Gene expression patterns underlying parasite-induced alterations in host behaviour and life history

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Abstract
Many parasites manipulate their hosts’ phenotype. In particular, parasites with complex life cycles take control of their intermediate hosts’ behaviour and life history to increase transmission to their definitive host. The proximate mechanisms underlying these parasite-induced alterations are poorly understood. The cestode Anomotaenia brevis affects the behaviour, life history and morphology of parasitized Temnothorax nylanderi ants and indirectly of their unparasitized nestmates. To gain insights on how parasites alter host phenotypes, we contrast brain gene expression patterns of T. nylanderi workers parasitized with the cestode, their unparasitized nestmates and unparasitized workers from unparasitized colonies. Over 400 differentially expressed genes between the three groups were identified, with most uniquely expressed genes detected in parasitized workers. Among these are genes that can be linked to the increased lifespan of parasitized workers. Furthermore, many muscle (functionality) genes are downregulated in these workers, potentially causing the observed muscular deformations and their inactive behaviour. Alterations in lifespan and activity could be adaptive for the parasite by increasing the likelihood that infected workers residing in acorns are eaten by their definitive host, a woodpecker. Our transcriptome analysis reveals numerous gene expression changes in parasitized workers and their uninfected nestmates and indicates possible routes of parasite manipulation. Although causality still needs to be established, parasite-induced alterations in lifespan and host behaviour appear to be partly explained by morphological muscle atrophy instead of central nervous system interference, which is often the core of behavioural regulation. Results of this study will shed light upon the molecular basis of antagonistic species interactions.

Keywords: behavioural manipulation, extended phenotype, host–parasite interactions, insects, transcriptome

Received 11 September 2015; revision received 16 November 2015; accepted 18 November 2015

Introduction
Parasites can manipulate the behaviour, morphology, physiology and life history of their hosts (Poulin & Thomas 1999; Thomas et al. 2010). This is particularly true for parasites with complex life cycles that are known to take control over the behaviour of their intermediate hosts in order to increase transmission to the definitive host. These host manipulations can range from slight changes in pre-existing traits to the display of entirely novel behaviours (Poulin 1994; Thomas et al. 2002), a phenomenon also called the ‘extended phenotype’ of a parasite (Dawkins 1982). Classical examples
of parasite-manipulated host behaviour include ants that climb to the apex of grasses and remain there, facilitating their parasites’ transmission to grazers such as sheep (Hohorst & Graefe 1961; Carney 1969; Libersat et al. 2009), or terrestrial insects that ‘commit suicide’ by jumping into water where the parasite can complete its life cycle (Moore 1995; Biron et al. 2005). The proximate mechanism of these fascinating behavioural manipulations is still poorly understood (Thomas et al. 2010; Biron et al. 2013; de Bekker et al. 2014) and is a hot topic in evolutionary biology (Van Houte et al. 2013).

The various mechanisms of parasite manipulation of host behaviour most likely evolved in the context of manipulation of other host traits required for the parasite’s survival, especially the immune system (Adamo 2013). To manipulate their hosts, parasites may use a suite of different strategies: (i) destruction of sensory structures and muscles (Beckage 1997), (ii) genomic and proteomic alterations, (iii) psychoneuroimmunological and (iv) neuropharmacological mechanisms (Adamo 2013). The tight connection between the immune and the nervous system facilitates carry-over effects. Behavioural changes might thus be due to neuronal consequences of parasites addressing the hosts’ immune system (Dantzer et al. 2008; Adamo 2013). Moreover, immune responses to parasite infection can have a suite of indirect effects on host phenotype, like reduced fertility, changes in melanization and increased lifespan, often based on resource trade-offs (Moret & Schmid-Hempel 2000; Ahmed et al. 2002). Parásite-induced alterations in host behaviour are expected to have a molecular basis in the central nervous system, which processes sensory information and transforms this into muscle activity (Biron et al. 2013). In some host–parasite systems, the parasite excretes neuro-effectors that directly induce behavioural changes in the host (Hakimi & Cannella 2011; McDonough & Rodriguez 2012). In other cases, parasites induce the host to secrete neuromodulators itself (Reilly et al. 1992), which is more effective for the parasite as it outsources the production costs to the host (Adamo 2013). Host–parasite genotype interactions seem to play an important role in host gene expression response upon infection and can be genotype specific (Barribeau et al. 2014).

Many manipulative parasites are known to exploit ants as intermediate hosts (e.g. Carney 1969; Moore 1995; Yanoviak et al. 2008). This is not only due to the ubiquity of ants, but also their social life style increases transmission probabilities of the parasite and thus makes them attractive targets. When parasites exploit social insects, they can influence not only their direct hosts, but also the entire society (Cremer et al. 2007; Konrad et al. 2012). This also holds true for our focal parasite–host association with the parasitic cestode Anomotaenia brevis (Plateaux 1972; Scharf et al. 2012) and its intermediate host, the ant Temnothorax nylanderi. To complete its life cycle A. brevis relies on predation of its intermediate host by its definitive hosts, the woodpeckers Dendrocopos major and Dendrocopos minor (Trabalon et al. 2000) (Fig. 1). Ants become infected during the larval stage by being fed with bird faeces containing the tapeworm eggs (Trabalon et al. 2000). These eggs develop into larvae, penetrate the ants’ gut wall and enter the haemocoel, where they transform into cysticercoids. The emerging infected adult ants exhibit a less pigmented, soft cuticle and are smaller than their uninfected brown nestmates (Fig. 2a; Trabalon et al. 2000; Scharf et al. 2012). Moreover, these workers are mainly inactive and remain in the nest, even when disturbed (Beros et al. 2015)—a behaviour that could increase parasite transmission to the definitive woodpecker host, which is known to feed on wood-boring insects and acorns. Despite their deviant chemical profile (Trabalon et al. 2000), these parasitized workers are accepted and well cared for by their unparasitized nestmates. Indeed, they are even more often fed and survive longer than unparasitized workers (Beros et al. 2015). Behavioural changes extend to all workers from parasitized colonies. Unparasitized workers in parasitized colonies showed behavioural patterns intermediate to those of parasitized workers and unparasitized workers from unparasitized colonies (Scharf et al. 2012). Interestingly, these unparasitized nestmates suffer from a reduced survivorship (Beros et al. 2015). Experimental manipulations further demonstrated that the presence of parasitized workers lowers the aggression of unparasitized workers towards intruders, indicating that parasites exploiting social animals cannot only induce phenotypic changes in their direct individual hosts, but also in unparasitized group members (Beros et al. 2015). Whether these reported parasite-induced changes are due to direct parasite manipulation of the hosts’ phenotype, host defences against parasite infection, or simple by-products of infection, benefitting neither parasite nor host still needs to be clarified. To gain more insights into the proximate basis of parasite-induced phenotypic changes, a first step is to understand how gene expression changes with infection status. Here, we investigate brain gene expression patterns of parasitized workers and unparasitized workers from parasitized and unparasitized nests. In particular, as we are interested in genes underlying the behavioural changes such as the inactivity of parasitized workers and the lower aggression levels of their nestmates.
Materials and methods

Ant collection

Temnothorax nylanderi colonies were collected from July to September 2013 from three sites close to Wiesbaden, Germany [Kloster Eberbach (KLE; n = 52): N50°02.357’, E8°02.983’; Lennebergwald (LBW; n = 79): N50°00.672’, E8°10.906’; Neroberg (NB; n = 35): N50°05.919’, E8°13.987’]. Ant colonies contained one queen and on average 81 workers (range: 32–150 workers). To exclude any bias due to differences in colony size, we included an unparasitized colony of similar size (±15 workers), for each parasitized colony. Parasitized colonies contained a mean of 12% parasitized workers (range: 4–37%) and at least ten unparasitized workers. The total number of workers per colony differed neither between replicates nor between treatments (Kruskal–Wallis tests, P = 0.90). Furthermore, the proportion and number of parasitized and unparasitized workers of parasitized colonies were not significantly different between replicates (Kruskal–Wallis tests, P = 0.85). All colonies were kept under standardized conditions (i.e. 20 °C and 12 h day/night cycle) for at least 3 weeks to minimize sample site effects. The ants were fed twice weekly with honey and crickets.

Sample preparation and sequencing

To investigate the parasites’ influence on gene expression, we compared three worker groups: unparasitized workers from ant colonies without any parasitized workers (UU); parasitized workers (PP, yellow, Fig. 2a) and unparasitized workers from parasitized nests (UP, brown, Fig. 2b). PP and UP workers were taken from the same colonies. Ant workers differ in behaviour

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were confirmed to contain cysticercoids. In two cases, all workers with the yellow, parasitized phenotype for the occurrence of cysticercoids of Anomotaenia brevis. The interpretation of any expression differences, especially of longevity genes, should take these presumably parasite-induced age differences between worker groups into account.

We extracted total RNA from ant brains for two reasons: (i) we were mainly interested in the causes of behavioural differences between workers of the different parasitism states, (ii) to exclude contamination with cestode RNA. In adult ants, the cestode resides in its cysticercoid stage attached to the ants’ intestine in the gaster (Plateaux 1972). To obtain a sufficient RNA yield, we pooled brains from 20 workers per replicate. We handled samples in batches of 10 brains to ensure RNA stability during brain preparation. Per sample, two batches were merged before RNA extraction. For each of the three worker groups, we prepared four biological replicates, that is, in total, we dissected 240 ant brains from 40 colonies. Each replicate from each treatment contained brains of workers from five colonies. On average, four workers (range 3–6) were removed per unparasitized colony and the same number for each worker type from parasitized colonies.

Each colony was disturbed only once and all required ants were moved to a petri dish with a small, wet paper tissue for humidity. Once the ants calmed down, brain dissections started. We tried to keep handling times as short as possible with about 10 min on average. We cannot rule out gene expression changes due to stress from worker isolation and the time needed for brain dissection. However, these factors should have affected all worker groups to the same degree. We focussed on brain tissue specifically, as we were interested in the molecular basis of the altered host behaviours. The head of a single worker was fixated in Flexaponal® dental wax (Dentaurum, Ispringen, Germany) in a new, sterile petri dish, which was cooled on ice during the entire procedure. Under a stereomicroscope, the head was cut off with a sterile lancet and the torso moved to a second petri dish. The gaster was dissected to check for the occurrence of cysticercoids of Anomotaenia brevis. All workers with the yellow, parasitized phenotype were confirmed to contain cysticercoids. In two cases, workers with unparasitized phenotypes were found to be infested by the parasite, which occasionally happens in parasitized nests as noted before (Scharf et al. 2011); these individuals were excluded from further analyses.

The number of cysticercoids of dissected ants was counted, and ovary development was quantified. Temnothorax workers normally have two ovarioles, but no spermatheca; hence, they can only produce male offspring, which they only do in the absence of the queen (Konrad et al. 2012). Workers were categorized either as (i) fertile, if we detected eggs in development in the ovarioles, (ii) infertile, when the ovaries clearly contained no eggs or (iii) undetermined, when the ovaries were undeveloped or destroyed by dissection. The latter individuals were removed from the further fertility analyses. In total, we successfully dissected the ovaries of 116 workers.

For brain dissection, the head was opened with a cut between the eyes and antennae. The brain was removed using a barbed needle and transferred into a tube containing 75 µL TRIzol (Life Technologies). After ten brain dissections, the brains were homogenized and stored at −80 °C.

For RNA isolation of each replicate, two batches with 10 brains each were merged. Then, 150 µL chloroform was added to the tube and the mixture was vigorously shaken for 5 and 15 min centrifuged at 4 °C at 11 500 g. The upper aqueous phase was transferred to RNase-free microcentrifuge tubes, and RNA was precipitated by adding 60 µL absolute ethanol. For subsequent RNA isolation, we used the RNeasy Mini Kit (Qiagen) and followed manufacturers’ instructions. Isolated total RNA was eluted in 30 µL RNA-free water and stored at −80 °C.

Libraries were constructed at Genterprise GmbH Mainz following the standard Illumina protocol, and each library was individually tagged. All 12 libraries were pooled and sequenced with 100 bp paired-end on 1.5 lanes of an Illumina HiSeq 2500. Adapter remains were removed using the 

De novo assembly and expression analyses

The trimmed reads from all 12 replicates were used for the de novo assembly of the reference transcriptome as follows. First, subassemblies based on reads from the four libraries for each of the three worker groups were generated with the 

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contigs, average contig length), we decided to use contigs of word size 45 from all three worker-type sub-assemblies for a subsequent meta-assembly with MIRA (Chevreux et al. 1999) (settings: job = de novo, genome, accurate, sanger).

These contigs were used as reference for the subsequent expression analyses in EDGER 3.4.2 (Robinson et al. 2010). Reads were aligned to the contigs using TOPHAT V2.0.10 (Trapnell et al. 2009) in combination with BOWTIE2 V2.1.0 (http://bowtie-bio.sourceforge.net/). Read counts were obtained using EXPRESS V1.5.1 (http://bio.math.berkeley.edu/eXpress/). Differentially expressed genes shared between groups, as well as uniquely expressed genes per group, were visualized using the online tool Venny 2.0 (http://bioinfogp.cnb.csic.es/tools/venny).

Functional annotation and enrichment analyses

All contigs were searched against the nonredundant arthropod protein database (NCBI, state December 2013) using BLASTX (Altschul et al. 1990). Functional annotation and enrichment analyses were performed using the Blast2Go online tool with default parameters (Conesa et al. 2005).

Cluster analysis

Genes belonging to the same gene network and function are assumed to show similar expression patterns (Langfelder & Horvath 2008). We therefore conducted a weighted gene co-expression network analysis, which clusters genes according to correlation in expression patterns using the R package WGCNA (Langfelder & Horvath 2008). This analysis was based on the 414 genes found to be differentially expressed between the three worker types. We adjusted the soft-threshold (β) values to ensure an approximate scale-free topology (Zhang & Horvath 2005) and set the minimum module size to 30 and a dynamic tree cut height to 0.2 to ensure a larger number of genes in each module to assess intramodule dynamics. Default settings were used for all other WGCNA parameters.

Behaviour, immunology and longevity candidate genes

Longevity candidate genes were obtained by literature search and the HAGR GENAGE database (http://geonomics.senescence.info/download.html), which features longevity genes for humans and other model organisms. Immunology-related candidate genes were obtained from the immunodb (http://cegg.uni-ge.ch/Insecta/immunodb) and Terrapon et al. (2014). Behavioural candidate genes were collected from numerous publications. (A list of all candidate genes and according references can be found in Table S1, Supporting information.)

Morphometric analysis

As our transcriptome analysis revealed lower expression of muscular genes in the brain of parasitized workers, we analysed by confocal microscopy the structure of the mandibular closer muscle located in the head. The mandibular closer muscle is one of the strongest ant muscles and much larger than the leg muscles, which are rather difficult to examine in 2 mm large workers. We compared workers of the parasitized phenotype (PP, N = 10) with their unparasitized nestmates (UP, N = 9) from 10 parasitized colonies. For this, ants were decapitated, the head capsule was fixed in a dental wax-coated dish and covered with a droplet of cooled ant-saline solution (127 mM NaCl, 7 mM KCl, 1.5 mM CaCl2, 0.8 mM Na2HPO4, 0.4 mM KH2PO4, 4.8 mM TES, 3.2 m Trehalose pH 7.0), adapted by Christine Dittrich and Manuel Nagel, University of Konstanz, during dissection. We opened the head capsule by cutting a window between the eyes above the brain and removed the cuticle. To avoid muscle destruction, the brain and all glands, trachea and fat tissue were left inside the head capsule. Henceforth, heads were immediately transferred to an ice-cold fixative (4% paraformaldehyde) containing additional 4% glutaraldehyde to increase tissue autofluorescence and stored at 4 °C for 4 days. No further staining was performed. Heads were then washed in a phosphate-buffered saline solution (PBS, pH 7.2, 5 × 20 min), followed by an ascending dehydration series of ethanol (50, 70, 90 and 100%; 20 min each) and finally cleared in methylsalicylic acid (M-2047, Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

Heads were viewed as whole-mount preparations using a laser-scanning confocal microscope (LEICA TCS SP8; Leica Microsystems AG, Wetzlar, Germany). Confocal images were taken at a resolution of 1024 × 1024 pixels using the customized settings for background fluorescence and scanned with an optical depth of 3.12 μm. Confocal image stacks were visually inspected and analysed using the free Leica LAS AF LITE v2.6.3 viewer. We used the stack profile tool to mark and calculate (report of pixel count values) the area of mandible closer muscles and intramuscular gaps. We used distinct landmarks (Fig. 5a,b) to select comparable sections of the mandibular closer muscle. At the selected layer, this muscle contains some regular gaps, which were present in all individuals. These gaps and their area were deducted when calculating the total number of gaps and the total area of the mandibular closer muscle for all individuals.
Statistical analyses

All statistical analyses were performed in the R v. 2.15.2. (R Development Core Team 2008).

Chi-squared tests were performed to test for differences in the frequency of fertile individuals between parasitized and unparasitized workers, and Wilcoxon tests to test for differences in the total number of gaps and total area of the mandibular closer muscle. Gene expression differences between the three worker types were analysed using the R package EDGER V3.4.2 (Robinson et al. 2010). Genes with significant expression differences after FDR-correction (p-FDR ≤ 0.05) (Benjamini et al. 1995) were classified as differentially expressed.

Results

In total, we obtained 442 808 496 raw reads after sequencing, of which 361 828 351 remained after quality trimming. The three CLC subassemblies resulted in 56 038–62 047 contigs with an average contig length of 757 bp (detailed summary statistics on reads and sub-assemblies can be found in Table S2, Supporting information). The subsequent MIRA meta-assembly resulted in 34 934 contigs, with a remaining un-assembled 14 275 original CLC-contigs (‘debris’). As the CLC sub-assemblies were context specific, these remaining CLC-contigs are assumed to contain valuable information for each of the three worker states. We thus added the ‘debris’ to the MIRA contigs, resulting in a total of 49 209 contigs, with an average length of 1376 bp and a N50 of 2643 bp. A BLASTX of these contigs vs. the non-redundant protein database (December 2013) gave 18 418 hits with ≤ e−5, of which 11 517 were single gene hits. Of the Blast hits, 65% were found in ants followed by 26% in other hymenopteran species (data not shown).

Gene expression analyses and functional enrichment

The expression analyses revealed a total of 414 differentially expressed genes (FDR ≤ 0.05) in pairwise comparisons between the three worker types (Table S3, Supporting information). In total, we identified 198 genes to be overexpressed in parasitized workers (PP) compared to the other two worker types, of which 177 were uniquely overexpressed in parasitized workers uniquely expressed (Fig. 2b). Unparasitized workers from unparasitized colonies (UU) had 168 overexpressed genes and 73 uniquely expressed genes. Unparasitized workers from parasitized colonies (UP) had the smallest number of overexpressed genes (156) in pairwise comparisons, as well as the smallest number of uniquely expressed genes (56). These workers also shared the highest number of genes (87) with the ULI workers and 13 genes with the PP workers. ULI and PP workers had eight overexpressed genes in common (Fig. 2b).

Within the set of shared genes among the two unparasitized worker types, we identified the cuticular protein 14 precursor, which could be involved in the hardening and pigmentation of the cuticle of unparasitized individuals. Moreover, we identified three enzymes (Esterase E4, Esterase FE4 and Fatty acyl-CoA reductase 2) among the uniquely expressed genes of PP workers, which might be involved in cuticular hydrocarbon synthesis (Blomquist & Bagnères 2010), potentially explaining chemical profile changes with parasitism status (Trabalon et al. 2000).

The functional enrichment analyses revealed the functions ‘ribosome’, ‘structural constituent of ribosome’ and ‘translation’ to be the only overrepresented functional categories in PP workers in comparison with the shared overexpressed genes between the two unparasitized worker types (Fig. 3). In the unparasitized worker types, several metabolic functions as well as actin binding was overrepresented in comparison with the uniquely expressed gene set of parasitized workers. Enrichment analyses based on uniquely expressed genes of the unparasitized worker types and the other sets with shared genes did not lead to the identification of additional overrepresented functional categories.

Candidate genes

We identified 36 immune candidate genes in our data of the 175 genes obtained from the immunology database, none of which was differentially expressed between the three worker types. The absence of differentially expressed immune genes might be due to our focus on brain tissue; however, immunity genes have been found to be differently expressed in brains of honeybee workers and queens (Grozinger et al. 2007). Comparable expression levels between the three worker types might suggest that the immune system of parasitized workers is neither impaired nor activated through cestode infection.

The same holds for genes related to aggressive behaviour. Here, 11 of a data set of 87 could be identified, but none of them was differentially expressed. Within the 857 genes spanning data set of longevity candidate genes, 119 were expressed in the brain of Temnothorax workers and two of these were differentially expressed between worker types: Cytochrome C was downregulated in parasitized workers in comparison with both unparasitized worker types, and Tropomysosine was downregulated in PP workers in comparison with ULI workers. Of six genes, which were shown to play a role in parasite-mediated host behaviour (Van Houte et al.
2013), the gene \textit{Tachykinin} could be identified as being overexpressed in \textit{UP} workers in comparison with \textit{PP} workers. Of 172 genes upregulated in fungus-infected workers vs. control workers (de Bekker \textit{et al.} 2015), 21 could be identified in our contig list. Of these, the gene \textit{Transferrin} was upregulated in \textit{PP} workers. Moreover, the genes \textit{Actin} and again \textit{Tropomyosin} were upregulated in unparasitized vs. parasitized workers in both studies. Expression levels of all mentioned candidate genes are shown in Table S4 (Supporting information).

\textit{Cluster analysis}

Weighted gene co-expression network analysis (WGCNA) (Langfelder & Horvath 2008) was used to identify modules (clusters) of correlated transcripts. This analysis not only allows to make inferences on genes associated with the three worker types (similar to the expression analysis), but it also associates contigs without annotation to a broad functionality as co-expressed genes are assumed to share the same function. The analysis resulted in five modules (Fig. 4; Table S5, Supporting information), which are associated with one of the three worker types. Three modules (yellow, blue and brown) are positively correlated with parasitized individuals. An enrichment analysis of the corresponding contigs falling in these modules identified the functions ‘ribosome’, ‘structural constituent of ribosome’ and ‘translation’ in all three modules plus ‘ribosome assembly’ as well as ‘small ribosomal subunit’ in the

\textbf{Fig. 3} Functional categories overrepresented in the uniquely expressed gene set of parasitized workers vs. the shared overexpressed genes of the two unparasitized worker types (most specific terms are depicted).

\textbf{Fig. 4} Module trait relationships of differentially expressed genes between the three different worker types. Rows correspond to modules and columns to worker types (Upper value: \(r = \) correlations of the corresponding module eigengenes by worker type; corresponding \(P\)-values printed below in parentheses).
brown and yellow module, respectively. The turquoise module, which is negatively correlated with the parasitized worker type, had the functions ‘troponin complex’, ‘muscle contraction’ and ‘actin binding’ enriched. No enriched functions could be detected for the grey module, which is highly positively correlated with the unparasitized workers from parasitized colonies. Only 47% of the contigs falling into this module had annotations in contrast to 67–88% in the other modules.

**Worker fertility**

Cestode infection was associated with a lower worker fertility; 36 and 37 unparasitized workers (81% and 77%) from parasitized and unparasitized nests had eggs in development in their ovarioles, whereas only 6 (25%) of the parasitized workers (chi-squared tests: overall: $\chi^2 = 26.1; \ P < 0.001$; UU – PP: $\chi^2 = 21.2; \ P < 0.001$, UP – PP: $\chi^2 = 18.0; \ P < 0.001$). There was no difference in fertility between the two unparasitized worker types (UII – UP: $\chi^2 = 0.3; \ P = 0.58$).

**Muscle structure**

Cestode infection was associated with a degradation of the mandible closer muscle. In comparison with their unparasitized nestmates, the mandible closer muscles of parasitized workers were less densely packed (Fig. 5a, b). Parasitized ants had less muscle tissue (Wilcoxon test: $W = 18, P = 0.003$, Fig. 5c) and more intramuscular gaps between muscle fibres (Wilcoxon test: $W = 84; P = 0.002$, Fig. 5d).

**Discussion**

Parasites often induce changes in their hosts’ phenotype, which can be due to host defences, by-products of infection, or parasite manipulation. Most fascinating are cases in which parasites take on the role of a puppet master and actively manipulate the behaviour of their host to increase transmission (Carney 1969; Eberhard 2000). The molecular mechanisms behind these behavioural manipulations often remain obscure, although...
first advances have been made (Biron et al. 2005, 2013; Van Houte et al. 2013; de Bekker et al. 2014, 2015). In the ant Temnothorax nylanderi, a suite of behavioural, morphological, chemical and life-history traits vary with infection by the parasitic cestode Anomotaenia brevis (Trabalon et al. 2000; Scharf et al. 2012; Beros et al. 2015). The parasite not only influences its direct host, but also unparasitized nestmates, leading to complex changes in the colony phenotype (Scharf et al. 2012; Beros et al. 2015). Here we investigate brain gene expression patterns associated with parasite-induced alterations in behaviour and life history. We identified several differentially expressed genes between parasitized workers and their uninfected nestmates. Among these are genes that can be linked to the increased lifespan of parasitized workers. Furthermore, many muscle (-functionality) genes are downregulated in these workers, which might be the cause for the observed muscular deformations and their behavioural inactivity.

**Muscle atrophy in parasitized individuals**

How do parasites manipulate host behaviour? In some systems, parasites make use of neuropharmacological substances such as dopamine, octopamine and serotonin to alter host behaviour (Beckage 1997; Klein 2003; Adamo 2013; Hari Dass & Vyas 2014). In our cestode system, however, the parasite seems to impair muscle development and functioning, rather than manipulate neurological pathways. Alongside with Tropomyosin, we found many actin and myosin genes to be downregulated in parasitized workers. The Tropomyosin family of actin-binding proteins plays a crucial role in the function of actin filaments by regulating the interaction between actin and myosin (Gunning et al. 2008). These filaments not only occur in muscle cells as parts of the contractile apparatus, but also in the cytoskeleton of any cell type (Gunning et al. 2008). A deficiency in Tropomyosin leads to weak muscles (Corbett et al. 2001), and low expression levels are generally associated with old age in mice (Bodyak et al. 2002). These traits therefore fit the phenotype of inactive parasitized workers, which are presumably older than their uninfected nest mates due to their higher survival rate. Further, we could show by confocal microscopy that indeed the structure of the mandibular closer muscle showed strong morphological aberrations in parasitized workers in comparison with unparasitized workers. A downregulation of Actin (de Bekker et al. 2015), in combination with extensive atrophy of the mandibular muscles was also observed in Camponotus leonardi infected by the fungus Ophiocordyceps unilateralis s.l. (Hughes et al. 2011). Parasite-induced muscle reduction in hosts is a common phenomenon (Beckage 1997). For example, the Trichinella parasite induces an inflammatory response in mice comprising an activation of oxidative stress, measurable in surrounding muscle fibres (Bruschi & Chiumiento 2011). Direct damage to the abdominal muscle of the host Gryllus rubens is caused by feeding of a parasitoid fly larva (Adamo et al. 1995). However, in these examples parasites directly and locally affect muscle tissue by either feeding on it or through inflammatory responses. In our system the parasitic cysticercoids are attached to the hosts’ gut, whereas the observed morphological and gene expression changes occur in the ants’ head, out of the direct range of the parasite. Furthermore, we would not expect a downregulation of a suite of muscle associated genes if the observed muscle degeneration would be due to lower resource availability, that is resources absorbed by the parasite.

**Longevity and fertility**

Recent analyses show that parasitized workers show a higher survival rate than their unparasitized nestmates and might thus be older (Beros et al. 2015). Their increased lifespan might either be due to parasite-induced upregulation of longevity genes or be a by-product of other alterations, for example the lower activity level of infected workers. Future analyses controlling for age will allow to disentangle cause and effect in respect to the changed expression of longevity genes in parasitized workers. Among the differentially expressed genes according to parasitism status were Cytochrome C and Tropomyosin, which are known to be involved in oxidative processes (Corbett et al. 2001; Sohal et al. 2008; Klichko et al. 2014). Cytochrome C was underexpressed in parasitized workers compared to both unparasitized worker types and has been shown to be associated with decreased walking speed and lifespan in Drosophila (Klichko et al. 2014). This fits the long-lived phenotype observed in cestode-infected T. nylanderi workers (Scharf et al. 2012). Moreover, among the uniquely expressed genes of parasitized workers, there were two additional genes (Phospholipid hydroperoxide glutathione peroxidase and Putative oxidoreductase ybE) associated with oxidation reduction (Holliday 2006), possibly contributing to the increased survival of parasitized workers.

Among the most abundant functional categories of differentially expressed genes between parasitized and unparasitized workers were those associated with ribosomal and translational genes. In yeast, worms, fruit flies and mice, a decrease in TOR (target of rapamycin) activity leads to a lower ribosomal biogenesis and translational activity, which in turn increases lifespan (Kaeberlein et al. 2007; Hands et al. 2009; Johnson et al. 2013).
and authors therein). Ribosomal activity can vary between developmental stages, cell and tissue types (Xue & Barna 2012), and different copies of ribosomal proteins or subunits take over diverse functions (Xue & Barna 2012). In contrast to the patterns described in the above-mentioned model organisms, we find ribosomal protein genes overexpressed in longer lived parasitized workers. As we did not control for age and parasitized workers survive much better (Beros et al. 2015), it is plausible that parasitized workers in our study are older than unparasitized ones and therefore have higher expression levels of ribosomal proteins. However, there are numerous genes with opposing expression patterns known in ants in comparison with other organisms. For example, an upregulation of the foraging gene leads to high motility in Drosophila larvae (rovers) (Dmfor) (Kent et al. 2009) and to foraging behaviour in honeybees (Amfor) (Ben-Shahar 2005), whereas in the harvester ant Pogonomyrmex barbatus, young callow workers are characterized by a higher Pbfor expression in comparison with foragers (Ingram et al. 2005). At the same time, we also have a reversal in the longevity-fecundity trade-off (Keller & Genoud 1997) in social hymenopterans where the secund queen outlives the sterile workers by up to decades. Alternative regulation of conserved molecular pathways associated with ageing and fecundity has recently been shown for ant queens (von Wyschetzki et al. 2015). It is thus conceivable that ageing-related mechanisms and pathways are differently regulated in ant workers then in the model organisms studied to date.

We would like to stress that even if age might seem like a confounding factor here, increased longevity is parasite-induced, and thus, the older age of infected workers is also an (indirect) effect of the parasite. Furthermore, fungus-infected Camponotus castaneus foragers (Hughes et al. 2011) show a similar muscle atrophy phenotype compared to noninfected foragers (of similar age), indicating that the parasite-induced muscle atrophy is not necessarily age related.

The increased longevity with infected workers could also be explained by resource allocation trade-off, according to which parasitized individuals invest less in fecundity and are thus longer lived. This hypothesis has been suggested in another tapeworm system, where infection by the rat tapeworm, Hymenolepis diminuta, leads to an increase in lifespan and lower fecundity in the female beetle host (Hurd et al. 2001). We observed the same pattern with parasitized workers having fewer eggs in their ovaries. Lower fecundity is also reflected in the expression pattern of the fertility candidate gene vitellogenin 3, which is down-regulated in cestode-infested individuals compared to unparasitized workers. Vitellogenin occurs in multiple copies in ants and is responsible for its original function in yolk protein production as well as for caste differentiation (Wurm et al. 2010; Feldmeyer et al. 2014; Morandin et al. 2014).

Effect on unparasitized nestmates

The gene Tachykinin was found to be upregulated in unparasitized workers from parasitized colonies in comparison with parasitized workers. This gene has been linked to aggressive behaviour in Drosophila and a range of other organisms (Pavlou et al. 2014 and authors therein). Tachykinin-related peptide (TKRP)-deficient flies display aberrant spatial orientation (Kahsai et al. 2010) and olfactory perception as well as enhanced locomotion, pointing to a role of TKRP in the modulation of locomotion activity in insects (Nässel & Winther 2010). TKRP also functions as release factor for the metabolic neuropeptide adipokinetic hormone (Nässel 2002), which has been suggested to be a prime target for parasite manipulation via host metabolic processes (Van Houte et al. 2013). It is therefore possible that the reduced aggression observed in nestmates of parasitized workers is modulated by the expression changes of Tachykinin. Whether this is caused by active manipulation of the parasite, an indirect effect of contact to parasitized individuals, or rather due to increased workforce of the unparasitized nestmates needs further investigation.

Conclusions and implications

According to the extended phenotype hypothesis (Dawkins 1982), the phenotype of infected hosts can be the consequence of parasite gene(s) being expressed. The altered phenotype can either be (i) adaptive for the parasite, by either increasing the parasites fitness through enhanced host-to-host transmission, or increased chances of finding a mate (Van Houte et al. 2013), (ii) it can result from the hosts efforts to reduce fitness costs of infection, or (iii) simply be a pathological side effect (Thomas et al. 2010). In our study system, the observed phenotypic changes in the host, mainly the reduced activity and longevity of parasitized workers, could be adaptive for the parasite as it increases the transmission probability to the definite woodpecker host (Trabalon et al. 2000). Inactive ant workers remain in the nest and do not participate in foraging and, through increased survival rate, have a four times higher likelihood of still being alive (data from Beros et al. 2015) when an acorn is eaten by a woodpecker. Even more so, as infected workers were shown to remain in their nest site even when under attack. At this moment we cannot disentangle the cause and consequence of increased survival and muscle atrophy. As we only determined the hosts’
side of the story so far, future studies should aim to identify the metabolites produced and released by the parasite to determine which genes and pathways are directly targeted. A recent study on the ant-fungus system Camponotus castaneus and Ophiocordyceps unilateralis sensu lato uncovered metabolites which are excreted by the fungus (de Bekker et al. 2014). Infection by the fungus leads to changes in activity patterns, as well as muscle atrophy in parasitized foragers vs. unparasitized foragers within the same age class (Hughes et al. 2011). Together, these results suggest that muscle atrophy might be yet another possible route for parasite-induced changes in host behaviour. Future studies, including RNAi-mediated gene knockdown and/or proteomics, may elucidate the causal link between the observed changes in gene expression and the alterations in behaviour and life history of parasitized individuals. These results will shed light on the molecular basis of antagonistic species interactions.

Acknowledgements

Collection permits were obtained at the local forestry department. We thank Heike Stypa for her help in ant dissection and ovary preparation. We thank Carsten Duch and Stefanie Ryglewski for permission and help with the use of the confocal microscope. We are grateful to Christoph Kleineidam, Manuel Nagel and Christina Dittrich for providing us with information and materials to conduct the brain dissections.

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Data accessibility

Original reads and .bam files were uploaded on NCBI (BioProject accession PRJNA287658; study accession SRP059789). De novo transcriptome assembly (contigs), read counts and morphometric data are available on Dryad (doi: 10.5061/dryad.c69n0).

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Lists of candidate genes from publications and databases.

Table S2 Summary of reads and pre-assembly contig summary statistics.

Table S3 Summary expression analyses.

Table S4 Expression profiles of candidate genes.

Table S5 Network analysis (WGCNA) module classifications.

B.F., S.F., H.L., J.M. and H.B. contributed to the design of the study. H.L., B.F., S.B. and S.F. collected the ant colonies. H.L. isolated the brains and prepared the samples for sequencing. J.M. and B.F. conducted the gene expression analysis. S.B. took the confocal microscope images. All authors contributed to writing the manuscript.