). The abundance of certain copies of *Vg* in honeybees positively correlates with worker lifespan (Corona et al., 2007; Münch & Amdam, 2010). *Vg* is preferentially carbonylated and thus functions as a buffer to oxidative damage for other proteins (de Verges & Nehring, 2016), which facilitates higher oxidative stress resistance in the honeybee (Seehuus et al., 2006). We found a higher *Vg*-like A abundance in infected individuals, which might contribute to protection from oxidative damage and an extended lifespan. Since *Vg*-like A specifically has been shown to control nursing behaviour and interest in chemical brood cues in *Temnothorax* workers (Kohlmeier et al., 2018), a higher abundance of this protein might explain the preference of infected workers to stay close to the brood (although they exhibit little brood care behaviour). This in turn could help increase the likelihood of transmission to the final host by keeping infected individuals within the colony. The fact that this protein is more abundant in both queens and infected workers underlines its putative role in facilitating the queen-like phenotype of infected individuals regarding both behaviour and lifespan.

We furthermore found two proteins exclusively in infected workers and healthy queens: fatty acid-binding protein 1 and facilitated trehalose transporter Tret1-like. Especially the latter is another interesting candidate for further elucidation of the long-lived phenotype of infected workers, as this protein is responsible for transporting trehalose, the main source of energy for insects, from the fat body into the haemolymph. There, one of trehalose's functions is

the protection from different environmental stressors, among them oxidative stress (Elbein et al., 2003), against which infected workers seem to be particularly resistant (Beros et al., unpublished data).

Our study system is special in many ways, as one might argue that on an individual level, an infection might be beneficial (longer lifespan, increased care received from nestmates and increased reproductive potential; Beros et al., 2019); however, the colony as a whole suffers fitness losses (Beros et al., 2015; Scharf et al., 2012). Following and expanding on Dawkins' theory of the *extended phenotype* Dawkins (1982), in which, classically, one party influences the expressed phenotype of another party beyond the reach of their normal physical constraints, we find consequences of parasitism on the level of the whole colony. Previous studies showed that infected colonies are less aggressive and uninfected workers live shorter (Beros et al., 2015, 2021), and furthermore, they exhibit signs of stress by raising more queen–worker intercastes and displaying a male-biased sex ratio (Beros et al., 2021). Also on a level of gene expression, the influence of a cestode infection on uninfected nestmates could be shown (Feldmeyer et al., 2016). It is thus not surprising that we found alterations in the proteome of uninfected nestmates compared to workers from uninfected colonies. Markedly, the proteome of uninfected nestmates showed an enrichment of processes that are responsible for detoxification and immune response. This could be a consequence of the observed shifts in the colonies' general stress level (Beros et al., 2021) that could lead to a higher susceptibility to pathogens, which would explain the higher expression of those pathways. We would like to point out again here that we used naturally infected ants and colonies, so some of our results could be due to confounding factors related to infection.

5 | CONCLUSION

We were able to identify proteins actively secreted into the host with functions in oxidation reduction and putative immune escape properties that may (i) explain how the cestode is able to maintain host manipulation and that (ii) likely contribute to the exhibition of the observed infection phenotype of their host with a substantially prolonged lifespan. On another level, we add to studies that show that not only the infected individual carries the consequences of an infection, but that the colony as a whole is affected. For most proteins secreted by the cestode into the host, annotated orthologues could not be found, which on the one hand severely limits our interpretation of the data but on the other hand is indicative of potential novel functions and a history of close adaptation to the host. Especially for the most abundant proteins, no matches in sequence similarity could be found. We found a marker for exosome activity, suggesting cell–cell communication from parasite to host. Within the host's proteome, we identified proteins that are subject to parasite-induced shifts in abundance and that are prime candidates to explain the infection phenotype with its extended lifespan and behavioural alterations. Among them is vitellogenin-like A, a protein with functions in the division of labour and fecundity. Future studies should focus on the identification of unknown novel proteins, either by structural predictions or knockouts.

AUTHOR CONTRIBUTIONS

Susanne Foitzik and Falk Butter conceptualised the study. Marion Kever and Jenny Fuchs prepared the samples. Alejandro Ceron-Noriega conducted the proteomic measurements. Juliane Hartke, Marah Stoldt and Tom Sistermans conducted the data analysis. Juliane Hartke and Susanne Foitzik drafted the manuscript. Juliane Hartke, Marah Stoldt and Susanne Foitzik edited the manuscript. Susanne Foitzik acquired the funding used for this research.

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CONFLICT OF INTEREST STATEMENT

The authors report no conflict of interest.

DATA AVAILABILITY STATEMENT

Processed label-free quantitative proteomics data are provided in Data S4 and raw peptide counts can be found in Data S5. Raw mass spectrometry data have been deposited to the ProteomeXchange Consortium via the PRIDE Archive database under the Project Name 'Cestode and ant infection proteome' and the Accession number PXD040025. The R scripts used for the analysis are publicly available on the first author's GitHub (github.com/jubiology/parasite-proteomics).

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