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Infection by the castrating parasitic nematode *Sphaerularia bombi* changes gene expression in *Bombus terrestris* bumblebee queens

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Short running title: Nematode alters bumblebee queen gene expression.

Abstract

Parasitism can result in dramatic changes in host phenotype, which are themselves underpinned by genes and their expression. Understanding how hosts respond at the molecular level to parasites can therefore reveal the molecular architecture of an altered host phenotype. The entomoparasitic nematode *Sphaerularia bombi* is a parasite of bumblebee (*Bombus*) hosts where it induces complex behavioural changes and host castration. To examine this interaction at the molecular level, we performed genome-wide transcriptional profiling using RNA-Seq of *S. bombi*-infected *Bombus terrestris* queens at two critical time-points: during and just after overwintering diapause. We found that infection by *S. bombi* affects the transcription of genes underlying host biological processes associated with energy usage, translation, and circadian rhythm. We also found that the parasite affects the expression of immune genes, including members of the Toll signaling pathway providing evidence for a novel interaction between the parasite and the host immune response. Taken together, our results identify host biological processes and genes affected by an entomoparasitic nematode providing the first steps towards a molecular understanding of this ecologically important host-parasite interaction.

Keywords: pollinator health, insect immunity, molecular parasitism, extended phenotype.

Introduction

Host-parasite interactions are amongst the most complex in the biological world (Poulin 1995). Both host and parasite can exert enormous selective pressures upon the other, and these have shaped their individual and co-evolutionary trajectories (Combes 2001). Central to this process is the genome, and the products it encodes for. Whilst the characterisation of selection signatures within host and parasite genomes can provide an insight into the evolutionary relationship between them (Combes 2001), investigating the expression of the genomes can identify the genes and genetic systems involved in these dynamic interactions (Biron & Loxdale 2013).

Parasitism can result in dramatic changes in the host phenotype, which may be a direct or indirect consequence of the host-parasite interaction. The expression of parasite genes can directly modify their hosts, resulting in an "extended phenotype" (Dawkins 1982). For example, parasites of humans secrete molecules that manipulate aspects of the host immune system (Maizels & Yazdanbakhsh 2003; Hewitson *et al.* 2009; McSorley *et al.* 2013; Buck *et al.* 2014), in some cases through effector proteins that have evolved to mimic or alter host functions (Sacks & Sher 2002). Dramatic examples of parasite-determined host phenotypes are also evident in insect hosts. Proteomic profiling identified candidate grasshopper host proteins with roles in neurogenesis that were impacted by infection with the nematomorph, *Spinochordodes tellinii* (Biron et al. 2005), and induced hydrophilic behaviour to enable completion of the parasite lifecycle, which resulted in host death through drowning (Thomas *et al.* 2002). High throughput transcriptomics has similarly uncovered genes underlying complex altered host phenotypes in response to parasite infections (de Bekker *et al.* 2015; Geffre *et al.* 2017; Guo *et al.* 2017).

Altered host phenotypes may be an indirect response to infection or present a parasite adaptation to increase parasite fitness. The entomoparasitic nematode, *Sphaerularia bombi* (Fig. 1B), infects and castrates queens in multiple species of bumblebee (*Bombus*) (Fig. 1A); it is found throughout the northern hemisphere (Khan 1957; Alford 1969a; McCorquodale *et al.* 1998; Rutrecht & Brown 2008; Maxfield-Taylor *et al.* 2011), South America (Plischuk & Lange 2012), and has been introduced to New Zealand (Macfarlane & Griffin 1990). *S. bombi* infection induces complex changes in the host phenotype, which have been suggested to increase parasite transmission (Poinar & Van Der Laan 1972; Lundberg & Svensson 1975). Infection of *Bombus* queens occurs in overwintering sites during host diapause (Pouvreau 1962; Madel 1966; Poinar & Van Der Laan 1972)(Fig. 1C). The exact site of host entry is unknown but the infective female adult stage is suggested to be able to enter through the mouth, anus or between the tegumental plates of the host (Poinar & Van Der Laan 1972). Upon entry, the nematode migrates to the host haemocoel and begins to evert its uterus and associated reproductive tract (Poinar & Van Der Laan 1972). Eversion is paused and the nematode enters a dormant state, overwintering within the diapausing host. When the infected host emerges from diapause, the nematode resumes eversion of its reproductive tract, which expands to a volume 300 times larger than that of the nematodes' body size. The nematode absorbs nutrients directly from the host haemolymph via invaginations present on the everted uterus (Poinar & Hess 1972). Eggs, containing larval stage 1 (L1) juveniles, are released into the haemocoel of the host, which undergo two further moults before emerging as stage 3 larvae (L3). L3 juveniles remain within the host for a period of time before actively burrowing into the digestive tract and exiting via the anus. L3 juveniles enter the soil and undergo two further moults before reaching sexual maturity. Females are fertilised and reside in the soil until contact with new hosts (Madel 1966; Poinar & Van Der Laan 1972).

Parasitised queens display altered behaviour compared to uninfected queens. Post-diapause, uninfected queens forage for resources before locating a nesting site and establishing a colony (Alford 1975) (Fig. 1). In comparison, parasitised queens forage but do not establish a colony, instead they investigate prospective overwintering sites, where parasite offspring are actively deposited (Poinar & Van Der Laan 1972; Lundberg & Svensson 1975). This behaviour coincides with a lack of development of the corpora allata (Palm 1948; Röseler & Röseler 1973; Röseler 2002), an endocrine organ that develops in uninfected queens post-diapause to produce juvenile hormone, a key regulator of ovarian development. This restriction has been suggested to be caused by secretions released by the parasite (Palm 1948; Pouvreau 1962) although examination and characterisation of secretory molecules by *S. bombi* has not yet been performed. Such morphological and behavioural changes are thought to represent a parasite adaptation that facilitates the dispersal of parasite progeny across locations to increase the probability of novel host encounter (Lundberg & Svensson 1975).

In addition to the direct effects of *S. bombi* on bumblebee queens, the nematode can have indirect ecological effects through the displacement of uninfected workers at foraging sites by *S. bombi*-infected queens (Kadoya & Ishii 2015). Given that parasite prevalence can be as high as 50% within certain geographical regions (Kelly 2009), the nematode may also impact host population dynamics. Consequently, understanding the interaction between *S. bombi* and its bumblebee hosts may help us elucidate drivers of host population dynamics in these globally declining pollinators (Brown & Paxton 2009).

Functional genomic techniques, such as RNA-Seq, provide an unbiased view into genome-wide transcriptional changes and have been applied to macroparasite-insect systems to elucidate genes underlying complex altered host phenotypes (Choi *et al.* 2014; Geffre *et al.* 2017). For the bumblebee, recent developments in genomics and transcriptomics have provided the tools to

explore important aspects of host biology, including phenotypic polymorphism (Colgan *et al.* 2011; Harrison *et al.* 2015), caste differentiation (Woodard *et al.* 2014; Collins *et al.* 2017), mating success (Manfredini *et al.* 2017) and diapause regulation (Amsalem *et al.* 2015a). In relation to pathogen response, previous transcriptomic studies have identified changes in host immune expression in response to the trypanosomatid *Crithidia bombi* (Barribeau et al. 2014) and bacterial challenge (Barribeau *et al.* 2016). However, investigation of changes in host gene expression in response to a macroparasite such as *S. bombi* has not been reported. Here we conduct a quantitative transcriptomic analysis using RNA-Seq to identify changes within the host transcriptome in response to *S. bombi* infection at two critical time-points: during diapause, when parasite infection initially occurs, and post-diapause, when the pathway towards the alternative host phenotype begins. We sampled queens at six hours post-diapause to allow for successful confirmation of diapause survival while providing a short period for recovery prior to sampling. Furthermore, we investigated whether the presence of *S. bombi* results in gene expression changes in queen bumblebees and, more specifically, given the significance of the host immune response within host-parasite dynamics, whether changes in host immune expression occur at either stage in response to *S. bombi*.

Results

Sphaerularia bombi affects gene expression during host diapause

For the diapause time-point, three of twenty seven *S. bombi*-challenged bumblebee queens were confirmed as infected using a diagnostic PCR. During host diapause, we identified ten significantly differentially expressed genes (DEGs) (Benjamini-Hochberg (BH) adjusted $p < 0.05$) between *S. bombi*-infected and uninfected bumblebee queens (Fig. 2A). Across these, there was a general signature of suppressed host expression in infected queens, with only a

single gene having elevated expression in response to nematode infection (binomial test, p = 0.02). Functional annotation identified these genes to have potential involvement in biological processes such as transcriptional regulation (LOC100643402: *PHD and RING finger domain*, LOC100647279: *Bromo Adjacent Homology Domain-containing*), energy metabolism (LOC100643871: *glucosylceramidase*; LOC100644055: *glucose dehydrogenase*; LOC100645043: *iron-sulfur cluster assembly enzyme ISCU*), venom (LOC100648490: *cysteine-rich venom protein 6*), and diapause regulation (LOC100644382: *regucalcin*) (Table S2a). Gene ontology term enrichment analysis (BH adjusted $p < 0.05$) identified the enrichment of seven GO terms within differentially expressed genes, including terms associated with cytoplasmic translation and endopeptidase inhibitor activity (Fig. 3A; Table S3a).

Sphaerularia bombi results in greater transcriptional changes post-diapause

At six hours post-diapause, seven of thirty *S. bombi*-challenged bumblebee queens were confirmed as infected using a diagnostic PCR. We identified 347 DEGs (BH adjusted $p < 0.05$) in *S. bombi*-infected queens at six hours post-diapause (Fig. 2B, Table S2b). In contrast to during diapause, there was no significant general pattern of expression, with genes showing both increased (n = 172 DEGs) and reduced (n = 175 DEGs) expression (binomial test, $p > 0.9$) There was no overlap between these genes and those identified during diapause. To determine whether this pattern of more DEGs post-diapause was biologically genuine, or just a function of the increased sample size at this time-point (which would enhance the probability of detecting DEGs), we performed differential expression analyses (n = 30) using three randomly subsampled queens per treatment from the post-diapause samples. This confirmed that more genes were significantly affected by *S. bombi* post-diapause (mean 79 genes; one-sample t-test, $p < 0.002$). Gene ontology term enrichment analysis identified the significant enrichment

(BH adjusted $p < 0.05$) of 23 GO terms within these DEGs, including terms associated with circadian rhythm, the Toll signaling pathway, and mitochondrial function (Fig. 3B; Table S3b).

Immunological response to *Sphaerularia* infection

We investigated whether immune genes were differentially expressed in response to parasitism using a list of previously characterised immune-associated genes (Barribeau et al. 2015). While we found no differential expression in these genes during host diapause, we identified fourteen putative immune-associated DEGs within *S. bombi*-infected queens at six hours post-diapause, including genes involved in immune recognition (LOC100651194: *scavenger receptor*), immune signaling (LOC100651716: *protein Toll*; LOC100644648: *toll-like receptor Tollo*; LOC100631067: *toll-like receptor 6*; LOC100644873: *domeless*; LOC100631093: *TGF-beta activated kinase 1*), immune regulation (LOC100647974: *leukocyte elastase inhibitor*), phagocytosis (LOC100649004: *draper*), effector molecules (LOC100631073: *thioester-containing protein A*; LOC100643365: *macrophage mannose receptor 1*; LOC100644559: *C-type lectin*), and antioxidant activity (LOC100646933: *haem peroxidase*). We identified a significant signature of elevated immune expression (binomial test, $p = 0.013$) with 12 of the 14 putative immune genes having increased expression in response to *S. bombi* presence.

Discussion

S. bombi is a highly virulent parasite of bumblebee queens (Poinar & Van Der Laan 1972). Here we provide novel insights into the molecular basis of its interaction with the buff-tailed bumblebee, *B. terrestris*, a common Eurasian species and important agricultural and ecological pollinator. Using RNA-Seq technology, we identified nematode infection to affect host transcription at two important time-points of the host life-cycle: during and post-diapause. During diapause, we identified a general trend of suppressed expression affecting genes involved in

cytoplasmic translation, ribosomal functioning and endopeptidase inhibitor activity within infected hosts. Post-diapause, we detected greater changes in gene expression with *S. bombi* affecting the expression of genes associated with mitochondrial functioning and circadian rhythm. Lastly, we identified differential immune expression within post-diapause queens, including enrichment of genes involved in the Toll signaling pathway, an important immune signaling pathway involved in the production of key effector molecules of the humoral immune response. Our findings highlight a compendium of genes involved in processes and pathways that may be directly targeted by *S. bombi* or represent off-target processes affected by parasite presence.

Bumblebee diapause is a prolonged period of arrested development lasting up to 6-9 months (Alford 1969b, 1975). Diapausing queens do not feed during this life-stage, placing pressure on nutritional reserves. Indeed, queens infected with the trypanosome *Crithidia bombi* have greater loss of biomass during diapause in comparison to uninfected queens (Brown *et al.* 2003b). Host diapause is also the stage when *S. bombi* infection occurs, although the timing or entry location are currently not known. Here, we identified a general trend of suppressed gene expression within infected hosts during host diapause, with nine out of ten genes having reduced expression. These genes have potential roles in transcription, such as a potential zinc-finger domain containing gene, and energy metabolism, including a *glucose dehydrogenase* and a *glucosylceramidase*, which function in oxidation-reduction processes (Ferri *et al.* 2011) and sphingolipid metabolism activities (Gault *et al.* 2010), respectively. While the exact role of these genes has not been characterised within *B. terrestris*, changes in transcription underlying host energy metabolism may be in response to parasite presence. *S. bombi* overwinters within the host during diapause, a hormonally and neurologically tightly controlled period of the queen's life-cycle (Amsalem *et al.* 2015b). However, our understanding of parasite activities, as well as potential consequences of such activities, during host diapause is poor. One explanation for

these results might be that the parasite imposes indirect metabolic costs on the host through detoxification of parasite by-products by host machinery.

One gene that surprisingly showed differential expression was a putative venom-associated gene (LOC100648490) coding for a cysteine-rich venom protein. An abundance of cysteine-rich proteins have been characterised within snake venoms (Yamazaki *et al.* 2003) but this specific gene is expressed within the venom gland and fat bodies of *B. terrestris* workers (Qiu *et al.* 2012). The presence of a trypsin-inhibitor domain within the predicted protein sequence suggests a general role within serine protease inhibition, which may function in ion channel blocking, blood coagulation and inflammation (Qiu *et al.* 2012). However, why infection by the nematode parasite results in downregulation of this gene remains unclear. An additional gene of particular interest is regucalcin (LOC100644382), due to its association with *Drosophila* diapause (Arboleda-Bustos & Segarra 2011; Reis *et al.* 2011; Vesala *et al.* 2012). Interestingly, transcriptional profiling of bumblebee queen fat bodies found increased expression of regucalcin within post-diapause foundress queens but not within diapausing queens (Amsalem *et al.* 2015a). Consequently, if the expression of this gene relates to metabolism in the fat body, one explanation for the downregulation of this gene in infected diapausing queens could be due to the costs of parasitism. Again, further studies are needed to investigate this in more detail.

Upon the termination of diapause, uninfected bumblebee queens seek nutritional resources before investigating potential sites for colony establishment (Alford 1975). As with diapause, this period is a vulnerable time within the life-stage of the queen. Queens infected with the generally benign gut parasite, *C. bombi*, are 40% less likely to establish a colony (Brown *et al.* 2003b). Here, we identified greater transcriptional changes within queens at six hours post-diapause in response to *S. bombi* presence. We identified differentially expressed genes to be enriched for gene ontology terms related to mitochondrial functioning and translation, providing evidence that

parasite presence impacts important cellular processes. The fact that host translation is impacted in both diapausing and post-diapause queens suggests that it is a conserved response to nematode parasitism across host life-stages. Manipulation of host translation machinery by parasites has been observed for viruses (Walsh & Mohr 2011), bacteria (Fontana *et al.* 2011) and protozoa (Jaramillo *et al.* 2011), with this manipulation potentially supporting parasite replication and/or avoidance of the host immune response. Reduced expression of proteins involved in translation has also been identified within the liver of mice infected with the parasitic nematode, *Ascaris suum* (Deslyper *et al.* 2016), indicating infection by macroparasites, such as nematodes, can affect host translation. Future research will be required to elucidate the role of translation within the development of this host-parasite interaction.

Aside from the effects that parasites can have on host cellular processes and metabolism, parasites can also affect host immunity. While the host may evolve behaviours or physical defences to avoid parasite infection, the host immune system represents an important barrier to parasite establishment within the host (Schmid-Hempel 2009). This point is further highlighted by the ability of parasites, such as parasitic nematodes, to suppress or evade aspects of the host immune response (Blaxter *et al.* 1992; Grencis & Entwistle 1997; Castillo *et al.* 2011). Host immunity can also be metabolically costly to maintain and produce (Schmid-Hempel 2003), especially during periods of nutritional restriction (Brown *et al.* 2003a; Brunner *et al.* 2014). Here we found elevated expression of immune genes post-diapause, including enrichment of the Toll signaling pathway, an important pathway in the generation and synthesis of effector molecules against bacteria and fungi (Hoffmann & Reichhart 2002; Tanji *et al.* 2007; Beckage 2008). This is interesting given the lack of observed host immune activity against *S. bombi*. Only one case of an encapsulated *S. bombi* has been documented (Kelly 2009), which is surprising given that the nematode actively resides in and feeds off the host haemolymph (Poinar & Hess 1972), a plasma medium full of immune proteins (Sadd *et al.* 2015). The lack of observed encapsulated

parasites would suggest that the nematode has evolved mechanisms to evade or suppress the host immune response, similar to those documented for other parasitic nematodes (Castillo *et al.* 2011). The differential expression of putative receptor genes of the Toll signaling pathway but not of downstream effector molecules, such as antimicrobial peptides, may possibly be due to suppression at some point of this pathway. However, such immunosuppression may also impose costs on the parasite, as *S. bombi* has a prolonged interaction with its host and is reliant on the host for dispersal of progeny. An immunocompromised host may be more likely to acquire, or allow to propagate, a secondary infection, which could reduce the amount of resources accessible to *S. bombi* or result in host mortality. As bumblebee queens are hosts to a taxonomically diverse group of pathogens (Alford 1975; Rutrecht & Brown 2008), any infection by other parasites could directly or indirectly affect the fitness of *S. bombi*. Whether the differences in gene expression we identified here actually reflect immunosuppression and, if so, what are the relative benefits and costs to the worm, remain open questions.

An alternative explanation for these differences in immune expression could be that they are driven by an as yet undocumented symbiont of the parasite, as has been seen in other nematode-insect systems (Hallem *et al.* 2007; Eleftherianos *et al.* 2010). Bacterial symbionts of entomopathogenic nematodes are released into the host haemolymph, multiply and kill the host through sepsis (Kaya & Gaugler 1993). These symbionts can also interact with and inhibit aspects of the host immune response (Ji & Kim 2004). However, host killing represents a different strategy from the *Sphaerularia-Bombus* dynamic, where premature host death would negatively affect parasite fitness. Alternatively, a potential symbiont of *S. bombi* could perform a similar role to that of *Wolbachia* bacterial symbionts of filarial nematodes (Fenn and Blaxter 2004). In these systems, *Wolbachia* are obligate symbionts providing key metabolites for their host (Wu et al. 2009). However, *Wolbachia* can also trigger host immune responses without resulting in host death (Taylor et al. 2001). Genomic sequencing has revealed the presence of

Wolbachia in tylenchid plant-parasitic nematodes (Brown et al. 2016) but there is no evidence for *Wolbachia* or other symbionts in *S. bombi*.

Aside from castration, parasitism results in dramatic changes in the host behavioural phenotype (Poinar & Van Der Laan 1972; Lundberg & Svensson 1975). Post-diapause, uninfected queens obtain resources before locating a nesting site to form a colony (Alford 1975). In contrast, *S. bombi* parasitised hosts investigate prospective overwintering sites where nematode larvae are deposited. This altered host phenotype is suggested to be a parasite adaptation that increases the probability of novel host encounter (Lundberg & Svensson 1975). Here we identified an enrichment of differentially expressed genes associated with circadian rhythm. Within host-parasite interactions, parasites must contend with and can affect host circadian rhythms (Reece *et al.* 2017; Westwood *et al.* 2019). This is best documented in parasites of medical importance, where symptoms of infection include irregular circadian rhythms, such as in the case of human infections by parasitic species, such as *Plasmodium* (Kwiatkowski & Greenwood 1989) and *Trypanosoma* species (Rijo-Ferreira *et al.* 2018). These rhythms in host pathology can be the result of parasite development, such as in cycles of asexual replication in *Plasmodium* species (Kwiatkowski & Nowak 1991) but the host immune system can also be regulated by circadian cycles (Lee & Edery 2008; Stone *et al.* 2012; Scheiermann *et al.* 2013). Uninfected bumblebee queens exhibit robust circadian rhythms before and after diapause (Eban-Rothschild *et al.* 2011), but whether this is true for *S. bombi*-infected bumblebees remains unknown. As infected queens do not found or raise colonies, differences in circadian rhythms from uninfected queens might be expected. Thus, the changes in gene expression associated with circadian rhythms identified here, may represent an early state of divergence away from the weak circadian rhythms normal queens develop as they establish colonies (Eban-Rothschild *et al.* 2011). *S. bombi* suppresses ovarian development in its host (Poinar & Van Der Laan 1972), and as

ovaries release hormones that can impact circadian rhythms (Mong *et al.* 2011), this could be one mechanism behind the differential expression of these genes in infected queens.

While there are overlaps in gene ontology terms enriched at both time-points, highlighting potential conserved mechanisms reacting to nematode presence, there were also dramatic differences in the number of differentially expressed genes identified at each time-point. Diapause represents a hormonally and neurologically tightly-controlled state within the insect life-cycle whereby transcription of certain genes may be suppressed as metabolic activity is reduced (Denlinger 2002; Sim & Denlinger 2013; Hand *et al.* 2016). Similarly, the nematode overwinters within its host during diapause (Poinar & Van Der Laan 1972), and therefore, the response of the host at the molecular-genetic level may be expected to be less pronounced. In contrast, post-diapause queens actively feed while the parasites also begin to evert their own reproductive tract (Poinar & Hess 1972), which may contribute to the increased number of host genes affected by nematode infection.

Future studies in this system should focus on tissue-specific profiling, as well as transcriptional profiling of additional time-points to improve our understanding of changes in both host and, in parallel, parasite gene expression. In the present study, our primary focus was on changes in expression within the host to better understand molecular signatures underlying host pathology. However, dual approaches exploring changes in gene expression within both host and parasite have been applied in systems of medical importance where abundant genomic resources are available for both parties to elucidate mechanisms involved in dynamic host-parasite interactions (e.g. Choi *et al.* 2014). In contrast to the recent advancement in genomic resources for the host (Sadd *et al.* 2015), the genetic resources available for *S. bombi* are limited. Currently, the only available nucleotide sequences are for ribosomal 18S subunit, which can be used for species identification but little more. Therefore, increasing genomic resources for this

parasite is an important next step if we are to improve our understanding of this complex system.

Conclusions

S. bombi represents a fascinating parasite of ecologically and commercially important bumblebees. Here, using genome-wide transcriptional profiling, we show that infection by *S. bombi* results in changes within its bumblebee host. More specifically, nematode presence affects transcription at two time-points within the establishment and development of the host-parasite interaction, altering gene expression associated with important biological processes, such as mitochondrial function, circadian rhythm and immunity. Future research will be required to identify whether these genes were directly impacted by the parasite, or represent biomarkers of non-target processes affected indirectly by infection. Our findings provide additional support for the use of genomic tools for exploring the molecular mechanisms within host-parasite interactions whereby changes within host transcription may be signatures of "extended phenotypes", the mechanisms underlying which are increasingly being explored within insect systems (e.g. Biron *et al.* 2006; Geffre *et al.* 2017). Our understanding of the *S. bombi-Bombus* interaction will benefit further through future tissue-targeted sequencing approaches, analyses of additional time-points, as well as investigation of additional genomic levels, such as the proteome or epigenome, for molecular changes in the host. Similarly, investigations of this host-parasite interaction will greatly benefit through the generation of more genomic resources for *S. bombi* and other *Sphaerularia* species. Taken collectively, our study provides a novel insight into a fascinating host-parasite interaction, and identifies candidate genes for future research.

Experimental procedures

Nematode collection and maturation

Sphaerularia bombi is a natural parasite of wild bumblebee species in Ireland with a prevalence up to 50% within the Dublin area (Kelly 2009). To obtain *S. bombi* for experimental infections, we collected wild *B. terrestris* queens from Merrion Square Park, Dublin 2, Ireland (53.339657, -6.249165) in spring 2010. We maintained these queens in a temperature-controlled room (27±1°C, 45-50% RH) under red-light illumination and provided them with pollen and sugar water (ApiInvert) *ad libitum*. We kept queens in plastic boxes (dimensions: (L) 11.5cm: (W) 9cm: (H) 7cm) lined with autoclaved clean sand for the purpose of collecting L3 stage juveniles. Queens were retained for 14 days post-collection, after which the sand was periodically checked via sieving for the presence of L3 juveniles. The presence of juveniles was confirmed by light microscopy. Larvae collected from multiple hosts were mixed and housed in double-autoclaved sand for 80 days in the dark at room temperature to allow for sexual maturation and fertilisation of female adults (Poinar & Van Der Laan 1972).

Sphaerularia bombi infection protocol

We obtained *B. terrestris* colonies from a commercial supplier (Koppert, the Netherlands) and maintained them within a temperature-controlled room (27±1°C, 45% relative humidity (RH)) under red light illumination. Within the experiment, two natal colonies were used: one colony for investigating differential expression within the host during diapause; and a second colony for investigating transcriptional changes within the host in response to parasitism at six hours post-diapause: both time-points are critical for the development and establishment of the host-parasite interaction. From each colony, we collected virgin queens and mated them under standard laboratory conditions (Sauter & Brown 2001).

Diapause maintenance and sample collection

Post-mating, we transferred mated queens to a temperature-controlled room where they were maintained for 72 hours to allow for preparation for diapause. Prior to diapause, we randomly assigned mated queens to one of two treatments: i) the control treatment where queens were placed into a 50 ml centrifuge tube containing 5 ml of autoclaved sand; or ii) the challenged treatment where queens were placed into a 50 ml centrifuge tube containing 5 ml of autoclaved sand inoculated with approximately 200 fertilised female *S. bombi* adults. We initiated host diapause through the incubation of queens at 4°C (RH of 70%) in the dark. Host diapause was maintained for a period of 14 weeks to facilitate nematode infection. For the diapause collection time-point, after 14 weeks, we transferred diapausing queens into a 2 ml cryotube and immediately snap froze in liquid nitrogen. For this collection time-point, a total of 27 bumblebee queens were passively challenged with *S. bombi* and were collected for molecular detection of *S. bombi* presence. For the second time-point, we transferred diapausing queens to a temperature-controlled room (27±1°C, 45% RH) where we provided them with pollen and sugar water (ApiInvert) *ad libitum*. For this time-point, a total of 30 queens were exposed to *S. bombi*-infested sand. Queens were maintained in the temperature-controlled room for six hours before being transferred to 2 ml cryotubes and snap frozen using liquid nitrogen. All samples were stored within a -80°C freezer prior to RNA extraction.

RNA extraction, cDNA synthesis and diagnostic PCR

For each collection time-point, we extracted total RNA from whole-bodied queens using TRIzol reagent (Invitrogen, UK), following the manufacturer's instructions. We purified RNA, which included an on-column DNase step, using the GenElute Mammalian Total RNA kit (Sigma, Ireland). To ensure complete removal of residual DNA, we treated purified RNA with a second stringent external DNase, Turbo DNA-free (Ambion, USA). We quantified total RNA using the Qubit fluorometer (Invitrogen, UK) and we visualised each sample using gel electrophoresis to ensure samples were not degraded. To check for evidence of *S. bombi* transcripts within extracted total RNA, we synthesised complementary DNA (cDNA) for each sample. During this process, we primed the cDNA synthesis step using *S. bombi*-specific 18S ribosomal primers (SBom1F: 5'-CTTACATGCTCTGACCTGC; SBom1R: 5'-GATTTGTTCAAAGTAAAATCG). This was to ensure the amplification of parasite genetic material. We synthesised template cDNA using the SuperScript reverse transcriptase kit (Invitrogen, UK). We performed diagnostic PCRs for both control and putatively challenged queens (i.e., queens overwintered with *S. bombi*-infected soil) using GoTaq kits (Promega, USA). We visualised resultant PCR products using gel electrophoresis to confirm the presence of products of expected fragment size. To confirm the amplified PCR products were *S. bombi*, we purified each using the JetQuick PCR purification kit (Genomed, UK) and had each sequenced in both orientations (5' and 3' sequencing) at GATC Biotech, Germany. For each individual, we trimmed the individual sequence pairs, aligned them and generated a consensus sequence using the software tool, BioEdit (Hall 1999). Finally, to confirm *S. bombi* identity, we searched each consensus sequences against the NCBI nucleotide database using BLASTn identifying amplicons from three diapause and seven post-diapause queens to have top BLAST (e-value < 1e-100) matches against *S. bombi* 18S ribosomal mRNA sequence (AB250212.1). A multiple sequence

alignment of *S. bombi* 18S ribosomal sequences obtained from infected queens is provided in Supplemental File S1.

RNA library preparation and sequencing

For control and infection confirmed queens, we further assessed the quality of extracted total RNA using an Agilent 2100 Bioanalyzer with an Agilent RNA 6000 Nano Chip kit. Libraries were prepared using the TruSeq RNA Sample Prep kit v1 (Illumina, USA). The sequencing of each time-point was performed at two different time periods, which resulted in each time-point being sequenced on a different sequencing platform. The "diapause" libraries, which consisted of six queens (three control, three infected), were individually tagged, pooled and sequenced on a single lane (2*76 bp) of an Illumina GAIIx sequencer resulting in the generation of 35.4 million paired-end reads (min: 5.26 million paired-end; max: 6.46 million paired-end; mean: 5.89 million paired-end reads; Table S1). For the second group of samples (n = 14, seven control, seven infected), the "post-diapause" samples, we tagged each individually, pooled and then sequenced on a single lane (2*76 bp) on an Illumina HiSeq 2000. This sequencing run resulted in a total of 186.1 million PE reads (Table S1). Library preparation and sequencing were performed at the University of Edinburgh, UK.

Pseudoalignment, differential expression and gene ontology enrichment analysis

The analysis of RNA-Seq libraries was performed using scripts modified from those made publicly available by Colgan *et al.* (2019). Scripts for the present analysis are available at https://github.com/Joscolgan/sphaerularia_rnaseq. We quality-assessed raw sequences for each sample using FastQC (v.11.5; Andrews 2010). As an additional step, raw sequences were aligned against the *B. terrestris* reference genome using STAR (Dobin *et al.* 2013) identifying at least 87% of reads from each individual sample aligning uniquely to the bumblebee genome.

Using the raw sequences, we pseudo-aligned each sample against the latest *B. terrestris* RefSeq transcript dataset using kallisto (v. 43.1; Bray *et al.* 2016). We chose to use a pseudoaligner, such as kallisto, as they have greater accuracy and consistency in transcript quantification in comparison to traditional aligners (Sahraeian *et al.* 2017). For each sample, we computed gene-level count estimates using tximport (v. 1.2.0; Soneson *et al.* 2015) and loaded the count estimates into the R package, DESeq2 (v. 1.14.1; Love *et al.* 2014) to perform differential expression analysis. We performed differential expression analysis for both time-points independently, comparing expression between *S. bombi*-infected and uninfected queens at each time-point. While the inclusion of all samples into a single linear model could be expected to increase power to detect differentially expressed genes, due to the extensive differences in expression profiles between samples collected at each time-point (Supp. Fig. S1), which could be influenced by inter-colony genotypic variation, as well as technical variation due to different sequencing runs, we decided to analyse the samples individually. Although our experimental design does not allow for quantifying differences between the two time-points, we investigated qualitative differences. As differences in throughput for the two time-points could result in differences in gene detection, we quantified gene expression for both datasets identifying each to detect expression for a similar number of genes (diapause samples, n = 11518 out of 11654 (98.8%) genes; post-diapause samples, n = 11610 out of 11654 (99.6%) genes). Furthermore, as the difference in sample size between the two time-points might affect the number of genes reported as differentially expressed at each time-point, we randomly subsampled three post-diapause queens for each treatment and performed a differential gene expression analysis using this reduced number. We replicated random subsampling and subsequent DE analysis 30 times. We compared the mean number of DEGs identified across these analyses with the number of DEGs detected during host diapause using a one sample t-test. For gene ontology term enrichment, we used gene ontology terms of *Drosophila melanogaster* assigned to *B. terrestris* orthologues. We obtained the gene ontology terms from

the Ensembl Metazoa BioMarts database (Kinsella *et al.* 2011). For each time-point, we performed gene ontology term enrichment using topGO (v. 2.34.0; Alexa and Rahnenfuhrer 2018); Kolmogorov-Smirnov test; algorithm = ”weight01”; nodeSize = 20) with significance assessed at $p < 0.05$ after Benjamini-Hochberg correction for multiple testing.

Conflict of Interest

There is no conflict of interest.

Ethical Guidelines

There are no ethical issues with the related work.

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Author contributions

SS, MLB and MJFB conceived the project. TJC, JCC, SS, MLB and MJFB designed the experiment. TJC and JCC ran the experiment. TJC analysed the data. All authors wrote and agreed on the final manuscript.

Data availability

We have archived short read data at the NCBI Short Read Archive Database (BioProject Accession ID: PRJNA533942). We have also archived scripts for quality assessment, differential expression, gene ontology enrichment analysis and data visualisation on an open-access repository hosted on Github. Scripts are provided at https://github.com/Joscolgan/sphaerularia_rnaseq.

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Figures

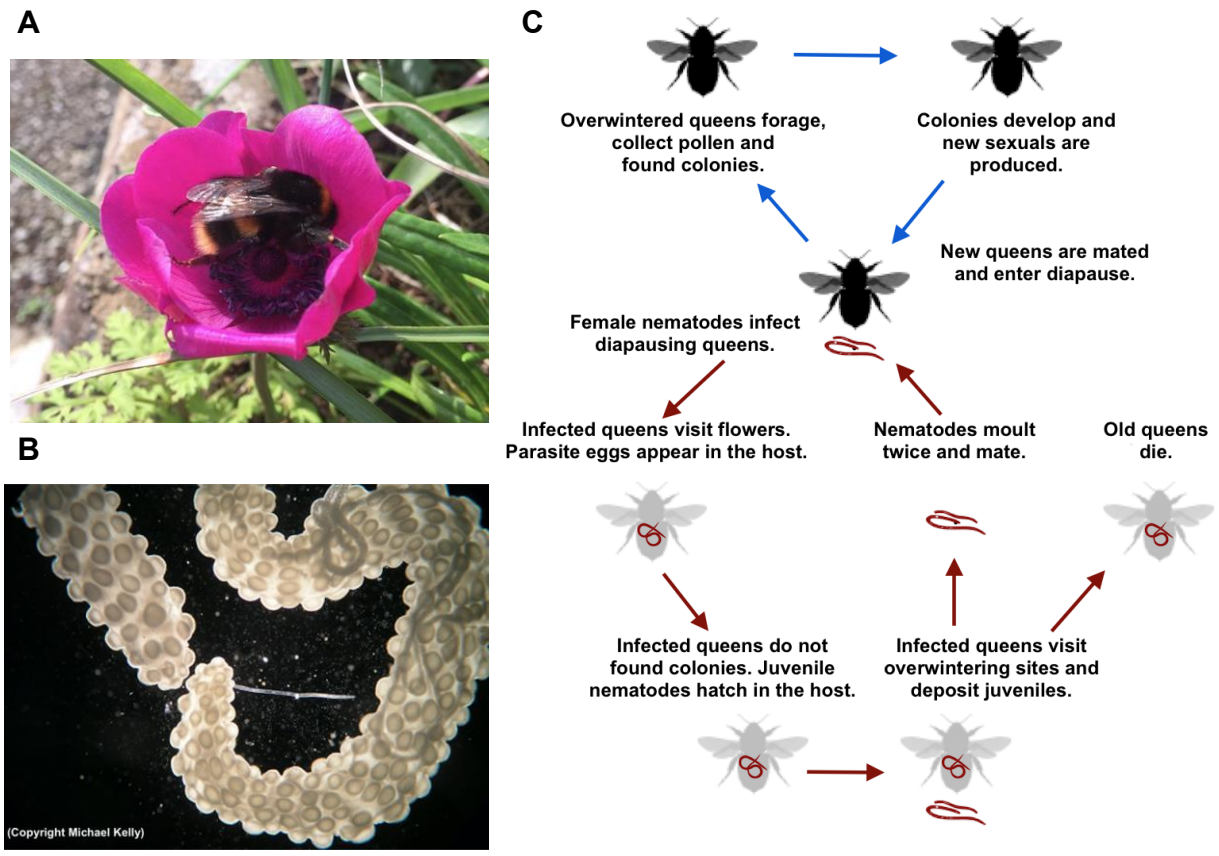


Figure 1. Host-parasite interaction between an entomoparasitic nematode and a bumblebee host. Bumblebee *Bombus* queens (A) are infected by fertilised *Sphaerularia bombi* (B) during host diapause. The host-parasite life-cycle (C) shown is adapted from Poinar and Van der Laan **(**1972). (Photos: Dr. Thomas J. Colgan; Dr. Michael Kelly).



Figure 2. *Sphaerularia* parasitism affects host gene expression. For each gene differentially expressed within *B. terrestris* queens in response to nematode presence, we show log fold changes amongst infected and control queens during diapause (A) and post-diapause (B). While a total of 347 genes were significantly expressed (BH adjusted $p < 0.05$) between *S. bombi* infected ('INF') and uninfected ('CON') bumblebee queens post-diapause, here we show the most significant genes (BH adjusted $p < 0.01$). For each plot, each column represents an independent queen sample, while each row represents a significantly differentially expressed gene. For each differentially expressed gene, NCBI RefSeq gene ID and gene description are provided.

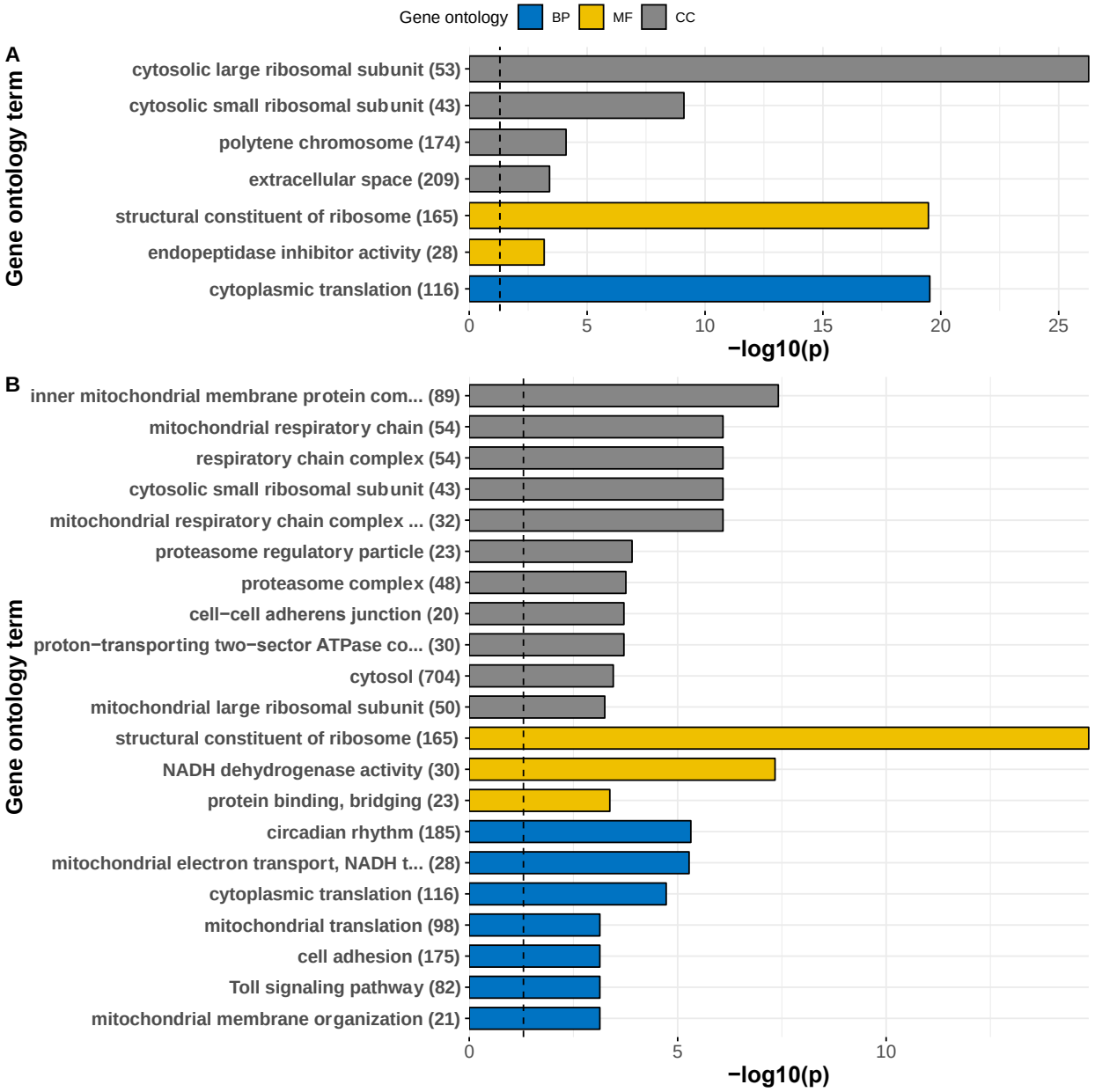


Figure 3. Gene ontology terms enriched across genes differentially expressed in *Sphaerularia bombi* infected queens during and post-diapause. Bar plots for gene ontology terms enriched during (A) diapause and (B) post-diapause queens displaying -log10 transformed BH-adjusted *p* values of significance for each gene ontology term within each ontology class: 'cellular component (CC)' (grey); 'molecular function (MF)' (yellow); and 'biological process (BP)' (blue). For each significant GO term, the term annotation, as well as the total number of annotated terms within the *B. terrestris* predicted proteome are shown.