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Ivan Antolic-Soban April 15th, 2014

Opening *Pandora*'s Box: Toward an Anti-Fungal Defense Mechanism

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Abstract

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The study of host-pathogen interactions has been a central focus of both ecological and evolutionary research. The discovery of diverse bacterial communities in many animals, including humans, has spurred new research into the interactions between host, pathogens and beneficial microbes. Here I present new insights into the relationship between a fungal pathogen, *Pandora neoaphidis*, its insect host, *Acyrthosiphon pisum*, and a facultative, defensive, bacterial aphid symbiont, *Regiella insectola*. The *Regiella* bacterium has been shown to confer protection onto its host against a variety of pathogen and predators including the *Pandora* fungus and parasitoid wasps. Specific mechanisms of action in this defense are currently unknown. The *Pandora* fungus is often cited as a potential bio control mechanism for aphid agricultural infestations; however, there are few genomic resources available for the study of *Pandora* infections. Using next generation RNA sequencing reads, I assembled a draft *de novo* transcriptome of *Pandora* during an infection. I then examined the differential transcript expression of the fungus in the absence and presense of the protective symbiont as well as in unwinged and winged aphid morphs, which differ in their susceptibility to fungal pathogens as well. The presence of the protective symbiont led to the decrease in the expression of *Pandora* transcripts containing domains associated with pathogenesis and fungal proliferation suggesting that the bacterium interferes with these processes. Differential expression of transcripts associated with fungal growth, stress response, and sporulation was noted between *Pandora* infections of winged aphids and those of unwinged aphids. It has been demonstrated that the production of winged morphology results in an energy deficit that decreases the efficacy of an immune response. These findings suggest that unwinged individuals are better able to suppress the production of fungal genetic elements involved in the destructive processes of fungal growth and sporulation. This leads to a lowering in mortality and less fungal dispersion after death. Future research will be required to investigate the specifics of these infection mechanisms.

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Chapter I

Introduction

The results of parasite infection are often dependent on the ecological context. Abiotic factors, such as temperature, can influence the outcome of host-parasite interactions (Vale *et. al* 2008). Biotic factors can play an important role as well. The outcome of interactions between monarch butterflies (*Danaus plexippus*) and a protozoan parasite, for example, is mediated by the host plant species on which the butterfly larvae feed; butterflies that feed on plants with higher levels of toxic cardenolides have increased resistance relative to individuals that feed on less toxic plants (de Roode *et. al.,* 2008). Little is known about how such alternations in environmental context of host-parasite interactions could change the responses of the parasite during the infection process. Here, I briefly review two important ecological factors that can mediate aphid-parasite interactions: symbiosis and host morphology. I then overview insect pathogens (entomopathogens), with specific reference to aphid infection by the fungal pathogen *Pandora neoaphidis.* In later chapters, I will focus on *Pandora* transcriptional responses during aphid infection in the context of these two important biological phenomena.

Symbiotic Bacteria and their Hosts

Research examining interactions between host organisms and their endosymbionts has demonstrated that symbiotic bacteria are an integral part of the

lives of many animal and plant hosts. Because of this, investigation of complex and dynamic biological processes, such as immunity and metabolism, can benefit from considering the host-symbiont complex as a singular super-organism rather than ignoring the symbiont contribution and focusing on responses and reactions of the host in isolation (Loker *et. al.* 2004). Humans are no exception. The diverse microbial communities that inhabit our bodies outnumber our somatic cells by an order of magnitude (Savage 1977). These communities make up the microbiome of a host organism, and recent evidence has shown that the composition of the human gut microbiome greatly influences the health of the host and may even play a role in body composition (Human Microbiome Project Consortium 2012). For example, there are differences in the composition of the microbiome between obese and lean individuals (Turnbaugh *et al.*, 2009, Turnbaugh *et al.* 2006). We have only recently appreciated and focused on the role that these organisms play. Studying the effects of these symbiotic microorganisms in aggregate, however, is complicated by the extreme diversity of the human microbiome both within and between individuals.

In contrast, microbial communities of some insects are more tractable due to the relative simplicity of their microbial communities. Many species only harbor a core set of microbial partners. Through these insect-microbe systems we are able to study how these endosymbionts affect the metabolism and immune system of their hosts (Moran 2006).

In the case of metabolism, there are several examples of a symbiont producing necessary amino acids and other nutrients for their host. The obligate symbiotic bacteria *Buchnera aphidicola* produces essential amino acids and vitamins

for its host, *Acyrthosiphon pisum*, which feeds on nutrient-poor plant phloem. In return for the amino acids and vitamins, the bacteria is passed from mother to offspring, and the microbe receives metabolites that it is unable to synthesize (Douglas 1998). Such nutrient provisioning may allow a host to utilize resources (i.e., host plants) that it could not use in the absence of the symbiont. For example, the mid-gut of the invasive stinkbug *Megacopta cribraria*, is colonized by the bacteria *Ishikawaella capsulata*, which has been shown to allow its host to utilize soy as a host plant (Hosokawa *et al.* 2007). These examples highlight the fact that the metabolism of an organism is not simply a system consisting of a single species and its diet but is instead a suite of cooperating species utilizing a food source in a mutualistic relationship.

As was the case in metabolic systems, viewing the host and symbionts as a single symbiotic unit is useful when considering immunological systems as well. A classical view of immunology focuses on interactions between a host and one or more pathogens attempting to exploit the resources of the host. Thus, instead of cooperating, they are at odds with one another, and as a result the host will attempt to mount an immune response to rid itself of the pathogen. The pathogen will, in its own response, attempt to combat these defenses. This leads to an evolutionary arms race between the attacks of the immune response and those of the pathogen's invasion. We now know that this is, in many cases, a simplistic model that ignores any symbionts present in the system (McFall-Ngai 2007). The assumption made is that only the host organism faces selective pressures; however, the symbiont has an interest in keeping the host alive because the host provides the microbe with a

stable environment for proliferation and, in many cases, the symbiont is unable to live outside the host. Selection favors the continuity of a symbiotic relationship if the costs of maintaining such a relationship are exceeded by the benefits. Any vertically-transmitted microbe that can aid in the defense of the host will be passed on to the next generation, leading to fitness benefits for both parties (Jones *et al.,* 2007).

In many systems, we have discovered that the symbiont plays an active role in the immune and defense response. Aphids that harbor *H. defensa* have significantly increased survival compared to aphids without the symbiont when infected by parasitoid wasps (Oliver *et al.* 2008). The mechanism of protection is thought to be a toxin expressed by APSE-2, a bacteriophage. This phage is an obligate part of the *H. defensa* life cycle, and the toxin it produces is known to disrupt the eukaryotic cell cycle (Moran *et al.,* 2005). The bacterial species *Wolbachia pipientis* commonly infects a variety of arthropod species. In most of these species, *Wolbachia* have been shown to manipulate the host reproductive system in order to ensure transmission (Werren *et al.,* 2008). However, in *Drosophila melanogaster,* this parasitic action does not occur and, rather, the symbiont provides the host with protection against viral infection (Hedges *et al.,* 2008). *Palaemon macrodactylus* and *Homarus americanus*, two crustacean species, harbor symbionts that synthesize anti-microbial chemicals to defend against the fungal pathogen *Lagenidium callinectes* (Gil-Turnes *et. al.* 1989; Gil-Turnes *et. al.,* 1992).

A common insect system for the study of host-symbiont interactions is *Acyrthosiphon pisum*, the pea aphid. *A. pisum* harbor an obligate symbiotic bacteria,

Buchnera aphidicola, in specialized cells called mycetocytes or bacteriocytes (Douglas 1998). *B. aphidicola* is incapable of living outside of the host and has been co-evolving as a primary symbiont of the aphid for approximately 160-280 million years (Moran *et al.* 1993). In addition to *B. aphidicola*, *A. pisum* can also exhibit symbiotic relationships with a number of non-essential, facultative symbionts. These symbionts are able to provide protection against heat-shock, parasitoids, and fungal pathogens (Oliver *et. al.* 2010).

A. pisum has a surprisingly weak immune system. Several major and canonical immunological pathways found in other insect genomes are missing in the aphid genome (Gerardo *et al.,* 2010). Interestingly, the symbiotic bacteria harbored by the insect can play a role in defense. In some cases, the genotype of the aphid is inconsequential with respect to the level of resistance to a particular threat while the genotype of the symbiont accounts for all the variation in resistance. For instance, the bacterial symbiont *Hamiltonella defensa* is able to defend its host against parasitism by *Aphidius ervi*. Oliver *et al.* (2005) demonstrated that the host's genomic defenses had no significant effect in fighting off the wasp, and variation in survival was due to the genotype of the bacteria. Similarly, aphids can be protected from fungal entomopathogens (discussed further, below) by a variety of different symbionts. The specific mechanisms of protection vary between bacteria. Infection with *Rickettsia* and *Rickettsiella* bacteria, for example, lead to a significant increase in survival after exposure to fungal spores. *Spiroplasma* bacteria, on the other hand, delay the onset of sporulation or cause aphids to drop off the plant in order to sporulate farther away from their clonal offspring (Lukasik *et al.,* 2013). These

methods serve to protect individuals surrounding the infected aphid who might also be carrying an identical symbiont (Scarborough *et al.,* 2005). A theme evident through these interactions is a clear cooperation between these insects and their symbionts. By ensuring survival of the host, the bacteria are able to increase their own fitness.

Phenotypic Plasticity and Alternative Morphs

Phenotypic plasticity is the ability of organisms to change their phenotype in response to the environment. Many examples involve the change of an organism's morphology in response to the availability of certain foods or the presence of predators. For example, the structure of a grasshopper's mandible will develop differently depending on the type and quality of the food available. A low quality and fibrous diet will result in larger mandibles. Additionally, ant colonies will increase the production of soldier ants when they sense a lack of bodyguards (Whitman and Agrawal 2009).

Depending on environmental pressure, aphids can be one of two distinct morphs, winged (a.k.a., alate) or un-winged (a.k.a., apterous). When aphids are exposed to stressful environments, they tend to produce a greater proportion of winged offspring (Kunert *et al.* 2005). This mechanism presumably allows their descendants to disperse and colonize a new plant that hopefully provides a less stressful environment. However, it has been shown that the development of the winged morphology is costly relative to the development of the un-winged morphology. Given this, as expected to maximize fitness, in the absence of stressful

stimuli, aphids produce more un-winged than winged offspring (Artacho *et. al.* 2011). This developmental switch also has important consequences for aspects of the aphids beyond their ability to fly. Recent work suggests that winged aphids have a reduced ability to mount and maintain effective immune responses towards fungal pathogens than un-winged aphids (Parker et al., in prep).

Entomopathogens

Entomopathogens are an actively studied group of organisms because of their applications in pest management as well as models for the study of pathogenesis. Because they can target their host more specifically than an insecticide can, they are used as a more environmentally safe alternative for the control of pest species (Shah and Pell 2003). One entomopathogen commonly used to control pest insect populations is the bacterium *Bacillus thuringiensis*. Various strains of this species have been shown to synthesize toxins that target specific insect orders. Genes that encode these toxins are often found on transmittable plasmids. This allows for the evolution of novel toxins that can be used to target specific insects (Feitelson *et al.,* 1992). In addition to bacteria, entomopathogenic fungi also attack insect hosts. Some fungi such as *Beauveria bassiana* are generalists that can exploit a diverse range of host insects. Others, like *Pandora neoaphidis*, are specialists that have evolved to specifically target the system of their host.

Entomopathogenic fungi are typically transmitted through airborne spores that attach and burrow through the cuticle of the insect (Roy *et al., 2006*). This is unlike bacteria, which typically must be ingested (Feitelson *et. al.,* 1992). Once

inside the cuticle, the fungus will multiply at the expense of the host, frequently killing the host, and then using the carcass as a launching point for new spores or resting until an opportunity for infection presents itself (Roy *et. al.,* 2006).

Aphids and *Pandora*

Pandora neoaphidis is a common aphid fungal pathogen in temperate regions and has been suggested as a possible bio-control mechanism against aphids, which are common agricultural pests (Glare & Milner 1991). The aphid specialist infects by degrading the cuticle and colonizing the gut. During development, the fungal colony will construct conidia, which burst out from the body of the aphid, killing the host and aiding in the dispersal of further spores. The force of the spore burst is sufficient to send them into the free air where they can be dispersed to other aphid colonies (Hemmati *et. al.*, 2001). The likelihood of an infection being transferred between colonies in this manner is relatively low, and it has been demonstrated that the beetle, *Coccinella septempunctata* can be a passive vector for transmission between aphid colonies (Roy *et. al.* 2001). Once the fungus has infiltrated a new colony, there is potential for an epizootic. This, however, is fairly unpredictable (Feng *et. al.* 1999).

As mentioned previously, in addition to the obligate endosymbiont, *Buchnera aphidicola*, *A. pisum* is capable of harboring a variety of facultative bacterial symbionts. Many of these bacteria protect the host from a variety of predators, pathogens, and environmental conditions (Oliver 2010). Of interest here is the bacteria *Regiella insecticola*, which increases aphid survival upon infection with

several fungal entomopathogens (Scarborough 2005, Parker 2013). Aphids harboring *R. insecticola*, regardless of aphid genotype, have significantly higher survival rates when infected by *Pandora,* suggesting that the bacteria itself is responsible for protection. In addition to increasing the chances of survival, the bacteria also delay and reduce sporulation (Scarborough 2005). However, *R. insecticola* is not a panacea for aphid fungal infections. Parker *et al.* (2013) demonstrated that *R. insecticola* could also protect against several other aphid-specific fungal entomopathogens but not against the generalist pathogen *Beauveria bassiana.*

Overview of Thesis

Here I investigate interactions of aphids with fungal pathogens through comparison of fungal gene expression in two different ecological contexts. In chapter 2, I discuss the assembly and annotation of a draft *Pandora neoaphidis* transcriptome used in later chapters for further analyses. In chapter 3, I compare fungal gene expression in the presence and absence of a protective *Regiella* symbiont, which is known to confer protection against fungal invasion. In chapter 4, I compare fungal gene expression in two alternative host morphs, winged and unwinged aphids, which are known to respond differently to fungal invasion (Parker *et al,* 2013 in prep). I then briefly conclude with comparisons between the two sets of data and emphasize future directions.

Chapter II

De Novo Transcriptome Assembly for the Fungal Entomopathogen, *Pandora neoaphidis*.

Introduction

Entomopathogens are organisms that target insects for pathogenic exploitation. Many of these species have been extensively studied for their application in bio-control mechanisms, which is facilitated by the host specificity demonstrated by some of these species (Shah and Pell 2003). Various strains of the bacterium *Bacillus thuringiensis*, for instance, are able to target specific hosts by evolving novel toxin variants (Feitelson *et al.,* 1992). Their specificity allows for a more targeted insecticide with fewer environmental consequences than traditional insecticides.

Entomopathogenic fungi are of additional interest to bio-control researchers because of their convenient dispersal mechanisms. While bacterial entomopathogens must generally be ingested in order to infect the host, fungi typically spread via airborne spores that need only land on the host in order to attempt an infection (Roy *et al., 2006,* Feitelson 1992). Once the infection is successful and the host is killed, the fungal spores will burst from the cadaver and attempt further infections (Roy 2006).

Pandora neoaphidis, an aphid specialist pathogen, is frequently cited as a possible bio-control agent for aphid agricultural pests (Pell *et. al.* 2001, Hemmati *et.*

al. 2000). However, at the time of writing, a query of "*Pandora neoaphidis*" on the NCBI Nucleotide database yielded only 133 results, with the majority of these being 18S ribosomal RNA sequences. Further development of genomic resources for this fungus could facilitate study of how this potential biocontrol agent responds to alternative environmental conditions and ultimately could facilitate genetic manipulation of the pathogen. Here I present the assembly of a draft *Pandora neoaphidis* transcriptome from RNA-Seq data collected from fungus infected aphids.

Methods

Aphid Infection

Pandora spores were harvested from aphid cadavers by placing cadavers on 1% Tap Water Agar overnight (12-15hrs) at 20C. The TWA plate with sporulation cadavers was then inverted over groups of 9-day-old aphids in a tube for 90 minutes. After conidial exposure, aphids were exposed to high relative humidity by placing the aphids on plants enclosed in an unventilated cage, with the bottom of the pot submerged in water. These conditions were maintained for four days. After this period of high humidity, the aphids were changed to a ventilated cup cage. Aphids of two genotypes (LSR1, 313) were frozen at 48 and 72 hours post-infection for later use (see Chapters 3 and 4 for further details on aphids used for infections). A subset of aphids was maintained to verify successful infection (data not shown).

Pandora RNA Extraction and Sequencing

RNA was extracted from *Pandora*-infected aphids frozen at 48 and 72 hours post-infection using a standard Trizol and isopropanol - based precipitation. The extracted RNA from each time-point was then pooled, and libraries were constructed using Illumina kit B. cDNA libraries were sequenced on an Illumina Hi-Seq machine.

***De novo* Transcriptome Assembly**

RNA-Seq data was initially mapped to the *A. pisum* genome using Bowtie in order to filter potential *Pandora neoaphidis* reads from those of the host. Additionally, reads were mapped against the *Buchnera aphidicola* and *Regiella insectola* genomes in order to filter away bacterial transcripts. Reads that remained unmapped to these genomes were retained and used for assembly. These reads were split into separate files containing left and right paired-end reads and then normalized *in silico* using Trinity's normalization by k-mer coverage script. Following normalization, the Trinity RNA-Seq *de novo* Assembler (2013-08-14 Release) was used to assemble the transcriptome.

Following assembly, final filtering was conducted by blasting transcripts against a database of bacterial genomes as well as a database of fungal genomes (Table 2, Table 3). Those transcripts that blasted to the bacterial database with a higher bit score than the fungal database were discarded. Using the aphid genome

and the fungal genome database, this procedure was repeated to filter out probable aphid transcripts.

Functional analysis was performed using the assembled transcripts and the Trinotate utility (2013-11-10 Release). Trinotate aggregates functional annotation data from several software packages. The software packages used here are blastp, blastx (BLAST 2.2.28+), SignalP (v4.1), and hmmscan (v3.1b1) for identification of protein domains, secreted proteins, and GO term analysis. The Trinotate analysis was conducted according to the Trinotate protocol (http://trinotate.sourceforge.net/). All Parameters are listed in Table 1.

bowtie	-f –x aphid -U Trinity.fasta –S map.sam
normalize_by_kmer_coverage.pl	--seqType fq --JM 70G --max_cov 300 --left left.fastq --right right.fastq --JELLY_CPU 11 --KMER_SIZE 31 --pairs_together --PARALLEL_STATS --output normalized_reads
Trinity.pl	--seqType fq --JM 75G --left left.fastq --right right.fastq --CPU 11 --output trinity_out

Table 1. Parameters used to assemble the transcriptome. Version numbers are shown in Supplemental Data.

Results

Basic Metrics. The assembly contains 19,977 unique transcripts and 14,797 trinity components with a contig N50 value of 1,392 bp. Blasting the transcripts against the SwissProt protein database showed that the majority of the transcripts

had a low match to their top hit, indicating many unique transcripts compared to those available for comparison (Figure 1).

Transcript Categorization. 49.2% of transcripts were assigned a GO term. Figure 2 shows the ten most frequent GO terms assignments for the assembly partitioned between the three high level GO annotations: biological process, cellular component, and molecular function.

In order to study host-pathogen interactions, focus was placed on transcripts annotated with GO terms corresponding to pathogenic activity. The three GO terms chosen were GO:0009405 (pathogenesis), GO:0004568 (chitinase activity), and GO:0006508 (proteolysis). Transcripts with these GO terms accounted for 2.00% of the total transcripts and 4.06% of annotated transcripts.

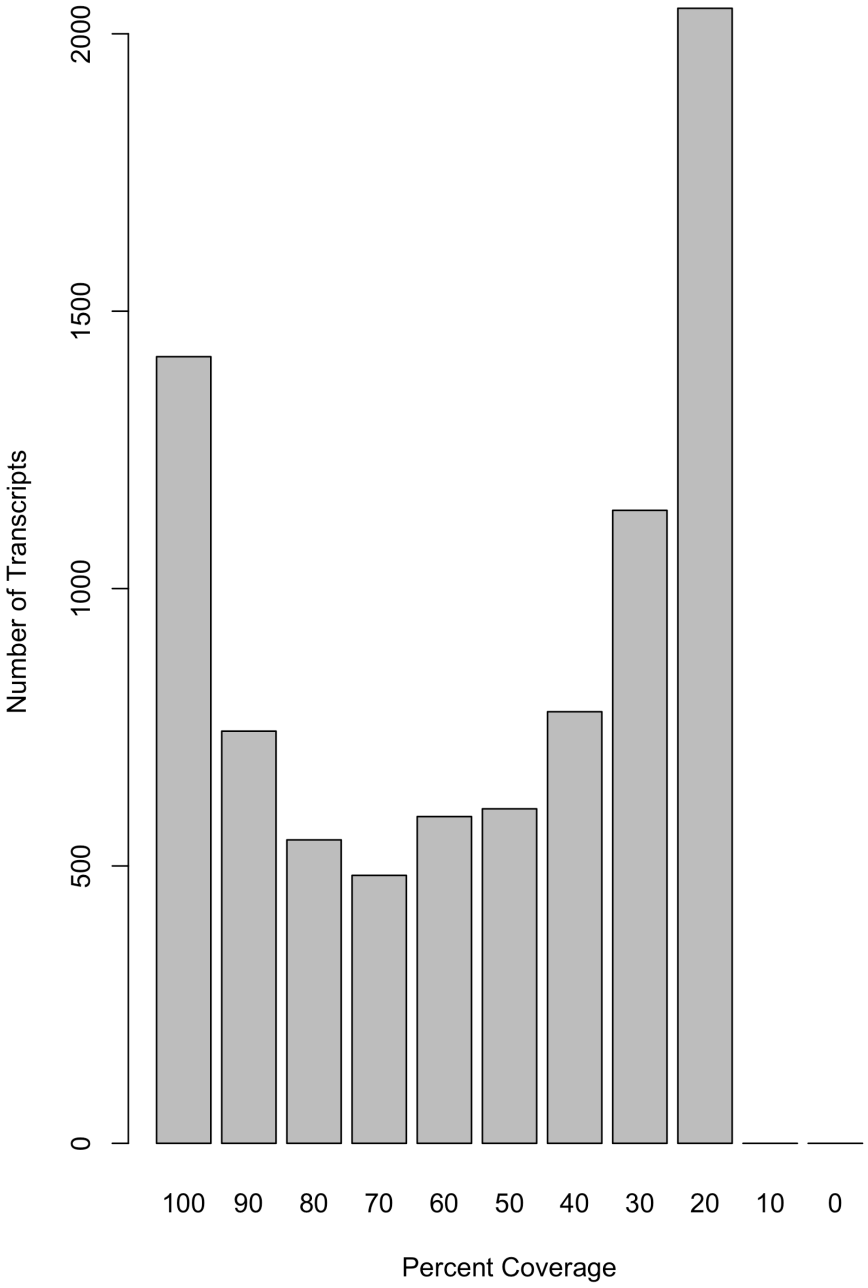
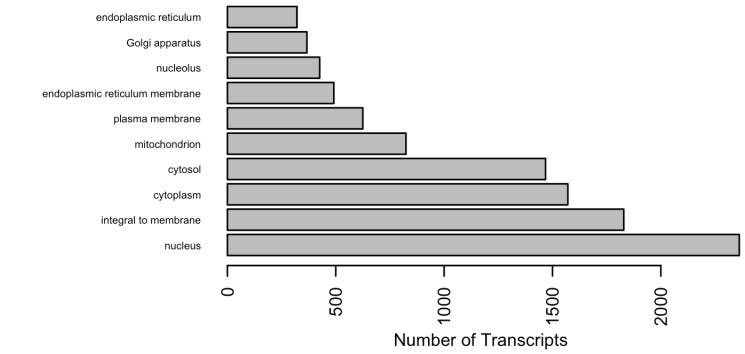
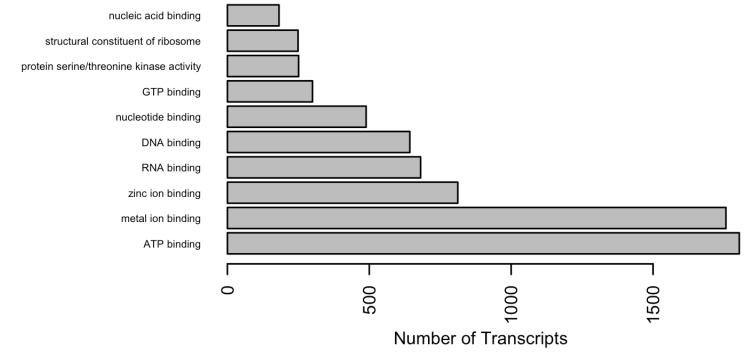


Figure 1. Distribution of the coverage of the transcripts over their top SwissProt hit. A large percent coverage implies that the protein is highly conserved or is available in the database. Lower values imply conserved domains interspaced with less conserved domains and more species specific coding regions.

a



b



c

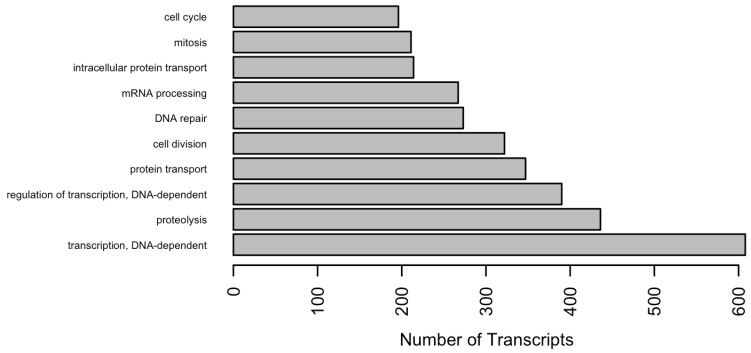


Figure 2. Most frequent GO annotations in each high-level annotation group. Transcripts were annotated with GO terms belonging to (**a**) components of the cell, (**b**) molecular functions, or (**c**) biological processes.

Fungal Genomes

Ajellomyces capsulatus G186AR
Ajellomyces capsulatus NAm1
Ascosphaera apis USDA-ARSEF 7405
Ashbya gossypii ATCC 10895
Aspergillus clavatus NRRL
Aspergillus clavatus NRRL 1
Aspergillus flavus NRRL3357
Aspergillus fumigatus A1163
Aspergillus fumigatus Af293
Aspergillus nidulans A4
Aspergillus niger CBS 51388
Aspergillus oryzae RIB40
Aspergillus terreus ATCC 20542
Aspergillus terreus NIH2624
Batrachochytrium dendrobatidis JAM81
Batrachochytrium dendrobatidis JEL423
Beauveria bassiana ARSEF 2860
Botryotinia fuckeliana B0510
Botrytis cinerea
Candida albicans SC5314
Candida albicans WO-1
Candida glabrata CBS 138
Candida guilliermondii
Candida lusitaniae
Candida tropicalis
Candida tropicalis MYA-3404
Chaetomium globosum CBS 14851
Clavispora lusitaniae ATCC 42720
Coccidioides immitis H5384
Coccidioides immitis RMSCC 2394
Coccidioides immitis RMSCC 3703
Coccidioides immitis RS
Coccidioides posadasii RMSCC 3488
Coccidioides posadasii str Silveira
Coprinus cinereus
Cordyceps militaris CM01
Cryptococcus neoformans B-3501A
Cryptococcus neoformans grubii
Cryptococcus neoformans var neoformans B-3501A
Cryptococcus neoformans var neoformans JEC21
Debaryomyces hansenii CBS767
Emericella nidulans FGSC A4
Encephalitozoon cuniculi GB-M1
Eremothecium gossypii ATCC 10895
Filobasidiella neoformans r grubii H99
Filobasidiella neoformans R265
Fusarium graminearum
Fusarium oxysporum f sp lycopersici 4286
Fusarium oxysporum lycopersici
Fusarium solani
Fusarium verticillioides
Gibberella moniliformis 7600
Gibberella zeae PH-1
Grosmannia clavigera kw1407
Histoplasma capsulatum WU24
Hypocrea jecorina QM6a
Hypocrea virens ns Gv29-8
Kluyveromyces lactis NRRL Y-1140
Kluyveromyces waltii NCYC 2644
Laccaria bicolor
Laccaria bicolor S238N-H82
Lachancea kluyveri eri NRRL Y-12651
Lodderomyces elongisporus NRRL YB-4239
Magnaporthe grisea 70-15
Malassezia globosa 20071102
Malassezia globosa CBS 7966
Malassezia restricta CBS 7877
Metarhizium robertsii strain ARSEF 2575
Neosartorya fischeri NRRL 181
Neurospora crassa OR74A
Penicillium chrysogenum Wisconsin 54-1255
Penicillium marneffei ATCC 18224
Phaeosphaeria nodorum SN15
Phanerochaete chrysosporium
Phycomyces blakesleeanus
Pichia guilliermondii ATCC 6260
Pichia stipitis
Pichia stipitis CBS 6054
Podospora anserina DSM 980
Postia placenta
Puccinia graminis f sp tritici CRL 75-36-700-3
Puccinia graminis tritici
Pyrenophora tritici-repentis Pt-1C-BFP
Rhizopus oryzae
Rhizopus oryzae RA 99-880
Saccharomyces bayanus 623-6C
Saccharomyces bayanus MCYC 623
Saccharomyces castellii NRRL Y-12630
Saccharomyces cerevisiae RM11-1a
Saccharomyces cerevisiae S288C
Saccharomyces cerevisiae YJM789
Saccharomyces kudriavzevii IFO 1802
Saccharomyces mikatae IFO 1815
Saccharomyces paradoxus NRRL Y-17217
Schizosaccharomyces japonicus yFS275
Schizosaccharomyces pombe 20071109
Schizosaccharomyces pombe 972h-
Sclerotinia sclerotiorum 1980
Sporobolomyces roseus
Stagonospora nodorum
Talaromyces stipitatus ATCC 10500
Trichoderma atroviride IMI 206040
Trichoderma reesei
Ustilago maydis
Ustilago maydis 521
Vanderwaltozyma polyspora DSM 70294
Yarrowia lipolytica CLIB122

Table 2. Fungal genomes used for filtering transcripts. The fungal genomes were compiled into a blast database and used after blasting against non-fungal databases. Transcripts with higher bits scored in the fungal database were retained while those with lower bit scores were discarded.

Bacterial Genomes	
Agrobacterium fabrum str. C58	Nitrosomonas europaea ATCC 19718
Bacillus clausii KSM-K16	Pectobacterium atrosepticum SCRI1043
Bacillus subtilis subsp. subtilis str. 168	Photorhabdus luminescens subsp. laumondii TTO1
Baumannia cicadellinicola str. Hc	Pseudomonas aeruginosa PAO1
Brucella melitensis bv. 1 str. 16M	Ralstonia solanacearum GMI1000
Buchnera aphidicola str. APS	Regiella insecticola
Buchnera aphidicola str. Bp	Rickettsia conorii str. Malish 7
Buchnera aphidicola str. Cc	Rickettsia prowazekii str. Madrid E
Buchnera aphidicola str. Sg	Salmonella enterica subsp. enterica serovar Typhimurium
Candidatus Blochmannia floridanu	Shewanella oneidensis MR-1
Candidatus Blochmannia pennsylvanicus str. BPEN	Sodalis glossinidius str. 'morsitans'
Candidatus Carsonella ruddii PV	Staphylococcus aureus subsp. aureus COL
Caulobacter crescentus CB15	Vibrio cholerae O1 biovar El Tor str. N16961
Escherichia coli O157:H7 str. Sakai	Vibrio cholerae O1 biovar El Tor str. N16961
Escherichia coli str. K-12 substr. MG1655	Wigglesworthia glossinidia
Haemophilus influenzae Rd KW20	Wolbachia endosymbiont of Drosophila melanogaster
Mesorhizobium loti MAFF303099	Wolbachia endosymbiont strain TRS of Brugia malayi
Neisseria gonorrhoeae FA 1090	Xanthomonas axonopodis pv. citri str. 306
	Yersinia pestis CO92

Table 3. Bacterial genomes used for filtering transcripts. The bacterial genomes were compiled into a blast database and used to filter away probably bacterial transcripts.

Discussion

Assembly identified 19,977 unique transcripts with a contig N50 value of 1,392 bp. An N50 value of 1,392 bp is consistent with the average length of a fungal coding sequence, which is between 1.3 and 1.9 kb (Galagan *et al.* 2005). Compared to the *Beauveria bassiana* genome assembly by Xiao *et. al.* (2012) which identified 13,412 EST sequences and predicted 10,366 protein encoding genes, this *Pandora* assembly is slightly larger. Because transcripts were assembled, and not genes, there will be a greater number of genetic elements due to the presence of splice variants. Additionally, another factor that may have influenced the number of assembled transcripts is the sequencing and assembly of two closely related but different strains of *Pandora*. The strains infecting the LSR1 and 313 aphid lines were collected from different geographic locations: the United States and Europe, respectively. Sufficient genetic differences could result in orthologous transcripts being assembles separately. Assemblies of other entomopathogenic fungi *Metarhizium anisopliae* and *M. acridum*, yielded a similar number of genes with 10,582 and 9,849 respectively (Goa *et. al.* 2011).

Compared to *B. bassiana, M. anisoplia,* and *M. acridum*, this *Pandora* assembly was predicted to contain fewer secreted proteins by SignalP. Of the assembled *Pandora* transcripts, 3% were predicted by SignalP to be secreted proteins. This is compared to 18.2%, 17.6%, and 15.1% of protein encoding genes in the genomes of *B. bassiana, M. anisoplia, and M. acridum* respectively (Goa *et. al.* 2011, Xiao *et. al.* 2012). This low abundance of secreted proteins may be explained by *Pandora*'s status as a specialist of aphids. Both *B. bassiana* and *M. anisoplia* are generalist

entomopathogens while *M. acridum*, the fungi with the lowest proportion of the three, is a specialist of acridids. Additionally, this transcriptome, due to its specificity, may not contain all possible secreted proteins encoded by the *Pandora* genome.

Figure 2 shows how the transcripts in the assembly cover their top SwissProt database hits. High coverage implies that the transcript is either a coding sequence for a gene in the database or that the transcript is highly conserved among proteins in the database. Lower coverage implies that part of the transcript may contain a conserved domain. In fact, as we see here, we would expect the distribution of an organism without a sequenced genome and few verified proteins to be skewed toward lower coverage limited to conserved domains only. The absence of a significant number of transcripts whose blast hits are covered by less than 10% of the transcript implies that the assembly produced few transcripts that did not contain biologically valid sequence.

The *Pandora* transcriptome has a variety of transcripts that could be involved in the attack and exploitation of the host organism. Because the fungus is spread through the air, rather than ingested, the fungus needs an array of proteins for degradation of the chitin making up the cuticle of the insect. Once inside, the fungus has mechanisms for producing virulence factors and promoting growth. Several transcripts were cataloged that contained domains associated with such growth and with the attachment of the fungal cells to the host substrate. Future work is needed to provide more detailed information about the function of some of the proteins encoded by the transcripts. Analysis of these transcripts using

orthologous proteins in closely related organisms as well as the sequencing of the *Pandora* genome will be useful in future studies of the pathogenesis of the fungus.

There is a present need for high quality transcriptomes because of their potential use in the study of host-pathogen interactions. Genomic tools like these can provide a way to find underlying mechanisms of pathogenesis and host defense. By using these transcriptomes to study differences in the gene expression of organisms involved in these interactions, specific genes and other genetic elements can be targeted as being involved in these mechanisms. Insight into particular genes and transcripts that are being repressed in the pathogen or upregulated in the host allows for fine-grain observations of defense and exploitation at transcriptional and genetic levels. Of course, differences in the count of a transcript alone are not going to allow for the confirmation of entire pathways but they do give specific directions for future research.

Chapter III

Gene expression of *Pandora neoaphidis* during attack of pea aphids with and without protective symbionts

Introduction

Pandora neoaphidis is a common aphid fungal pathogen in temperate regions and has been suggested as a possible bio-control mechanism against aphid agricultural pests (Glare & Milner, 1991). The aphid specialist infects by degrading the cuticle and colonizing the gut. During development, the fungal colony will construct conidia, which burst out from the body of the aphid, killing the host and aiding in the dispersal of further spores.

The pea aphid, *Acythrosiphon pisum,* is capable of harboring facultative bacterial symbionts that protect it from a variety of predators, pathogens, and environmental conditions (Oliver 2010). The bacteria *Regiella insectola* has been demonstrated to increase the rate of aphid survival upon infection with several fungal entomopathogens, include *P. neoaphidis* (Scarbourough 2005, Parker 2013). Aphids harboring *R. insecticola*, regardless of aphid genotype, have significantly higher survival rates when infected by *P. neoaphidis,* suggesting that the bacteria itself is responsible for protection. It is not yet known how *Regiella* protects aphids from fungal pathogen, and there has been no investigation of how the presence of *Regiella* alters pathogen development, transcription or other processes. Here, I

explore *Pandora* transcriptional responses during attack of aphids with and without the protective symbiont, *Regiella insecticola.*

Methods

RNA-Seq data from four *Pandora neoaphidis*-infected aphid lines was used for differential expression analysis: LSR1-genotype *A. pisum* without any secondary symbionts (LSR1-01), LSR1-genotype *A. pisum* with the secondary symbiont *Regiella insecticola* (LSR1-Ri), 313-genotype *A. pisum* without any secondary symbionts (313_O), and 313-genotype *A. pisum* with the protective symbiont *Regiella* 313. As aphids clonally reproduce, all LSR1 are presumed to be genetically identical, and all 313 aphids are presumed to be genetically identical. Experimental infections with LSR1 and 313 treatments were conducted separately using different strains of *Pandora*. Infections, sample preparation and sequencing were carried out as outlined in Chapter II.

In order to determine differentially expressed transcripts, the RNA-Seq reads were mapped onto a Trinity generated *Pandora* transcriptome using Tophat. Following the initial alignment, transcript abundance for each treatment was estimated using the RSEM utility. Once the abundance was estimated and the RSEM data was merged, differential expression analysis was conducted pairwise between the treatments containing a protective bacterial symbiont and those lacking such a symbiont. The result was two expression comparisons: LSR1-01 vs LSR-Ri and 313-O vs 313-313. This analysis was done using the EdgeR bioconductor package. The

scripts used for this pipeline are available in the Trinity *de novo* Assembler utilities package (Table 1).

alignReads.pl	--seqType fq --left ../paired_$i.fastq.1 --right ../paired_$i.fastq.2 --retain_intermediate_files --aligner tophat2 --target ../Pandora_Transcriptome.fa -- -p 14
run_RSEM_align_n_estimate.pl	--transcripts ../Pandora_Transcriptome.fa --left left.fastq --right right.fastq --seqType fq --prefix prefix
merge_RSEM_frag_counts_single_table.pl	*.abundance.results
run_DE_analysis.pl	--matrix counts.matrix --method edgeR

Table 1 Parameters used for differential expression analysis.

Results

RNAseq Analysis. In both host genetic backgrounds, there were a large number of significantly differentially expressed transcripts between aphids with and without *Regiella*. Between LSR1-01 and LSR1-Ri, there were a total of 2,335 differentially expressed transcripts (Figure 1). Of those transcripts, 1,594 were more expressed in the LSR1-01 aphids, and 741 were more expressed in the LSR1-Ri aphids. Between 313-O and 313-313, there were a total of 9,433 differentially expressed transcripts. Of these transcripts, 6,403 were more expressed in the 313-O aphids and 3,030 were more expressed in the 313-313 aphids.

To focus on differences in transcripts that most likely play a role in pathogenic processes, those differentially expressed transcripts that were annotated with the Gene Ontology terms 'Pathogenesis', 'Chitinase', and 'Proteolysis' were selected for further analysis (Figure 2). In general, presence of a protective symbiont decreased expression of these pathogen-associated transcripts in the attacking fungus. There were however a few transcripts that were more highly expressed by the fungus in the presence of the protective symbiont. Specifically, in 313, Chitinase 1, Chitinase A1, and cuticle-degrading protease transcripts were significantly more expressed in the presence of *Regiella.*

Only one transcript was differentially expressed in the two comparisons (LSR1 and 313). In both comparisons, the Chitinase A1 transcript was expressed differently; however, in the LSR1 comparison the transcript was less expressed in the presence of *Regiella* while in the 313 comparison the transcript was more expressed in the presence of *Regiella*. The remaining transcripts were differentially expressed exclusively in one or the other genotype set.

Of the Chitinase 1 transcripts, comp32679_c0_seq1 was less expressed in the presence of the 313 *Regiella* by a log fold change of -2.699. Transcript comp55311_c0_seq1 was more expressed in the presence of the 313 *Regiella* with a log fold change of 5.298. Neither transcript was differentially expressed in the LSR1 comparison.

Of the Chitinase A1 transcripts, comp23970_c0_seq1 and comp26111_c0_seq1 were less expressed in the presence of the 313 *Regiella* by a log fold change of -10.006 and -4.940 respectively. Chitinase A1 transcript

comp58163_c0_seq1 was more expressed in the presence of the 313 *Regiella* with a log fold change of 5.153 and less expressed in the presence of *Regiella* in the LSR1 comparison with a log fold change of -4.563.

Transcript comp8157_c0_seq1, blasting to Alkaline protease 2, was expressed less in the presence of the 313 *Regiella* with a log fold change of -7.901. The transcript was not differentially expressed in the LSR1 comparison.

Transcript comp37731_c0_seq3 blasted to Dolichyl-phosphate-mannose--protein mannosyltransferase 1. Transcript comp37731_c0_seq3 was expressed less in the presence of *Regiella* with a log fold change of -8.328 in the 313 comparison only.

Transcript comp42502_c0_seq1, blasting to Extracellular metalloproteinase 5, was less expressed in the presence of *Regiella* in the 313 comparison with a log fold change of -11.031. There was no differential expression in the LSR1 comparison.

Transcript comp42068_c0_seq2, blasting to Glycolipid 2-alpha-mannosyltransferase 1, was less expressed in the presence of *Regiella* in the LSR1 comparison with a log fold change of -7.263. There was no differential expression in the 313 comparison.

Transcript comp15000_c0_seq1, blasting to Cuticle-degrading protease, was more expressed in the presence of the 313 *Regiella* by a log fold change of 4.282. The transcript was not differentially expressed in the LSR1 comparison.

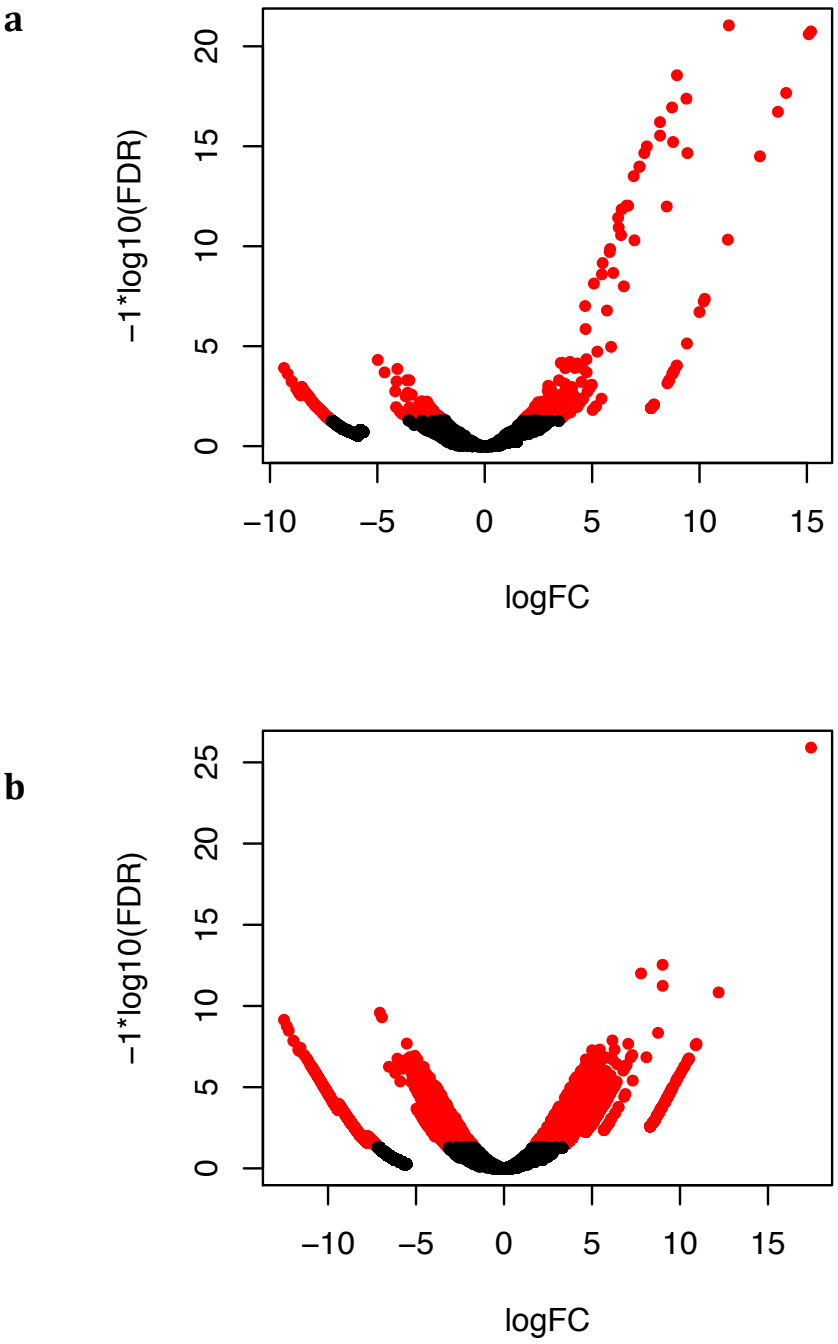


Figure 1. Differential transcript expression. Transcripts with differential expression in the (**a**) LSR1 comparison and the (**b**) 313 comparison are considered differentially expressed with a p-value < 0.05 and FDR < 0.05 (points in red). Negative logFC (left side of distribution) values indicate genes expressed less in presence of *Regiella*. Positive logFC (right side of distribution) values indicate gene expressed more in the presence of *Regiella*. Values higher on the y-axis indicate a lower FDR and thus more confidence being correctly called as differentially expressed.

Transcript ID	Annotation	Top BLAST Hit	313_O vs 313_313	LSR01 vs LSRRi
comp32679_c0_seq1	chitinase	Chitinase 1	-2.699	-
comp55311_c0_seq1	chitinase	Chitinase 1	5.298	-
comp23970_c0_seq1	chitinase	Chitinase A1	-10.066	-
comp26111_c0_seq1	chitinase	Chitinase A1	-4.94	-
comp58163_c0_seq1	chitinase	Chitinase A1	5.153	-4.563
comp8157_c0_seq1	pathogenesis	Alkaline protease 2	-7.901	-
comp37731_c0_seq3	pathogenesis	Dolichyl-phosphate-mannose--protein mannosyltransferase 1	-8.328	-
comp42502_c0_seq1	pathogenesis	Extracellular metalloproteinase 5	-11.031	-
comp42068_c0_seq2	pathogenesis	Glycolipid 2-alpha-mannosyltransferase 1	-	-7.263
comp15000_c0_seq1	proteolysis	Cuticle-degrading protease	4.282	-

Table 2. Transcripts related to pathogenesis that are significantly differentially expressed in aphids with and without protective bacteria. Transcripts in this set had a log fold-change p-value < 0.05 and an FDR < 0.05. Here a negative log fold-change can be interpreted as a decrease in transcript number in the presence of the protective symbiont.

Transcript	BLAST	LogFC
comp57395_c0_seq1	UNKNOWN	15.191
comp51737_c0_seq1	UNKNOWN	15.086
comp30512_c0_seq1	UNKNOWN	14.034
comp13574_c0_seq1	UNKNOWN	13.650
comp1867_c0_seq1	UNKNOWN	12.813
comp7511_c0_seq1	UNKNOWN	11.366
comp49420_c0_seq2	Pre-mRNA-processing-splicing factor 8	11.319
comp23098_c0_seq1	UNKNOWN	10.246
comp39894_c0_seq4	UNKNOWN	10.194
comp52259_c0_seq1	Uncharacterized protein YLR154C-G	9.998

Table 3. Most positively significantly differentially expressed transcripts in LSR01 aphids with and without protective bacteria. Transcripts in this set were more expressed in the presence of the protective *Regiella* symbiont.

Transcript	BLAST	LogFC
comp38136_c0_seq1	60S ribosomal protein L12	-9.346
comp45618_c0_seq2	Histone H3	-9.173
comp36287_c0_seq1	Mitochondrial protein import protein mas5	-8.989
comp43377_c0_seq1	ATP-dependent RNA helicase eIF4A	-8.989
comp26012_c0_seq2	60S ribosomal protein L6	-8.779
comp50034_c0_seq1	Mitochondrial acidic protein mam33	-8.678
comp41488_c0_seq8	Retrotransposable Tf2 155 kDa protein type 2	-8.625
comp44199_c0_seq1	ATP synthase subunit alpha, mitochondrial	-8.625
comp49951_c0_seq1	Probable mitochondrial membrane porin	-8.570
comp35564_c0_seq1	Vesicular inhibitory amino acid transporter	-8.532
comp38136_c0_seq1	60S ribosomal protein L12	-9.346

Table 4. Most negatively significantly differentially expressed transcripts in LSR01 aphids with and without protective bacteria. Transcripts in this set were less expressed in the presence of the protective *Regiella* symbiont.

Transcript	BLAST	LogFC
comp64476_c0_seq1	Cytochrome c oxidase subunit 1	17.445
comp5172_c0_seq1	NADH-ubiquinone oxidoreductase chain 4	12.193
comp15026_c0_seq1	UNKNOWN	10.938
comp24221_c0_seq1	UNKNOWN	10.915
comp28001_c0_seq3	Proline dehydrogenase 1, mitochondrial	10.891
comp70111_c0_seq1	UNKNOWN	10.508
comp43024_c0_seq7	UNKNOWN	10.508
comp9502_c0_seq1	UNKNOWN	10.476
comp22528_c0_seq1	Elongation factor 1-alpha	10.411
comp57105_c0_seq1	UNKNOWN	10.270

Table 5. Most positively significantly differentially expressed transcripts in 313 aphids with and without protective bacteria. Transcripts in this set were more expressed in the presence of the protective *Regiella* symbiont.

Transcript	BLAST	LogFC
comp37027_c0_seq1	Glycine-rich RNA-binding protein 2, mitochondrial	-12.503
comp41635_c1_seq1	Argininosuccinate synthase	-12.346
comp49945_c0_seq1	Monocarboxylate transporter 2	-12.234
comp44199_c0_seq2	ATP synthase subunit alpha, mitochondrial	-11.973
comp42518_c0_seq1	Allergen Tha p 2	-11.955
comp8854_c0_seq1	Granzyme C	-11.695
comp26012_c0_seq2	60S ribosomal protein L6	-11.648
comp32383_c0_seq1	Adenosine kinase 2	-11.563
comp8939_c0_seq1	UNKNOWN	-11.347
comp24521_c0_seq1	H/ACA ribonucleoprotein complex subunit 1	-11.206

Table 6. Most negatively significantly differentially expressed transcripts in 313 aphids with and without protective bacteria. Transcripts in this set were less expressed in the presence of the protective *Regiella* symbiont.

Discussion

A symbiont can protect the host organism through a variety of mechanisms. One possible approach to defense is giving aid to the host's own immune system and helping to clear the host of the pathogen. Another possible approach is to contain or mitigate the effect that the pathogen has on the host. The majority of the *Pandora* transcripts profiled were less expressed in the presence of a *Regiella* strain. These data suggest that the bacteria have some effect on the expression of pathogenic transcripts. By influencing the production of pathogenic genes, the symbiont is able to bolster defense against the fungus.

Transcript comp37731_c0_seq3 was annotated with the pathogenesis Gene Ontology (GO) term. The transcript was less expressed in the presence of the 313 *Regiella* in the 313 aphids and contains domains associated with cell-substrate adhesion, fungal cell wall organization, and filamentous growth. Decreased expression of genes involved in the growth of the fungus could be a method by which *Regiella* is able to protect the host and itself from a lethal infection.

Beyond direct protection of the infected individual, because aphids live in close proximity to their genetically identical offspring, siblings and other clonal descendants, they could improve their inclusive fitness substantially if the could suppress sporulation, the process by which the fungus escapes the host to find other hosts. Interestingly, transcript comp8157_c0_seq1, which was less expressed in the presence of *Regiella,* was annotated with a domain associated with sporulation. Decreasing expression of a transcript that plays a role in the mechanism of the pathogen's spread would have significant inclusive fitness benefits for the aphids as

well as the symbiotic bacteria because of the density of closely related individuals around the infected aphid.

Transcript comp42502_c0_seq1 contained domains associated with extracellular metalloproteases. These are secreted proteins that can function as virulence factors. There was more than a 11-fold decrease in the expression of this probable virulence factor in the presence of the symbiont in the 313 comparison. Suppression of a pathogen's virulence factors would provide a direct fitness benefit to the insect-symbiont complex. Interestingly, this direct effect was not seen in the LSR1 comparison.

Transcript comp42068_c0_seq2 contained domains associated with the evasion or tolerance of host defenses. Such domains are involved in any active or passive process used by an organism to avoid or tolerate the effects of another organism's defenses. Typically, these two organisms are in a symbiotic interaction. For example, such process can either be induced by the context or exist *a priori* in the case of physical barriers like cell walls. Additionally, the transcript contained domains associated with filamentous growth and fungal cell wall organization. This transcript was only differentially expressed in the LSR1 comparison. There would exist selective pressure for the fungal pathogen to evade host defenses in much the same way that pressure is placed on any symbionts to evade host defenses. The switching off of this defense in the pathogen by the symbiont would carry a significant advantage for the host-bacteria complex. It is unlikely that this transcript is expressed by the symbiont itself due to the presence of domains associated with fungal cell wall organization.

Transcript comp15000_c0_seq1 was annotated with the Gene Ontology terms corresponding to proteolysis and the negative regulation of catalytic activity. This transcript corresponds to a gene encoding a chitin-degrading protease found previously in the *Pandora* secretome (Grell *et. al.* 2010). Interestingly, there was increased expression of this transcript in the presence of the 313 protective symbiont. The presence of a domain that corresponds to negative regulation could suggest that this expression limits the impact of chitin-degradation. According to Grell *et. al.,* this protein contains features associated with the inhibition of peptidase activity (2010). An increase in the expression of such an inhibitor may be a mechanism by which the virulence of the pathogen is decreased by the presence of the *Regiella*.

The decreased expression of chitinase transcripts is consistent with previous findings that aphids are less susceptible to *Pandora* spores when they harbor a protective *Regiella*. Such a decrease would be able to provide a significant defense against a *Pandora* infection as *Pandora* spores are transmitted through the air and must burrow through the cuticle of the insect in order to colonize and exploit the host. Two of the five chitinase transcripts however were found to have increased expression. This may be as a result of the pathogen's response to defenses used by the symbiont. Further study is needed to elucidate differences between these transcripts and their expression.

There were marked differences in how the presence of the protective symbiont affected expression during infection of each of the two aphid genotypes. In general, more transcripts were differentially expressed in the 313 comparison.

Differences could be due to the pathogen genotype or the symbiont genotype, which both differed in the two host backgrounds, due to host genetics, or do to some combination of the three, and future work will need to look for the impact of these players on pathogen expression. Despite this, there were some overall patterns. In both analyses, there existed at least one chitinase transcript that was more expressed when the protective symbiont was absent. Likewise, different transcripts with similar functions were differentially expressed between the two comparisons. Transcripts comp37731_c0_seq3 and comp42068_c0_seq2, for example, were both annotated with GO terms associated with fungal cell wall construction and organization. The former was more expressed in the 313 system in the absence of the protective symbiont and the latter was more expressed in the LSR1 system in the absence of the protective symbiont. These data may suggest that, though these symbionts both act to protect the host-symbiont complex, they act in distinct ways and act on distinct transcripts to achieve this. However, because these are two genetically different strains of *Pandora*, the differences in expression reported here might be due to orthologous transcripts being assembled separately. For example, transcripts comp37731_c0_seq3 and comp42068_c0_seq2 might be different isoforms of the same coding sequence but they may also be transcripts orthologous between the two *Pandora* strains. Future studies will be required to tease apart the mechanistic differences between the two strains.

In addition to analyzing transcripts that had been annotated with GO terms of interest, the transcripts with the largest absolute value fold changes were noted (Table 3, Table 4, Table 5, Table 6). Several of these transcripts, especially those that

were expressed more in the presence of *Regiella*, were not able to be annotated and thus have unknown functions. More in depth functional analysis needs to be performed in order to elucidate the functions of these highly differentially expressed transcripts, which may provide novel insight into the biology of this and other fungal entomopathogens.

Chapter IV

Gene expression of *Pandora neoaphidis* during attack of pea aphids of two alternative morphs

Introduction

Pandora neoaphidis is an aphid specific fungal entomopathogen commonly suggested as a possible bio-control mechanism against aphids, agricultural pests. The fungus is the most common aphid fungal pathogen in temperate regions (Glare & Milner, 1991). Most recently, it is has been the focus of studies investigating the role that symbiotic bacteria play in defending their pea aphid hosts from fungal infections (Scarborough *et. al.* 2005, Parker *et. al.* 2013). It has also been used to investigate the costs aphids pay for mounting an immune response (Barribeau et al. 2014).

Here, I compare *Pandora* transcriptomes assembled from pea aphids of two alternative morphs, winged and un-winged. These phenotypic morphs are genetically identical and can be born to the same mothers. Mothers exposed to stressful conditions (e.g., pathogens, predators, crowding) tend to produce more winged than un-winged offspring. It is presumed that this would allow offspring to escape these stressful conditions. In the absence of stressful conditions, aphids tend to produce only un-winged offspring, which require fewer resources in terms of developing the wings and associated, energetically expensive musculature (reviewed in Brisson and Stern 2006).

Recent work suggests that winged aphids are less resistant to *Pandora* infection than un-winged aphids, and that they also display greater fitness costs upon inoculation with heat-killed *Pandora* than un-winged aphids (Parker *et. al*., 2013). Little is known about the underlying mechanisms responsible for these differences nor about how these differences impact the pathogen during infection. To investigate this, we are analyzing host and pathogen transcriptional responses. Here, I investigate whether the pathogen is differentially responding to these alternative host forms.

Data and Methods

RNA-Seq data from one *Pandora neoaphidis*-infected aphid line was used for differential expression analysis: LSR1-genotype *A. pisum* without any secondary symbionts (LSR1-01). As aphids clonally reproduce, all LSR1 are presumed to be genetically identical. The developing aphids were exposed to the alarm pheromone (E)-*β*-farnesene (EBF) at a dose that causes them as adults to give birth to approximately 50% winged and 50% un-winged offspring (dose based on preliminary experiments, 5 µL of 1000 ng/µL EBF every other day for 10 days). Infections, sample preparation and sequencing were carried out as outlined in Chapter II.

In order to determine differentially expressed transcripts, the RNA-Seq reads were mapped onto a Trinity generated *Pandora* transcriptome (Chapter II) using Tophat (v2.0.3). Following the initial alignment, transcript abundance for each

treatment was estimated using the RSEM (v1.2.11) utility. Once the abundance was estimated and the RSEM data was merged, differential expression analysis was conducted pairwise between the winged and un-winged aphid treatments. This analysis was done using the edgeR bioconductor package (v2.12). The scripts used for this pipeline are available in the Trinity *de novo* Assembler utilities package. Discussion is focused on transcripts with domains that corresponded to functions involved in stress responses, fungal growth, and sporulation. Transcripts of interest were determined by their Gene Ontology (GO) annotations.

alignReads.pl	--seqType fq --left left.fastq --right right.fastq --retain_intermediate_files --aligner tophat2 --target Pandora_Transcriptome.fa -- -p 14
run_RSEM_align_n_estimate.pl	--transcripts Pandora_Transcriptome.fa --left left.fastq --right right.fastq --seqType fq --prefix prefix
merge_RSEM_frag_counts_single_table.pl	*.abundance.results
run_DE_analysis.pl	--matrix counts.matrix --method edgeR

Table 1 shows the parameters used for differential expression analysis

Results

RNAseq Analysis. Between the winged and un-winged aphid morphs, 3,102 Pandora transcripts were significantly differentially expressed. Of these transcripts, 2,547 were more highly expressed in the winged morph and 555 were more highly expressed in the un-winged morph (Figure 1). Focus was placed on three classes of

transcripts -- those involved with fungal growth, fungal stress response, and sporulation -- every differentially expressed transcript belonging to one of these groups was more expressed when the fungus was infecting the winged aphid morph (Table 2), which is more resistant to fungal infection than the un-winged morph (Parker et al. 2013).

Three enzymes with primary functions involved in the creation and maintenance of fungal cell walls exhibited increased expression in winged aphids. Transcripts comp42068_c0_seq2 (Glycolipid 2-alpha-mannosyltransferase 1), comp50078_c0_seq1 (Glutamine-fructose-6-phosphate aminotransferase), and comp9684_c0_seq1 (MAP kinase kinase skh1/pek1) were annotated with Gene Ontology terms associated with fungal cell wall proteins and exhibited log fold changes of 8.346, 8.463, and 7.549 respectively (positive values indicated increased expression in winged compared to un-winged aphids). Glycolipid 2-alpha-mannosyltransferase 1 catalyzes the creation of mannoproteins in the cell walls of the fungus and is integral to virulence and to the adherence of the fungus to host cells (Murno *et. al.,* 2005). Such proteins are important structural components in cell walls. Glutamine-fructose-6-phosphate aminotransferase functions as a catalyst in the biosynthesis of chitin, the main polysaccharide and structural molecule making up the cell walls of fungi. Finally, MAP kinase kinase skh1/pek1 is involved in the mkh1 signal transduction pathway. This pathway plays a role in cell wall integrity.

Proteins that play a role in *Pandora's* stress responses also exhibited increased expression in winged aphids. The majority of the differentially expressed

genes that fall into this category are heat shock proteins. The exception is transcript comp8990_c0_seq1, which blasts to an inorganic pyrophosphatase, an enzyme involved in osmoadaptation. The heat shock transcripts were observed to all have log fold differences between 6.800 and 8.959.

Transcripts that contained domains associated with sporulation were also differentially expressed. Transcript comp49879_c1_seq1 is annotated with a domain involved in spore wall formation and exhibited a log fold change of 3.995. The transcript blasts to Meiotic expression up-regulated protein 10, a protein that is required for the proper construction and maturation of the fungal spore wall.

Interestingly, genes putatively involved in reproductive mode were also differentially expressed. Transcript comp50451_c0_seq1 blasts to MYND-type zinc finger protein samB and exhibited a log fold change of 7.549. This protein plays a role in fungal sexual spore formation. Additionally, transcript comp36760_c0_seq1 blasts to Transcriptional repressor rco-1 and exhibited a log fold change of 3.557. This protein is also involved in the regulation of sexual and asexual spore pathway genes.

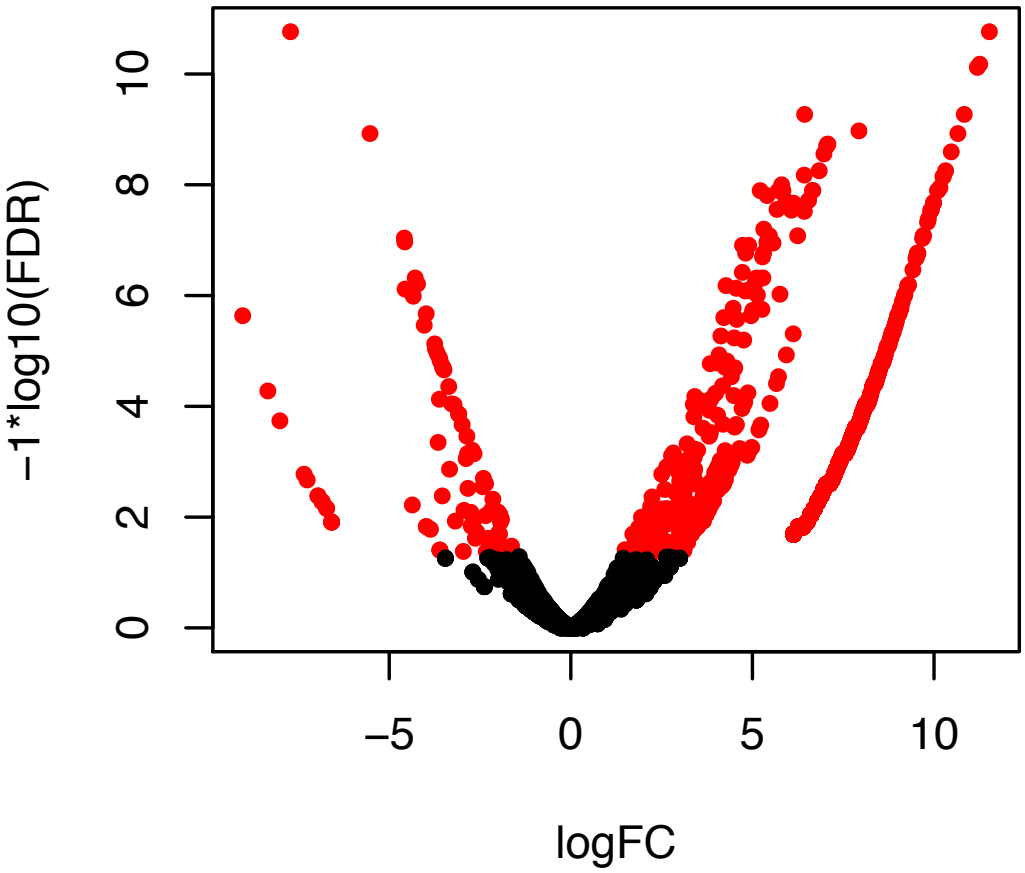


Figure 1. Differential transcript expression. Transcripts with differential expression in the aphid morphology comparison are considered differentially expressed with a p-value < 0.05 and FDR < 0.05 (points in red). Positive logFC (right side of distribution) values indicate transcript expressed more in the winged morph. Negative logFC (left side of distribution) values indicate transcript expressed more in the un-winged morph. Values higher on the y-axis indicate a lower FDR and thus more confidence being correctly called as differentially expressed.

Transcript ID	Annotation	Top BLAST Hit	Unwinged vs Winged
comp42068_c0_seq2	filamentous growth	Glycolipid 2-alpha-mannosyltransferase 1	8.346
comp50078_c0_seq1	fungal cell wall biosynthetic	Glutamine--fructose-6-phosphate aminotransferase	8.463
comp9684_c0_seq1	fungal cell wall organization	MAP kinase kinase skh1/pek1	7.549
comp49804_c0_seq9	response to stress	Heat shock 70 kDa protein 1	6.8
comp49804_c0_seq21	response to stress	Heat shock 70 kDa protein 2	7.319
comp49804_c0_seq12	response to stress	Heat shock 70 kDa protein 2	7.837
comp24228_c0_seq1	response to stress	Heat shock protein Hsp88	8.767
comp43079_c3_seq4	response to stress	Heat shock protein HSS1	6.968
comp9458_c0_seq1	response to stress	Heat shock protein sti1 homolog	8.959
comp8990_c0_seq1	response to stress	Inorganic pyrophosphatase	4.982
comp49879_c1_seq1	spore wall	Meiotic expression up-regulated protein 10	3.995
comp50451_c0_seq1	sporulation	MYND-type zinc finger protein samB	7.549
comp36760_c0_seq1	sporulation	Transcriptional repressor rco-1	3.557

Table 2. Transcripts with putative pathogen relevance significantly differentially expressed in winged and un-winged aphid morphs. Transcripts in this set had a log fold-change p-value < 0.05 and an FDR < 0.05. Here a positive log fold-change can be interpreted as an increase in transcript number in winged aphids.

Transcript	BLAST	LogFC
comp45618_c0_seq6	Histone H3.1	11.525
comp49873_c0_seq1	40S ribosomal protein S10-B	11.259
comp34493_c0_seq1	60S ribosomal protein L35	11.192
comp39173_c0_seq3	Histone H2A type 1	10.832
comp33974_c0_seq2	ATP-dependent RNA helicase DBP2	10.658
comp37749_c0_seq2	ATP synthase subunit a	10.474
comp26012_c0_seq1	60S ribosomal protein L6	10.319
comp49937_c0_seq1	40S ribosomal protein S21	10.255
comp25624_c0_seq1	29 kDa ribonucleoprotein B, chloroplastic	10.247
comp20136_c0_seq1	UNKNOWN	10.163

Table 3. Most positively significantly differentially expressed transcripts in winged and un-winged aphids. Transcripts in this set were more expressed when infecting a winged aphid.

Transcript	BLAST	LogFC
comp38136_c0_seq1	60S ribosomal protein L12	-9.346
comp45618_c0_seq2	Histone H3	-9.173
comp36287_c0_seq1	Mitochondrial protein import protein mas5	-8.989
comp43377_c0_seq1	ATP-dependent RNA helicase eIF4A	-8.989
comp26012_c0_seq2	60S ribosomal protein L6	-8.779
comp50034_c0_seq1	Mitochondrial acidic protein mam33	-8.678
comp41488_c0_seq8	Retrotransposable Tf2 155 kDa protein type 2	-8.625
comp44199_c0_seq1	ATP synthase subunit alpha, mitochondrial	-8.625
comp49951_c0_seq1	Probable mitochondrial membrane porin	-8.570
comp35564_c0_seq1	Vesicular inhibitory amino acid transporter	-8.532
comp38136_c0_seq1	60S ribosomal protein L12	-9.346

Table 4. Most negatively significantly differentially expressed transcripts in winged and un-winged aphids. Transcripts in this set were more expressed when infecting an un-winged aphid.

Discussion

For aphids, the production and maintenance of wings and the associated musculature is energetically expensive (Hatano *et al.*, 2010). Thus, it is necessary for these organisms to make trade-offs in the allocation of their energy resources. The production of wings has an opportunity cost that leaves fewer energy resources for other biological activities and mechanisms including immunological processes (Parker 2013). Lower energy allocated to immunological functions allows the *Pandora* fungus to proliferate more quickly. This is consistent with my finding that expression differences between *Pandora* infected winged and un-winged aphids were associated with pathogen growth and development, specifically fungal cell wall construction and organization, stress responses, and sporulation. The transcriptional differences in the fungus between the two treatments did not occur in transcripts associated with virulence and pathogenesis, which is in contrast with results of infecting aphids with and without protective bacterial symbionts (Chapter 3).

Transcripts comp42068_c0_seq2 (Glycolipid 2-alpha-mannosyltransferase 1), comp50078_c0_seq1 (Glutamine-fructose-6-phosphate aminotransferase), and comp9684_c0_seq1 (MAP kinase kinase skh1/pek1) are involved in the organization, construction, and maintenance of fungal cell walls. The MAP kinase kinase in particular is part of a pathway that regulates the growth of fungal cells (Krantz *et. al.* 2005). An increase in the expression of these transcripts suggests that the growth of the fungal colonies is increasing at a greater rate in the winged aphids relative to the un-winged aphids, consistent with previous findings (Parker 2013).

The weaker immune response from the energy deficient winged aphids would allow for a greater proliferation of fungal colonies.

Transcripts comp49879_c1_seq1 (Meiotic expression up-regulated protein 10) and comp36760_c0_seq1 (Transcriptional repressor rco-1) were both annotated with domains associated with the sporulation process. Parker (2013) demonstrated that winged aphid morphs infected with *Pandora* were more likely to produce a sporulating cadaver after being killed by the infection. The increase in the expression of transcripts associated with the process corroborates these findings and suggests that the un-winged aphids may be able to suppress the production of these spores with a stronger immune response.

The remaining transcripts reported here are associated with the pathogen's stress response. Transcript comp8990_c0_seq1 (Inorganic pyrophosphatase) is associated with osmoadaptation. In filamentous fungi, growth of the cell is aided by the adjusting of turgor pressures within the cell wall (Lew and Kapishon *et. al.,* 2009). An increase in the expression of this transcript signals an increase in the demand for cell growth. Such growth is more possible in winged aphids.

The remaining differentially expressed transcripts were annotated with domains associated with heat shock proteins. These are highly conserved proteins that aid in the refolding of denaturing protein structures. They are expressed in response to a variety of stressful stimuli. Fungal heat shock proteins have been demonstrated to play an integral role in virulence and fungal growth (Lamoth *et. al.* 2013, Znaidi *et. al.,* 2013). Experimental repression of a heat shock protein 90 results in a significant decrease in the virulence of the pathogenic fungus *Aspergillus*

fumigatus as well as an increase in the sensitivity of the fungus to anti-fungal compounds (Lamoth *et. al.* 2013). Znaidi *et. al.* (2013) demonstrated that proteins with heat shock factor type domains are involved in the transcriptional regulation of virulence factors in *Candida albicans*. It is possible that the differences in expression reported here are as a result of the difference in immune response strength between winged and un-winged morphs. A stronger immune response in the un-winged aphids would be able to suppress transcripts that resulted in the up-regulation of virulence and growth factors.

In addition to analyzing transcripts that had been annotated with GO terms of interest, the transcripts with the largest absolute valued fold changes were noted (Table 3, Table 4). Future studies of pathway analysis using these transcripts is needed to elucidate the role of these highly differentially expressed transcripts in the fungal infection of aphids.

The differing expression of the fungus between the two treatments implies that the fungus is able to more easily exploit the resources of its host if the aphid suffers an energy deficit due to wing production. As with everything in biology, this is a trade-off. When an aphid has the ability to fly away and find a better environment, it risks greater damage from microbes that are able to exploit it.

Chapter V

Conclusions

A rigorous study of *Pandora neoaphidis*, an organism often suggested and even implemented as a bio-control mechanism for aphid infestations, requires the availability of high quality genomic tools. At the time of writing, a query for *Pandora neoaphidis* on the NCBI Nucleotide database returned only 133 sequences, all of which were 18S rRNA encoding genes. As these sequences can only aid in the identification of *Pandora*, and do not provide any information on biological mechanisms, it is important to develop tools that can give insight into *Pandora*'s biology. Future research will require new transcriptome analyses and perhaps even a sequenced genome for the fungus if we are to be sure that *Pandora* is a viable and effective aphid bio-control mechanism.

With a transcriptome of *Pandora* during infection, I was able to study the impact of ecological context on the fungus' attack on its aphid host. My findings suggest that ecology plays an integral role in host-pathogen interactions and, rather than viewing these systems as two-way interactions, its more accurate to consider them as n-way interactions between hosts, pathogens, beneficial microbes, predators, abiotic influences, etc.. Specifically, here, the presence of a protective *Regiella* symbiont is shown to significantly change the RNA expression of the infecting fungus (Chapter 3). This change is associated with the bacteria's status as a defensive symbiont of aphids. Additionally, the fungus was shown to also differentially express certain transcripts when infecting winged and un-winged

aphids (Chapter 4). This finding suggests that even predators and abiotic factors that result in a stressed mother aphid can affect the interactions between her offspring and pathogens that try to exploit them.

It is clear that the current and convenient two-way architecture of immunology and host-pathogen interactions is too simplistic. Any mechanism for accurately understanding these phenomena must place these interactions within an ecological context, a set of biotic and abiotic factors that can play a role in these interactions. Previous studies, as well as those reported here, suggest that a variety of factors, which could easily be overlooked, can influence the way a pathogen infects its host and the way a host defends against a pathogen. These factors can also have very different influences and may be dependent on host and pathogen genotypes, and the interactions between the two. This may be reflected in the fact that gene expression difference depended both on conditions across experiments here (Table 1). This highlights the need for careful consideration of ecological factors mediating host-microbe interactions and dynamics.

Transcript ID	Annotation	Top BLAST Hit	Unwinged vs. Winged	313_O vs. 313_313	LSR01 vs. LSRRi
comp32679_c0_seq1	chitinase	Chitinase 1	-	-2.699	-
comp55311_c0_seq1	chitinase	Chitinase 1	-	5.298	-
comp23970_c0_seq1	chitinase	Chitinase A1	-	-10.066	-
comp26111_c0_seq1	chitinase	Chitinase A1	-	-4.94	-
comp58163_c0_seq1	chitinase	Chitinase A1	-	5.153	-4.563
comp8157_c0_seq1	pathogenesis	Alkaline protease 2	-	-7.901	-
comp37731_c0_seq3	pathogenesis	mannosyltransferase 1	-	-8.328	-
comp42502_c0_seq1	pathogenesis	Extracellular metalloproteinase 5	-	-11.031	-
comp42068_c0_seq2	pathogenesis	Glycolipid 2-alpha-mannosyltransferase 1	8.346	-	-7.263
comp15000_c0_seq1	proteolysis	Cuticle-degrading protease	-	4.282	-
comp50078_c0_seq1	cell wall biosynthetic	aminotransferase	8.463	-	-
comp9684_c0_seq1	cell wall organization	MAP kinase kinase skh1/pek1	7.549	-	-
comp49804_c0_seq9	stress response	Heat shock 70 kDa protein 1	6.8	-	-
comp49804_c0_seq21	stress response	Heat shock 70 kDa protein 2	7.319	-	-
comp49804_c0_seq12	stress response	Heat shock 70 kDa protein 2	7.837	-	-
comp24228_c0_seq1	stress response	Heat shock protein Hsp88	8.767	-	-
comp43079_c3_seq4	stress response	Heat shock protein HSS1	6.968	-	-
comp9458_c0_seq1	stress response	Heat shock protein sti1 homolog	7.593	-	-
comp8990_c0_seq1	stress response	Inorganic pyrophosphatase	4.982	-	-
comp49879_c1_seq1	spore wall	Meiotic expression up-regulated protein 10	3.995	-	-
comp50451_c0_seq1	sporulation	MYND-type zinc finger protein samB	7.549	-	-
comp36760_c0_seq1	sporulation	Transcriptional repressor rco-1	3.557	-	-

Table 1. All significantly differentially expressed genes of interest referred to in the chapters above. A positive logFC for an A vs. B comparison indicates an increase in transcript expression in the A treatment. A negative logFC for an A vs. B comparison indicates a decrease in transcript expression in the A treatment.

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