Beauveria bassiana infection in *Drosophila melanogaster*: survival, transcriptional response and variability among populations and host conditions

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1 SUMMARY

Drosophila melanogaster is a model organism to study innate immunity in invertebrates. Temperate and tropical *D. melanogaster* populations, being exposed to different environments, potentially face distinct parasites and parasite pressure. Indeed, there is experimental evidence suggesting that tropical *D. melanogaster* populations survive longer than temperate ones to infection by the fungal parasite *Beauveria bassiana*. In the present work we test the generality of this conclusion and investigate if host populations differ in their molecular response to infection.

We first exposed to *B. bassiana* infection two tropical (from Africa and South-East Asia) and two temperate (from Europe and North America) *D. melanogaster* out-crossed populations. We consistently found a significant effect of *B. bassiana* on *Drosophila* mortality, but we were not able to identify a significant difference in survival to infection among populations. These results indicate that tropical populations may not always survive better than temperate ones, and suggest that other environmental factors, such as humidity or local species richness may be more accurate predictors of immune competence.

Subsequently, we recorded transcriptional response to *B. bassiana* in all *D. melanogaster* populations, both by microarray and RNA sequencing. To our knowledge this is the first time that transcriptional response to fungal infection has been determined in multiple *D. melanogaster* out-crossed populations. We found few or no genes significantly induced 8 hours after infection. On the other hand, we identified between 200 and 1,300 genes induced 24 hours after infection depending on the population. This means that transcriptional response to *B. bassiana* begins between 8 and 24 hours after infection. We reveal here that host populations respond differently at the molecular level, as shown by the large variation in the number of induced genes. We report that gene ontology categories related to translation, biosynthesis and reproduction are enriched in genes down-regulated upon infection, suggesting a metabolic cost of mounting the defence response.

Next, we wanted to assess the selective pressures acting on induced candidate genes. We compared the genes induced in all populations to the ones induced specifically in each population and computed population genetic statistics for a subset of genes in each category. We noticed higher conservation at non-synonymous sites for commonly induced genes compared to population specific ones. This hints that common genes are under stronger selective constraints.

Another topic we addressed in the present work is the effect of endosymbionts and trans-generational immune priming on *D. melanogaster* survival to *B. bassiana*. We tested for a protective effect of the endosymbiont *Wolbachia pipientis* in two *D. melanogaster* inbred lines. We did not find an effect of *Wolbachia* on survival to infection in two independent experimental replicates. In absence of infection, flies bearing *Wolbachia* had a lower fitness than cured ones. Therefore *W. pipientis* appears to have a negative effect on *Drosophila* general vigour, but no effect on mortality upon infection.

Finally, we tested if flies whose parents were exposed to *B. bassiana* were less susceptible when infected by the same parasite. This would imply a transfer of immune memory from parents to offspring, which is called trans-generational immune priming. However, no evidence of immune transfer for two *D. melanogaster* out-crossed populations could be found. Yet, as trans-generational immune priming depends on host and parasite genotype, more experiments are needed to determine its generality in the *D. melanogaster – B. bassiana* system.

2 INTRODUCTION

2.1 GENERALITY OF HOST-PARASITE INTERACTIONS

Species interact widely both in antagonistic (predation, parasitism, competition) and in synergistic ways (cooperation, symbiosis). These interactions define the biotic environment of each species, exert selection and promote evolution and co-evolution through time. Host-parasite interactions appear to have the greatest importance in this regard, as most of the species on Earth are parasites (Windsor 1998). Parasites (or pathogens) depend on their host for growth and reproduction and are at the same time a source of selection on their host for reduced damage and/or clearance. This interplay is far from being static, as both parts change – *evolve* and sometimes *co-evolve* – through time (see the next section for more details). The effect of parasites is manifold, as they have been claimed to be responsible for the evolution of sex (Hamilton et al. 1990; Morran et al. 2011), the evolution of male ornaments and female choice (Hamilton and Zuk 1982), populations diversification (Brockhurst et al. 2004), the maintenance of genetic variation (Berenos et al. 2011) especially at MHC genes (Eizaguirre et al. 2012a), the acceleration of molecular evolution (Paterson et al. 2010) and the increase of host recombination frequency (Fischer and Schmid-Hempel 2005).

Unfortunately parasites are not only a challenging topic in evolutionary biology but remain one of the main threats for human health, causing every year around 13 millions deaths (WHO 1999). It has been recently advocated that an evolutionary perspective can help designing more effective vaccines (Gandon et al. 2001) and antibiotics with a lower potential for resistance development (Andersson 2006), as well as choosing the appropriate clinical setting and antibiotic treatment to prevent for example the spread of resistance strains in hospitals (Perron et al. 2007).

While some parasites rely only on one host to complete their life cycle, others infect in succession one or more intermediate hosts, where they grow and develop, and a final host where they usually reproduce sexually (Schmid-Hempel 2011). As parasites rely on infection to survive, hosts defend themselves to minimize fitness loss. The first defence strategy is avoidance. For example, the bumblebee *Bombus terrestris*, is able to recognize and avoid flowers contaminated with the

pathogenic bacteria *Crithidia bombi* and *Escherichia coli* (Fouks and Lattorff 2011) and the ladybird *Cocinella settempuncatata* avoids leaf surfaces and soil inoculated with spores of the parasitic fungus *Beauveria bassiana* as well as sporulating cadavers (Ormond et al. 2011).

When infection occurs, the host can defend itself by mounting an immune response. The first line of defence is the so-called innate immunity (Ferrandon et al. 2007; Janeway 2011). In innate immunity host receptors recognize conserved microbial patterns, triggering a set of non-specific defence mechanisms. Innate immunity is present both in plants (Jones and Dangl 2006) and in animals, where it is considered an old evolutionary feature highly conserved at the molecular level (Kimbrell and Beutler 2001).

In vertebrates an adaptive (or acquired) immunity is present besides the innate one. The main novelty of adaptive immunity is the ability to build up a specific response against an infectious agent. This is achieved by selection and clonal expansion of defensive cells (B and T lymphocytes in jawed vertebrates) that recognize a specific motif, or antigen, of the infecting pathogen. An adaptive response needs more time to unfold compared to an innate one, but, as memory cells are formed, a second exposure to the same pathogen leads to a faster, stronger and more effective response (Janeway 2011; Schmid-Hempel 2011).

For a long time adaptive immunity was regarded as exclusive to vertebrates, but the last decade saw the discovery of adaptive defence in bacteria (Koonin and Makarova 2009) and increasing evidences for immune memory in invertebrates (Kurtz and Franz 2003; Luna et al. 2012; Luna and Ton 2012) and in plants (Conrath et al. 2006). This kind of immune memory, also called *priming*, shows varying degrees of specificity: from non-specific, so that a challenge with one pathogen or even a physical stress, can protect against multiple pathogens, to species – or strains – specific (Rowley and Powell 2007). The protective effect lasts in general for part or the whole life of a host, but can in some cases extend to the next generation; this case being referred to as *trans-generational immune priming* (Rowley and Powell 2007; Sadd and Schmid-Hempel 2007; Sadd and Schmid-Hempel 2009; Roth et al. 2010; Luna et al. 2012; Slaughter et al. 2012).

The mechanisms behind priming are not clear in invertebrates, although it has been shown that

immune memory in *Drosophila* is dependent on phagocytes (Pham et al. 2007) and trans-generational protection is mediated through factors inside the egg in bumblebees (Sadd and Schmid-Hempel 2007). In plants, epigenetic changes and protein kinases accumulation seem to be related with immune memory (Conrath 2011) and epigenetic change appears mainly responsible for trans-generational immune priming (Luna and Ton 2012). More research is needed in order to better characterise the generality of immune priming and its molecular mechanisms.

Social insect colonies have evolved collective defences against pathogens. Among them are allogrooming, the use of antimicrobial compounds to disinfect the nest and social fever in honeybees (Cremer et al. 2007). The fungal parasite *Metarhizium anisopliae*, for example, is unable to spread through a colony of termites (*Coptotermes formosanus)* because the nest mates cannibalise or bury infected or dead individuals in order to kill fungal spores (Chouvenc and Su 2012).

On the other hand, parasites are under selective pressure to avoid host defences. They can elude them by modifying their antigenic surface, e.g. by expressing successively different surface variants, or, in long-lived infections like HIV, by mutation of epitopes. Another possibility is to actively subvert host immune response by secreting compounds that block or interfere with some of its steps (Schmid-Hempel 2011). A virulent strain of the bacteria *Pseudomonas aeruginosa* for example is able to suppress *Drosophila* defence by limiting antimicrobial peptides gene expression (Apidianakis et al. 2005).

Some parasites modify host behaviour in order to increase their transmission and/or survival. For example the ant *Camponotus leonardi* infected by the fungus *Ophiocordyceps unilateralis* descends from its canopy nest in the Thai rainforest to reach a leaf in the low vegetation at around 25 cm from the soil. At that spot, where the conditions for fungal development are optimal, the ant bites the leaf veins with its mandibles and dies attached to the leaf. The parasite then develops a spore dispersal structure from the head of the dead ant (Andersen et al. 2009).

Parasites transmitted via predation can increase transmission probability by acting on the central nervous system of the intermediate host to reduce predation fear and escaping behaviour (Kaushik

et al. 2012). An extreme case is the amphipod *Gammarus pulex* infected by the acanthocephalan parasite *Pomphorhynchus laevis*. Hosts bearing the non-infective stage of the parasite hide more than non-infected animals and are less likely to get caught. When the parasite enters the infective stage, the behaviour of the host changes: infected individuals hide less than uninfected ones and are more hunted. In this way the parasite modifies host behaviour to maximize its transmission to the final fish host species at the correct developmental stage (Dianne et al. 2011).

As we have seen host and parasite interplay ranges from the behavioural to the molecular level with various degrees of specificity. Its far reaching consequences and the overwhelming prevalence of the parasitic lifestyle make host-parasite interaction one of the most interesting topics in modern evolutionary biology.

2.2 ECOLOGY AND EVOLUTION OF HOST-PARASITE INTERACTIONS

We will explore here the different evolutionary and ecological forces influencing the outcome of given host-parasite interactions, i.e. the success of an infection and the extent of fitness loss for the host and fitness gain for the parasite. The ability to resist a parasite increases host fitness, because it lowers the probability of infection, and in case of successful infection reduces damages. However, in absence of infection other traits, such as survival, fecundity, competitiveness and the ability to resist different parasites are more important. As each trait is costly, its actual level will be the result of a trade-off between conflicting needs. Zuk and Stoehr identify three possible costs of defence (Zuk and Stoehr 2002).

1) A “resource cost” is due to limited physiological and metabolic resources and can be paid upon evolution and maintenance of immune defence. This is the case in *Drosophila melanogaster* selected for increased survival to the endoparasitoid *Asobara tabida*. Selected flies show reduced larval competitive ability in absence of infection (Kraaijeveld and Godfray 1997). Immune system deployment can also incur a resource cost, as shown in bumblebees (*Bombus terrestris*) injected with LPS, a bacterial outer membrane molecule with antigenic activity. Injected individuals exhibited a reduced survival rate in food limited conditions (Moret and Schmid-Hempel 2000).

2) An “option cost” occurs if a change in a receptor molecule increases the ability to recognize one pathogen but decreases the ability to recognize a different one, as has been shown for MHC haplotypes (Eizaguirre et al. 2012b).

3) An “immunopathology cost” is the result of an unspecific immune response that either is getting out of control (such as auto-immune diseases) or that is diverted by the parasite causing damage to the host (Sorci and Faivre 2009; Belloni et al. 2010).

A trade-off is present between two different components of defence: tolerance and resistance. Resistance is the ability to actively reduce the parasite burden, while tolerance is the ability to limit the damage caused by a given pathogen load (Raberg et al. 2009). For example a single mutation in a gene encoding a protease in *Drosophila melanogaster* affects resistance and tolerance to infection in a microbe-dependent way (Ayres and Schneider 2008). Resistance and tolerance have quite different effects on parasite evolution. While resistance imposes a selection on the parasite to overcame host defence, and can lead to antagonistic co-evolution between the host and the parasite, tolerance is not predicted to exercise any selection on the parasite (Raberg et al. 2009). The ability to resist a pathogen is always dependent on a trade-off against other physiological and defence components, and thus cannot be studied independently from host life-history and ecology (Zuk and Stoehr 2002; Schulenburg et al. 2009). Also parasites incur in trade-offs. For example there is some evidence that parasites with a broad host range have lower infection intensity and prevalence on each host compared with more specialized parasites (Poulin 2002; Garamszegi 2006). The reason for this is the negative genetic correlation between fitness on different hosts. Evidences for the opposite trend are also observed though, with generalist parasites showing higher prevalences (Cleaveland et al. 2001; Woolhouse et al. 2001). Parasites could also pay an evolutionary cost for being generalist, as they adapt more slowly to each host compared to specialists (Whitlock 1996; Kawecki 1998).

Host and parasites species and populations are not homogeneous entities, but show at least some degree of genetic variability. Different host or parasite genotypes can address the same trade-off in different ways, following distinct evolutionary strategies (van Baalen 1998). The outcome of host parasite interactions depends on the genotypes of the two partners, the so-called Genotype x Genotype interaction (G x G). For example when the crustacean *Daphnia magna* is exposed to its

bacterial microparasite *Pasteuria ramosa*, the percentage of infected individuals depends on the combination of host and parasite genotypes (Carius et al. 2001). The same is true for the expression level of immunity genes in *Bombus terrestris* infected with the gut trypanosome *Crithidia bombi* (Riddell et al. 2009). The molecular basis for such specificity will be explained below in this page.

Another important factor that determines the outcome of host-parasite interactions is the environment. For example *D. melanogaster* survival rate after infection with the bacteria *Pseudomonas aeruginosa* depends on the time of the day when the infection took place (Yang et al. 2008). Temperature fluctuations influence also the development time of the parasite *Plasmodium chaubadi*, the causing agent of rodent malaria, in the vector host *Anopheles stephensi* (Paaijmans et al. 2010).

The effect of the environment can be complex and modify the outcome of infection for given combination of host and parasite genotypes, so that the same environmental change increases parasite fitness in some cases and host fitness in others (Wolinska and King 2009). This is the so-called Genotype x Genotype x Environment interaction (G x G x E). Caution therefore is needed in interpreting the results of infection experiments performed in the lab, as they depend on the genotypes and the environmental conditions used.

The interaction between host and parasite genotypes and the selection pressure they exert on one another set up the scene for antagonistic co-evolution: host and parasite continuously evolve in response to each other. It is thus possible to study co-evolution by observing changes in phenotype and genotype frequencies in time in both species.

Frequency-dependent selection (FDS) is thought to be the major force driving host-parasite co-evolution (Haldane 1954; Clarke 1964). Under FDS, rare host alleles are favoured because the corresponding parasite alleles, able to infect these host types, are also at low frequencies. As the host alleles become more common, the corresponding parasite alleles are selected for and thus increase in frequency, with a slight time lag. This type of selection is also called negative frequency-dependent selection (nFDS) because alleles at high frequencies are selected against (Frank 1992), or negative indirect frequency-dependent selection (ndFDS), (Tellier and Brown

2007).

Parasites are usually considered to have a shorter generation time and therefore to be ahead in the co-evolutionary race, although this is not always true. For example parasitoids have to kill their host in order to reproduce (Godfray 1994), thus generation times are in this case synchronised.

Host-parasite co-evolution generates two types of evolutionary dynamics at loci under selection: recurrent fixation of new genetic variants or evolutionary cycles with fluctuation in allele frequencies (Stahl et al. 1999; Bergelson et al. 2001; Holub 2001). These two extreme scenarios based on FDS models involve different outcomes, namely positive directional and balancing selection, and are expected to leave different footprints at the genetic and genomic levels (Woolhouse et al. 2002; Brown and Tellier 2011). Besides studying the signatures of co-evolution at the genomic level (see below for further details), empirical studies have mainly focused on monitoring the changes in phenotype over time in both natural and experimental populations (Jokela et al. 2009; Eizaguirre et al. 2012b).

The advantage of exhibiting rare alleles and of generating more diverse offspring, that would not be infected by common parasites, increases when parasite prevalence is high (Koskella and Lively 2009). The *Red Queen Hypothesis* (Van Valen 1973) postulates that high infection rates should select for sexual reproduction and meiotic recombination in order to increase offspring variability. These predictions have been verified in different systems such as the snail *Potamopygrus antipodarum* (Jokela et al. 2009), and the nematode *Caenorhabditis elegans* (Morran et al. 2011).

Antagonistic co-evolution is expected to be stronger between closely associated host and parasite populations. Hosts and parasites interact and co-evolve locally and one naively expects that adaptation should be evident at a local scale. Indeed local adaptation has been demonstrated in several systems, although it is not always easy to detect or may not be present at all, because it can occur over several geographic scales and is influenced by host and parasite migration rates and parasite specificity and virulence (Kaltz and Shykoff 1998; Gandon 2002; Kawecki and Ebert 2004; Vos et al. 2009).

Environmental (biotic and abiotic) heterogeneity adds a new level of complexity (Lopez Pascua et al. 2012). According to the “geographic mosaic theory of co-evolution” (Thompson 2005) and to some experimental evidence, host and parasite interact and co-evolve more intensively at some locations “hot-spots” than at others “cold-spots” (Thompson 1999; Gomulkiewicz et al. 2000; King et al. 2009); migration from a hot-spot to a cold-spot intensifies co-evolution at the cold-spot, while the opposite is true in case of migration from a cold spot to a hot-spot (Vogwill et al. 2009). This again indicates the importance of a holistic view in studying host-parasite interactions and of understanding the environmental and geographic dimension in which they unfold.

Furthermore a general effect of latitude has been claimed to influence host-parasite interactions. Due to the higher species richness at low latitudes, parasite prevalence and host investment in immune defence should be higher in tropical compared to temperate populations. Evidence for this hypothesis exists, although some exceptions have also been reported (Schemske 2009).

In the last decade the importance of symbionts in host-parasite interaction has been increasingly recognized (Gross et al. 2009). More specifically some facultative maternally transmitted endosymbionts of insects have been shown to help protecting their host from pathogens (Scarborough et al. 2005; Hedges et al. 2008; Jaenike and Brekke 2011). Host acquisition of endosymbionts can indeed deeply influence the evolution of both host and parasite (Jaenike et al. 2010; Dion et al. 2011; Jiggins and Hurst 2011) and a parallelism between endosymbionts acquisition and the occurrence of a beneficial nuclear mutation has been proposed (Jaenike 2012). A defensive endosymbiont can be advantageous to the host in presence of a parasite but detrimental if the parasite is absent, representing therefore another example of trade-off. This has been shown for the pea aphid *Acyrthosiphon pisum* and its natural endosymbionts *Hamiltonella defensa* (Oliver et al. 2008). In this case the protective phenotype is actually dependent on the presence of a bacteriophage in the symbiont genome (Oliver et al. 2009). Therefore symbionts, and mobile genetic elements of symbionts, add an extra level of complexity to host-parasite interactions, especially in combination with the “geographic mosaic theory of co-evolution”.

2.3 GENETICS AND GENOMICS OF HOST-PARASITE INTERACTIONS IN *DROSOPHILA MELANOGASTER*

Genes that are important in host-parasite interactions are expected to be under natural selection. It is possible to determine the selection pressure acting on them by looking at the molecular signature it leaves (Nielsen et al. 2005; Pavlidis et al. 2008). One classical example is the major histocompatibility complex (MHC) genes in vertebrates. The extremely high level of intra- and trans-species polymorphisms at these genes is maintained by balancing selection (Hughes and Yeager 1998; Bernatchez and Landry 2003; Spurgin and Richardson 2010).

Several studies have assessed the selection forces acting on defence genes in human and vertebrates, mainly on MHC genes and innate immunity receptors (Edwards et al. 1997; Barreiro et al. 2009; Barreiro and Quintana-Murci 2010) and in plants (Stahl et al. 1999; Holub 2001; Tian et al. 2002; Horger et al. 2012). On the other hand, only a few have focused on infectivity genes of parasites (Raffaele et al. 2010; Cadar et al. 2012).

While the majority of these studies looked for evidence of selection in the coding regions of genes, the importance of regulatory evolution has being increasingly recognized in the last decade (Wittkopp et al. 2004; Gibson and Weir 2005; Wray 2007; Fay and Wittkopp 2008).

Changes in the regulatory region of genes can modify their expression pattern and prompt evolutionary adaptation (Lopez-Maury et al. 2008; Fraser et al. 2010). For example *Drosophila melanogaster* experimentally evolved for increased survival to the bacteria *Pseudomonas aeruginosa* shows higher infection-induced expression of immunity genes than non-evolved lines (Ye and McGraw 2011).

Transcriptional response to infection can be a major determinant of the ability to resist a pathogen (Polesani et al. 2010; Moscou et al. 2011). For example a *Drosophila melanogaster* line resistant to the bacteria *Listeria monocytogenes* shows a stronger activation of immune genes early after infection compared to a susceptible line (Okado et al. 2009).

On the other hand, transcriptional response is a target for parasite interference. For example only a virulent strain of the bacteria *Pseudomonas aeruginosa*, suppresses antimicrobial peptide gene expression in *Drosophila melanogaster* (Apidianakis et al. 2005). It appears that more studies are needed to assess the selective forces acting on the regulatory region of defence and infectivity genes.

Drosophila melanogaster is the model organism to study immunity in invertebrates. For this reason I will briefly introduce *Drosophila* immune pathways before focusing on what is known at the genetic and genomic levels. The immune defence in *Drosophila melanogaster* is based on two main components: the humoral, or systemic, immunity and the cellular immunity (Lemaitre and Hoffmann 2007) (figure 1).

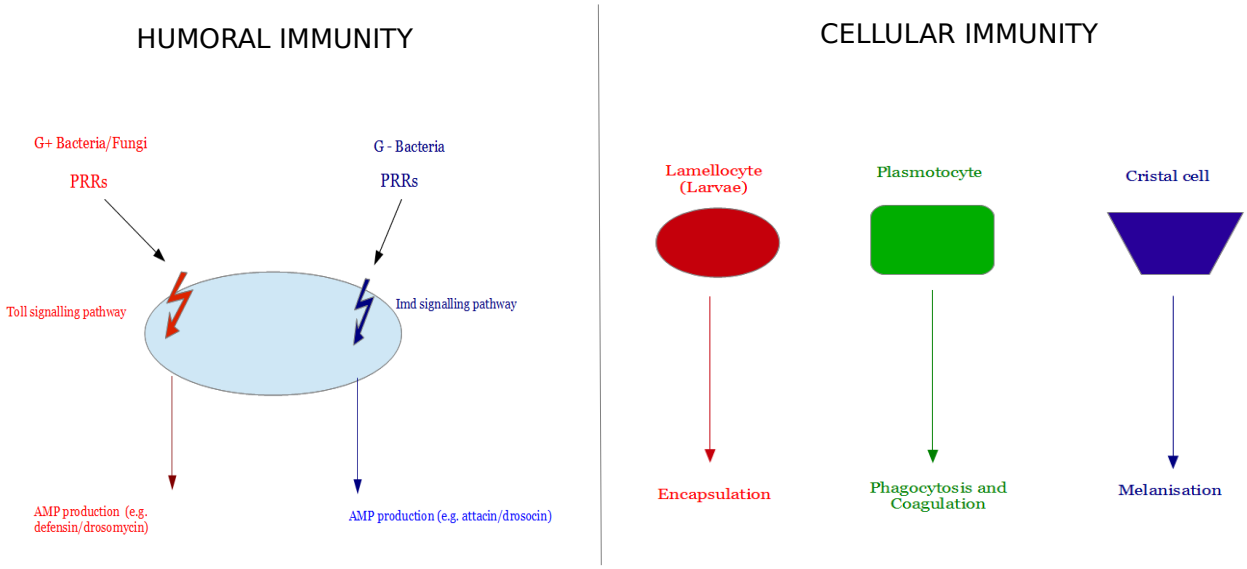

Figure 1 Schematic representation of *Drosophila melanogaster* immunity (see main text)

In humoral immunity, antimicrobial peptides (AMPs) are produced by a specialized tissue, the fat body, and subsequently secreted into the body cavity. This response is triggered by the presence of pathogens in the body cavity and shows some degree of genus specificity, as different genera of pathogens trigger the expression of different AMPs.

Microbial detection relies on direct contact between a host pattern recognition receptor protein (PPR) and a pathogen molecule. Bacteria recognition is achieved through peptidoglycan recognition proteins (PGRPs) that sense peptidoglycan (PGN), an essential component of bacteria cell wall.

PGRP-LC isoforms and PGRP-LE are specialized in recognizing Gram-negative PGN, while PGRP-SA, PGRP-SD and GNBP1 in sensing Gram-positive PGN. Fungal recognition is thought to depend on GNBP3 that binds beta(1,3)-glucan, a component of fungal cell wall, and on Persephone that is activated by fungal proteases.

Fungi and Gram-positive bacteria recognition triggers a serine protease cascade that activates the Toll signalling pathway (Lemaitre and Hoffmann 2007). This results in the expression of some AMPs, like defensin, active against Gram-positive bacteria, and drosomycin and metchnikowin, active against fungi. On the other hand Gram-negative bacteria recognition triggers the Imd signalling pathway with the consequent expression of AMPs like diptericin, attacin and drosocin. Other signalling cascades that activate immune genes in *Drosophila* fat body have been recognized, as the JAK/STAT and the JNK pathways, although their precise contribution to immune defence is not clear.

The cellular response relies on blood cells (hemocytes) present in the body cavity. Plasmatocytes represent 90-95% of all hemocytes in *Drosophila* and are responsible for the phagocytosis of microorganisms and apoptotic cells. Several receptors have been shown to be involved in phagocytosis, the most studied are Eater and Dscam. A group of six thioester-containing proteins (TEPs) has an important role in this defence mechanism. TEPs are secreted proteins and three of them are up-regulated upon infection. There is evidence that they bind to pathogens and promote phagocytosis in a similar fashion as the complement proteins in vertebrates (Blandin et al. 2004; Stroschein-Stevenson et al. 2006).

Plasmotocyte also promote coagulation (or clotting) consisting in the production of fibers at injury site that trap bacteria and promote their killing (Lemaitre and Hoffmann 2007). *Hemolectin*, a plasmotocyte specific gene, codes for the main component of the clotting fibres and is required for coagulation in *D. melanogaster*. Other genes required for proper coagulation response are *fondue*, that codes for a protein involved in the cross-linked of the clotting fibres, and *transglitaminase* that is involved in the first stage of clotting formation (Lindgren et al. 2008).

Lamellocytes, another category of hemocytes, are specialized in encapsulating and killing parasitoid

wasp eggs in *Drosophila* larvae, possibly by local cytotoxic products. While the molecular mechanism of encapsulation are virtually unknown, recent evidence suggests that N-glycosilation of lamellocyte membrane components has a key role in the encapsulation response (Mortimer et al. 2012).

Crystal cells, the last group of hemocytes, are responsible for melanization, a defence mechanism consisting in melanin production at cuticular injury sites and on the surface of parasites invading the body cavity. Many genes have been implicated in the melanization cascade, like *MP1*, with antibacterial and antifungal activity and *MP2*, shown to be specific against fungi.

A third and more recently discovered component of *Drosophila* innate immunity is antiviral RNA interference (Wang et al. 2006; Obbard et al. 2009a; Saleh et al. 2009). In this pathway double-strand RNA from viruses is recognized by Dicer that cuts it into short fragments of 21-24 nucleotides. These fragments activate an Argonaute-containing complex that slices viral RNA with complementary sequence, stopping the virus infection.

Many studies have looked for signature of selection at *Drosophila* immunity genes (Clark and Wang 1997; Jiggins and Hurst 2003; Schlenke and Begun 2005; Jiggins and Kim 2006; Jiggins and Kim 2007; Sackton et al. 2007; Lazzaro 2008; Obbard et al. 2011). A first remarkable point is that immunity genes have a significantly higher rate of adaptive substitution compared to control genes in *Drosophila* species (Sackton et al. 2007; Obbard et al. 2009a). This means that parasites indeed exercise a strong selection pressure on *Drosophila*.

Going down to single defence genes categories, Obbard and colleagues found higher rates of adaptive substitution for genes in the antiviral RNA interference pathway and in the Imd signalling pathway (Obbard et al. 2009b) (figure 2). Evidence of positive selection has also been reported for the TEP proteins, especially TEP1 (Jiggins and Kim 2006; Sackton et al. 2007). However, AMP and recognition genes of humoral immunity do not show evidence of positive selection. The most likely explanation is that both are constrained as they bind to highly conserved targets.

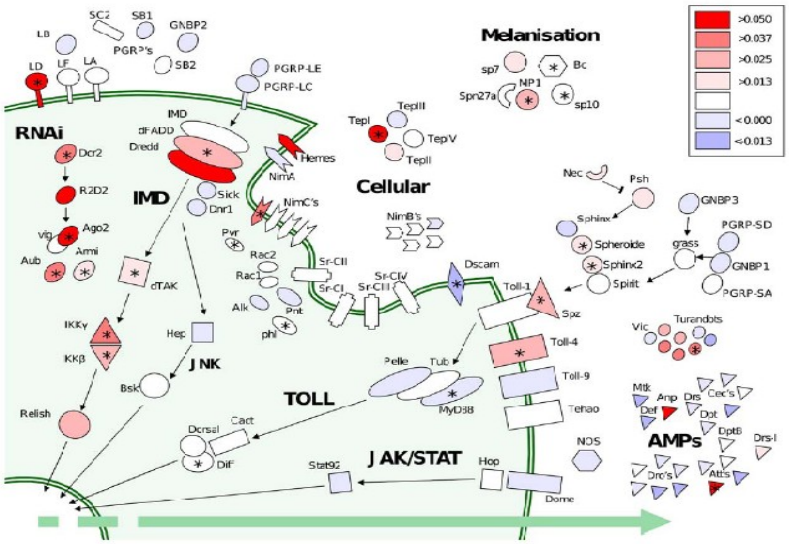

Figure 2 Evidence of positive selection at *Drosophila melanogaster* immunity genes. Genes are coloured according to the estimated rate of adaptive substitution as indicated in the legend. Reprinted from (Obbard et al. 2009b).

Sackton and colleagues reported high levels of gene duplication and turnover for AMP genes in *Drosophila* species (Sackton et al. 2007). On the other hand, signalling genes show strong copy number and function conservation. This could make them a more suitable target for pathogens interference and explain the higher rate of adaptive substitution they experienced (Lazzaro 2008).

Evidence of recent fixation of positively selected variants has been observed at some *Drosophila melanogaster* immune genes. For example, *Argonaute-2*, a member of the antiviral RNAi pathway, has experienced recurrent spreads of selected alleles in three *Drosophila* species (Obbard et al. 2011). Similarly, at the *CHKov1* locus of *Drosophila melanogaster* an allele conferring resistance to the sigma virus (Rhabdoviridae) has recently spread to become the common form in natural populations (Magwire et al. 2011).

Interestingly, no evidence of balancing selection has been reported to date for *Drosophila melanogaster* immunity genes. This is a clear difference with vertebrates immune system receptors of adaptive immunity like MHC (see above) or plant resistance genes (Barreiro et al. 2009) that have been shown to evolve under balancing selection. Indeed it has been proposed that the unspecific nature of *Drosophila* immunity could explain the absence of genes evolving under balancing selection (Schlenke and Begun 2003). The observation that genes of the vertebrates innate immunity evolve under positive or negative, but not balancing, selection corroborates this

hypothesis (Barreiro et al. 2009).

All the studies cited above focus on coding sequence evolution and look for past selection that acted at the species level. Therefore research on regulatory evolution and recent selection acting at the population level is required.

Several studies have assessed *Drosophila* transcriptional response to different pathogens (Lemaitre et al. 1997; De Gregorio et al. 2001; Roxstrom-Lindquist et al. 2004; Wertheim et al. 2005; Wertheim et al. 2011). For example De Gregorio and colleagues (De Gregorio et al. 2001) surveyed transcriptional response to *Beauveria bassiana* in *Drosophila melanogaster* at four time points, from 12 to 96 hours after infection. Among the genes that displayed a significant change in gene expression 32 were already known as immune genes, while 368 had previously not been associated with immunity. Therefore transcriptome analysis can help us to identify new candidate immune genes and get a better understanding of how immune response unfolds.

Roxström-Lindquist and collaborators assessed gene expression in *D. melanogaster* 24 hours after infection by *B. bassiana*, the protozoan parasite *Octosporea muscaedomesticae*, the Gram-negative bacterium *Serratia marcensens* and *Drosophila* C virus (Edwards et al. 1997; Roxstrom-Lindquist et al. 2004). They found a high degree of microbe specificity, with the fungal infection generating the strongest response with 298 genes induced.

Wertheim and co-workers investigated transcriptional response following parasitoid wasp attack in *D. melanogaster* larvae (Wertheim et al. 2005). Most genes found in this study differ from the ones induced by antimicrobial immune response and were not previously reported as immune genes. Their results improved our knowledge of the encapsulation defence mechanism.

A common characteristic of the studies cited above is that in all of them only one inbred line of *D. melanogaster* was used. A problem with this approach is that the outcome of host-parasite interaction and host transcriptional response are dependent on the interacting genotypes (G x G, see previous section). For example different genes are found to be induced after *B. bassiana* infection in De Gregorio et al. (2001) and Roxstrom-Lindquist et al. (2004). A likely explanation is that they

used two different *D. melanogaster* strains, although discrepancies in the infection procedure and in data analysis could also play a role.

Another drawback of using inbred lines is that inbreeding reduces fitness and resistance to parasite in *D. melanogaster* (Luong et al. 2007). Therefore the use of out-cross populations would be closer to the natural situation. In addition, because differences in the set-up can make two experiments difficult to compare, having multiple populations in the same experiment allows to assess between populations variability. This could also help to identify genes facing contrasting selective pressure for gene regulation among populations. The present work tries to address some of these issues.

In comparison to what we know on the genomics of *Drosophila* immune response, our knowledge of what happens at the protein level is still in its infancy. As infection affects gene expression, it also changes proteins concentration (Engstrom et al. 2004; Levy et al. 2004). More studies and new technologies are needed to achieve a better comprehension of immune response at the proteomic level and of how this correlates with changes in gene expression.

2.4 *BEAUVERIA BASSIANA*: A MODEL FUNGAL ENTOMOPATHOGEN

Fungal parasites of insects (fungal entomoptahogens) gain access to the host via cuticle penetration (Vega and Kaya 2012). As a first step, spores need to adhere to host surface through mucilage and enzymes production. Penetration is then achieved both by enzymes secretion and the development of specialized structures called appresoria that exert mechanical pressure. When the fungus reaches the body cavity of the host it produces hydrolytic enzymes to assimilate nutrients and toxins with immunosuppressive activity (Gillespie et al. 2000). If the host fails to clear the infection the fungus eventually kills the insect and transmission is achieved by sporulation from the cadaver (figure 3).

Figure 3 Grasshoppers killed by the entomopathogenic fungus *Beauveria bassiana*. The sporulating fungus has a white colour (Wikimedia Commons).

Beauveria bassiana (Clavipitaceae family, Hypocreales) is an entomopathogenic fungus with a global geographic distribution (Rehner and Buckley 2005; Devi et al. 2006). Its extremely broad host range (> 700 insect hosts) makes it a natural candidate as pest control (Samish et al. 2004; Akbar et al. 2005; Dara 2008). Together with *Metarhizium anisopliae*, another related entomopathogenic fungus, it has also been proposed as bio-control against malaria mosquitoes (Bukhari et al. 2011; Fang et al. 2011).

To further improve *B. bassiana* insecticidal activity and bio-control potential, several studies have assessed the effect of growth medium and spores suspension on fungal virulence, viability and thermotolerance (Daoust et al. 1983; Ying and Feng 2004; Ying and Feng 2006; Safavi et al. 2007). Other studies have focused on isolating secreted compounds with toxic activity that could be used as active principles in bio-insecticides (Vey et al. 1993; Quesada-Moraga 2003; Ortiz-Urquiza et al. 2010). A consequent research effort has been done to isolate and characterise toxic compounds produced by *B. bassiana* and other entomopathogenic fungi (Fuguet et al. 2004; Kaur and Padmaja 2009; Xu et al. 2009; Valencia et al. 2011). Destruxins play a specific role in this context, as they are able to suppress AMP gene expression (Pal et al. 2007).

In spite of their prevalent parasitic way of life, *B. bassiana* and other entomopathogenic fungi could live in association with plants and grow in plant tissues as endophytes (Ownley et al. 2008; Vega 2008). Interestingly this association results in plant feeding deterrence, as the fungus inhibits phytopathogenic fungi mycelial growth and kills herbivorous insects.

The phylogeny of the genus *Beauveria* has been deeply revised with the appearance of molecular markers as taxonomical informative morphological traits are scarce (St Leger 1992; Rehner and Buckley 2005; Devi et al. 2006; Rehner et al. 2011). *B. bassiana* represents a species-complex and it is not clear if genetic structure is associated with geographic distance, host range or neither of them (Maurer 1997; Gaitan 2002; Wang et al. 2003; Rehner et al. 2006; Fernandes et al. 2009; Meyling et al. 2009). At the same time there is contrasting evidence on the association between thermal growth preference and climatic origin of isolates (Fargues 1997; Bidochka et al. 2002). Overall, studies are so far inconclusive on whether local adaptation to some host or environment occurs in *B. bassiana*. More research is therefore needed to better characterize *B. bassiana* phylogeny and factors determining genetic structure in this species.

The genome of one *B. bassiana* strain has been recently sequenced, together with its transcriptome in different environmental conditions (Xiao et al. 2012). This study has shed new light on the physiology and evolution of *B. bassiana* by comparing its genome with the ones of the fungal insect pathogens *Metarhizium robertsii*, *M. acridum* and *Cordyceps militaris*.

B. bassiana contains more bacterial-like toxins than other fungal entomoptahogens, suggesting a possible oral toxicity. The four species have more proteases than plant pathogenic fungi. This expansion is dramatic in *B. bassiana* and *M. robertsii* that have a broad host range and less marked in *M. acridum* and *C. militaris* that are more specialized pathogens. Therefore the increased number of proteases may reflect an adaptation to infect insects and is possibly influenced by host range. Another common feature of insect pathogenic fungi is the abundance of chitinases compared with plants pathogens. This is likely an adaptation to the amount of chitin in the insect cuticle.

The transcriptome analysis identifies transcription factors, G protein coupled receptors and kinases involved in signal transduction that are differently expressed on root exudates, insect cuticle and insect body cavity. Furthermore genes involved in metabolism show an induction pattern that is environment specific.

The genome and transcriptome analysis therefore suggests that *B. bassiana* is well adapted to its prevalent parasitic way of life. A deeper understanding of the genes and molecular mechanisms

involved in infection is required also in order to better characterise the targets for parasite interference inside the host.

Many studies have used *B. bassiana* as a model fungal pathogen to gain insight in different aspects of insect immunity (Lemaitre et al. 1997; De Gregorio et al. 2001; Levy et al. 2004; Roxstrom-Lindquist et al. 2004; Tinsley et al. 2006; Pham et al. 2007; Kraaijeveld and Godfray 2008; Le Bourg et al. 2009; Reber and Chapuisat 2012). Tinsley and co-workers found variation in survival to *B. bassiana* infection both within and between *D. melanogaster* populations (Tinsley et al. 2006) and reported higher survival in tropical host populations compared to temperate ones. This last finding supports the theory of higher immune investment at tropics (see above). Given *B. bassiana* broad host range, it seems unlikely that it is engaging a strict co-evolutionary arms-race with a particular host species or population (Kawecki 1998). Therefore *B. bassiana* use in infection experiments gives us the possibility to study how the host responds to a generalist fungal pathogen and to assess if variability among host populations is present, possibly due to different life history strategies.

2.5 OBJECTIVES OF THIS THESIS

The general aim of this work is to determine the extent of variation in survival and transcriptional response to a generalist pathogen among host populations. We further investigate the effect of endosymbionts and trans-generational immune priming on host survival. We use the *Drosophila melanogaster – Beauveria bassiana* model system to address these questions.

In the first part of the present study we test if susceptibility to *B. bassiana* varies among *D. melanogaster* populations. The presence of variability can provide information on how host populations adjust their immune investment and defence strategy depending on the environment in which they live and/or evolved. Tinsley and co-workers (Tinsley et al. 2006) found lower susceptibility to *B. bassiana* in tropical *D. melanogaster* populations. We assessed two tropical and two temperate host populations in order to test the generality of such geographical trend. To avoid the immune depressive effect of inbreeding we created out-cross populations starting from lab inbred lines. We also investigated if parasite genotype can affect the outcome of infection, possibly

due to G x G interactions. To this purpose we used in separate experiments two distinct *B. bassiana* strains: strain 547 isolated from Coleptera (Malaysia, 1986) and strain 1630 isolated from Diptera (France, 1984).

In the second part of this work we assess *D. melanogaster* transcriptional response to *B. bassiana* and its variability among host populations. At least two studies have already surveyed gene expression change in a *D. melanogaster* inbred line upon *B. bassiana* infection (De Gregorio et al. 2001; Roxstrom-Lindquist et al. 2004). However, inbred lines could differ in the response they mount from natural populations that are genetic variable. We used the same *D. melanogaster* out-cross populations as for the survival experiment and infected them with *B. bassiana* strain 1630. As we tested multiple populations in the same experiment, we were able to compare their transcriptional response directly.

Early response to infection has been identified as an important predictor of survival ability in *D. melanogaster* (Okado et al. 2009). Evidences indicate that *D. melanogaster* changes in gene expression occur already few hours after *B. bassiana* infection (Lemaitre et al. 1997; De Gregorio et al. 2001). On this basis we choose two time points for our analysis: 8 and 24 hours post infection. We performed two independent experiments. In the first one we used a full genome microarray approach at 8 hours post infection, while in the second RNA sequencing (RNAseq) both at 8 and 24 hours following infection. Combining these data with the ones from the survival analysis allows us to have a better picture of the defence mechanisms that different host populations unfold and of their efficacy.

Finally, in the last part of our study we look for the effect of endosymbionts and trans-generational immune priming on *D. melanogaster* susceptibility to *B. bassiana*. In the first sub-section we focused on the role of the endosymbiont *Wolbachia pipientis*. As we have seen above an endosymbiont can protect its host from parasites. *W. pipientis* is a natural intracellular symbiont of many arthropods (Werren et al. 2008) and nematodes (Taylor et al. 2012) and exhibits varying prevalence in *D. melanogaster* populations (Riegler et al. 2005; Ilinskii and Zakharov 2007; Verspoor and Haddrill 2011). *W. pipientis* increases survival of *D. melanogaster* against RNA viruses (Hedges et al. 2008; Teixeira et al. 2008) and a previous study has hinted a potential protective effect against *B. bassiana* in one *D. melanogaster* inbred line (Panteleev et al. 2007).

However, the outcome of such triple complex interactions may depend on the genotype of the host, the parasite and the endosymbiont.

We cured one European and one African *D. melanogaster* inbred lines from *W. pipientis* by tetracycline treatment and exposed them together with the original lines to *B. bassiana* 1630 strain. We controlled for the effect of the antibiotic by treating a *Wolbachia*-free African line with tetracycline. Flies from this line, both tetracycline treated and not, were infected together with the other two lines described above. We repeated the infection experiment twice in order to gain a clearer picture.

In the second sub-section we wanted to verify if trans-generational immune priming plays a role in the study system. As we have seen above there is increasing evidence of immune memory in invertebrates. This could be limited to one generation or extend to the offspring. In this last case it is called "trans-generational immune priming" (see above). There is evidence that *D. melanogaster* injected with heat-killed *B. bassiana* spores survives longer when later exposed to the living parasite (Pham et al. 2007). However, no study to our knowledge has tested if immune memory to *B. bassiana* extends to the next generation in *D. melanogaster.*

No evidence of trans-generational immune priming was found in *D. melanogaster* infected with the bacteria *Lactococcus lactis* and *Pseudomonas aeruginosa* (Riegler et al. 2005). However, a possible drawback of this study is that the flies used for the experiment were not virgin and this could leave few place for priming if it happens at the eggs level (Sadd and Schmid-Hempel 2007; Zanchi et al. 2012).

We used the fungal strain 1630 and the European and the American out-cross populations described above in two separate experiments. Male and virgin female flies were exposed separately for 2-3 days to the parasite or to a control treatment. After exposure male and female flies were pooled and allowed to reproduce. The offspring were collected and infected with the same fungal strain as the parents. We tested if flies whose parents were exposed to the parasite exhibit a lower susceptibility than flies whose parents were exposed to the control treatment, indicating the presence of trans-generational immune priming.

3 MATERIALS AND METHODS

3.1 ASSESSMENT OF SURVIVAL TO *B. BASSIANA* IN *D. MELANOGASTER POPULATIONS*

3.1.1 *Drosophila melanogaster* out-crossed populations

In order to build out-cross populations we crossed inbred lines that have been kept in the lab since several years. We used 10 inbred lines derived from an African population (Lake Kariba, Zimbabwe), 12 from an European population (Leiden, the Netherlands), 12 from an Asian population (Kuala Lumpur, Malaysia) and 12 from a North American population (Raleigh, North Carolina) (Glinka et al. 2003; Laurent et al. 2011; Mackay et al, 2012). These populations were chosen to represent major geographic regions in the worldwide distribution of *D. melanogaster* (Stephan and Li 2007). Flies were reared at 23°C on standard fly media (see Appendix, 6.1), with a 14 hours light and 10 hours dark cycle. The lines used are reported in Appendix (6.2).

Initial differences in fecundity among the lines could bias the genetic composition of the out-crossed populations. In order to reduce this effect we followed a two steps approach. In the first step the inbred lines from the same population were crossed in pairs. Reciprocal crosses were performed so that the Y chromosome of each line was represented (figure 4). For example if there were 12 lines, it was necessary to perform 12 crosses: males from line 1 with females from line 2 produced the F1 1.2; females from line 1 with males from line 2 produced the F1 2.1; males from line 3 with females from line 4 produced the F1 3.4 ; males from line 4 with females from line produced the F1 4.3 and so on to get 12 F1s: 1.2, 2.1, 3.4, 4.3, 5.6, 6.5, 7.8, 8.7, 9.10, 10.9, 11.12, 12.11. In the second step F1 flies from each population were pooled for out-crossing.

In the first step 20 males and 20 virgin females were used for each cross. *D. melanogaster* females need 12-14 hours to be sexually mature (Ashburner 1989). Therefore the adult flies were removed every day in the morning from the bottles where they were reared and virgin females were collected 6 hours later. In the second step 50 male and 50 female flies from each F1 were pooled. In total this represents 1,000 to 1,200 flies for each population. The offspring from these crosses are regarded as the first out-crossed generation in the following.

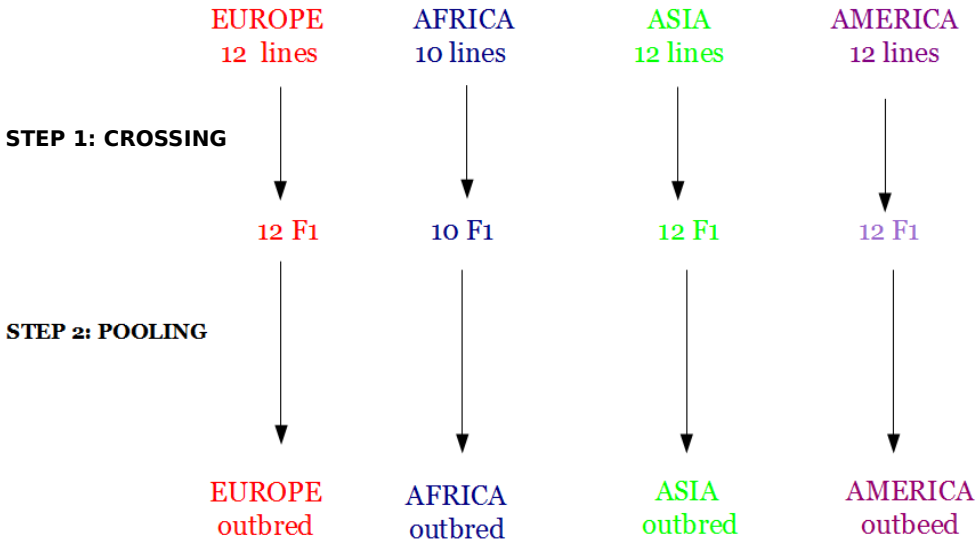

Figure 4 Schematic representation of the approach used to generate the out-crossed populations. In the first step, lines are crossed in pairs in both directions to obtain F1 lines. In the second step the F1s are pooled for out-crossing.

In order to maintain the out-crossed populations at a constant size of around 1,000 flies, the appropriate number of eggs was collected every two weeks and a new generation was started following Clancy and Kennington (2001). At the second out-crossed generation each population, except for the African one, was split in two sub-populations with the same size of about 1,000 flies. For the African population, it was necessary to wait until the fourth generation to get enough eggs for building two sub-populations. At the 34th out-crossed generation each sub-population pair was merged and the resulting populations have been maintained at the same census size of 1,000 flies since (figure 5).

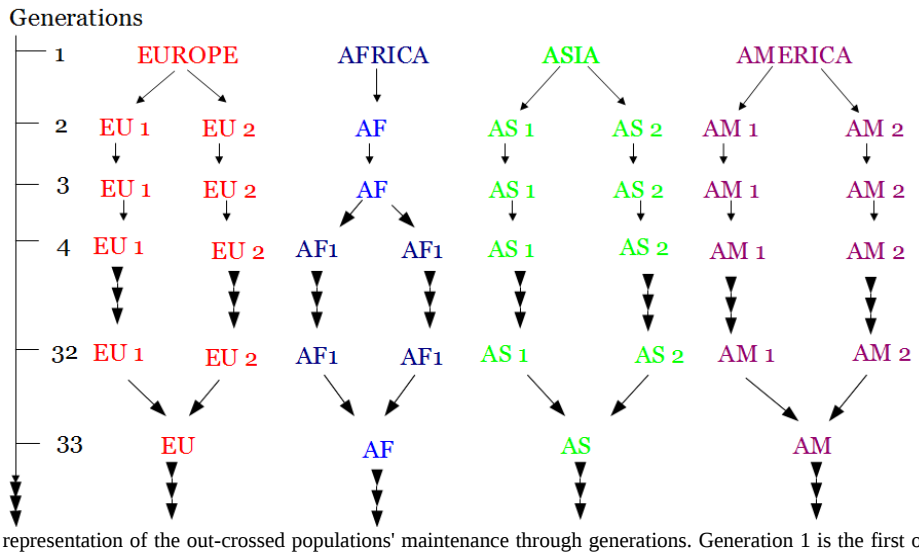

Figure 5 Schematic representation of the out-crossed populations' maintenance through generations. Generation 1 is the first out-crossed generation (see main text); generations increase from top to bottom (left arrow).

3.1.2 *Beauveria bassiana* strains

B. bassiana strains 547 and 1630 were ordered from USDA ARSEF Collection Center (http://www.ars.usda.gov/Main/docs.htm?docid=12125&page=2) (table 1). Both strains have been collected several years ago and since then have been maintained in the lab.

STRAIN	ORIGIN	HOST	YEAR
547	Malaysia	Coleoptera: Chrysomelidae	1986
1630	France	Diptera: Calliphoridae	1984

Table 1 Strains used for the infection experiments; strains number, place and date of collection and host from which they have been isolated are reported.

During this time the strains' virulence may have attenuated. In any case, to select for increased virulence against *Drosophila,* both strains were passed through a *Drosophila yacuba* inbreed line as in Tinsley et al. (2006). This allows us to increase the virulence of the strain, while avoiding fungal adaptation to a specific *D. melanogaster* line. We proceeded as in Tinsley et al. (2006) with minor modifications. Flies were sprayed in a mesh cage with a fungal/oil suspension (see below), Petri dishes with agar-molasses medium were changed every day and cadavers were collected for the following 10 days. Dead flies were incubated two weeks at 25°C at 24 hours dark. Sporulating cadavers were homogenised in Shellsol T oil and plated onto potato dextrose agar containing chloramphenicol antibiotic (5×10^{-5} g/ml).

Plates were incubated 2 weeks at 25 °C at 24 hours dark and then dried at room temperature for 1 week. Sporulating material was collected from each plate, dried in silica gel and stored in the fridge suspended in oil (87.5% Shellsol T, 12.5% Ondina El). The spore concentration was adjusted to 10^{-8} spores/ml by mixing approximately one volume of spores with four volumes of oil. The suspension was agitated using a probe sonicator at medium power for 30-45 seconds to avoid spore clustering. In order to determine spore concentration an aliquot of the suspension was diluted 100 times in Shellsol T oil. The spores were counted using a haemocytometer and 10 independent measurements were performed.

An airbrush was used to spray the spores/oil suspension on transparency film. After 2 weeks each transparency film was cut into stripes (approximately 5 cm wide and 7.5 cm long). Each stripe was

then rolled and inserted into a standard *Drosophila* vial in order to cover its walls from below the food surface up to the stopper. Mean spore density was determined by washing 10 stripes with 5 ml Shellsol T oil in a centrifuge tube. Tubes were inverted several times and then centrifuged for 5 minutes at maximum speed. Spore concentration was assessed using a haemocytometer and spore density on the paper was thus determined.

3.1.3 Infection procedures

Three to five days old male flies coming from the same cohort were collected using CO_2 anaesthesia and placed in vials with standard *Drosophila* food (see Appendix, table 1). Fifteen flies were placed in each vial. Flies for the infection treatment were transferred 1-3 days after collection to vials containing the transparency film sprayed with the oil/spores suspension, while flies for the control treatment to vials containing transparency film sprayed with oil. A number was assigned randomly to each vial. Flies were exposed 3 days to infection/control before being moved to fresh vials free of any transparency film.

For the whole duration of the experiment, the standard *Drosophila* food was amended to exclude the anti-fungal agent Nipagin and the anti-fungal/anti-bacterial compound propanoic acid. In order to assure homogeneity in the experimental procedure, similar food was used both for infection and control treatments. Every 3 days flies were changed to fresh vials and the number of dead flies was recorded. A possible cause of loss of flies independent of infection and natural mortality was also recorded, namely escaped flies from the vial.

As no preservatives were present in the food, contamination from unrecognised bacteria and molds was observed in around 2% - 5% of the vials every day. In such cases flies were immediately changed to fresh vials, and when severe contamination occurred, the entire vials were discarded. The experiment ended on the 21st day after treatment, although it was possible that some infection treatment flies were still alive. Vials were kept in an incubator at 25°C, 14 hours light – 10 hours dark for the whole duration of the experiment.

Several infection experiments were conducted with the out-crossed populations. In the first

experiment, flies from the 5th generation of the out-cross populations were infected with *B. bassiana* strain 547. As both out-crossed populations from each continent were used, there were 8 populations in total. For each population 20 infection and 5 control vials were started, for a total of 200 vials.

In the second experiment flies from the 8th generation of the out-crossed populations described above were infected with *B. bassiana* strain 1630. As both out-crossed populations from each continent were used, there were 8 populations in total. For each population 25 infection and 10 control vials were started, for a total of 280 vials. For practical reasons, flies were exposed to infection/control treatment in two randomised groups with one day difference.

In the third experiment, flies from the 17th generation of the out-crossed populations were infected with *B. bassiana* strain 1630. As both out-crossed populations from each continent were used, there were 8 populations in total. For each population 25 infection and 10 control vials were started, for a total of 280 vials.

3.1.4 Statistical analyses

All statistical analyses were performed twice: considering the pairs of replicate populations together as continents "Continent analysis" or each population independently "Population analysis". This allows us to compare the outcome of the two sub-populations (i.e. the replicates of each population). In all experiments we observed a high proportion of flies dying in the first 3 days following exposure to infection/control (29%, 12% and 27% in the 1st to 3rd experiment, respectively).

Interestingly this percentage was constantly higher in controls than in infected flies (see Results). A likely explanation is that the flies died because of the oil and not because the infection. The reason for the higher mortality in controls is probably that in the absence of spores the oil takes longer to dry and more flies could soak or stick to the vial walls (M. C. Tinsley, personal communication). To assess the effect of treatment and continent/population of origin three days after infection, the data

were both graphically summarised and a generalized linear mixed model was fitted (see below). In order to avoid the confounding effect due to the oil, the early mortality rate was excluded from all subsequent analyses

3.1.4.1 Generalized linear mixed models

The data were analysed using generalised linear mixed models (GLLM) (Bolker et al. 2009). These models are able to deal with random factors and are especially good for data from ecology and evolution that usually do not follow a normal distribution. The number of flies dead and alive in each vial at each time interval was chosen as dependent variable and the effect of covariates on mortality was assessed. A binomial distribution was used to fit the data. Flies that escaped and vials that were lost due to contamination were discarded from the analysis.

The following covariates were used: “Time”, “Logarithm of time”, “Time squared”, “Treatment”, “Continents” (or “Populations”) and the interaction between “Treatment” and “Continents” (or “Treatment” and “Populations”). In addition the random factor “Vial” was used to account for the repeated measurements from the same vial. For the second experiment the additional covariate “Group” was introduced to account for the fact that flies were infected in two separate groups (see above).

The time covariates have six levels. For example “Time” has the following levels: “6”, “9”, “12”, “15”, “18” and “21”, that represent days after infection. The covariate “Treatment” has two levels: “infected” and “controls”. The covariate “Continents” has four levels: “Africa”, “Asia”, “America” and “Europe”. The covariate “Populations” has eight levels: “Africa 1”, “Africa 2”, “America 1”, “America 2”, “Asia 1”, “Asia 2”, “Europe 1” and “Europe 2”. “Vial” has a number of levels equal to the number of vials used in the analysis. “Group” has two levels, “Group 1” and “Group 2”.

“Continents” and “Populations” are categorical covariates. In these cases one level was chosen as reference and all the others were compared to it. For example in the “Continents” analysis the difference between “Africa” and the other three continents was assessed; in the “Populations” analysis the difference between “Africa 1” and the other seven populations was assessed. In the case

of the random factor “Vial” we were not interested in its effect on mortality, as for the others covariates, and the experimental variance explained by this factor was determined and removed from the analysis.

We specified multiple time covariates because we did not make do any assumption on how time affects mortality. To determine which time covariate or combination of time covariates described better the data, a likelihood ratio test was performed (Steiger et al. 1985). In a first step three models were fitted each with a different time covariate and the model with the higher likelihood (L) was selected. In the next step a new time covariate was added to this model and the likelihood of the new model (L') was assessed. The likelihoods of the more general model (L') and of the nested one (L) were then compared. For large samples the following relation holds:

$$W = (-2\log(L)) - (-2\log(L')) \sim \chi^2 \quad ,$$

meaning that the statistic W follows a chi squared distribution with a number of degrees of freedom equal to the difference in covariates number between the two models (one in this case). If the test was significant, then the model with two time covariates was selected and the same approach was repeated for the third time covariate. Otherwise the simpler model was retained. The same approach was used in the second infection experiment for the covariate “Group”.

To analyse the mortality data three days after treatment the following covariates were used: “Treatment”, “Continents” or “Populations” and, in the case of the second experiment, “Group”. A generalised linear model (GLM) was used instead of a generalised linear mixed model (GLMM) because no random factor was present. The data were fitted using a pseudo-binomial distribution that allows for over-dispersion. Both the GLMM and GLM were fitted using the R library “lme4”.

3.1.4.2 Survival analysis

We additionally used survival analysis methods to examine our data. In survival analysis the time to death of each individual (in this case of each single fly) is used as dependent variable and the effect

of covariates on survival time is assessed (Klein and Moeschberger 2005). A characteristic of survival data is that some subjects could escape or be removed from the experiment for some reason, here for example due to vial contamination. Another common situation is that not all subjects die by the end of the experiment. Survival analysis does not discard this information but takes it into account in the following way.

The time from the beginning of the experiment (t) at which each individual dies is recorded. If a subject is lost at time t = k than it is considered to have died during the time interval $t \in [k, \infty)$. Similarly the subjects still alive at the end of the experiment (e.g. t = z) are considered to have died during the time interval $t \in [z, \infty)$. There are several ways to analyse survival data. Here we graphically visualise the Kaplan-Meier estimator of the survivor function and used Cox proportional hazard models to assess the effect of covariates on survival (see below).

3.1.4.2.1 Graphical representation of the data

Data were graphically represented using the Kaplan-Meier estimator (Goel et al. 2010). The Kaplan-Meier survival curve gives the probability of surviving to a given time point. Time is divided in observation (or counting) intervals and survival probability is computed for each interval. The probability (St) of surviving to a given time interval is given by:

St= (Number of individuals surviving to that time interval -

Number of individuals at risk)/ Number of individuals at risk.

In this case the number of individuals at risk is equal to the number of flies that are still alive before the beginning of that time interval minus the number of flies that are lost in that time interval. The total probability of surviving to a given time interval is the product of the survival probabilities at all preceding time intervals. To obtain the Kaplan-Meier survival curve, the R package “survival” was used.

3.1.4.2.2 Cox proportion hazard models

Proportional hazards models (Bollen 1989) estimate the effect of covariates and of their interaction on survival. In these models a baseline hazard function $h_0(t)$ defining the instantaneous rate of death is first fitted. The hazard function $h_i(t)$ for an individual i with specific covariates values (for example: infected and American) is given by the baseline hazard function multiplied by a constant Φ_i that depends on the covariates values. If x_{i1} is the value of the first covariate for the individual i, x_{i2} the value of the second covariate for the individual i (and so on) and there are p covariates, then the relationship between hazard function and covariates is:

$$h_i(t) = \Phi_i * h_0(t)$$

$$\Phi_i = \exp(h_i)$$

$$\eta_i = \beta_1 * x_{i1} + \beta_2 * x_{i2} + \beta_3 * x_{i3} + \beta_p * x_{ip} \quad .$$

The additive form means that each covariate affects the hazard independently. Using maximum likelihood is it possible to estimate the coefficients β. A covariate k has a significant effect on the hazard if β_k is significantly different from 0. A drawback of proportional hazard models is that the distribution of the baseline hazard function has to be specified.

Cox models (Cox 1972) are a special kind of proportional hazard models that do not make any assumption on the baseline hazard distribution. The significance of the covariates is assessed using a partial likelihood maximisation. This feature makes Cox proportional hazard model the most widespread model in survival analysis. The Cox proportional hazard model was fitted using the R library “coxme”. For the “Continent” analysis, the following fixed effects were specified: “Treatment”, “Continent” and their interaction “Treatment x Continent”. For the “Population analysis” the following fixed effects were specified: “Treatment”, “Population” and their interaction “Treatment x Population”. In the case of the second experiment, where flies were infected in two independent groups, the extra fixed effect “Group” was specified. The random factor “Vial” was introduced to account for the vial to which each fly belonged.

3.2 *D. MELANOGASTER* TRANSCRIPTIONAL RESPONSE TO *B. BASSIANA*

3.2.1 Micro-array analysis

3.2.1.1 Infection experiment

Flies for the microarray experiment originated from the 17th generation of the out-crossed populations described above. Flies were collected and exposed to infection or control treatment in the same time as the ones used for the third infection experiment described in 3.1.3. The *B. bassiana* strain 1630 was used (see above). For each out-crossed population 15 infection treatment and 15 control treatment vials were used, each one containing around 15 male flies. Vials were placed at 25° C under a 14 hours light and 10 hours dark cycle. 24 hours after infection flies from each vial were anaesthetised using CO_2, transferred to an Eppendorf tube previously labelled and frozen in liquid nitrogen. Frozen tubes were stored at -80° C for 3-7 months before proceeding to RNA extraction.

3.2.1.2 RNA extraction, sample preparation and array hybridisation

RNA was extracted from groups of 65 - 75 flies (pooling of 5 vials) from the same out-crossed population and treatment. RNA was retro-transcribed to cDNA and labelled with a green or a red fluorescent dye. Labelled cDNAs from two different samples were competitively hybridised on the array. Full genome *D. melanogaster* microarrays D14k3 (UHN Microarray Centre) were used. For more details and protocols see Appendix (6.3).

3.2.1.3 Hybridisation scheme

Competitive hybridisations were performed following a ring design (figure 6, Appendix table 2). For each comparison two hybridisations were done. In the first one, one sample was labelled with the red dye and the second with the green one, while in the respective dye-swap the opposite sample-dye combination was used. This was done in order to avoid a bias in the case a dye gives a stronger signal than the other. The full ring design was repeated twice, once for each out-crossed population replication (figure 6, Appendix table 2). In total 24 comparisons and 48 hybridisations were performed.

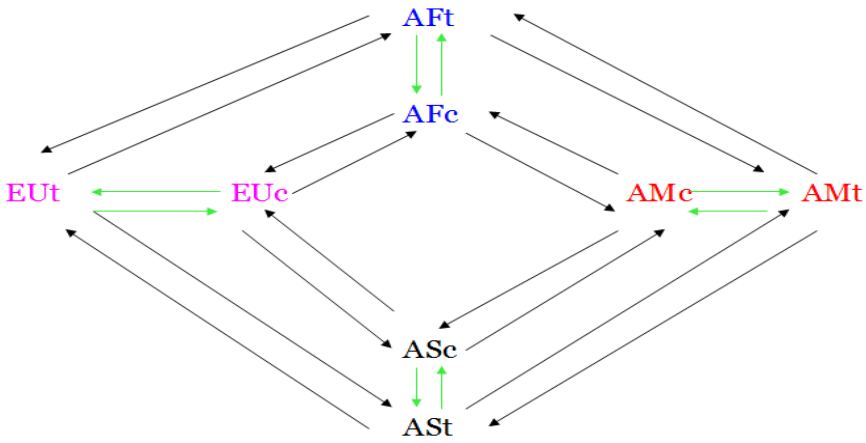

Figure 6 Experimental design scheme. Each pair of coloured arrows pointing in opposite direction represents the two dye swaps for each comparison. The following hybridisation scheme was repeated twice, once for each out-crossed population replication. "AF" stands for Africa,"AM" for America, "AS" for "Asia" and "EU" for Europe. "t" stands for treatment, while "c" stands for control.

3.2.1.4 Array scanning

Signal intensity on the hybridised arrays was assessed using the scanner Aquire (Genetix). This machine uses one channel to detect the signal of the red dye and another one to detect the signal of the green dye. For each spot on the array the signal intensity of one dye is proportional to the RNA quantity of the corresponding transcript in the sample marked with that given dye.

3.2.1.5 Spot annotation and editing

Scanned arrays were analysed using the program Qscan (Genetix). The annotation file “CMDCoD14Kv3.gal”, provided by the UHN Microarray Centre, was used in order to assign each spot to a gene. Spot position was manually edited to better fit the spot signal on the array. Finally, red and green foreground and background signal intensities were computed for each spot on the array and exported together with the annotation information as a text file.

3.2.1.6 Normalisation

Row data were normalised using the CARMAweb 1.5 online service (https://carmaweb.Genome.tugraz.at/carma/). Text files were converted to the “.gpr” format (the input format for CARMAweb) using the Munich Microarray Analysis Tool (http://10.153.163.103/MuMAT/index.html). Several normalisation steps were followed for each slide. First, background correction was carried out: the

intensity of each spot was normalised against the unspecific background intensity around the spot. The “minimum” correction mode was chosen (for details see CARMAweb users guide: https://carmaweb.genome.tugraz.at/carma/UsersGuide.pdf). Then, within-array normalisation was performed in order to correct for spatial trends in signal intensity on the array. The “print tip loess” normalisation mode was selected (Smyth and Speed 2003). The next step was normalization between the two dye-swaps for each comparison. The “quantile” normalisation mode was chosen (Yang and Thorne 2003).

Finally, for each array, spots whose signal intensity in at least one channel was higher than the 95% of the distribution of the negative (blank) control spots were retained for statistical analysis.

3.2.1.7 Statistical analysis

Two analyses were performed: one to compare all possible population – treatment combinations (in the following “Eight nodes analysis”) and the other one to compare infected and control flies independently on a per population basis (in the following “Two nodes analysis”).

In the “Eight nodes analysis” each population – treatment combination was a node in the ring design shown in figure 6. All nodes are connected directly or through one or more intermediate nodes. Using the program BAGEL (Townsend and Hartl 2002) it was possible to estimate genes differently expressed for each pair of nodes.

BAGEL accepts as input the normalised red/green ratios of the retained spots for each array and the description of the experimental design (figure 6). As output the software returns for each pair of nodes the p-value of each gene to exhibit a higher expression in one or in the other node. When data are lacking for one gene, for example if the corresponding spot has been discarded for many arrays, then BAGEL excludes this gene from the analysis.

In order to correct for multiple testing, the association among red/green ratios and the description of the samples hybridised on the array was randomised. BAGEL was run a second time with the randomised data set and new p-values were computed for each pair of nodes. We calculated the false discovery rate (FDR) as the number of genes in the randomised data set whose p-value was lower than an arbitrary threshold, divided by the number of genes in the real data-set whose p-value was lower than the same threshold. By choosing the p-value threshold we got the significant genes

at the desired FDR. In the second analysis the same procedure described above was followed assuming only two possible nodes: control and infected flies.

3.2.1.8 Gene ontology analysis

Gene ontology analysis was carried out using the online tool GOrilla (http://cbl-gorilla.cs.technion.ac.il/) (Eden et al. 2009). GOrilla compares a set of target genes, in this case the genes up- or down-regulated after infection, to a background list of genes, in this case all the genes retained in the BAGEL analysis. The program performs an enrichment analysis to identify gene ontology categories more commonly associated with the target genes than expected by chance, if genes were randomly drawn from the background set.

As all the categories associated with at least one gene in the target set are tested, the Benjaminin and Hochberg correction for multiple test (Benjamin and Hochberg 1995) is used to produce a FDR corrected q-value for each significant category. GOrilla allows to choose ontology categories related to molecular function, biological process or cellular component (cellular, tissue or organ location). All types of categories were used in our analyses.

3.2.1.9 Comparison among populations and with previous studies

For the “Eight nodes analysis”, genes differently expressed in infected vs control flies for each within population comparison were compared among populations and with the results of De Gregorio et al. (2001) and Roxstrom-Lindquist et al. (2004). For the “Two nodes analysis” genes differently expressed in infected vs control flies were compared with the results of (De Gregorio et al. 2001; Roxstrom-Lindquist et al. 2004). To obtain functional and molecular information for the genes shared among two or more populations and also among this study and those of De Gregorio et al. (2001) and Roxstrom-Lindquist et al. (2004), Flybase was used (http://flybase.org/) (Bettencourt et al. 2004).

3.2.2 RNA sequencing analysis

3.2.2.1 Infection experiment

Flies were reared at 23°C on standard fly media (see Appendix, table 1) with a 14 hours light and 10 hours dark cycle. Flies from the 36th generation of the out-crossed populations described above were employed. Four populations were used in the experiment, as the pairs of replicate populations were pooled at the 31th generation after out-crossing (see 3.1.1). Flies were infected with the fungal strain 1630 (see 3.1.2).

The experimental procedure was the same as outlined above (see 3.1.3) with the major difference that there were three treatments: control, 8 hours infection and 24 hours infection. Control flies were exposed 8 hours to mock treatment (i.e. oil), while infected flies were exposed 8 and 24 hours to the spores. During the experiment flies were kept at 25°C under a 14 hours light and 10 hours dark cycle. For each population 6 vials, with 15 male flies in each, were exposed to each treatment, for a total of 72 vials (see Appendix, table 3). All treatments were started simultaneously.

Eight hours after the beginning of the experiment control flies and infected ones were anaesthetised using CO_2. Flies from the same vial were transferred to an Eppendorf tube previously labelled, and frozen in liquid nitrogen. Frozen tubes were then stored at -80° C. The same procedure was followed for 24 hours infected flies. Flies were kept at -80° C for 5-7 days before proceeding to RNA extraction.

3.2.2.2 RNA extraction

For each population – treatment combination, three independent RNA extractions were performed, each from two vials (around 30 male flies) and treated as biological replicates. Therefore RNA was extracted from a total of 36 samples. The Master Pure RNA Purification kit from Epicentre was used for extraction of total RNA according to the manufacturer's instructions.

3.2.2.3 RNA Sequencing

RNA from the 36 samples was sent on dry ice to GATC Biotech AG for sequencing. Each sample had an approximate amount of 3.5 - 5 μg prepared in RNAse free water with a concentration of

around 200 ng/µl. For each sample GATC Biotech AG performed a quality control to assess if RNA was not degraded. The mRNA of samples that passed the quality control was isolated from total RNA, fragmented, retro-transcribed to cDNA and sequenced via Illumina technology to generate 50-base pairs single end sequences.

3.2.2.4 Reads mapping

Sequencing reads were assigned to *D. melanogaster* genes using the program Stampy (version 1.0.20) (Lunter and Goodson 2011). Reads were mapped against *D. melanogaster* transcriptome (version 5.48). In case of a gene with multiple transcripts, the reads assigned to each transcript were summed up. A count table with the number of reads for each sample assigned to *D. melanogaster* genes was produced. Around 15,000,000 - 27,000,000 reads were mapped to *D. melanogaster* genes for sample.

3.2.2.5 Statistical analysis

Genes differently expressed among control and 8 hours infected flies and among control and 24 hours infected flies were identified. Row count data were normalised, bringing reads number to a common scale among samples, and analysed in R using the "DESeq" package (Anders and Huber 2010). For each time point both a "population analysis" and a "treatment analysis" were performed.

In the "population analysis" control and infected flies were compared independently for each population. In the "treatment analysis" two models were fitted, a full model containing both "population" and "treatment" as factors and a reduced one containing only the factor "population". For each gene the two models were compared and the genes for which the specification of treatment significantly increased the fit of the model, i.e. "treatment" had a significant effect, were identified.

3.2.2.6 Gene ontology analysis and comparison with previous studies

Gene ontology (GOs) analysis was performed using the online tool GOrilla (Eden et al. 2009). Results were compared with previous studies (De Gregorio et al. 2001, Roxstrom-Lindquist et al. 2004) and with the micro-array analysis described above. If not differently stated, molecular and functional information for interesting genes were obtained from the internet database Flybase (Bettencourt et al. 2004).

3.2.2.7 Immunity related induced genes

In order to find immune related genes whose change in gene expression had a biological meaning, an approach similar to the one proposed in Mortazavi et al. (2008) was followed. Normalised genes count data (see above) were divided by the length of their longest transcript (in thousand of base pairs) and averaged among all treatments and populations. In this way a value proportional to the average gene expression level was obtained. This value is called thereafter "estimated average gene expression level" (EAGEL). Only immune related genes with an EAGEL value in the 60% higher quantile of the distribution were considered for this analysis. The rationale is that for genes expressed on average at low levels, a change in gene expression, although significant, is not likely to be biologically meaningful.

3.2.3 Genetic analysis of candidate genes

3.2.3.1 Subset of interesting genes

A subset of candidate genes is shown in table 2. “Shared” genes are induced in at least two different analyses and can be divided in three groups. The first group comprises genes induced in all populations at 24 hours after infection and in at least one of De Gregorio et al. (2001) and Roxstrom-Lindquist et al. (2004). The second group comprises 2 genes induced in one ore more populations both at 8 and 24 hours after infection in the RNA sequencing. These genes are CG2064, that is induced at 8 hours in the American population and at 24 hours in the African, American and European ones, and *Cyp6d2* (CG4373) that is induced in the Asian and European population at 8 hours and in all populations at 24 hours. The last group is constituted of only one gene: CG10247, that is induced both in the European, African, and American populations 24 hours after infection and in the African population in the microarray study. In addition the three private genes showing the highest up-regulation and the two-three private genes showing the highest down-regulation at 24 hours post-infection were examined for each population.

SHARED	SHARED	AFRICA	AMERICA	ASIA	EUROPE
CG14516	CG2064	CG16772	CG8346	CG32356	CG6653
CG10118	CG4373	CG13840	CG14585	CG16879	CG15589
CG10247	CG6667	CG3047	CG14356	CG17226	CG15253
CG11073	CG6816	CG15919	CG4178	CG4739	CG6578

CG11951	CG7171	CG31954	CG12763	CG31922	CG17820
CG12766	CG7592	CG34305			CG13026
CG17560	CG8453				
	CG9649				

Table 2 Analysed genes are reported. “SHARED” stands for “SHARED” genes, “AFRICA” stands for African privately induced genes, “AMERICA” for American privately induced genes, “ASIA” for Asian privately induced genes and “EUROPE” for European privately induced genes.

3.2.3.2 Genetic sequences

Fully sequenced *D. melanogaster* lines from France, Netherlands, North American, Malaysia, Rwanda, and Zambia-Zimbabwe populations were analysed. Note that some of these Netherlands and North American lines were used to build our out-crossed populations (see 3.1.1). Along with each gene (exons + introns), 2,000 base pairs up-stream and down-stream, or less in case of flanking genes nearer than 2,000 base pairs, were assessed.

3.2.3.3 Coding sequence evolution

In order to determine the level of coding sequence evolution, the ratio of non-synonymous over synonymous substitutions and the ratio of synonymous over non-synonymous polymorphisms were computed in DnaSP (v 5.10) (Librado and Rozas 2009) using *D. simulans* as out-group. In the case of privately induced genes, the sequences of the population (or populations) from the same continent were used. In the case of “shared” genes, the sequences from all populations were pooled and analysed together.

3.3 EFFECT OF *WOLBACHIA* AND PARENTAL PARASITE EXPOSURE ON *D. MELANOGASTER* SURVIVAL TO *B. BASSIANA*

3.3.1 Effect of the endosymbiont *Wolbachia pipientis*

3.3.1.1 *Drosophila melanogaster* inbred lines

Flies were reared at 23°C on standard fly media (see Appendix, table 1), with a 14 hours light and 10 hours dark cycle. The *Drosophila melanogaster* European line E20 (Leiden, the Netherlands) and the Egyptians lines Eg25 and Eg57 (Cairo, Egypt) were used. The lines E20 and Eg25 were already identified as bearing *Wolbachia pipientis*, while the line Eg57 was found free from the endosymbiont (Jancke 2011).

The lines Eg25 and E20 were cured from *Wolbachia* by exposure to the antibiotic tetracycline: flies were reared on standard fly media supplemented with 0.5 mg/ml of tetracycline for 4 generations. The line Eg57 was exposed to tetracycline to test for a general effect of the antibiotic on fly fitness and survival to infection. Absence or presence of *W. pipientis* was assessed by PCR (see below). Flies treated with tetracycline were exposed for two days to media coming from bottles of the respective not exposed line to allow them to recover the normal fly gut microbiome (Sharon et al. 2011; Charroux and Royet 2012). Antibiotic exposed flies were subsequently reared on standard fly media without the antibiotic for at least two generation before being exposed to fungal infection.

3.3.1.2 PCR assessment of *Wolbachia pipientis*

To determine if flies were bearing *W. pipientis*, the *Wolbachia* gene *Wsp* coding for a surface protein was amplified by PCR. Primers commonly used in similar studies were chosen (Zhou et al. 1998; Teixeira et al. 2008; Rottschaefer and Lazzaro 2012) and visible in Appendix table 4. The PCR protocol is given in Appendix tables 5 and 6. The *D. melanogaster* ribosomal gene *28S* was amplified as a control using the primers indicated in Appendix table 4 and the PCR protocol reported in Appendix tables 5 and 6. Two repetitions of the PCR were performed, the first one just after the tetracycline exposure and the second one 10 generation after exposure.

DNA was extracted from samples of 10 male and 10 female flies for each line, both for antibiotic exposed and not exposed flies. DNA extraction was performed using the MasterPure DNA Purification kit from Epicentre according to the manufacturer's instructions. PCR products were run on 1.5% agarose gel at 100 V for 40 minutes.

3.3.1.3 Infection experiment

Flies from lines E20, Eg25 and Eg57, both exposed and not exposed to tetracycline, were infected with the *B. bassiana* strain 1630 (see above). Two replicates of the infection were performed, the first one using flies from the third generation after the tetracycline exposure and the second one using flies from the sixth generation after the antibiotic treatment. The infection procedure was the same as outlined above with the major difference that flies were reared at the temperature of 21° C instead of 25° C during the experiment.

The first experiment was performed only with male flies. As there were both flies exposed to

tetracycline and not exposed flies and 3 different *D. melanogaster* inbred lines, in total 6 groups of flies were present. For each fly group 25 vials, with approximately 15 flies per vial, were exposed to infection, while 10 vials, again with approximately 15 flies per vial, were exposed to control. In total 210 vials were started. Mortality was assessed every third day after treatment for 21 days.
The second experiment was performed with both male and female flies. As both cured flies and flies bearing *W. pipientis* were used for each *D. melanogaster* inbred line, 6 fly groups were present, 12 considering flies' sex. For each fly group 26 vials (13 vials for each sex), containing approximately 15 males or females, were exposed to infection, while 10 vials (5 vials for each sex) were exposed to control treatment. In total of 210 vials were started. Mortality was assessed every day after treatment for 21 days.

3.3.1.4 Statistical analysis

Statistical analysis was performed using the same methods outlined above. As the aim was not to detect differences among *D. melanogaster* lines but to determine the effect of *W. pipientis* and of its interaction with the experimental treatment on mortality, in all analyses a separate model was fitted for each fly line (E20, Eg25, Eg57).

3.3.1.4.1 Generalized linear mixed models

The following covariates were used: “Time”, “Logarithm of time”, “Time squared”, “Treatment”, “Wolbachia” (or “Antibiotic” for the Drosophila line Eg57) and the interaction between “Treatment” and “Wolbachia” (or “Treatment” and “Antibiotic” for the Drosophila line Eg57). The covariate “Wolbachia” has two levels: “Wolbachia” and “Cured”, the covariate “Antibiotic” has two levels: “Antibiotic” and “Not Antibiotic”, the other covariates have already been described in the previous section. The random factor “Vial” was introduced to account for the repeated measurements from the same vial. For the second experiment the extra covariate “Sex” was introduced. To analyse the mortality data three days after treatment the following covariates were used: “Treatment” and “Wolbachia” and, in the case of the second experiment, “Sex”. Analysis was performed as outlined in the previous section.

3.3.1.4.2 Cox proportional hazards models

The same covariates specified for the other proportional hazard models (see above) were used and additionally the random factor “Vial” to account for the vial to which each fly belonged. Analysis was performed as outlined in the previous section

3.3.2 Effect of trans-generational immune priming

3.3.2.1 *Drosophila* out-crossed populations

Flies were reared at 21°C on standard fly media with a 14 hours light and 10 hours dark cycle. Two experiments were performed. In the first experiment flies from the 27th out-crossed generation of one of the two replicate European populations described above (see 3.1.1) were used. In the second experiment flies from the 30th out-crossed generation of one of the two replicate American populations described above (see 3.1.1) were used. In both experiments flies were infected with the fungal strain 1630 (see 3.1.2).

3.3.2.2 Parents priming

400 male and 400 virgin female flies were collected from the out-crossed population in the first experiment, 600 males and 600 females in the second experiment. In order to collect female virgin flies the same approach outlined above was followed (see 3.1.1). Male and female flies were exposed separately to infection and to control treatment. Half of the flies of each sex were exposed to each treatment. Flies exposed to infection treatment were divided into two groups of approximately the same size. Each group was transferred to a mating cage with walls covered with ink-jet paper sprayed with fungal spores (see above). The same approach was followed for control treatment, with the only difference that the ink-jet paper was sprayed with oil. Flies were kept on agar molasses media at 25°C with a 14 hours light and 10 hours dark cycle for 48 hours.

After 48 hours all male and female flies exposed to the same treatment were moved to a new mating cage free from sprayed ink-jet paper, placed at 21°C with a 14 hours light and 10 hours dark cycle and supplemented with agar molasses plates. Molasses plates were changed every day and eggs

were collected from infected and control flies for the next two days and placed in bottles (see above) on standard fly media at 21°C with a 14 hours light and 10 hours dark cycle.

3.3.2.3 Offspring infection

Male and female offspring of the infected and control parents were exposed to infection or control treatment (see above). For each parental group and offspring sex, 25 vials, with around 15 flies each, were exposed to infection and 10 vials to control treatment (see table 3). For each category 3 extra vials, with approximately 15 flies per vial, were exposed to infection and 3 to control treatment (see below). These extra-vials were used to replenish vials if needed due to the mortality of flies (see below). In total 140 vials were used plus the 24 extra ones. Infection and control treatment were carried out as outlined in the previous section with the main difference that flies were exposed to treatment for 48 hours. During treatment flies were kept at 25°C in the first experiment and at 21°C in the second experiment with a 14 hours light and 10 hours dark cycle.

After treatment, the flies were moved to vials free from ink-jet paper and kept at 21°C with a 14 hours light and 10 hours dark cycle for the rest of the experiment. As a few flies died during the 48 hours treatment, they were replaced by flies from the corresponding extra vials in order to keep approximately 15 flies in each vial. One day later, the flies were changed again to new vials and dead flies were recorded. The flies were changed to new vials and mortality was recorded every third day until the 21st day after the beginning of the experiment.

PARENTS TREATMENT	OFFSPRING SEX	OFFSPRING TREATMENT	NUMBER OF VIALS
Infection	Male	Infected	25 +3
		Control	10 +3
	Female	Infected	25 +3
		Control	10 +3
Control	Male	Infected	25 +3
		Control	10 +3
	Female	Infected	25 +3
		Control	10 +3

Table 3 The table shows parents treatment, offspring treatment and offspring sex combinations with the number of vials used in the experiment for each combination. “+ 3” indicate the three extra vials for each category.

3.3.2.4 Statistical analysis

The Statistical analysis of mortality data was performed using the same methods outlined above.

Note that dead flies were not recorded during the first two days. In the first experiment there was no significant difference among control and infected flies at this time point, while in the second one control flies suffered a significantly higher mortality than infected ones.

3.3.2.4.1 Generalized linear mixed models

The following covariates were used: “Time”, “Logarithm of time”, “Time squared”, "Sex", “Treatment”, “Parents" and the interaction between “Treatment” and “Parents”. The covariate “Parents” has two levels: “Infected” and “Control”, the other covariates have been already described in the previous section. The random factor “Vial” was introduced to account for the repeated measurements from the same vial. To analyse the mortality data three days after treatment the following covariates were used: “Treatment”, “Parents” and "Sex". The statistical analysis was performed in R as outlined in the previous section.

3.3.2.4.2 Cox proportional hazards models

The same covariates specified for the other proportional hazard models (see above) were used with additionally the random factor “Vial” to account for the vial to which each fly belonged. The statistical analysis was performed in R as outlined in the previous section.

4 RESULTS

4.1 ASSESSMENT OF SURVIVAL TO *B. BASSIANA* IN *D. MELANOGASTER* POPULATIONS

4.1.1 First infection experiment

The mean spore concentration in the oil/spores suspension was 1.7 x 10^8 spores/ml with a standard deviation of 0.95 x 10^8 spores/ml (10 independent measurements were performed). The mean spore density on the ink-jet transparency film was 2,520 spores/mm^2 with a standard deviation of 1,120 spores/mm^2 (10 independent measurements were performed). Twenty one vials were discarded due to contamination. As the exact date was not recorded, these vials were removed from all the statistical analyses.

4.1.1.1 Mortality at three days after treatment

Control flies suffered a higher mortality than infected ones (figure 7). Mortality was not homogeneous among continents and populations (figure 8). “Treatment” was significant both in the “Continent” (p-value = 0.00325) and in the “Population” analysis (p-value = 0.00348). In the “Continent” analysis “America” was significantly different from “Africa” (p-value = 0.02844). In the “Population” analysis “Asia 1” was significantly different from “Africa 1” (p-value = 0.03925).

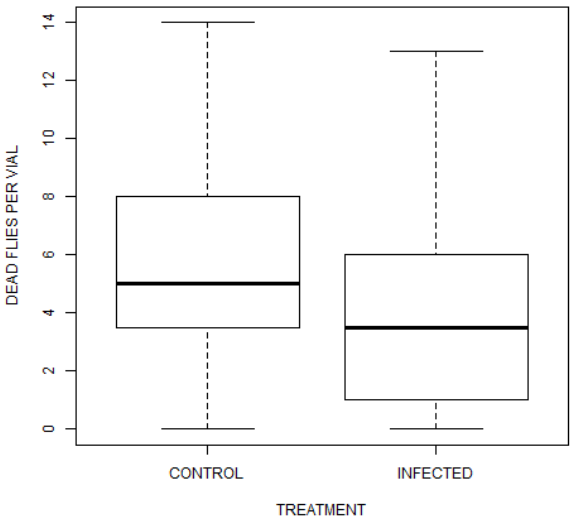

Figure 7 Box plot representing the number of dead flies per vial during the first three days after treatment.

The higher mortality of control flies was due to the effect of the oil: more flies got stuck to the vial walls or soaked in the oil (see Materials and Methods). Also the variability in mortality among continents and populations is of interest and reflects a difference in general vigour, highlighting the higher fitness of the American populations (see figure 8); conversely the “Asia 1” population exhibits an extremely pronounced mortality (see figure 8).

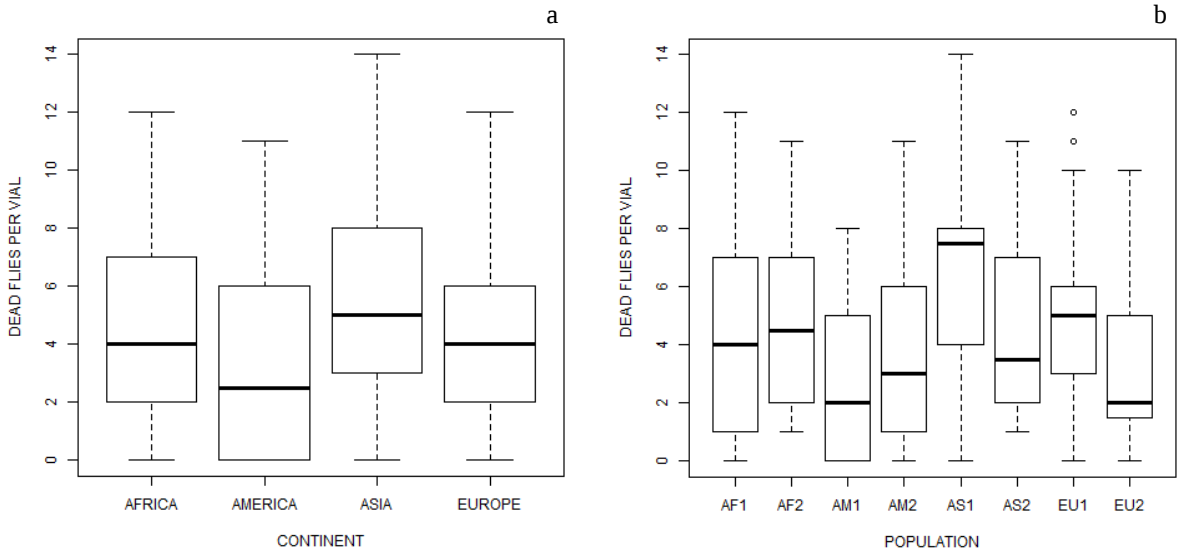

Figure 8 (a) Box plot representing the number of dead flies per vial during the first three days after treatment for each continent . (b) Box plot representing the number of dead flies per vial during the first three days after treatment for each population. (“AF1” = Africa 1, “AF2” = Africa 2, “AM1” = America 1, “AM2” = America 2, “AS1” = Asia 1, “AS2” = Asia 2, “EU1” = Europe 1, “EU2” = Europe 2).

4.1.1.2 Mortality at six days after treatment and later

4.1.1.2.1 Generalized linear model

All time covariates were retained and were significant both in the “Continent” and in the “Population” analysis (table 4, 5). In the “Continent” analysis “Treatment” was significant. In the “Population” analysis only “America 2” was significantly different from “Africa 1”.

	Time	Log Time	Time squared	Treatment
p-value	0.002833	0.048800	0.000684	0.002198

Table 4 P-values of the significant covariates in the “Continent” analysis.

These results do not support any difference in susceptibility to fungal infection among continents or populations. In the “Population” analysis “Treatment” was not significant, probably due to the relatively small increase in mortality in infected compared to control flies and to the big variability

among populations (figure 9, 10). The flies from the “America 2” population died less then the ones from the other populations (table 5, figure 10) reflecting higher vigour independently of the treatment.

	Time	Log Time	Time squared	America 2
p-value	0.002804	0.048280	0.000678	0.036002

Table 5 P-values of the significant covariates in the “Population” analysis.

4.1.1.2.2 Kaplan-Meier estimator

The Kaplan-Meier survival curve for each continent is shown in figure 9. As expected infected flies suffered higher mortality than control ones; we also observed that temperate populations performed better than tropical ones both for infected and the control flies. This plot again suggests no difference in susceptibility to fungal infection and a difference in general vigour among continents.

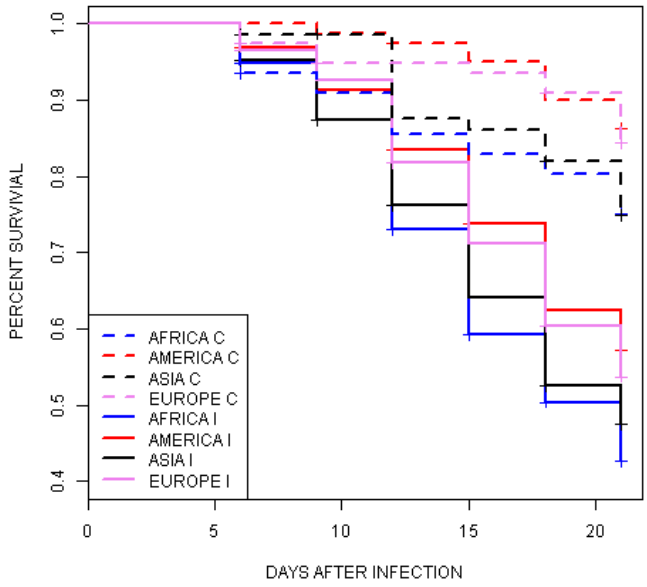

Figure 9 Kaplan-Meier plot representing survival curves from six days after treatment for all continents. Full lines represent infected flies (“I” in the legend), while dashed lines represent control flies (“C” in the legend). Colours are as follow: African flies in blue, American in red, Asian in black and European in pink.

The Kaplan-Meier survival curve for each pair of replicate populations is reported in figure 10. There is some variability among replicate populations, especially for the treatments of the two African populations. The American and European populations are the ones with the lowest mortality after infection.

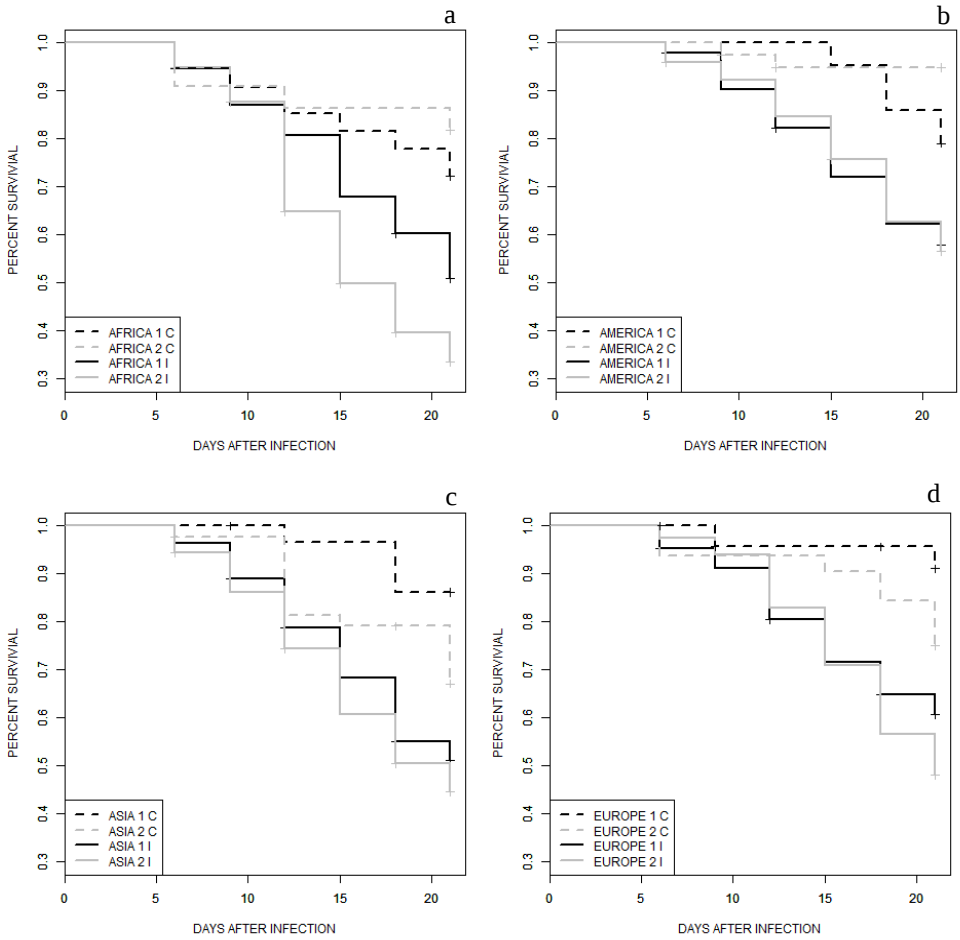

Figure 10 Kaplan-Meier plots for pairs of replicate populations. (a) represents the African replicates, (b) represents the American replicates, (c) represents the Asian populations and (d) represents the European populations.

4.1.1.2.3 Cox hazard model

In the "Continent" analysis "Treatment" was significant (p-value = 0.0035). In the "Population" analysis "America 2" was significantly different from "Africa 1" (p-value = 0.0340). These results once more reject the hypothesis of different susceptibility to fungal infection among continents and populations and suggest that the "America 2" population is more fit independently of the treatment.

4.1.2 Second infection experiment

The mean spore concentration in the oil/spore suspension was 2.2 x 10^8 spores/ml with a standard deviation of 0.7 x 10^8 spores/ml (10 independent measurements were performed). The mean spore density on the ink-jet transparency was not measured because a similar value as in the first experiment was expected. Eighty one vials were discarded due to contamination.

4.1.2.1 Mortality at three days after treatment

Control flies suffered higher mortality than infected ones (figure 11).

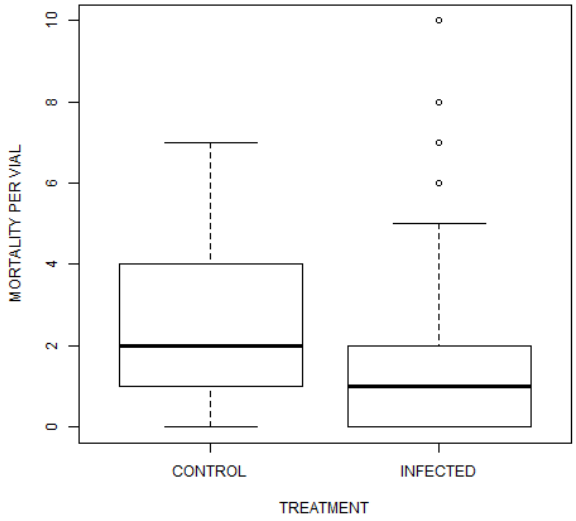

Figure 11 Box plot representing the number of dead flies per vial during the first three days after treatment.

Mortality was not homogeneous among continents and populations (figure 11, figure 12).

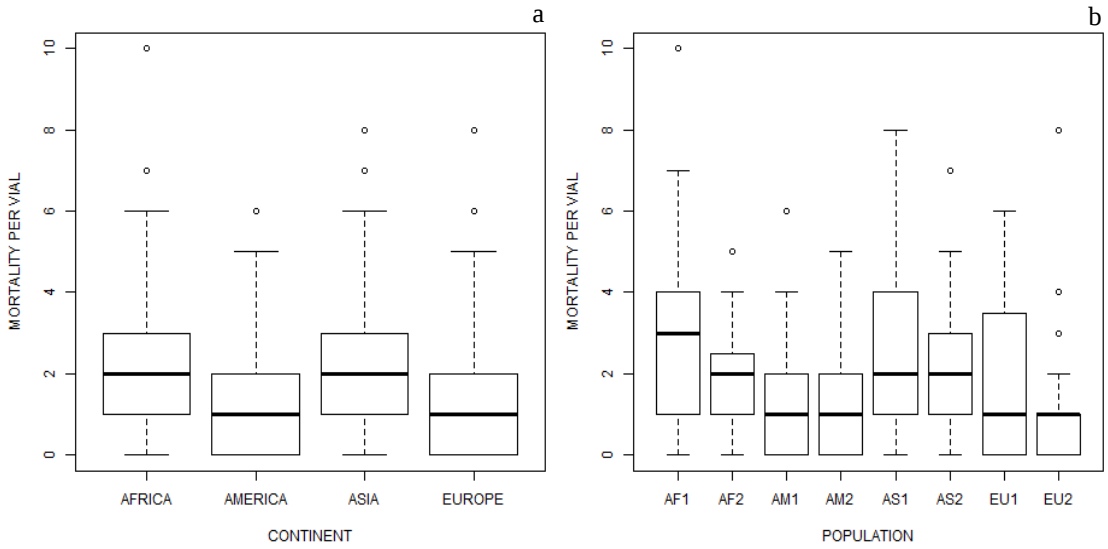

Figure 12 (a) Box plot representing the number of dead flies per vial during the first three days after treatment for each continent. (b) Box plot representing the number of dead flies per vial during the first three days after treatment for each population. There are initially approximately 15 flies per vial. (AF1 = Africa 1, AF2 = Africa 2, AM1 = America 1, AM2 = America 2, AS1 = Asia 1, AS2 = Asia 2, EU1 = Europe 1, EU2 = Europe 2).

"Group" was not significantly increasing the likelihood neither in the "Continent" nor in the "Population" analysis and therefore was dropped from the models. "Treatment" was significant both in the "Continent" and in the "Population" analysis. In the "Continent" analysis "America" and "Europe" were significantly different from "Africa" (table 6).

	Treatment	America	Europe
p-value	0.000478	0.001218	0.024138

Table 6 P-values of the significant covariates in the “Continent” analysis.

In the “Population” analysis the “Africa 2”, “America 1”, “America 2”, “Asia 2”, “Europe 1” and “Europe 2” were significantly different from “Africa 1” (table 7). Mortality was higher in control than in infected flies, most likely due to the negative effects of the oil (see above). “America” and “Europe” show the highest vigour and “America 1”, “America 2” and “Europe 2” are the populations with the lowest mortality (see figure 12).

	Treatment	Africa 2	America 1	America 2
p-value	0.000263	0.003636	0.00089	8.47E-05
	Asia 2	Europe 1	Europe 2	
p-value	0.020273	0.040411	9.72E-05	

Table 7 P-values of the significant covariates (p-value < 0.05) in the “Population” analysis.

4.1.2.2 Mortality from six days after treatment

4.1.2.2.1 Generalized linear model

All time covariates were retained. The covariate “Group” was not significantly increasing the likelihood neither in the “Continent” nor in the “Population” analysis and therefore was dropped from the models. All time covariates and “Treatment” were significant both in the “Continent” and in the “Population” analysis (table 8, 9).

	Time	Log Time	Time squared	Treatment
p-value	1.80e-12	0.000274	< 2e-16	2.64e-16

Table 8 P-values of the significant covariates in the “Continent” analysis.

Also in this case there is no evidence for a difference in susceptibility to fungal infection among continents and populations. “Europe 2” shows significantly higher vigour than “Africa 1” (see table 9).

	Time	Log Time	Time squared	Treatment	Europe 2
p-value	1.82e-12	0.000273	< 2e-16	3.69e-09	0.035993

Table 9 P-values of the significant covariates in the "Population" analysis.

4.1.2.2.2 Kaplan-Meier estimator

The Kaplan-Meier survival curve for each continent is shown in figure 13. Infected flies suffered a higher mortality rate than control ones. This plot does not suggest any difference in susceptibility among continents.

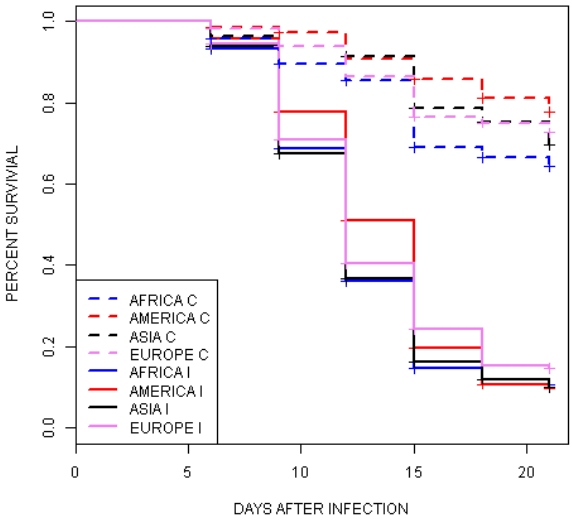

Figure 13 Kaplan-Meier plot representing survival curves from six days after infection for all continents.

The Kaplan-Meier survival curve for each pair of replicate populations is reported in figure 14. There is some variability among replicate populations, especially for the treatments of the two American and of the two Asian populations. However, note that the mortality is higher than for experiment 1 above (figure 9).

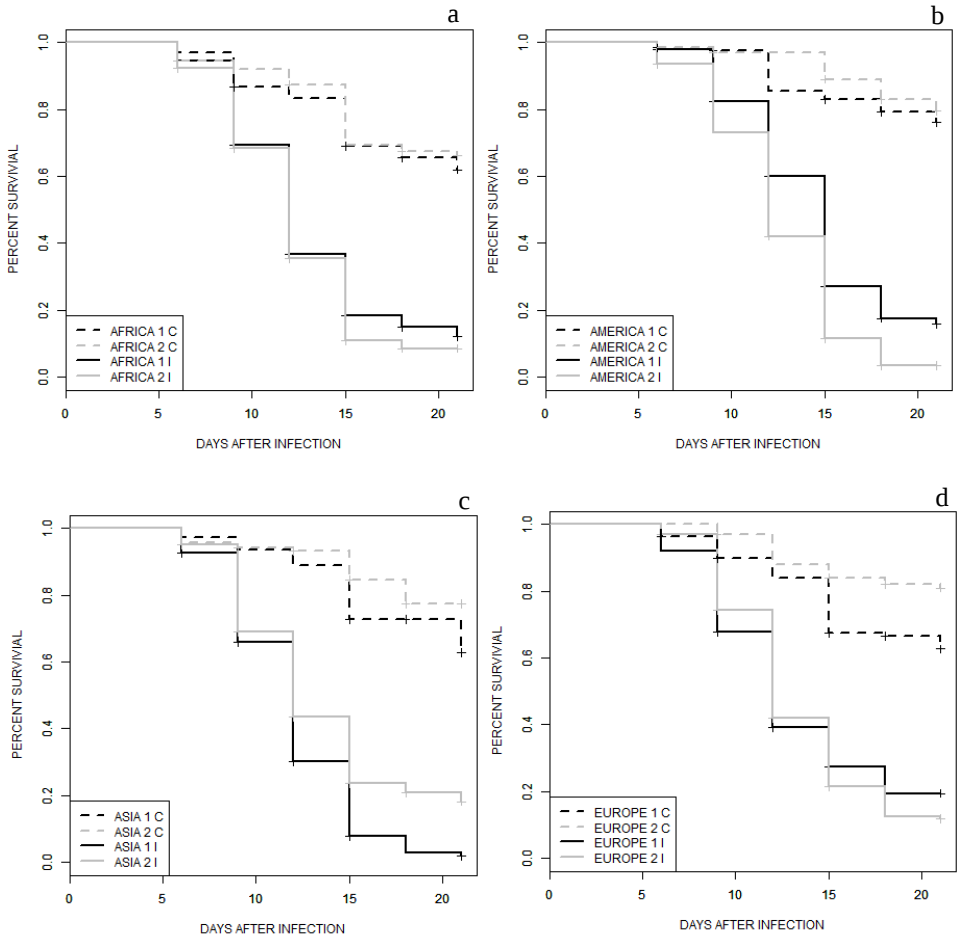

Figure 14 Kaplan-Meier plots for pairs of replicate populations. (a) represents the African replicates, (b) represents the American replicates, (c) represents the Asian populations and (d) represents the European populations.

4.1.2.2.3 Cox hazard model

"Group" did not significantly increase the likelihood neither in the "Continent" nor in the "Population" analysis and therefore was dropped from the models. "Treatment" was significant both in the "Continent" (p-value = 9.4e-14) and in the "Population" analysis (p-value = 2.2e-06). In the "Continent" analysis "America" was significantly different from "Africa" (2.3e-02). In the "Population" analysis "America 2" and "Europe 2" were significantly different from "Africa 1" (p-value = 3.6e-02 and p-value=2.8e-02, respectively). There is no evidence of different susceptibility to infection among continents and populations. "America" has a higher vigour than the other continents and "America 2" and "Europe 2" are more fit than the other populations independently of treatment.

4.1.3 Third experiment

The mean spores concentration in the oil/spore suspension was 1.7 x 10^8 spores/ml with a standard deviation of 0.95 x 10^8 spores/ml (10 independent measurements were performed). The mean spore density on the ink-jet transparency film was 13,693 spores/mm^2 with a standard deviation of 6,791 spores/mm^2 (10 independent measurements were performed). Note that 12 vials were discarded due to contamination.

4.1.3.1 Mortality three days after treatment

As expected control flies suffered higher mortality than infected ones (figure 15), but not significantly higher.

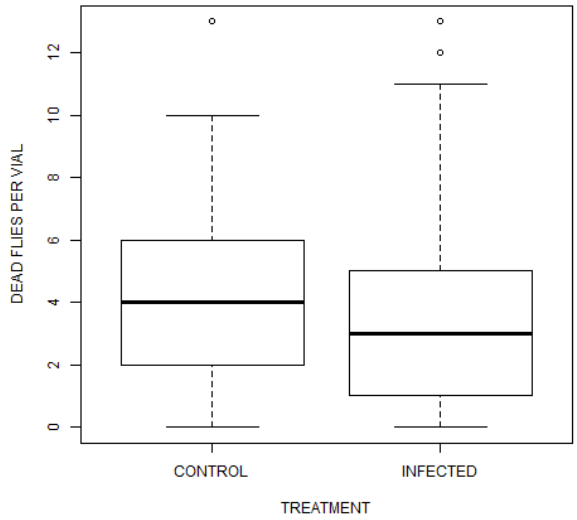

Figure 15 Box plot representing the number of dead flies per vial during the first three days after treatment.

Mortality was not homogeneous among continents and populations (figure 16). In the "Continent" analysis "America" was significantly different from "Africa" (p-value = 9.04e-09). In the "Population" analysis "Africa 2", "America 1", "America 2", "Asia 2", "Europe 1" and "Europe 2" were significantly different from "Africa 1" (table 10).

	Africa 2	America 1	America 2	Asia 2	Europe 1	Europe 2
p-value	5.63e-05	2.94e-10	2.50e-08	0.000151	0.042967	5.12e-06

Table 10 P-values of the significant covariates in the "Population" analysis.

Once again the higher mortality of the control flies has likely to do with the negative effects of the oil. Flies from “America” have higher vigour than the ones from other continents and the American populations are more fit than the others (see figure 16).

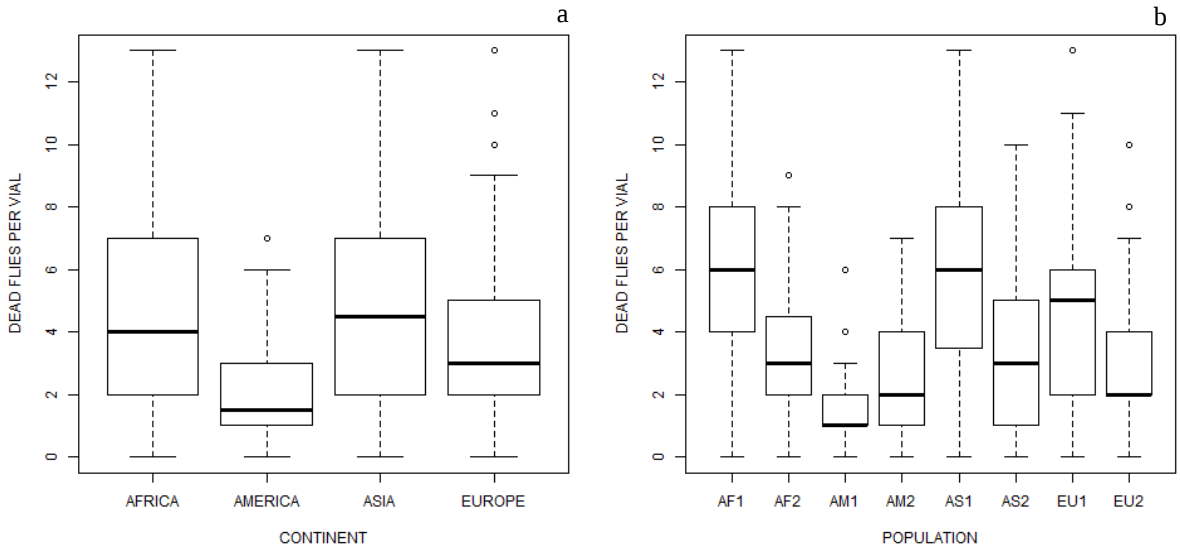

Figure 16 (a) Box plot representing the number of dead flies per vial during the first three days after treatment for each continent. (b) Box plot representing the number of dead flies per vial during the first three days for each population (AF1 = Africa 1, AF2 = Africa 2, AM1 = America 1, AM2 = America 2, AS1 = Asia 1, AS2 = Asia 2, EU1 = Europe 1, EU2 = Europe 2).

4.1.3.2 Mortality from six days after treatment

4.1.3.2.1 Generalized linear model

For the “Continent” and “Population” analyses two best models were found: one with “Log time” and “Time” as time covariates (model 1) and the other with “Log time” and “Time squared” as time covariates (model 2). The use of all three time covariates did not increase the likelihood significantly. “Treatment” and the time covariates were significant in each model both in the “Continent” and in the “Population” analysis (table 11, 12, 13, 14). In the “Continent” analysis “America” was significantly different from “Africa” (table 11, 12).

	Log time	Time	Treatment	America
p-value	1.56e-06	0.0358	1.43e-07	0.0465

Table 11 P-values of the significant covariates in the “Continent” analysis (model 1).

	Log time	Time squared	Treatment	America
p-value	4.63e-12	0.0416	1.43e-07	0.0465

Table 12 P-values of the significant covariates in the “Continent” analysis (model 2).

In the “Population” analysis “America 1” was significantly different from “Africa 1” (table 13, 14).

	Log time	Time	Treatment	America 1
p-value	1.58e-06	0.035930	0.000189	0.049468

Table 13 P-values of the significant covariates in the “Population” analysis (model 1).

	Log time	Time squared	Treatment	America 1
p-value	4.71e-12	0.041706	0.000189	0.049475

Table 14 P-values of the significant covariates in the “Population” analysis (model 2).

Based on these results there is no significant difference in susceptibility among continents and populations. “America” has a significantly higher vigour than the other continents, and “America 1” is significantly more fit than the other populations independently of treatment (see figures 17,18).

4.1.3.2.2 Kaplan-Meier estimator

The Kaplan-Meier survival curve for each continent is reported in figure 17. Infected flies suffered a higher mortality than control ones; the American population performed better than the other populations both for infected and control flies. The plot suggests no difference in susceptibility among continents and a reduced mortality in control and infected American flies compared to the ones from other continents. The Kaplan-Meier survival curve for each pair of replicate populations is reported in figure 18. There is some variability among replicate populations, especially for the infected flies of the two European populations.

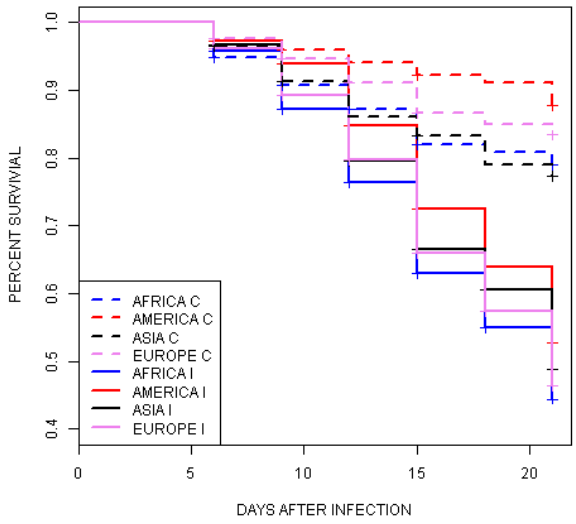

Figure 17 Kaplan-Meier plot representing survival curves from six days after infection for all continents.

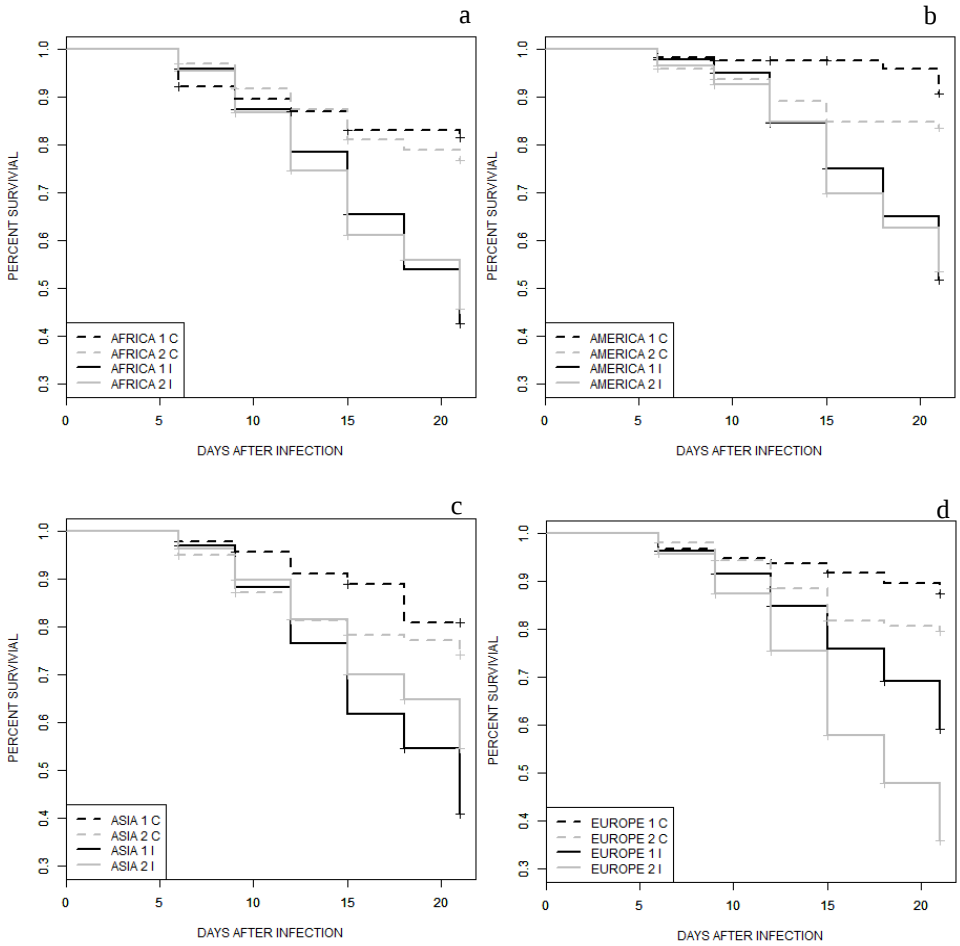

Figure 18 Kaplan-Meier plots for pairs of replicate populations. (a) represents the African replicates, (b) represents the American replicates, (c) represents the Asian populations and (d) represents the European populations.

4.1.3.2.3 Cox hazards model

"Treatment" was significant both in the "Continent" (p-value = 2.4e-07) and in the "Population" analysis (p-value = 0.00015). In the "Continent" analysis "America" was significantly different from "Africa" (p-value = 4.3e-02). In the "Population" analysis "America 1" was significantly different from "Africa 1" (p-value = 0.04800).

These results do not support any difference in susceptibility to fungal infection among continents and populations, but support the higher vigour of the flies from "America" and especially from the "America 1" population.

4.2 *D. MELANOGASTER* TRANSCRIPTIONAL RESPONSE TO *B. BASSIANA*

4.2.1 Micro-array analysis

4.2.1.1 Eight-nodes analysis

4.2.1.1.1 Induced genes

14,229 genes (from a total of 14,338) were retained in the BAGEL analysis. With a false discovery rate of 6.7 % we found between 18 and 30 genes induced in each population (table 15).

AFRICA UP	AFRICA DOWN	AMERICA UP	AMERICA DOWN	ASIA UP	ASIA DOWN	EUROPE UP	EUROPE DOWN
CG10637	CG12028	CG9094	CG1314	CG6342	CG14454	CG40339	CG14674
CG10247	CG9919	CG6342	CG30496	CG8833	CG4136	CG31415	CG10533
CG10174	CG3221	CG31422	CG7638	CG11373	CG11720	CG16848	CG2160
CG32450	CG5258	CG40002	CG7735	CG3000	CG10245	CG3301	CG10501
CG11373	CG1539	CG30361	CG11951	CG11354	CG12001	CG15313	CG12042
CG12758	CG9490	CG14095	CG6759	CG9108	CG8615	CG1244	CG6506
CG11663	CG15368	CG3600	CG12798	CG13361	CG12736	CG7321	CG18358
CG3878	CG40339	CG30273	CG15539	CG12699	CG40270	CG15414	CG15736
CG5475		CG12224	CG12278	CG31868	CG18478	CG12559	CG8884
CG1221		CG7833	CG5902			CG11329	CG15454
CG7535		CG15118	CG13195				CG17035
CG30154			CG30061				CG14095
CG3608			CG15381				CG11598
CG11425			CG4019				CG30496
CG12092			CG17136				CG17707
CG3245			CG12913				
CG7654			CG12050				
CG32476			CG5537				
			CG32476				

Table 15 Genes induced upon infection in each population (FDR: 6.7%). "UP" stays for genes up-regulated and "DOWN" for genes down-regulated following infection. For each gene the annotation symbol is reported.

4.2.1.1.2 Gene ontology enrichment

Genes down-regulated in the African population upon infection show enrichment for the GO category "ecdysteroid 22-hydroxylase activity" (p-value: 0.000579), but this was not significant after multiple tests correction (FDR q-value: 1). We did not find enrichment for any other GO category in genes over-expressed in infected or control flies for any other population.

4.2.1.1.3 Genes shared among populations

Genes up-regulated after infection in two or more populations are reported in table 16. Functional and biological information is available for the gene CG6342 (iron-responsive element binding;

aconitate hydratase activity; regulation of translational initiation by iron). The gene CG30496 is down-regulated upon infection in the American and European populations.

POPULATIONS	GENE
AFRICA_ASIA	CG11373
AMERICA_ASIA	CG6342

Table 16 Genes up-regulated after infection (FDR 6.7%) in two or more populations; for each gene the annotation number is reported.

4.2.1.1.4 Genes shared with other studies

We refer to Roxstrom-Lindquist et al. (2004) as “Roxs04” and to De Gregorio et al. (2001) as “DeGreg01” in the rest of the Results section.

The gene CG11425 is up-regulated both in the African population and in Roxs04 and is known to have phosphatidate phosphatase activity and to be involved in dephosphorylation. The gene CG3301 is up-regulated both in the European population and in DeGreg01 and is known to have oxidoreductase activity and to be involved in metabolic process.

No gene was down-regulated both in one or more populations of the present study and in Roxs04. The gene CG4019 is down-regulated both in the American population and in DeGreg01 and is known to have water channel activity and to be involved in renal system process.

4.2.1.1.5 Conclusions about the population analysis

Few genes are significantly induced in this study, less than in Roxs04 and in DeGreg01. The gene ontology analysis did not reveal enrichment for any meaningful GO category and no GO category was significant after multiple tests correction. Only few genes show the same expression pattern among populations or are shared with the two other studies cited above.

These results indicate that transcriptional response to infection is absent or just starting 8 hours after infection and no clear pattern appears from the data. These findings differ to a large extent from what we observed in the RNA sequencing analysis (see below). Also in the RNAseq analysis we found few genes induced 8 hours after infection but they have a more coherent function and are mainly involved in detoxification (see below). The difference among the two studies possibly relies on the higher power of the RNA sequencing technology and on the intrinsic noise of the micro-array

experiments (Wang et al. 2009) that may not be able to detect the low signal produced by infection on gene expression at an early time point.

4.2.1.2 Two-nodes analysis

4.2.1.2.1 Induced genes

14,229 genes (from a total of 14,338) were retained in the BAGEL analysis. There were 27 genes induced with a FDR of 7.4 % (see table 17).

UP	DOWN
CG40002	CG11430
CG8833	CG15690
CG6342	CG32297
CG2746	CG1555
CG40040	CG31200
CG8038	CG11951
CG11373	CG3221
CG18135	CG6283
CG31422	CG11502
CG10605	CG12249
CG10637	CG4841
CG1362	CG7735
CG12602	CG11893
CG10174	

Table 17 Induced genes (FDR 7.4 %). "UP" denotes genes up-regulated and "DOWN" genes down-regulated following infection.

4.2.1.2.2 Gene ontology enrichment

We did not find enrichment for any GO category in genes over-expressed in infected or control flies, or for any population and at any false discovery rate.

4.2.1.2.3 Genes shared with other studies

No gene was up-regulated following infection both in the present study and in Roxs04 or in DeGreg01. The gene CG6283 is down-regulated after infection both in the present study and in Roxs04 and is known to have triglyceride lipase activity and to be involved in lipid metabolic process. No gene was down-regulated upon infection both in the present study and in DeGreg01.

4.2.1.2.4 Conclusions about the two-nodes analysis

As for the “Eight-nodes analysis” few genes were found to be induced upon infection in comparison to DeGreg01 and Roxs04. The gene ontology analysis did not reveal enrichment for any GO

category, and no clear picture emerges from the analysis. Again the reason could be the inadequacy of the micro-array technology to detect the low signal present at the early time point after infection that was chosen (see above).

4.2.2 RNA sequencing analysis

4.2.2.1 Sample quality control

All samples, with the exception of one American control biological replicate, passed the RNA quality control. Therefore only two American control biological replicates were used in the following analyses.

4.2.2.2 Principal Components Analysis (PCA)

Principal component analysis of expression profile was performed and results are presented in figure 19. Control flies and 8 hours infected flies cluster together and are almost undistinguishable (figure 19a). This is in agreement with the micro-array results. On the other hand, 24 hours infected flies show a different pattern, meaning that infection has a significant effect on gene expression at this time point. Difference among populations is also present, with the American flies clustering apart from the others (figure 19b).

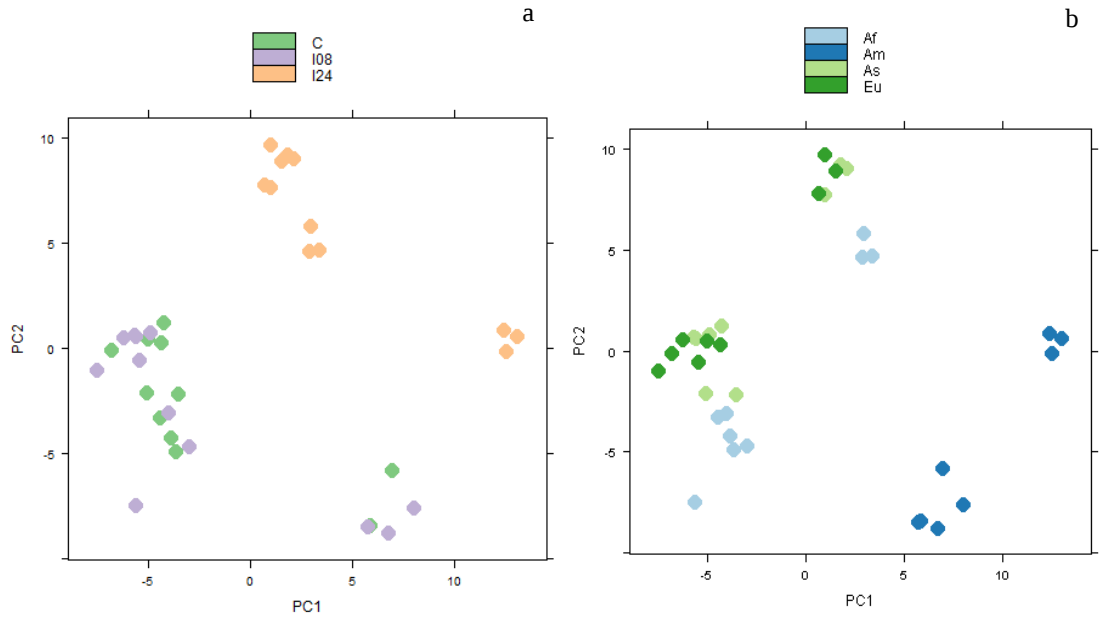

Figure 19 PCA plots. (a) colours define different treatments,"C" stands for control, "I08" for 8 hours infected and "I24" for 24 hours infected flies. (b) colours define different populations, "Af" stands for Africa, "Am" for America, "As" for Asia and "Eu" for Europe.

4.2.2.3 Control and eight hours infected flies

In the following sections transcriptional response eight hours after infection is described and the corresponding induced genes are discussed. First we start by describing the result of the expression analysis considering each population independently.

4.2.2.3.1 Volcano plots

Volcano plots, reporting FDR corrected p-values as a function of fold change for all genes, are presented in figure 20 for each population. The first notable observation is that few genes show a significant change in expression. This is in agreement with the PCA and the micro-array results (see above). Not all the populations show the same pattern of transcriptional response: the American flies have more genes significantly induced than all the other populations. On the other hand only one gene is significantly induced in the African population. These results suggest that the American population is the fastest to mount a transcriptional response to infection.

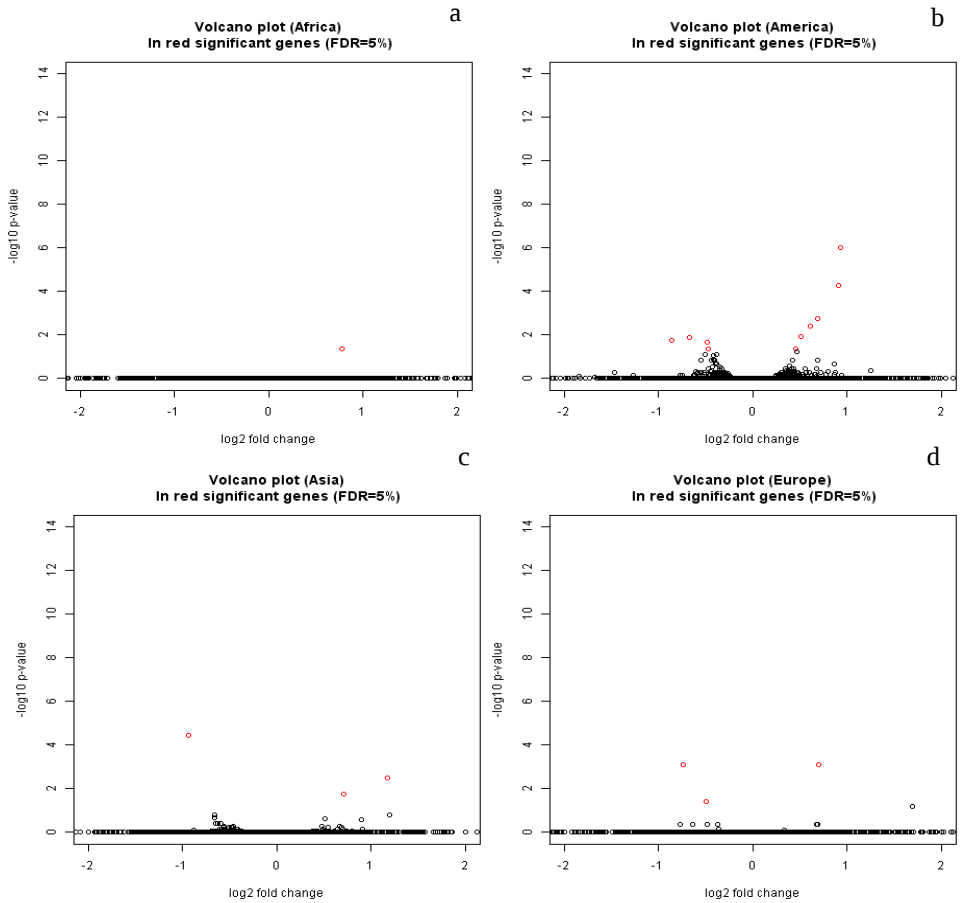

Figure 20 Volcano plots for genes induced 8 hours after infection in each population. The x axis represents gene expression in terms of log2 fold change: a positive value on this axis means that a gene is up-regulated upon infection, while a negative value indicates that is down-regulated. The y axis represents significance level in terms of -log10 of the Benjamin-Hochberg corrected p-value: a high value on this axis means that the difference in gene expression is highly significant; genes with significant p-value (FDR 5%) are indicated in red. (a) is for the African population, (b) is for the American population, (c) is for the Asian population and (d) is for the European population.

4.2.2.3.1 Induced genes

Significantly induced genes (FDR 5%) are reported in table 18. The first notable result is that in each population there is at least one up-regulated gene member of the cytochrome P450 family: Cyp6a8 (CG10248) in Africa, *Cyp6a2* (CG9438) and *Cyp4e2* (CG2060) in America and *Cyp6d2* (CG4373) in Asia and Europe. The cytochrome P450s family consists of 83 functional genes in *D. melanogaster* (Tijet et al. 2001). These genes code for enzymes that catalyze chemical reactions important in development, detoxification of foreign substances and insecticide resistance (Tijet et al. 2001, Thomas 2007, Chung et al. 2009, Giraudo et al. 2010, Kleinhesselink et al. 2011, Thomas et al. 2013). *Cyp6a8* is known to be expressed in *D. melanogaster* malpighian tubules, *Cyp6a2* and *Cyp4e2* both in larvae midgut and malpighian tubules and *Cyp6d2* in the tracheal system (Chung et al. 2009).

Malpighian tubules is an excretory organ (Beyenbach et al. 2010) important for hemolymph detoxification and has recently been proposed to play a role in insect immunity (Davies et al. 2012). On the other side the tracheal system serves in insects for the transport of oxygen through the cuticle and throughout the body (Cabernard et al. 2004) and is a hot spot for fungal penetration of host cuticle (Pekrul and Grula 1979, Gillespie et al. 2000, Sahayaraj et al. 2013).

The up-regulation of several cytochrome P450 genes early after infection suggests that detoxification of toxic compounds produced by the fungus is of primary importance during the first phase of defence response. Indeed entomopathogenic fungi are known to produce toxic compounds during infection (see Introduction). The prevalence of Cyp450s among the induced genes early after infection also prompts the intriguing hypothesis of their involvement in sensing the infection and triggering the defence response.

The metabolism of drugs and toxic compounds usually happens in three steps (Parvez and Reiss 2001). The first one is modification, meaning the insertion of a polar group in the target compound, and could happen by oxidation, reduction or hydrolisis. Cyp450s can catalyze both oxidative and reductive reactions. The second step is conjugation. In this step the modified toxic compounds are conjugated with negatively charged species as glutathione (GSH), sulfate, glycine, or glucuronic acid. In the third step conjugated compounds are eventually further metabolised and finally actively excreted.

Looking from this perspective CG2064 and CG9360 that have oxidoreductase activity could be involved in the first phase of toxic compounds metabolism. On the other hand CG5724 that has a glucuronosyltransferase activity is clearly involved in phase two metabolism, as could be the case for CG10182 that has an acyl group transfer activity. In conclusion it emerges the importance of detoxification in early response to infection.

AFRICA I08	AFRICA C	AMERICA I08	AMERICA C	ASIA I08	ASIA C	EUROPE I08	EUROPE C
CG10248		CG9438	CG34387	CG4373	CG15263	CG4373	CG6484
		CG2060	CG42795	CG10182			CG6385
		CG9360	CG14446				
		CG2064	CG9907				
		CG5724					
		CG31104					

Table 18 Significant induced genes in each population."I08" stays for genes over-expressed in 8 hours infected flies, "C" stays for gene over-expressed in control files.

4.2.2.3.1 Gene ontology analysis

In Appendix table 7 gene ontology categories showing enrichment in genes up- or down-regulated upon infection in each population are presented. The American population has more significant GOs, as is to be expected given the higher number of induced genes in this population. No GO category is significant after multiple tests correction, possibly due to the low number of induced genes at this time point.

4.2.2.3.1 Comparison among populations

Only one gene is up-regulated upon infection in two populations: *Cyp6d2* (CG4373). This gene is seen both in Asia and Europe and is up-regulated in the African and American populations, although not significantly after multiple tests correction. As already discussed above, *Cyp6d2* is known to be expressed in the tracheal system and could therefore be involved in early response to fungal infection.

4.2.2.3.1 Comparison with other studies

We could not find any gene induced both in this study and in Roxs04. Three genes are induced both in the present analysis and in DeGreg01: CG9360 that has an oxido-reducatase activity and is involved in metabolic process, *Cyp4e2* (CG2060) and CG15263, whose molecular and biological function are unknown. Interestingly we could not find any gene induced both in the present and in

the microarray analysis. This is quite surprising given that in both cases the same time point after infection was chosen (see Discussion).

4.2.2.3.2 Treatment analysis

In this section we show the results of the expression analysis considering the general effect of treatment.

4.2.2.3.2 Volcano plot

The volcano plot is presented in figure 21. As already observed in the "populations" analysis, the number of induced genes is low. Interestingly all induced genes are up-regulated upon infection.

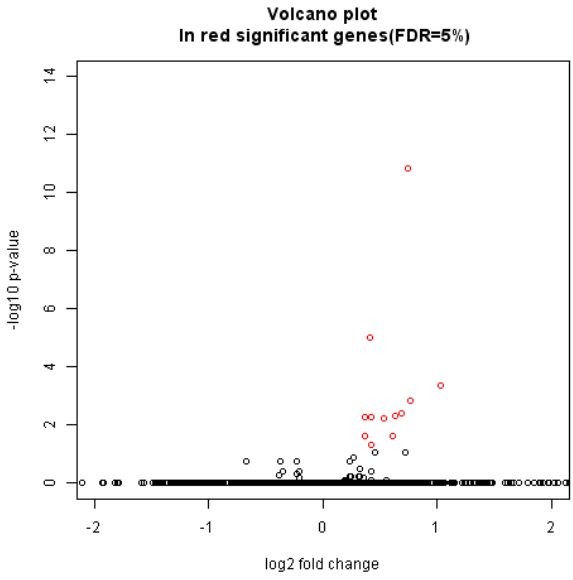

Figure 21 Volcano plot for treatment analysis.

4.2.2.3.2 Induced genes

Significantly induced genes (FDR 5%) are reported in table 19. In this analysis only one gene from the cytochrome P450 family was found: *Cyp6d2* (CG4373) (see above). The other Cyp450s found in the "population analysis" could be involved in population specific responses to early stage fungal infection. CG2064 has an oxido-reductase and CG5371 a reductase activity and could possibly be involved in the first step of toxic compounds metabolism.

Three genes likely involved in the second phase of toxic compounds metabolism are CG4371 that has a glutathione transferase activity, CG13270 with a glucuronosyltransferase activity, and possibly CG10182 that has an acyltransferase activity.

Smvt (CG2192) and CG30272 are important in transmembrane transport and could be involved in the excretion of metabolised xenobiotics.

Finally CG13160, CG33012 and *Jonah 65Aii* (CG6580) code for proteolytic enzymes and possibly take part in a not yet characterised defence mechanism.

I08	C
CG10182	
CG4371	
CG4373	
CG13160	
CG13270	
CG30272	
CG33012	
CG5371	
CG6580	
CG2064	
CG2069	
CG2191	

Table 19 Significantly induced genes in each population.

4.2.2.3.2 Gene ontology analysis

No gene ontology category was enriched in the induced gene set. This could be due to the low number of induced genes.

4.2.2.3.2 Comparison with other studies

We could not find any gene induced both in this analysis and in Roxs04, DeGreg01 or in the micro-array analysis (see above). Once again the difference could be due to the lower power of the micro-array analysis (see above).

4.2.2.4 Control and twenty-four hours infected flies

In the following part, we show the transcriptional response 24 hours after infection. First, the results of the expression analysis considering each population independently are shown.

4.2.2.4.1 Volcano plots

Volcano plots are presented in figure 22. The first evident result is that many more genes are induced at 24 than at 8 hours after infection. This indicates that strong transcriptional response to infection begins between these two time points. Interestingly there is quite a big variability in the number of induced genes among populations, from more than 1,000 in the African population to only 200 in the Asian one. The expression fold change of up- and down-regulated genes also varies and is especially high in the Asian population and low in the European one. These peculiarities may reflect different strategies of sensing and responding to fungal infection between *D. melanogaster* populations.

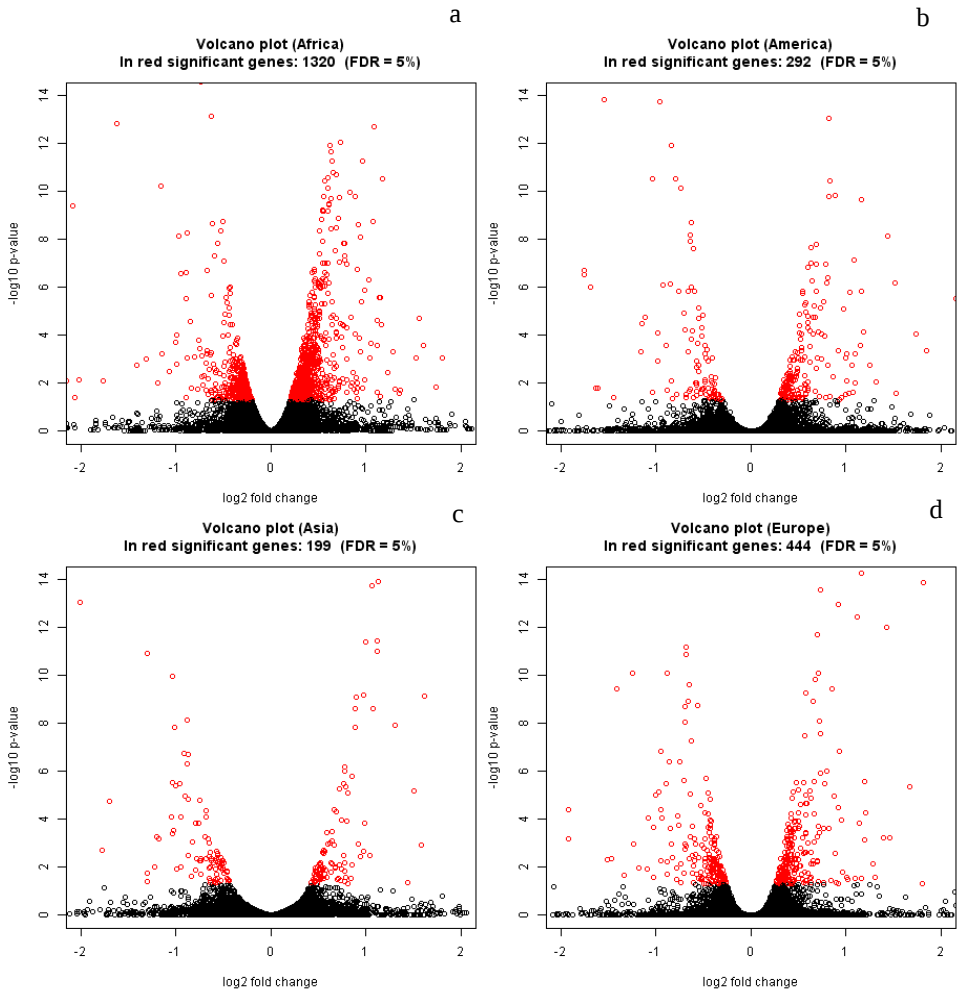

Figure 22 Volcano plots for genes induced 24 hours after infection in each population.

4.2.2.4.1 Induced genes

In table 20 the number of genes up- or down-regulated upon infection in each population is shown.

AFRICA I24	AFRICA C	AMERICA I24	AMERICA C	ASIA I24	ASIA C	EUROPE I24	EUROPE C
861	459	189	103	104	95	249	195

Table 20 Number of significantly induced genes in each population.

4.2.2.4.1 Gene ontology analysis

In the following sections gene ontology categories enriched in genes up- and -down regulated upon infection are reported for each population. Due to the high number of significant categories only the ones related to biological process (see 3.2.1.8) are reported here.

4.2.2.4.1 African population

274 GOs are significantly enriched in genes up-regulated upon infection. The ones involved in response to stimulus, immunity, circadian rhythm and tracheal system are reported in Appendix table 8. Several GOs enriched in up-regulated genes are involved in response to stimulus. These processes are possibly connected with sensing the infection. Some categories are involved in immunity and the tracheal system. This last group is of interest as fungi penetrate through the host cuticle and the trachea openings are an ideal spots for penetration (see above). Finally it is worth noting that gene ontology categories involved in circadian rhythm are also enriched. This is most likely due to the time when flies were sampled: control flies were sampled in the evening, while 24 hours infected flies in the morning.

Given their hign number, GOs significantly enriched in genes down-regulated upon infection are visible in Appendix table 9. The majority of these categories is involved in energy production, reproduction and biosynthesis. This suggests that infected flies may reduce non-essential metabolic and biological processes to mount a response to infection.

While we touch upon only a portion of all significant GOs, the high number of significant categories and the different biological processes in which they are involved suggest that fungal infections trigger a complex transcriptional response in *D. melanogaster*. The higher number of induced genes and enriched GOs in the African population is of particular interest and will be

addressed in the discussion.

4.2.2.4.1 American population

The GOs enriched in genes up-regulated after infection are reported in table 21. Among them there are: "Toll signalling pathway" that is known to be involved in humoral immunity (Lemaitre 1996), "response to organic substance", "response to abiotic stimulus" and "response to insecticide" that have likely to do with sensing the infection, and "circadian rhythm" that probably reflects the time at which flies were sampled (see above). None of these categories, however, is significant after multiple-testing correction (see table 21).

GO Term	Description	p-value	FDR q-value
GO:0055114	oxidation-reduction process	3.22E-06	1.71E-02
GO:0009072	aromatic amino acid family metabolic process	7.13E-06	1.89E-02
GO:0044710	single-organism metabolic process	7.43E-06	1.31E-02
GO:0008063	Toll signaling pathway	1.88E-04	2.49E-01
GO:0019752	carboxylic acid metabolic process	4.62E-04	4.90E-01
GO:0010033	response to organic substance	5.57E-04	4.92E-01
GO:0009628	response to abiotic stimulus	5.97E-04	4.52E-01
GO:0006573	valine metabolic process	6.69E-04	4.43E-01
GO:0006572	tyrosine catabolic process	6.69E-04	3.94E-01
GO:0043436	oxoacid metabolic process	6.76E-04	3.58E-01
GO:0006082	organic acid metabolic process	6.76E-04	3.26E-01
GO:0017085	response to insecticide	8.57E-04	3.79E-01
GO:0007623	circadian rhythm	9.07E-04	3.70E-01

Table 21 Gene ontology categories enriched in genes up-regulated after infection in the American population.

The GOs enriched in genes down-regulated after infection are reported in table 22. The significance of most categories, with the exception of "organic cation transport" and "transmembrane transport", is due to two circadian genes: *timeless* and *period* (data not shown). These categories may be a consequence of the sampling approach (morning sampling). The two other categories may not appear to be clearly connected with the infection process. No GO is significant after multiple-testing correction (see table 22).

GO Term	Description	p-value	FDR q-value
GO:0001659	temperature homeostasis	6.99E-05	3.70E-01
GO:0060086	circadian temperature homeostasis	6.99E-05	1.85E-01
GO:2000678	negative regulation of transcription regulatory region DNA binding	2.08E-04	3.68E-01
GO:2000677	regulation of transcription regulatory region DNA binding	2.08E-04	2.76E-01
GO:0043392	negative regulation of DNA binding	4.15E-04	4.40E-01
GO:0015695	organic cation transport	4.15E-04	3.66E-01
GO:0051100	negative regulation of binding	4.15E-04	3.14E-01
GO:0048871	multicellular organismal homeostasis	6.87E-04	4.56E-01
GO:0055085	transmembrane transport	7.01E-04	4.13E-01

Table 22 Gene ontology categories enriched in genes down-regulated after infection in the American population.

4.2.2.4.1 Asian population

The GOs enriched in genes up-regulated after infection are reported in table 23. Among them there are: "response to organic cyclic compound", "response to organic substance" and "response to alkaloid" that are possibly involved in sensing the fungal infection. Nevertheless only "response to organic compounds" is significant after multiple-tests correction (see table 23).

GO Term	Description	p-value	FDR q-value
GO:0014070	response to organic cyclic compound	7.90E-06	4.19E-02
GO:0050962	detection of light stimulus involved in sensory perception	6.82E-05	1.81E-01
GO:0010033	response to organic substance	3.53E-04	6.24E-01
GO:0055114	oxidation-reduction process	7.61E-04	1.00E+00
GO:0043279	response to alkaloid	9.29E-04	9.86E-01

Table 23 Gene ontology categories enriched in genes up-regulated after infection in the Asian population.

The GOs enriched in genes down-regulated upon infection are reported in table 24. We expect that most of them, with the exception of "mating", "multicellular organism reproduction, "reproduction", "multi-organism reproductive process" and "response to DDT", are due to the circadian genes: *timeless* and *period* (data not shown). These categories may appear due to the sampling approach (see above). The categories involved in reproduction may reflect the decrease of biological functions not essential for response to fungal infection (see above).

GO Term	Description	p-value	FDR q-value
GO:0032504	multicellular organism reproduction	1.70E-05	9.00E-02
GO:0000003	reproduction	2.34E-05	6.20E-02
GO:0007618	mating	3.55E-05	6.28E-02
GO:0001659	temperature homeostasis	4.61E-05	6.11E-02
GO:0060086	circadian temperature homeostasis	4.61E-05	4.89E-02
GO:0044703	multi-organism reproductive process	8.34E-05	7.37E-02
GO:2000678	negative regulation of transcription regulatory region DNA binding	1.38E-04	1.04E-01
GO:2000677	regulation of transcription regulatory region DNA binding	1.38E-04	9.12E-02
GO:0043392	negative regulation of DNA binding	2.74E-04	1.61E-01
GO:0051100	negative regulation of binding	2.74E-04	1.45E-01
GO:0046680	response to DDT	2.74E-04	1.32E-01
GO:0048871	multicellular organismal homeostasis	4.55E-04	2.01E-01
GO:0007620	copulation	9.46E-04	3.86E-01
GO:0009648	photoperiodism	9.46E-04	3.58E-01

Table 24 Gene ontology categories enriched in genes down-regulated after infection in the Asian population.

4.2.2.4.1 European population

The GOs enriched in genes up-regulated after infection are reported in table 25. Among them there are several categories involved in response to stimulus or chemical substance, although only "response to organic cyclic compounds" and "response to organic substance" are significant after multiple-testing correction. Several categories are related to immunity and some of them quite specifically against fungi. Nevertheless none of these categories is significant after multiple-testing

correction.

GO Term	Description	p-value	FDR q-value
GO:0014070	response to organic cyclic compound	7.34E-06	3.89E-02
GO:0010033	response to organic substance	1.63E-05	4.32E-02
GO:0051239	regulation of multicellular organismal process	1.07E-04	1.89E-01
GO:0048935	peripheral nervous system neuron development	1.48E-04	1.96E-01
GO:0045087	innate immune response	2.53E-04	2.69E-01
GO:0097305	response to alcohol	3.77E-04	3.33E-01
GO:0006967	positive regulation of antifungal peptide biosynthetic process	4.02E-04	3.04E-01
GO:0002810	regulation of antifungal peptide biosynthetic process	4.02E-04	2.66E-01
GO:0042221	response to chemical stimulus	4.20E-04	2.47E-01
GO:2000026	regulation of multicellular organismal development	4.36E-04	2.31E-01
GO:0050896	response to stimulus	5.62E-04	2.71E-01
GO:1900150	regulation of defense response to fungus	5.94E-04	2.62E-01
GO:0002788	regulation of antifungal peptide production	5.94E-04	2.42E-01
GO:0009605	response to external stimulus	6.58E-04	2.49E-01
GO:0008063	Toll signaling pathway	6.73E-04	2.38E-01
GO:0009636	response to toxic substance	6.94E-04	2.30E-01
GO:0050817	coagulation	8.36E-04	2.61E-01
GO:0050962	detection of light stimulus involved in sensory perception	8.36E-04	2.46E-01
GO:0042381	hemolymph coagulation	8.36E-04	2.33E-01
GO:0007599	hemostasis	8.36E-04	2.22E-01
GO:0006955	immune response	9.63E-04	2.43E-01
GO:0048731	system development	9.79E-04	2.36E-01
GO:0050793	regulation of developmental process	9.80E-04	2.26E-01
GO:0043279	response to alkaloid	9.95E-04	2.20E-01

Table 25 Gene ontology categories enriched in genes up-regulated after infection in the European population.

Given their high number, the GOs enriched in genes down-regulated upon infection are reported in Appendix table 10 Several of them are involved in biosynthetic/metabolic processes and in reproduction. This possibly reflects the decrease of non essential biological processes upon infection.

4.2.2.4.1 Comparison among populations

The number of genes induced privately in each population or jointly in two, three or all four populations is visible in figure 23. We note that the majority of genes is privately expressed in the African population, based on the higher number of induced genes in this population (see table 20). Interestingly 65 genes are induced in all populations, of them 47 are up- and 18 down-regulated upon infection. These genes are natural candidates to understand the *D. melanogaster* response to fungal infection.

Gene ontology categories which are enriched in genes up-regulated in all populations are shown in table 26. Interestingly these GOs do not seem directly involved in immunity. The only two categories significant after multiple-testing correction are "oxidoreducatese activity" and "fatty-acyl-CoA reductase (alcohol-forming activity)".

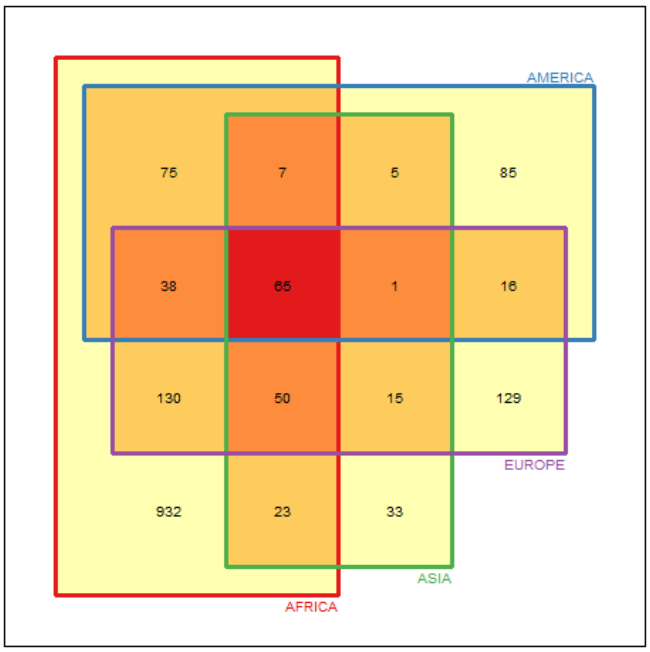

Figure 23 The Venn diagram represents the number of induced genes (both up- and down-regulated) privately in each population or jointly in two or more populations. The diagram was generated using the R library "Vennerable".

The GOs enriched in genes down-regulated upon infection do not appear interesting. Most of them are due to circadian genes *time*, *period* and *vrille* (see above).

BIOLOGICAL PROCESS			
GO Term	Description	p-value	FDR q-value
GO:0014070	response to organic cyclic compound	3.61E-04	1.00E+00
GO:0055114	oxidation-reduction process	5.40E-04	1.00E+00
GO:0050962	detection of light stimulus involved in sensory perception	6.79E-04	1.00E+00
MOLECULAR FUNCTION			
GO Term	Description	p-value	FDR q-value
GO:0016491	oxidoreductase activity	0.0000194	0.0471
GO:0080019	fatty-acyl-CoA reductase (alcohol-forming) activity	0.0000382	0.0463
GO:0003824	catalytic activity	0.000101	0.0821
GO:0016616	oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	0.000261	0.158
GO:0016620	oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	0.00032	0.156
GO:0016903	oxidoreductase activity, acting on the aldehyde or oxo group of donors	0.000645	0.261

Table 26 Gene ontology categories enriched in genes up-regulated after infection in all population. Both GOs involved in biological process and molecular function are reported.

A clearer pattern emerges, however, if, instead of looking at GOs, we focus on some specific induced genes. A subset of genes up-regulated upon infection in all populations is presented in table 27. A first category of interesting genes is composed of two Cyp450 genes. Cyp450 genes are known to be involved, among other things, in detoxification (see above). Interestingly *Cyp6d2* was already found to be induced 8 hours after infection and is known to be expressed in the tracheal system, a potential hotspot for fungal penetration. On the other side *Cyp6g1* is known to be involved in detoxification processes and is expressed specifically in *D. melanogaster* malpighian tubules (Chung et al. 2009). The importance of detoxification is also highlighted by the up-regulation of three genes that are involved in the phase II metabolism of toxic xenobiotics (see

above): CG6649, CG9363, CG18578.

The induction of several genes involved in transmembrane transport could be explained in this context by the transport processes necessary to excrete metabolised toxic substances (see above). The up-regulation of genes involved in oxidation-reduction processes could reflect reactions involved in the modification of toxic compounds (see above) or possibly the energetic needs of excretion (Davies et al. 2012).

CYTOCROMES P450		
GENE	BIOLOGICAL PROCESS	MOLECULAR FUNCTION
CG4373 (*Cyp6d2*)	response to camptothecin	electron carrier activity
CG8453 (*Cyp6g1*)	response to insecticide ...	electron carrier activity
PHASE 2 DETOXIFICATION		
GENE	BIOLOGICAL PROCESS	MOLECULAR FUNCTION
CG6649	glucuronosyltransferase activity	metabolic process
CG9363	glutathione transferase activity	glutathione metabolic process
CG18578	glucuronosyltransferase activity.	metabolic process
TRANSPORT		
GENE	BIOLOGICAL PROCESS	MOLECULAR FUNCTION
CG2191	sodium-dependent multivitamin transmembrane transporter activity	
CG12787	transporter activity	
CG1213	glucose transmembrane transporter activity.	transmembrane transport
CG3380	sodium-independent organic anion transmembrane transporter activity	response to methotrexate
CG4607	substrate-specific transmembrane transporter activity	transmembrane transport
CG31321		transmembrane transport.
OXID-REDUCTION PROCESSES		
GENE	BIOLOGICAL PROCESS	MOLECULAR FUNCTION
CG17560	nucleotide binding; fatty-acyl-CoA reductase (alcohol-forming) activity	
CG17562	nucleotide binding; fatty-acyl-CoA reductase (alcohol-forming) activity	
CG12766	alditol:NADP+ 1-oxidoreductase activity.	oxidation-reduction process
CG7171	urate oxidase activity	allantoin biosynthetic process
CG13091	nucleotide binding; fatty-acyl-CoA reductase (alcohol-forming) activity	
CG7724	oxidoreductase activity, acting on CH-OH group of donors	oxidation-reduction process
CUTICLE		
GENE	BIOLOGICAL PROCESS	MOLECULAR FUNCTION
CG4784	structural constituent of chitin-based cuticle	
CG14534	structural constituent of chitin-based cuticle	chitin-based cuticle development
IMMUNITY		
GENE	BIOLOGICAL PROCESS	MOLECULAR FUNCTION
CG9377		serine-type endopeptidase activity
CG10118 (*pale*)	pigmentation ...	tyrosine 3-monooxygenase activity
CG7356 (*transglutaminase*)	hemolymph coagulation; innate immune response	protein-glutamine gamma-glutamyltransferase activity
CG6667 (*dorsal*)	immune response ...	RNA polymerase II regulatory region sequence-specific DNA binding ...

Table 27 Subset of genes up-regulated upon infection in all populations. Biological process and molecular function information was obtained in Flybase. "..." means that only some term reported in Flybase are shown in the table.

A further interesting category is constituted by two genes that code for structural constituents of cuticle: CG4784 and CG14534, given the importance of cuticle to prevent parasite penetration (see above).

Finally four immunity genes are found. Among them *pale* (CG10118) and *transglutaminase* (CG7356) are involved in the melanization and coagulation process, respectively (see Introduction). The other two genes are *dorsal* (CG6667) that is part of the Toll signalling pathway and CG9377 that is possibly involved in the serine protease cascade up-stream of Toll signalling pathway (see

above).

Conversely no clear pattern appears when looking at genes down-regulated in all populations. Three genes: *timeless*, *period* and *vrille* are involved in circadian rhythm and could reflect the flies sampling approach (see above). One gene, *Cyp18a1* (CG6816) is a member of the Cyp450 family (see above) and is reported to have a "steroid hydroxylase activity", but could also be involved in phase one of toxic compounds metabolism. The other two genes, CG13309 and *Spds* (CG8327), are related to "multicellular organism reproduction" and to "spermidine biosynthetic process", possibly reflecting the decrease of biological processes, such as reproduction, which are not necessary for responding the infection (see above). The detoxification process appears to be the constituent of early transcriptional response to *B. bassiana* infections in *D. melanogaster*.

An interesting question is thus weather genes that are induced in all populations have on average a different expression fold change than genes induced privately in each population. In figure 24 the absolute fold change value of private and common induced genes is compared for each population.

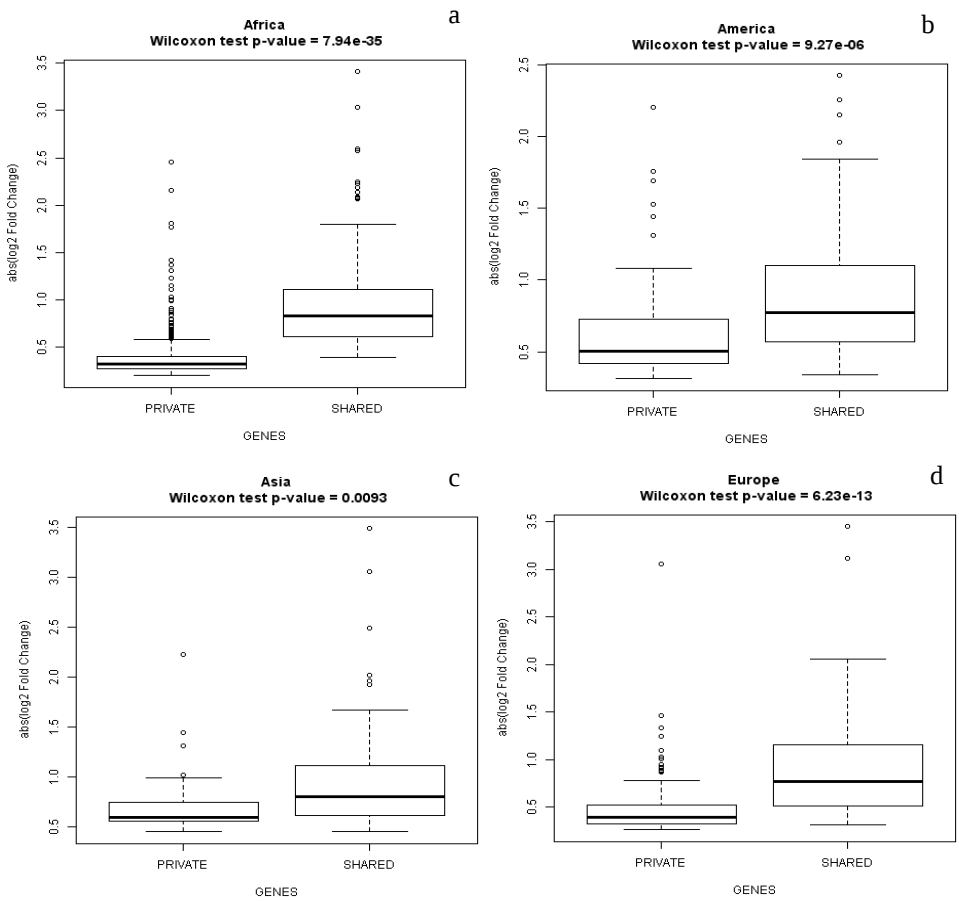

Figure 24 Box plots of the log2 fold change absolute value of privately and commonly induced genes for each population. (a) African population, (b) American population, (c) Asian population and (d) European population. The significance of the difference among the two categories of genes for each population was assessed by performing a Wilcoxon test and the resulting p-value is reported on the top of each graph.

In all populations common induced genes have on average a significantly higher fold change than private induced ones. This pattern is stronger in the African population, where many genes are induced with a low fold change, and weaker in the Asian population, where fewer genes are induced but with a comparably higher fold change (see figure 24).

One one hand, this is not completely unexpected, as genes with a higher fold change are more likely to be significant. On the other hand, it means that looking at genes commonly induced among populations allows us to identify the highly responsive ones (see Discussion).

4.2.2.4.1 Comparison with genes induced eight hours after infection

In table 28, genes induced in one or more populations both at 8 and 24 hours after infection are shown. In table 29 genes induced both in the treatment analysis 8 hours after infection and in one or more populations 24 hours after infection are reported.

	AFRICA 8	AMERICA 8	ASIA 8	EUROPE 8
AFRICA 24		CG9438 CG2064 **CG9907**	*CG4373*	*CG4373*
AMERICA 24		CG2064 CG5724 CG31104	*CG4373*	*CG4373*
ASIA 24			CG15263 *CG4373*	*CG4373*
EUROPE 24		CG9438 CG2064	CG15263 *CG4373*	**CG6484** **CG6385** *CG4373*

Table 28 The table reports genes induced in one or more populations both at 8 and 24 hours after infection; table rows correspond to populations 24 hours after infection, table columns correspond to population 8 hours after infection. Genes induced in all populations 24 hours after infection are indicated in italic; genes privately induced in the same population both at 8 and at 24 hours are indicated in bold.

We first note that *Cyp6d2* (CG4373) is expressed both in the African and Asian population at 8 hours after infection and in all populations at 24 hours after infection. This is of interest, given its possible role in detoxification and the reported expression in the tracheal system (see above).

Paralytic (CG9907) is expressed both at 8 and at 24 hours only in the African population, while CG6484 and CG6385 in the European. *Paralytic* has as molecular function "voltage-gated sodium channel activity" and is involved in several biological processes, among which we find "response to hypoxia", "response to mechanical stimulus" and "response to pyrethroid" (Flybase). CG6484 has as molecular function "glucose transmembrane transporter activity" and is involved in "transmembrae transport". CG6385 has as molecular function "sarcosine dehydrogenase activity" and is involved in "oxidation-reduction process". We suggest that that these genes are involved in

population specific responses to infection and may deserve further investigation.

Cyp6d2 (CG4373) and *Smvt* (CG2191) are induced both in the treatment analysis at 8 hours and in all populations at 24 hours after infection (table 29). Smvt has as biological function "sodium-dependent multivitamin transmembrane" and is involved in "transmembrane transport" (Flybase). It is possible that this gene is important in the transport processes related to excretion (see above).

AFRICA	AMERICA	ASIA	EUROPE
CG5371	CG2064	CG5371	CG5371
CG2064	*CG4373*	CG13160	CG2064
CG13160	*CG2191*	*CG4373*	CG13160
CG4373	CG13270	CG6580	*CG4373*
CG6580	CG30272	*CG2191*	CG6580
CG2191			*CG2191*
			CG13270

Table 29 The table reports genes induced both in the treatment analysis at 8 hours and in one or more populations at 24 hours after infection; table columns correspond to population 24 hours after infection. Genes induced in all populations 24 hours after infection are indicated in italic.

4.2.2.4.1 Comparison with genes induced in the micro-array

Genes that are induced both in this analysis and in the micro-array are reported in tables 30 and 31. In table 30 genes induced both in one or more populations in the present analysis and in the micro-array are shown. CG11951 is induced in the American population in the micro-array and in all populations in the present analysis. Few genes, in bold in table 30, are privately induced in the same population in the two studies.

	AFRICA M	AMERICA M	ASIA M	EUROPE M
	CG7535			
	CG10247			
	CG12758			
	CG3608			CG8884
	CG11373	CG6342	CG6342	CG1244
AFRICA 24	**CG1539**	*CG11951*	CG11373	CG31415
	CG10247	**CG12224**	CG10245	
AMERICA 24	CG12092	*CG11951*		
	CG3608			
ASIA 24	CG12092	*CG11951*		
EUROPE 24	CG10247	*CG11951*		

Table 30 The table reports genes induced in one or more populations both in the microarray and at 24 hours after infection in the RNA sequencing analysis; table rows correspond to populations 24 hours after infection in the RNA sequencing, table columns correspond to populations in the microarray study ("M" stands for microarray). Genes induced in all populations in the RNA sequencing are indicated in italic; genes privately induced in the same population in the two analyses are indicated in bold.

In table 31, we show genes induced both in one or more populations in the RNA sequencing and in the micro-array two-nodes analysis. Again CG11951 is induced in all populations in the RNA sequencing and in the micro-array two-nodes analysis and therefore may deserve further

investigation.

The African population in the present analysis has more genes in common with the micro-array study compared to the other populations. This may be due to the extremely high number of induced genes in the African population in the RNA sequencing analysis.

AFRICA	AMERICA	ASIA	EUROPE
CG11502	CG11893	CG11502	CG11502
CG6342	*CG11951*	*CG11951*	CG18135
CG18135	CG31200		*CG11951*
CG11951			
CG11373			
CG31200			

Table 31 Genes induced both in the micro-array treatment analysis and in one or more populations at 24 hours after infection in the RNA sequencing are reported; table columns correspond to populations in the RNA sequencing. Genes induced in all populations are indicated in italic.

4.2.2.4.1 Comparison with other studies

In table 32, the number of genes induced both in Roxs04 and in each population in the present analysis is reported. In table 33, we show genes induced both in DeGreg01 and in all populations in the present analysis. Looking at table 33 the only gene directly involved in immunity is *pale* (CG10118). It has a role in the melanization defence response (see above). CG14516 and CG11951 have a proteolitic activity and may be part of a not yet characterised general defence response to fungal infection. Interestingly CG11951 was already found to be induced in the two-nodes micro-array analysis (see above).

AFRICA	AMERICA	ASIA	EUROPE
62	39	17	25

Table 32 Number of genes induced both in Roxs04 and in each population in the present analysis.

Urate oxidase (CG7171) is specifically expressed in malpighian tubules where it converts uric acid into the more soluble compound allantoin (Wu et al. 1989, Wu et al. 1992). Interestingly this gene is also strongly up-regulated in *D. melanogaster* infected by the bacteria *Listeria monocytogenes*, although the biological meaning of its induction is not clear (Chambers et al. 2012). *Obp99b* (CG7592) is related to autophagic cell death, possibly suggesting a role for apoptosis in fungal

immunity.

GENE	BIOLOGICAL PROCESS	MOLECULAR FUNCTION
CG14516	proteolysis	aminopeptidase activity
CG7171 (*Urate oxidase*)	allantoin biosynthetic process	urate oxidase activity
CG9400		
CG10118 (*pale*)	melanization	
CG11951	proteolysis	aminopeptidase activity
CG11073		
CG7592 (*Obp99b*)	autophagic cell death	odorant binding

Table 33 Genes induced both in Roxs04 and in all populations in the present analysis; biological process and molecular function of each gene is reported as in Flybase when available.

In table 34, the number of genes induced both in DeGreg01 and in each population in the present analysis is indicated.

AFRICA	AMERICA	ASIA	EUROPE
88	50	21	54

Table 34 Number of genes induced both in DeGreg01 and in each population in the present analysis

In table 35, genes induced both in DeGreg01 and in all populations in the present analysis are reported. The only two genes directly involved in immunity are *pale* (CG10118) and *dorsal* (CG6667) (see above). *Cyp18a1* (CG6816) and *Cyp6g1* (CG8453) are members of the Cyp450 family and are likely important in detoxification. *Urate oxidase* (CG7171), *Obp99b* (CG7592) and *pale* (CG10118) are found in all populations in the present analysis and in Roxs04 and DeGreg01 and are therefore likely to be of key importance in response to fungal infection.

GENE	BIOLOGICAL PROCESS	MOLECULAR FUNCTION
CG11407	metabolic process	long-chain fatty acid transporter activity
CG8453 (Cyp6g1)	response to DDT ...	electron carrier activity
CG6667 (dorsal)	Toll signalling pathway	
CG17560		nucleotide binding; fatty-acyl-CoA reductase (alcohol-forming) activity
CG7171 (Urate oxidase)	allantoin biosynthetic process	urate oxidase activity
CG12766	oxidation-reduction process	alditol:NADP+ 1-oxidoreductase activity
CG10118 (pale)	pigmentation	
CG9649	neurogenesis	endopeptidase activity
CG7592 (Obp99b)	autophagic cell death	odorant binding
CG6816 (Cyp18a1)	pupation; metamorphosis	steroid hydroxylase activity

Table 35 Genes induced both in DeGreg01 and in all populations in the present analysis; biological process and molecular function of each gene is reported as in Flybase when available.

4.2.2.4.2 Treatment analysis

In this section we show the expression analysis considering the general effect of treatment.

4.2.2.4.2 Volcano plot

The volcano plot is shown in figure 25. We note that 3,262 are significantly induced (FDR = 5%), more than in the population analysis. This is due to the higher power of this analysis (see Materials and Methods).

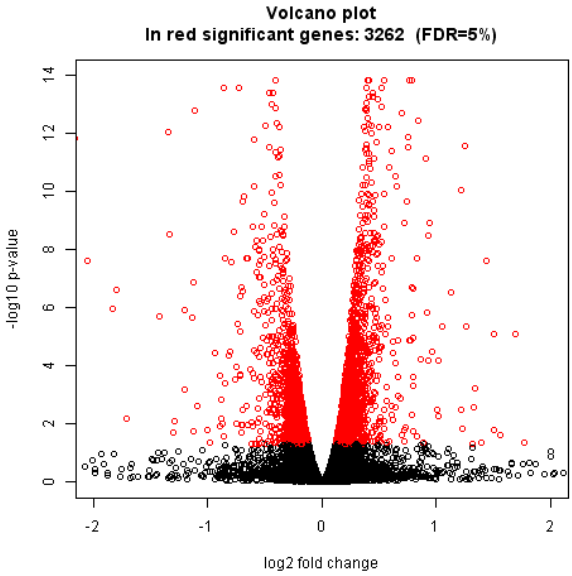

Figure 25 Volcano plots for genes induced 24 hours after infection in the treatment analysis. Genes with significant p-value (FDR 5%) are indicated in red.

4.2.2.4.2 Induced genes

Of the 3,262 induced genes, 1,837 are up- and 1,425 down-regulated upon infection.

4.2.2.4.2 Gene ontology analysis

Due to the high number of significant categories only the ones related to biological process (see 3.2.1.8) are reported here. After multiple testing correction 456 GOs are significantly enriched in genes up-regulated after infection. Among them 41 are involved in response to stimulus (table 36). The importance of sensing and responding to fungal infection is shown as the category "response to stimulus" has an extremely low corrected p-value (see table 36). We find two categories quite specific against fungal infection: "regulation of antifungal peptide biosynthetic process" and "positive regulation of antifungal peptide biosynthetic process". Nine categories are related to the tracheal system, supporting the idea that the trachea may be important for the infection and defence mechanisms. Finally some GOs relate to circadian rhythm, as expected due to the flies sampling

approach (see above). A subset of the GOs involved in response to stimulus or substance, immunity, tracheal system and circadian rhythm, are visible in table 36.

RESPONSE TO STIMULUS OR STRESS			
GO	Gene ontology category (Flybase)	p-value	FDR
GO:0050896	response to stimulus	1.97E-47	3.48E-44
GO:0048585	negative regulation of response to stimulus	1.07E-15	9.62E-14
GO:0048584	positive regulation of response to stimulus	7.74E-7	1.96E-5
GO:0006950	response to stress	4.58E-4	5.76E-3
GO:0032101	regulation of response to external stimulus	8.32E-4	9.83E-3
IMMUNITY			
GO	Gene ontology category (Flybase)	p-value	FDR
GO:0002810	regulation of antifungal peptide biosynthetic process	2.04E-4	2.92E-3
GO:0006967	positive regulation of antifungal peptide biosynthetic process	2.04E-004	2.93E- 3
GO:0045087	innate immune response	2.63E-4	3.67E-3
GO:0006952	defense response	3.43E-4	4.49E-3
GO:0006959	humoral immune response	6.89E-4	8.26E-3
TRACHEAL SYSTEM			
GO	Gene ontology category (Flybase)	p-value	FDR
GO:0007424	open tracheal system development	4.15E-9	1.56E-7
GO:0007427	epithelial cell migration, open tracheal system	1.92E-7	5.39E-6
GO:0035152	regulation of tube architecture, open tracheal system	2.44E-7	6.7E-6
CIRCADIAN RHYTHM			
GO	Gene ontology category (Flybase)	p-value	FDR
GO:0007623	circadian rhythm	1.46E-8	4.95E-7
GO:0048512	circadian behavior	1.5E-7	4.4E-6
GO:0042752	regulation of circadian rhythm	1.27E-5	2.59E-4

Table 36 Subset of gene ontology categories significantly enriched in genes up-regulated after infection; for each category p-value and FDR corrected q-value are reported.

On the other side 82 categories are significantly enriched in genes down-regulated upon infection after multiple testing correction. Interestingly 25 of them relate to biosynthesis, possibly reflecting the decrease of non-necessary metabolic processes in the presence of infection (see above). A subset of GOs involved in biosynthesis and reproduction are shown in table 37.

GO	Gene ontology category (Flybase)	p-value	FDR
GO:0044710	single-organism metabolic process	1.76E-20	9.32E-17
GO:0006412	translation	2.23E-17	5.92E-14
GO:0032504	multicellular organism reproduction	2.63E-17	4.66E-14
GO:0000003	reproduction	6.64E-17	8.81E-14
GO:0008152	metabolic process	6.54E-14	4.33E-11
GO:0009058	biosynthetic process	6.56E-12	2.90E-09
GO:0006091	generation of precursor metabolites and energy	1.42E-10	5.79E-08
GO:0009059	macromolecule biosynthetic process	1.33E-07	3.70E-05
GO:0006754	ATP biosynthetic process	2.83E-07	7.14E-05
GO:0044283	small molecule biosynthetic process	2.47E-05	3.12E-03
GO:0046390	ribose phosphate biosynthetic process	4.49E-05	4.96E-03
GO:0009260	ribonucleotide biosynthetic process	4.49E-05	4.86E-03
GO:0009152	purine ribonucleotide biosynthetic process	5.18E-05	5.50E-03
GO:0006164	purine nucleotide biosynthetic process	8.24E-05	7.28E-03

Table 37 Subset of gene ontology categories significantly enriched in genes down-regulated after infection.

4.2.2.4.2 Comparison with genes induced eight hours after infection

Genes found to be induced both 8 and 24 hours after infection are reported in table 38. Interestingly *Cyp6d2* (CG4373) is induced both in the 24 hours treatment analysis and in the Asian and European

populations and treatment analysis 8 hours after infection (see table 38).

AFRICA	AMERICA	ASIA	EUROPE	TREATMENT
	CG9438	CG4373	CG6385	CG30272
	CG2064		CG4373	CG4371
	CG31104			CG5371
	CG34387			CG2064
	CG14446			CG13160
	CG9907			CG4373
				CG6580
				CG2191
				CG13270
				CG33012

Table 38 Genes induced both in the present analysis and in the population and treatment analysis 8 hours after infection are reported. "TREATMENT" stands for treatment analysis.

4.2.2.4.2 Comparison with genes induced in the micro-array analysis

In table 39, we report the genes induced both in the present analysis and in the micro-array. Interestingly CG11951 is found to be induced both in the present analysis and in the American population and treatment analysis in the micro-array.

AFRICA	AMERICA	ASIA	EUROPE	TREATMENT
CG5258	CG5345	CG8288	CG15414	CG5345
CG3557	CG2050	CG11373	CG14966	CG4205
CG12602	CG9378	CG12699	CG11876	CG15429
CG3608	CG7380	CG4710	CG14048	CG12602
CG7654	CG5497	CG9108	CG11502	CG18135
CG11373	CG3803	CG6342	CG8884	CG11373
CG32450	CG5582	CG10245	CG1244	CG40002
CG12092	CG15539	CG15088		CG11502
CG10637	CG40002	CG7458		CG10637
CG7535	CG10605	CG5931		CG10605
CG1221	CG6342			CG6342
CG10247	CG6950			CG12758
CG12758	CG12224			CG11893
CG1090	CG7390			CG11951
CG11893	CG11951			CG31200
CG1539				

Table 39 Genes induced both in the present analysis and in the population and treatment analysis in the micro-array are reported.

4.2.2.4.2 Comparison with other studies

The number of genes induced in the present analysis, in Roxs04 and in DeGreg01 are shown in figure 26. We show that, due to the higher statistical power, more genes are found to be induced in our analysis than in the two other studies.

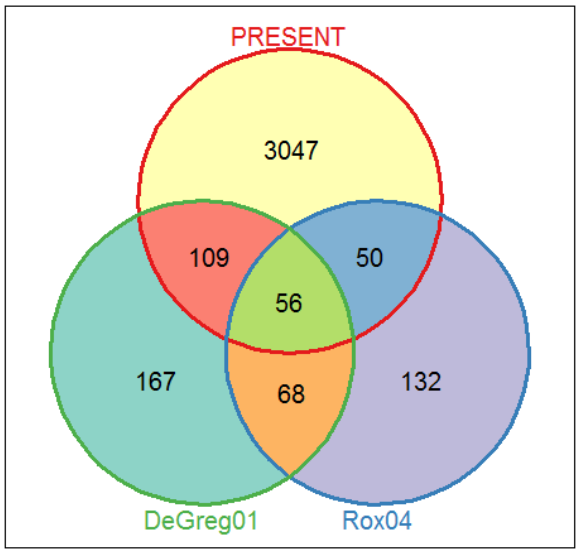

Figure 26 Venn diagram showing the number of genes induced privately in one or jointly in two or all studies; PRESENT stands for the present analysis, DeGreg01 for (De Gregorio et al. 2001) and Roxs04 for (Roxstrom-Lindquist et al. 2004).

Interestingly the majority of the 67 biological process GOs that are after multiple-testing correction significantly enriched in genes induced in all studies relates to immunity. The categories with lower corrected q-values are shown in table 40. Immunity GOs arise in our analysis, but not with major preponderance and significance than when studying genes induced in all studies. This is probably due to the fact that immunity GOs are the most significant and preponderant genes found both in Roxs04 and in De Greg01 (data not shown). The fact that less genes directly involved in immunity are found in our study than previously reported is of interest and will be addressed in the Discussion.

GO Term	Description	P-value	FDR q-value
GO:0006952	defense response	1.65E-17	8.78E-14
GO:0051707	response to other organism	4.87E-16	1.29E-12
GO:0009607	response to biotic stimulus	6.03E-16	1.07E-12
GO:0051704	multi-organism process	7.54E-15	1E-11
GO:0008063	Toll signaling pathway	2.1E-14	2.23E-11
GO:0019730	antimicrobial humoral response	2.8E-13	2.48E-10
GO:0006950	response to stress	3.4E-13	2.58E-10
GO:0031347	regulation of defense response	3.6E-13	2.39E-10
GO:0006955	immune response	4.43E-13	2.61E-10
GO:0009617	response to bacterium	9.41E-13	4.99E-10

Table 40 The 10 gene ontology categories with lower FDR corrected q-values are listed; for each GO the p-value and the FDR corrected q-value are reported.

4.2.2.4.3 Immunity related induced genes

In the present section, we discuss immunity or defence related genes found to be induced in the population and/or in the treatment analysis. We limit our survey to genes with an EAGEL value in

the 60% higher quantile (see Materials and Methods). This is done in order to focus on genes whose expression change is more likely to be biologically meaningful.

Starting with humoral immunity, induced genes coding for members of the Toll signalling pathway are shown in table 41. The first notable point is that, due to the higher statistical power, more genes are found in the treatment analysis than privately in each population. The population which exhibits the most genes is the African, as could be expected given the higher number of overall induced genes in this population. Conversely, in the Asian population only one gene is significantly induced, pointing to a low induction of the Toll pathway in this population at the time point under investigation. The only gene that is found in all populations is *dorsal* (CG6667). This gene is quite down-stream in the Toll signalling pathway. The up-regulation of *dorsal* is of key importance for the activation of humoral immunity (Lemaitre 1995).

Almost all genes reported in table 41 are induced also in Roxs04 and/or in DeGreg01 confirming the importance of the Toll pathway in anti-fungal immunity.

TOLL SIGNALLING PATHWAY (GO:0008063)								
GENE	SYMBOL	NAME	T	AF	AM	AS	EU	ALSO IN
CG15066	*IM23*	*Immune induced molecule 23*	1.7	1.73			1.81	DeGreg01 & Roxs04
CG16844	*IM3*	*Immune induced molecule 3*	0.8	0.93	1.01			Roxs04
CG18279	*IM10*	*Immune induced molecule 10*	0.76	1.05	1.16			DeGreg01 & Roxs04
CG6667	*dl*	*dorsal*	0.44	0.47	0.4	0.52	0.37	DeGreg01
CG6794	*Dif*	*Dorsal-related immunity factor*	0.39	0.47	0.35		0.33	DeGreg01
CG6134	*spz*	*spatzle*	0.38	0.4			0.36	DeGreg01 & Roxs04
CG11992	*Rel*	*Relish*	0.32	0.29			0.3	DeGreg01
CG1857	*nec*	*necrotic*	0.3	0.33	0.37			DeGreg01 & Roxs04
CG5848	*cact*	*cactus*	0.21	0.23				DeGreg01 & Roxs04
CG4261	*Hel89B*	*Helicase 89B*	0.16					
CG11709	*PGRP-SA*	*Peptidoglycan recognition protein SA*	-0.2					DeGreg01 & Roxs04
CG5212	*Pli*	*Pellino*	-0.21	-0.27				
CG5974	*pll*	*pelle*	-0.29				-0.43	DeGreg01 & Roxs04

Table 41 Genes of the Toll signalling pathway induced in one or more populations and/or in the treatment analysis are shown in the table. When a gene is significant the log2 fold change in expression is shown. “T” stands for Treatment, “AF” for Africa, “AM” for America, “AS” for Asia and “EU” for Europe. For each gene is also reported if is found to be induced also in Roxs04 and/or in DeGreg01. All genes are member of the gene ontology "Toll signalling pathway" (GO:0008063) as reported in Flybase.

Serine proteases and their inhibitors, Serpins, play an important role in immunity: a serine protease cascade is known to be involved in the activation of the Toll ligand Spätzle (Krem 2002) and could possibly be important in triggering the melanization and coagulation defense response (Tang 2009).

Given their high number, induced genes coding for serine proteases and Serpins are reported. in Appendix table 11 and table 12 Particularly one serine protease gene, CG9372, deserves further investigation, as it is up-regulated in the treatment analysis and in all populations and was observed also in DeGreg01.

In table 42 we show the induced genes coding for members of the Imd signalling pathway. As expected, fewer genes are found in comparison with the Toll pathway (see above). As discussed in the Imd pathway is responsive mainly to Gram negative bacteria. Nevertheless, and in agreement with Roxs04 and DeGreg01, some genes, among which the transcription factor *Relish*, are found to be induced in the treatment analysis and in the African and European populations. This indicates that cross-talks among the two pathways exist.

IMD SIGNALING PATHWAY (GO:0061057)								
GENE	SYMBOL	NAME	T	AF	AM	AS	EU	ALSO IN
CG11992	*Rel*	*Relish*	0.32	0.29			0.3	Roxs04 & DeGreg01
CG9080	*Listericin*	*Listericin*	0.26	0.54				Roxs04 & DeGreg01
CG7417	*Tab2*	*TAK1-associated binding protein 2*	0.15					
CG15917	*Gbp*	*Growth-blocking peptide*	-0.26	-0.33				

Table 42 Genes coding for members of the Imd pathway induced in one or more populations and/or in the “Treatment” analysis are shown in the table. When a gene is significant the log2 fold change in expression is shown. All genes are member of the gene ontology "Imd signalling pathway" (GO:0061057) as reported in Flybase.

To conclude the survey of humoral immunity we report induced genes coding for recognition proteins and antimicrobial peptides (table 43). Recognition proteins do not seem to be strongly induced and are detected almost only in the “Treatment” analysis.

Regarding the genes coding for antimicrobial peptides, we show the induction of *Drosomycin-like 5* (CG10812) in the “Treatment” analysis and at low levels. This protein is known to have anti-fungal activity (Yang 2006). The strong down-regulation of the *Drosocin* gene in the “Treatment” analysis and in the African and European populations and of the *Diptericin* gene in the “Treatment” and in the American population are also of interest. *Drosocin* and *Diptericin* are specific against Gram negative bacteria (Lemaitre and Hoffmann 2007) and their down-regulation in presence of fungal infection could indicate that resources may be saved to produce AMPs specific to fungi.

Regarding cellular immunity, we report induced genes coding for complement like proteins (table 44). These proteins recognize the pathogen and trigger the phagocytosis defence response (see Introduction).

CATEGORIES AND GENES	T	AF	AM	AS	EU	ALSO IN
RECOGNITION PROTEINS (PGRP)						
CG11709 (*PGRP-SA*)	-0.2					DeGreg01 & Roxs04
CG9681 (*PGRP-SB1*)	-0.24				-0.76	DeGreg01
CG32042 (*PGRP-LA*)	0.16					
RECOGNITION PROTEINS (GNBP)						
CG13422 (GNBP-like)	0.97	1.12				DeGreg01 & Roxs04
CG12780 (GNBP-like)	-0.37				-0.39	DeGreg01 & Roxs04
AMPs						
CG10812 (*Drosomycin-like 5*)	0.24					DeGreg01 & Roxs04
CG10816 (*Drosocin*)	-1.29	-1.19			-2.44	DeGreg01
CG12763 (*Diptericin*)	-1.3		-1.76			DeGreg01
CG1365 (*Cecropin A1*)		-0.34				DeGreg01 & Roxs04

Table 43 Genes coding for recognition proteins and antimicrobial peptides (AMP) that are induced in one or more populations and/or in the treatment analysis are shown in the table. When a gene is significant the log2 fold change in expression is shown.

These genes do not show a strong induction and are especially absent in the Asian and European populations, pointing possibly to a low activation of phagocytosis at the time point under investigation.

COMPLEMENT LIKE PROTEINS						
GENE	T	AF	AM	AS	EU	ALSO IN
CG7052 (*Tep2*)	0.18					DeGreg01 & Roxs04
CG7068 (*Tep3*)	0.25	0.37				
CG10363 (*Tep4*)	0.3	0.35	0.32			DeGreg01 & Roxs04
CG7586 (*Mcr,Tep VI*)	0.2	0.28				

Table 44 Genes coding for complement like proteins that are induced in one or more populations and/or in the treatment analysis are shown in the table. When a gene is significant the log2 fold change in expression is shown.

Given their high number, the induced genes coding for proteins involved in the phagocytosis defence response are shown in Appendix table 13. Once again almost no gene is induced in the Asian and European populations, with the interesting exception of *Pdh* (CG4899) and *inaC* (CG6518). These two genes are over-expressed in all populations and could therefore be up-stream in the phagocytosis defence response. Interestingly they are not induced either in DeGreg01 or in Roxs04.

The induced genes coding for proteins involved in the melanization defence response are shown in table 45. The gene *pale* (CG10118) is the most highly up-regulated in all populations and is up-stream in the melanin production pathway. This gene is up-regulated also in DeGreg01 and Roxs04 and therefore its induction is a hallmark of the activation of the melanization defence response. Dorsal, which is part of the Toll signaling pathway, is also involved in the activation of

the melanization defense pathway (Bettencourt et al. 2004) (table 45).

MELANIN METABOLIC PROCESS (GO:0006582) and MELANIZATION DEFENSE RESPONSE (GO:0035006)								
GENE	SYMBOL	NAME	T	AF	AM	AS	EU	ALSO IN
CG10118	*ple*	*pale*	0.58	0.61	0.59	0.6	0.51	DeGreg01&Roxs04
CG6667	*dl*	*dorsal*	0.44	0.47	0.4	0.52	0.37	DeGreg01
CG12120	*t*	*tan*	0.28	0.35				
CG1634	*Nrg*	*Neuroglian*	0.27	0.36				
CG3066	*Sp7*	*Serine protease 7*	0.27					DeGreg01&Roxs04
CG1768	*dia*	*diaphanous*	0.25	0.31				
CG1511	*Eph*	*Eph receptor tyrosine kinase*	0.18					
CG10697	*Ddc*	*Dopa decarboxylase*	0.17					DeGreg01&Roxs04
CG42783	*aPKC*	*atypical protein kinase C*	0.15					
CG9366	*RhoL*	*Rho-like*	-0.14					
CG8063	*yellow-f2*	*yellow-f2*	-0.2	-0.28				
CG9792	*yellow-e*	*yellow-e*	-0.21				-0.45	
CG9441	*Pu*	*Punch*	-0.26					DeGreg01
CG9889	*yellow-d*	*yellow-d*	-0.3	-0.33				

Table 45 Genes coding for proteins involved in the melanization defence response that are induced in one or more populations and/or in the treatment analysis are shown in the table. When a gene is significant the log2 fold change in expression is shown. All genes are member of the gene ontology "melanin metabolic process" (GO:0006582) and "melanization defense response" (GO:0035006) as reported in Flybase.

The genes coding for proteins playing a role in the coagulation defence response are shown in table 46. The gene *transglutaminase* (CG7356) is over-expressed in all populations, pointing to a key role of its induction in the coagulation process.

COAGULATION (GO:0050817)								
GENE	SYMBOL	NAME	T	AF	AM	AS	EU	ALSO IN
CG7356	*Tg*	*Transglutaminase*	0.55	0.59	0.57	0.65	0.4	
CG15825	*fon*	*fondue*	0.41	0.55			0.47	DeGreg01

Table 46 Genes coding for proteins involved in the coagulation defence response that are induced in one or more populations and/or in the treatment analysis are shown in the table. When a gene is significant the log2 fold change in expression is shown. (De Gregorio et al. 2001, Roxstrom-Lindquist et al. 2004). All genes are member of the gene ontology "coagulation" (GO:0050817) as reported in Flybase.

We assess two categories of genes which role in immunity has not been well defined. The first group is the iron binding proteins, mainly belonging to the Cyp450 family (see above) which are involved among other activities in detoxification of toxic compounds and possibly immunity. Due to their high number, induced genes coding for iron binding proteins are reported in Appendix table 14. Two Cyp450 genes are up-regulated in all populations: *Cyp6d2*, that is expressed in the tracheal system and *Cyp6g1*, that is specific of the malpighian tubules and also observed in DeGreg01. These two genes possibly play an important role in the detoxification of toxic compounds from the fungus. The gene pale (CG10118) that is part of the melanization defence response is also present in Appendix table 14 as it is an iron binding protein.

Finally several genes coding for proteins exhibiting lipase activity have been proposed to be involved in a not yet characterized defense mechanism DeGreg01. We find them to be induced in one or more populations (Appendix table 15). Of special interest is CG6675, that is up-regulated in the treatment analysis and in all populations except the European one and that shows up also in Roxs04 and DeGreg01. Additionally CG42237 is down-regulated in all populations.

4.2.3 Genetic analysis of candidate genes

4.2.3.1 Coding region conservation

The distribution of Ka/Ks and Pi(a)/Pi(s) ratios is shown in figure 27 both for shared and private induced genes. The Ka/Ks ratio is lower for shared genes than for private induced ones, with the exception of the American population. These results suggest that genes induced in all populations tend to be more conserved in the coding region than private induced ones. This indicates selective constraints at the species level due to functional reasons (Nielsen 2005).

A similar pattern is observed for the Pi(a)/Pi(s) ratios suggesting that the same evolutionary constraints act at the population level. The extremely low values for the Asian private induced genes is probably a consequence of the sequencing technique. These sequences show a high number of masked positions due to residual heterozygosity and therefore an almost zero level of polymorphisms. The analysis of more shared and private genes is needed in order to test the generality of the observed trends (figure 27).

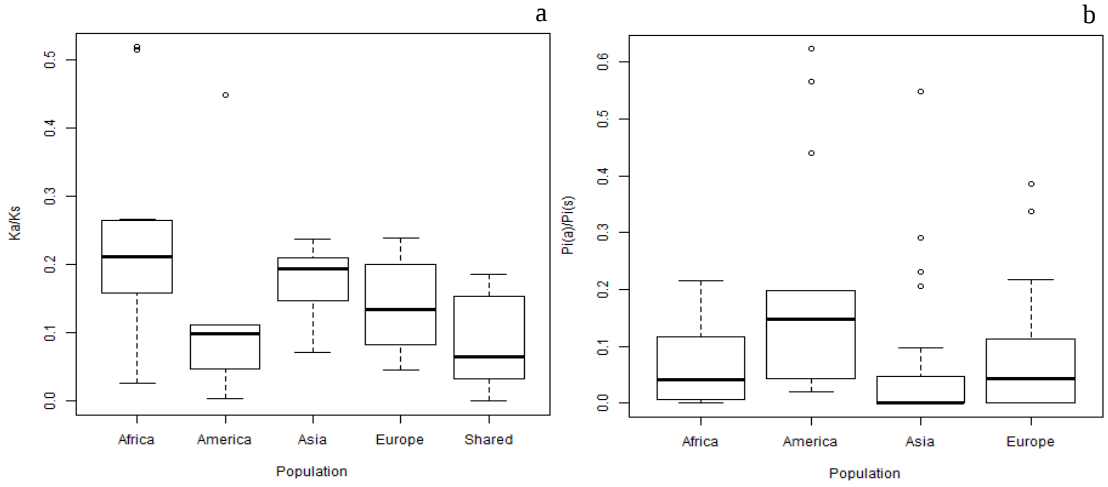

Figure 27 The box plots shows (a) the distribution of Ka/Ks ratios and (b) Pi(a)/Pi(s) ratios for privately and shared induced genes. "Africa" stands for African privately induced genes, "America" for American privately induced genes, "Asia" for Asian privately induced genes, "Europe" for the European privately induced genes, "Shared" for genes induced in all populations.

4.3 EFFECT OF *WOLBACHIA* AND PARENTAL PARASITE EXPOSURE ON *D. MELANOGASTER* SURVIVAL TO *B. BASSIANA*

4.3.1 Effect of the endosymbiont *Wolbachia pipientis*

4.3.1.1 *Wolbachia* assessment by PCR

The absence of *Wolbachia* in flies exposed to tetracycline was confirmed by PCR. The *Wsp W. pipientis* gene was amplified in the Eg25 and E20 not exposed flies, while no amplification was detected in tetracycline exposed flies and in the Eg57 not treated flies. On the other hand, the *28S D. melanogaster* ribosomal gene was amplified in cured and not exposed flies from all inbred lines. The same result was observed in both repetitions of the PCR analysis.

4.3.1.2 First infection experiment

The mean spore concentration in the oil/spore suspension was 3.0 x 10^8 spores/ml with a standard deviation of 0.7 x 10^8 spores/ml (10 independent measurements were performed). The mean spore density on the ink-jet transparency film was 8,933 spores/mm^2 with a standard deviation of 4,428 spores/mm^2 (10 independent measurements were performed). No vial was discarded due to contamination.

4.3.1.2.1 Mortality three days after treatment

4.3.1.2.1.1 Line Eg25

There was no difference in mortality among infected and control flies (figure 28a) (p-value = 0.255150). Flies bearing *Wolbachia* suffered significantly higher mortality than cured ones (figure 28b) (p-value = 0.005870). In total the percentage of dead flies was 4.43 %.

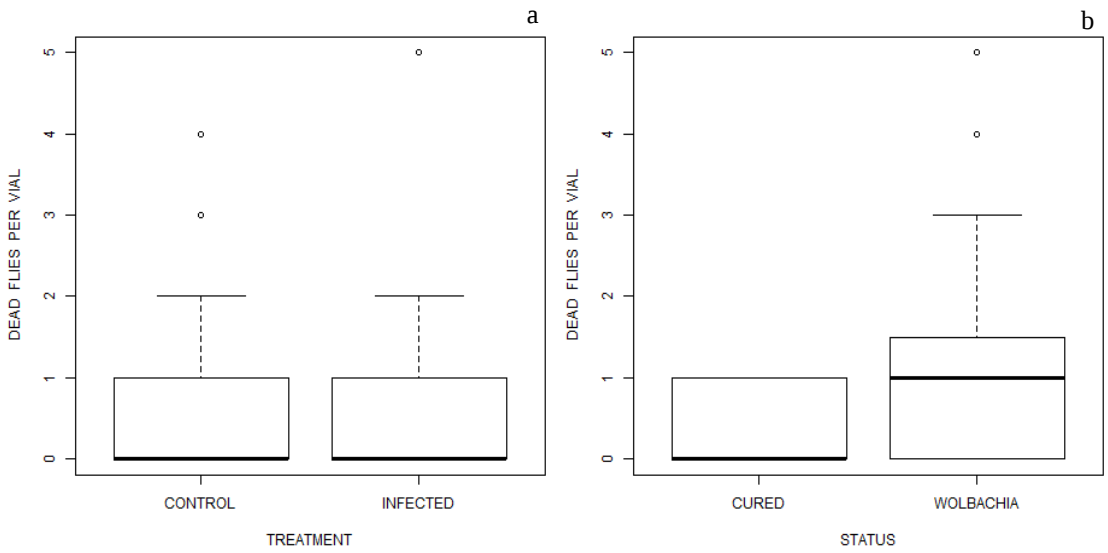

Figure 28 (a) Box plot representing the number of dead flies per vial during the first three days after treatment for control and infected flies. (b) Box plot representing the number of dead flies per vial during the first three days after treatment for flies bearing *Wolbachia* and cured flies.

4.3.1.2.1.2 Line E20

There was no difference in mortality among infected and control flies (figure 29a) (p-value = 0.20990). Flies bearing *Wolbachia* suffered a significantly higher mortality than cured ones (figure 29b) (p-value = 0.00190). In total the percentage of dead flies was 17.37%

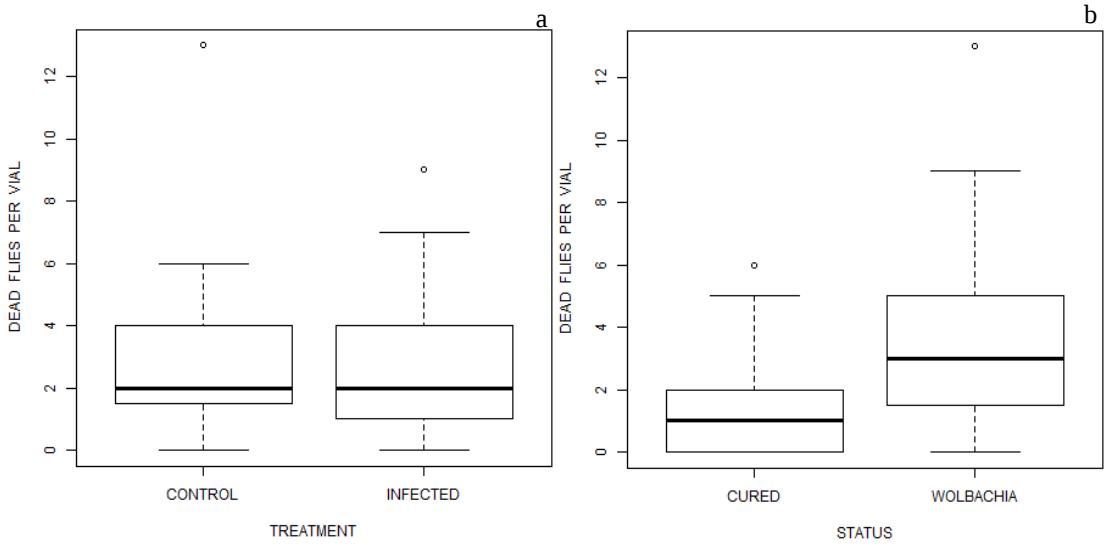

Figure 29 (a) Box plot representing the number of dead flies per vial during the first three days after treatment for control and infected flies. (b) Box plot representing the number of dead flies per vial during the first three days after treatment for flies bearing *Wolbachia* and cured flies.

4.3.1.2.1.3 Line Eg57

There was no significant difference in mortality among infected and control flies (figure 30a) (p-value = 0.153058), although more control flies died. Flies treated with tetracycline died significantly more than untreated ones (figure 30b) (p-value = 0.000102) . In total the percentage of dead flies was 5.97%

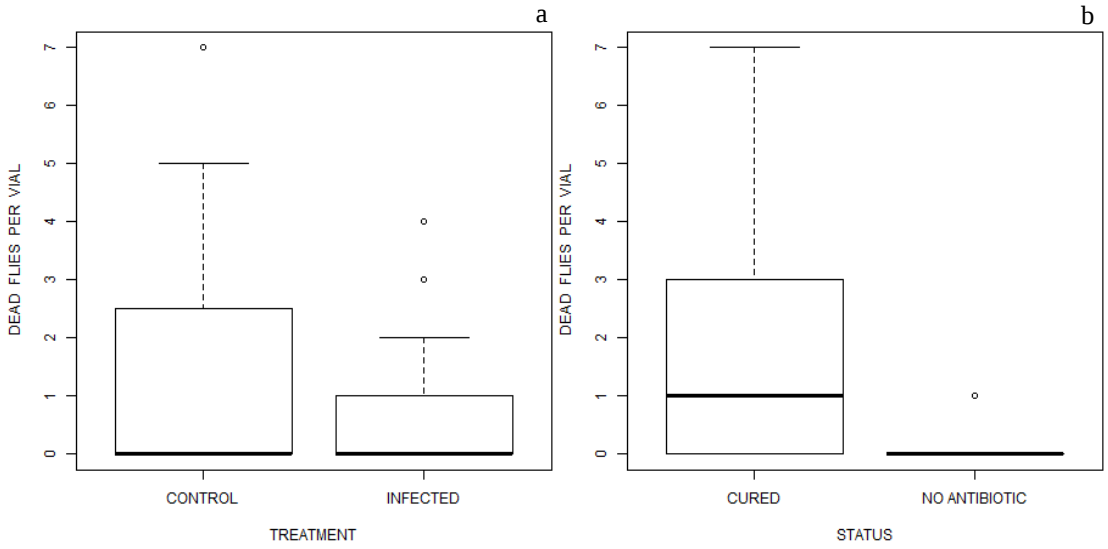

Figure 30 (a) Box plot representing the number of dead flies per vial during the first three days after treatment for control and infected flies. (b) Box plot representing the number of dead flies per vial during the first three days after treatment for flies bearing *Wolbachia* and cured flies.

4.3.1.2.1.4 Conclusions about mortality three days after treatment

These results are quite interesting. Treatment does not have a significant effect during the first three days, meaning that flies died most likely because they got stuck to the oil or to the food. Therefore mortality does not reflect susceptibility to the parasite, but is an indicator of flies fitness or vigour. Eg25 and E20 lines show reduced mortality after being cured, possibly pointing to a cost of *Wolbachia* in flies under stress. Indeed negative effect of *W. pipientis* in *D. melanogaster* exposed to lead has been reported (Wang et al. 2012). On the other side the *Wolbachia* free Eg57 line exhibits increased mortality after being exposed to tetracycline. This points to a negative effect of the antibiotic on fly fitness. An intriguing hypothesis is that by using the antibiotic we got rid of a different symbiont or of some components of *Drosophila* gut microbiota and so reduced the flies' fitness and vigour (Ridley et al. 2012).

4.3.1.2.2 Mortality at six days after treatment and later

4.3.1.2.2.1 Linear models

4.3.1.2.2.1.1 Line Eg25

"Log time" and "Time squared" were retained and significant, “Treatment” was significant (table 47).

	Log Time	Time squared	Treatment
p-value	5.75e-11	9.92e-06	9.75e-14

Table 47 P-values of the significant covariates.

4.3.1.2.2.1.2 Line E20

All time covariates were retained and all but "Log time" were significant (table 48). “Treatment” was significant.

	Time	Time squared	Treatment
p-value	0.0276	0.0162	<2e-16

Table 48 P-values of the significant covariates.

4.3.1.2.2.1.3 Line Eg57

All time covariates were retained and all were significant with the exception of "Log time" (table 49). “Treatment” was significant.

	Time	Time squared	Treatment
p-value	0.02141	0.00511	1.86e-15

Table 49 P-values of the significant covariates.

4.3.1.2.2.2 Kaplan-Meier survival curves

4.3.1.2.2.2.1 Line Eg25

The Kaplan-Meier survival curve for each flies group is shown in figure 31. Infected flies suffered higher mortality than control ones; control flies bearing *Wolbachia* performed worse than control flies cured from the endosymbiont.

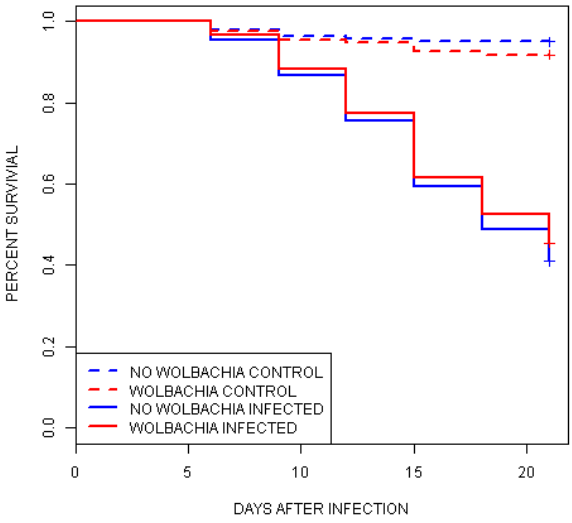

Figure 31 Kaplan-Meier plot representing survival curves for all flies groups. Full lines represent infected flies, while dashed lines represent control flies; red lines represents flies bearing *Wolbachia*, while blue lines represent cured flies (see legend).

4.3.1.2.2.2.2 Line E20

The Kaplan-Meier survival curve for each flies group is shown in figure 32. Infected flies suffered higher mortality than control ones; control flies bearing *Wolbachia* performed worse than control flies cured from the endosymbiont.

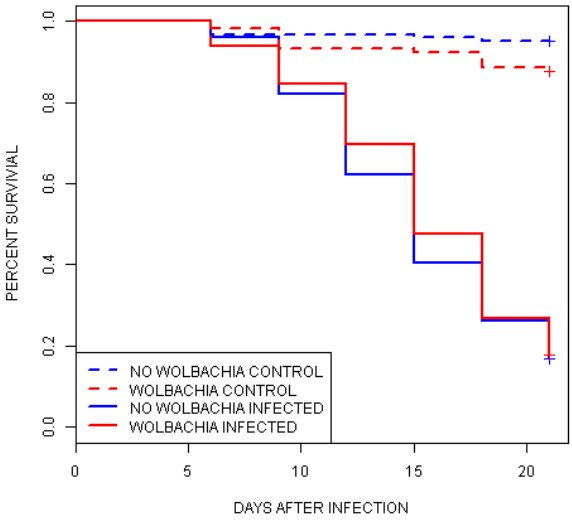

Figure 32 Kaplan-Meier plot representing survival curves for all flies groups. Full lines represent infected flies, while dashed lines represent control flies; red lines represents flies bearing *Wolbachia*, while blue lines represent cured flies (see legend).

4.3.1.2.2.3 Line Eg57

The Kaplan-Meier survival curve for each flies group is shown in figure 33. Infected flies suffered higher mortality than control ones; flies treated with tetracycline performed worse than untreated flies.

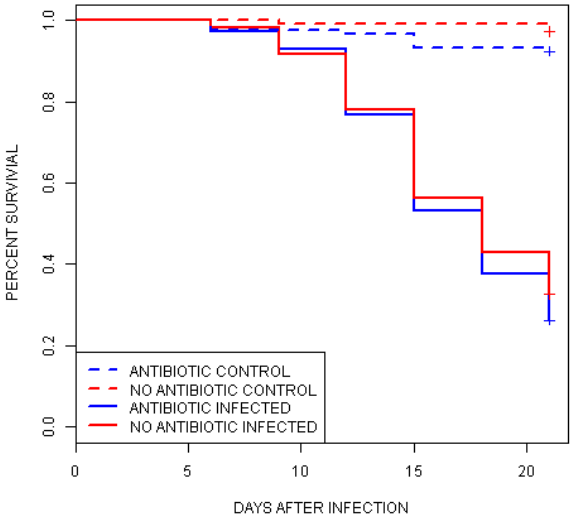

Figure 33 Kaplan-Meier plot representing survival curves for all flies groups. Full lines represent infected flies, while dashed lines represent control flies; the blue lines represent flies treated with tetracycline, while the red lines represent untreated flies (see legend).

4.3.1.2.2.3 Cox hazard model

4.3.1.2.2.3.1 Line Eg25

“Treatment” was significant (p-value = 2.5e-13).

4.3.1.2.2.3.2 Line E20

“Treatment” was significant (p-value = 1.1e-16).

4.3.1.2.2.3.3 Line Eg57

“Treatment” was significant (p-value = 3.1e-15).

4.3.1.2.2.4 Conclusions about mortality at six days after treatment and later

Wolbachia does not appear to have any protective effect against fungal infection in this study system. This is not the first case in which such a result is found, for example no effect of *W. pipientis* has been reported in *D. melanogaster* infected by a pathogenic bacteria (Rottschaefer and Lazzaro 2012). Indeed our results are at odds with a previous study showing a protective effect of *Wolbachia* in one *D. melanogaster* inbred line infected by *B. bassiana* (Panteleev et al. 2007). A possible explanation is that the *Wolbachia* strains used in the two studies are different. Indeed variation in the antiviral protection of different *Wolbachia* strains has been found in *D. simulans* (Osborne et al. 2009).

4.3.1.3 Second Infection experiment

The mean spore concentration in the oil/spore suspension was 3.0 x 10^8 spore/ml with a standard deviation of 2.0 x 10^8 spores/ml (10 independent measurements were performed). The mean spore density on the ink-jet transparency film was 20,927 spores/mm^2 with a standard deviation of 6,103 spores/mm^2 (10 independent measurements were performed). Three vials were discarded due to contamination.

4.3.1.3.1 Mortality three days after treatment

4.3.1.3.1.1 Line Eg25

There was no difference in mortality among infected and control flies (figure 34, table 50).

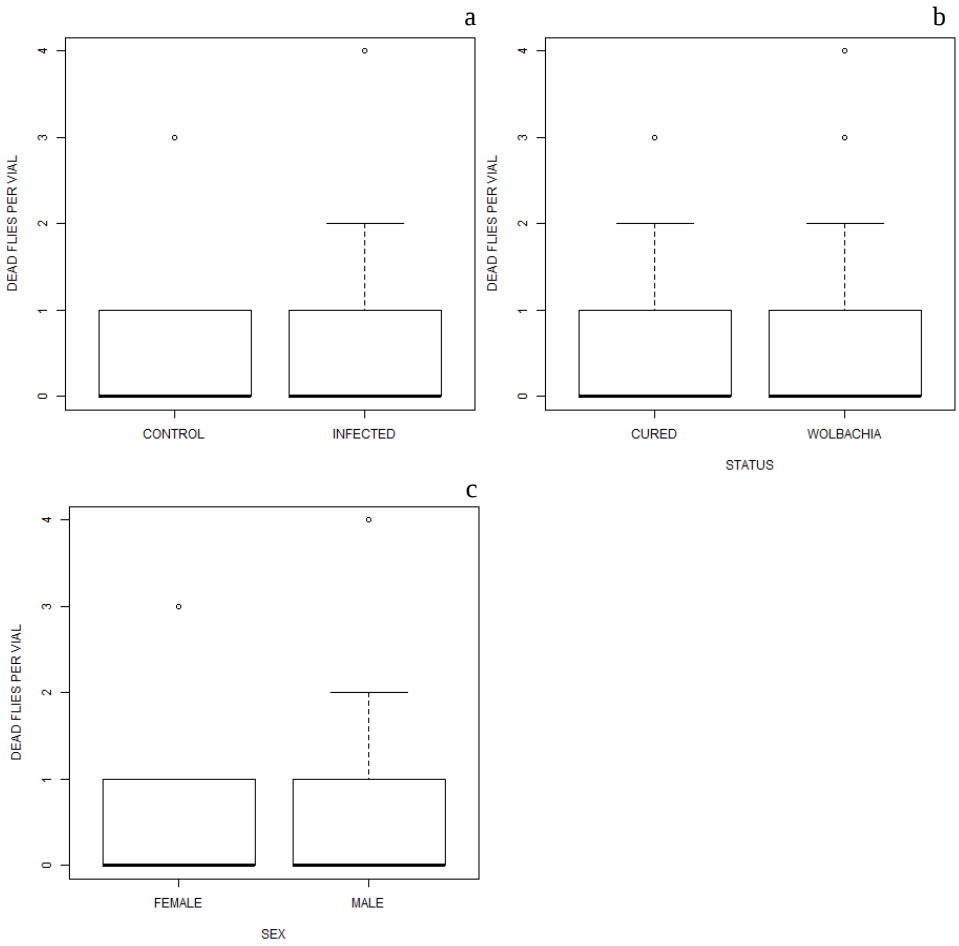

Figure 34 (a) Box plot representing the number of dead flies per vial during the first three days after treatment for control and infected flies. (b) Box plot representing the number of dead flies per vial during the first three days after treatment for flies bearing *Wolbachia* and cured flies. (c) Box plot representing the number of dead flies per vial during the first three days after treatment for female and male flies.

No difference in mortality was present among flies bearing *Wolbachia* and cured flies and among sexes (table 50). In total the percentage of dead flies was 3.81%.

	Treatment	Wolbachia	Sex
p-value	0.588000	0.490000	0.720000

Table 50 P-values of model covariates.

4.3.1.3.1.2 Line E20

There was no difference in mortality among infected and control flies (figure 35, table 51). Flies bearing *Wolbachia* suffered a significantly higher mortality than cured flues (figure 35, table 51).

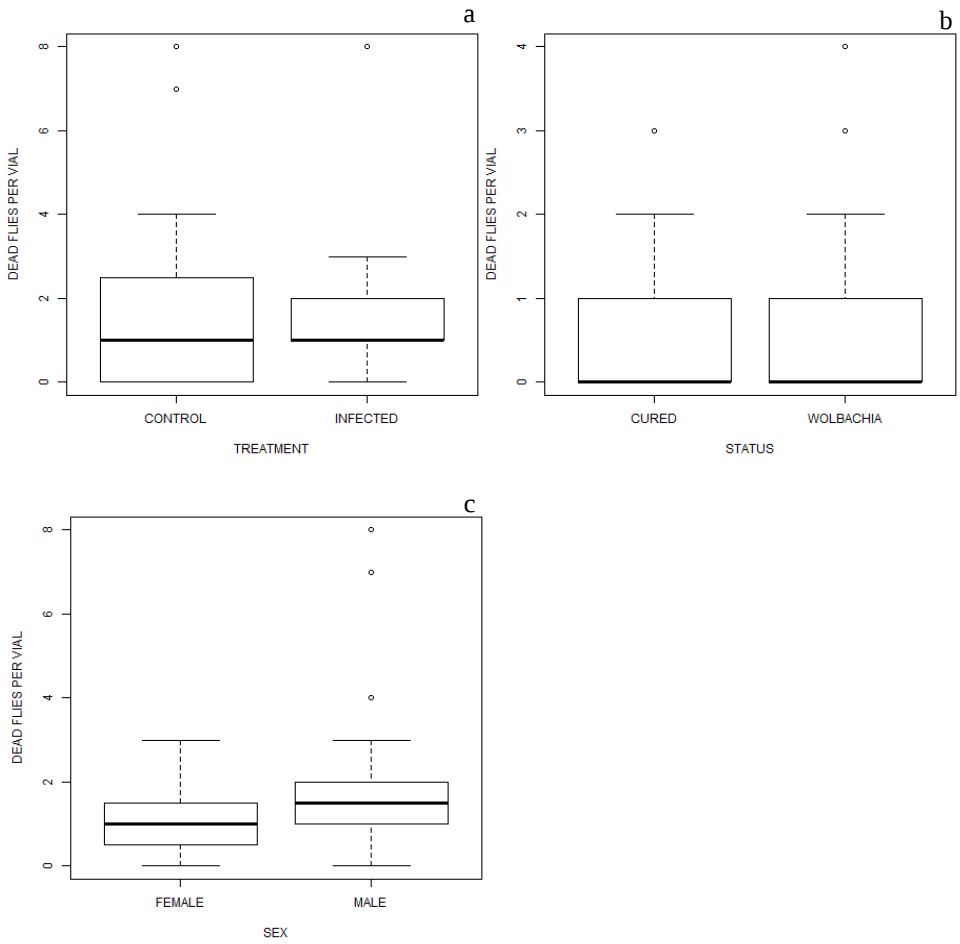

Figure 35 (a) Box plot representing the number of dead flies per vial during the first three days after treatment for control and infected flies. (b) Box plot representing the number of dead flies per vial during the first three days after treatment for flies bearing *Wolbachia* and cured flies. (c) Box plot representing the number of dead flies per vial during the first three days after treatment for female and male flies.

Male flies suffered a significantly higher mortality than female flies (figure 35, table 51). In total the percentage of dead flies was 9.98%.

	Treatment	Wolbachia	Sex
p-value	0.098763	0.000265	0.003282

Table 51 P-values of model covariates.

4.3.1.3.1.3 Line Eg57

There was no significant difference in mortality among infected and control flies, although control flies suffered a higher mortality than treated ones (figure 36, table 52).

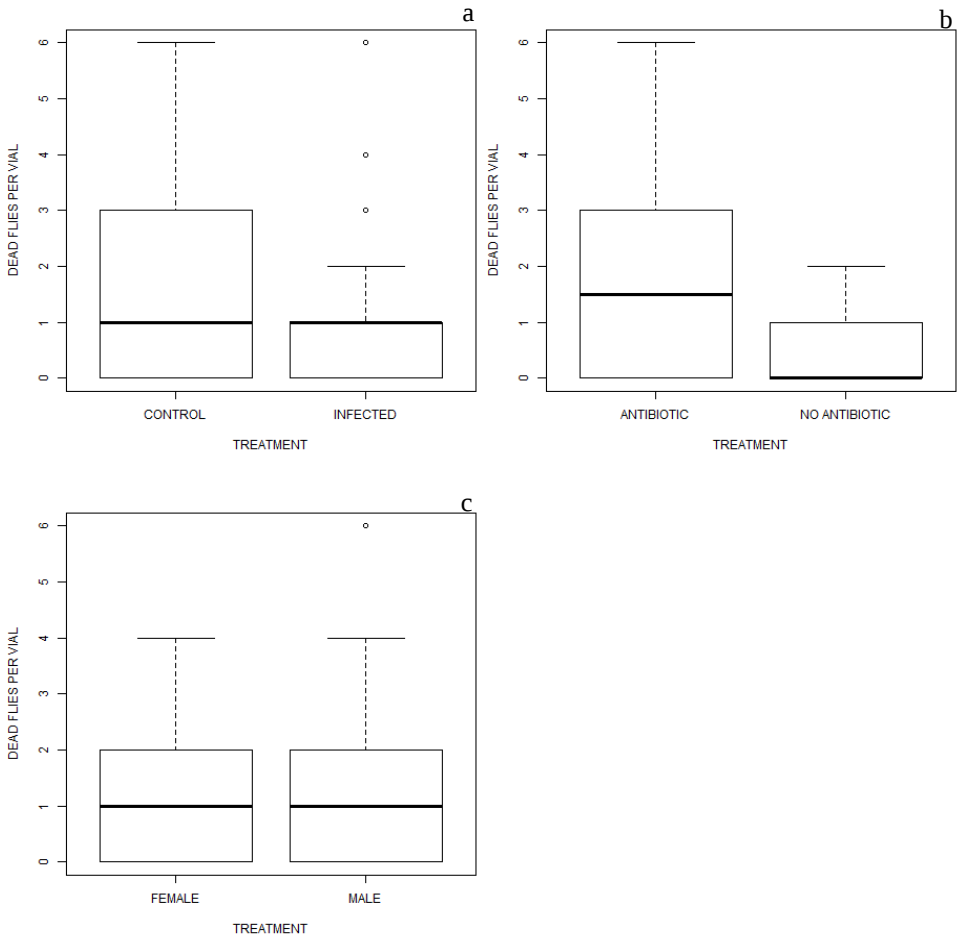

Figure 36 (a) Box plot representing the number of dead flies per vial during the first three days after treatment for control and infected flies. (b) Box plot representing the number of dead flies per vial during the first three days after treatment for flies bearing *Wolbachia* and cured flies. (c) Box plot representing the number of dead flies per vial during the first three days after treatment for female and male flies.

Flies treated with tetracycline died significantly more than untreated flies (figure 36, table 52). In total the percentage of dead flies was 8.17%.

	Treatment	Antibiotic	Sex
p-value	0.165000	1.84e-05	0.342000

Table 52 P-values of model covariates.

4.3.1.3.1.4 Conclusions about mortality three days after treatment

Again we do not find a significant effect of treatment. Interestingly E20 flies carrying *Wolbachia* show a significantly higher mortality than their cured counterpart and Eg57 flies exposed to the antibiotic die significantly more then the not treated ones. These results confirm the conclusions drawn from the previous experiment.

Conversely Eg25 flies carrying *Wolbachia* do not suffer a higher mortality than cured ones, as was the case in the first experiment. The reason for this difference could be the lower mortality (3.81% compared to 4.43% in the present study), although the difference is not too big.

Finally we see that E20 males suffer higher mortality than females from the same line.

4.3.1.3.2 Mortality at six days after treatment and later

4.3.1.3.2.1 Linear model

4.3.1.3.2.1.1 Line Eg25

All time covariates were retained and all were significant with the exception of "Log time" (table 53). “Treatment” was significant; “Wolbachia”, the interaction “Treatment” x “Wolbachia” and “Sex” were not significant.

	Time	Time squared	Treatment
p-value	0.01780	0.00192	5.25e-13

Table 53 P-values of significant covariates.

4.3.1.3.2.1.2 Line E20

There were two best models, the first one in which "log time" and "time" were retained and the second one in which "log time" and "time squared" were retained. As the second model has a higher likelihood, we report here the result for this model (table 54). They are not qualitatively different from the ones obtained using the other model. "Treatment", "Wolbachia", their interaction and "Sex" are significant.

	Log Time	Time squared	Treatment	Wolbachia	Sex	Wolbachia *Treatment
p-value	< 2e-16	3.29e-09	< 2e-16	0.0244	0.0335	0.0250

Table 54 P-values of significant covariates.

4.3.1.3.2.1.3 Eg57

There were two best models, the first one in which "log time" and "time" were retained and the

second one in which "log time" and "time squared" were retained. As the second model has a higher likelihood, we report here the result for this model (table 55). They are not qualitatively different from the ones obtained using the other model. “Treatment” and “Sex” were significant.

	Log Time	Time squared	Treatment	Sex
p-value	< 2e-16	1.98e-12	< 2e-16	0.00473

Table 55 P-values of significant covariates.

4.3.1.3.2.2 Kaplan-Meier survival curves

4.3.1.3.2.2.1 Line Eg25

The Kaplan-Meier survival curve for each fly group is shown in figure 37. Infected flies suffered a higher mortality than control ones.

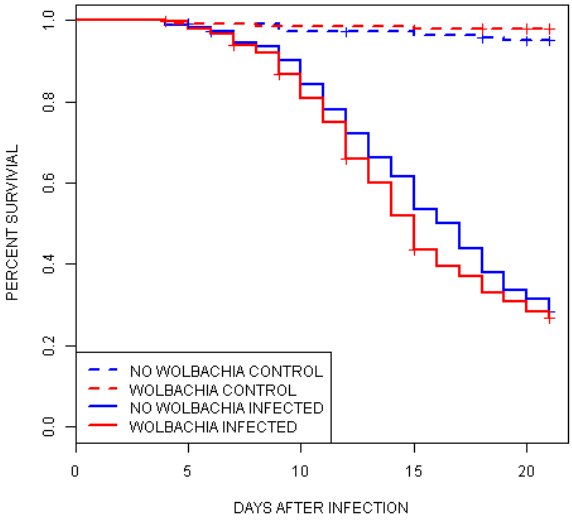

Figure 37 Kaplan-Meier plot representing survival curves for all flies groups. Full lines represent infected flies, while dashed lines represent control flies; red lines represents flies bearing *Wolbachia*, while blue lines represent cured flies (see legend).

4.3.1.3.2.2.2 Line E20

The Kaplan-Meier survival curve for each fly group is shown in figure 38. Infected flies suffered higher mortality than control ones; control flies bearing *Wolbachia* performed worse than control flies cured from the endosymbiont.

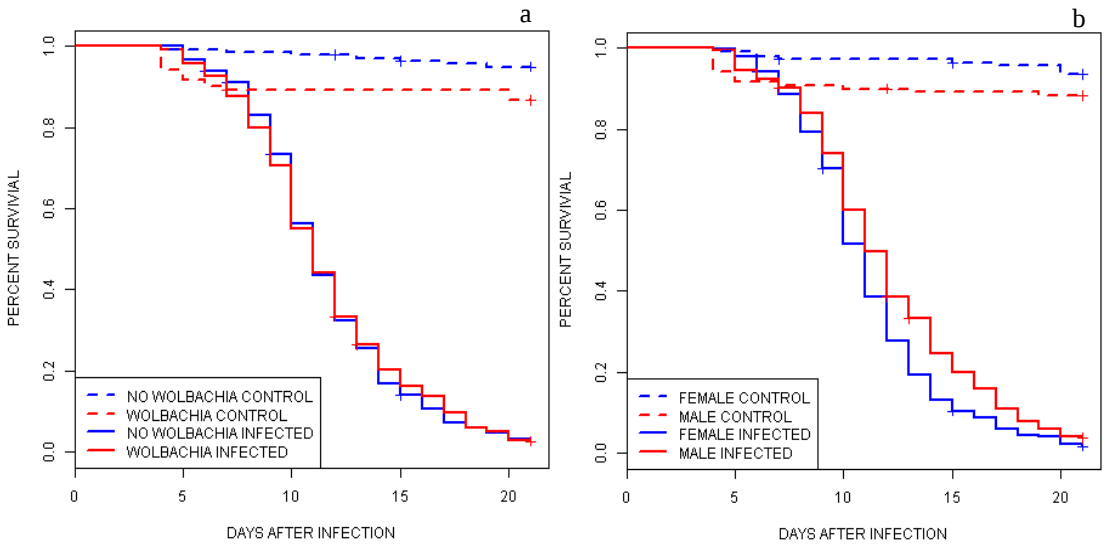

Figure 38 (a) Kaplan-Meier plot representing survival curves for all antibiotic treatment – experimental treatment combinations, full lines represent infected flies, while dashed lines represent control flies; blue lines represent flies treated with tetracycline, while red lines represent untreated flies (see legend). (b) Kaplan-Meier plot representing survival curves for all sex – experimental treatment combinations, full lines represent infected flies, while dashed lines represent control flies; red lines represents males, while blue lines represent females (see legend).

4.3.1.3.2.2.3 Line Eg57

The Kaplan-Meier survival curve for each flies group is shown in figure 39. Infected flies suffered higher mortality rate than control ones; flies that were treated with tetracycline performed worse than untreated flies. Male infected flies died more than female infected flies.

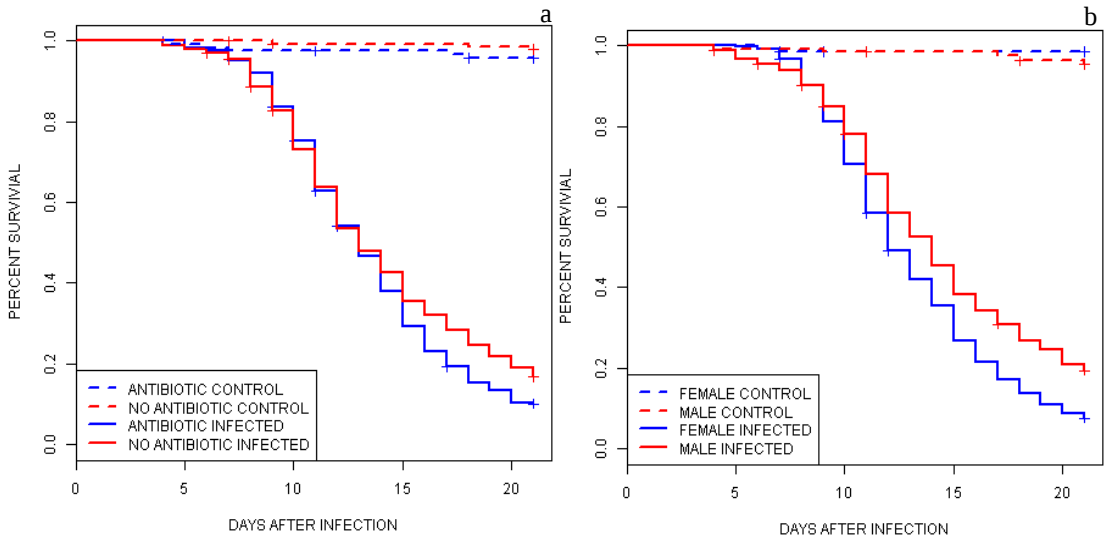

Figure 39 (a) Kaplan-Meier plot representing survival curves for all antibiotic treatment – experimental treatment combinations, full lines represent infected flies, while dashed lines represent control flies; blue lines represent flies treated with tetracycline, while red lines represent untreated flies (see legend). (b) Kaplan-Meier plot representing survival curves for all sex – experimental treatment combinations, full lines represent infected flies , while dashed lines represent control flies; red lines represents males, while blue lines represent females (see legend).

4.3.1.3.2.3 Cox hazard model

4.3.1.3.2.3.1 Line Eg25

“Treatment” was significant (p-value = 2.6e-15).

4.3.1.3.2.3.2 Line E20

“Treatment”, “Wolbachia” and their interaction were significant, “Sex” was not significant (table 56).

	Treatment	Wolbachia	Treatment x Wolbachia	Sex
p-value	0.000	0.023	0.023	0.028

Table 56 P-values of the significant covariates.

4.3.1.3.2.3.3 Line Eg57

“Treatment” was significant (p-value = 0.000). “Sex” was significant (p-value = 0.0069).

4.3.1.3.2.4 Conclusions about mortality at six days after treatment and later

We did not find a protective effect of "Wolbachia" for Eg25 flies. On the other hand "Wolbachia" and "Wolbachia" x "Treatment" interaction were significant for E20 flies. Rather than indicating a protective effect of *Wolbachia* this is likely a consequence of the high mortality rate of control flies bearing the endosymbiont. In fact if we look at figure 38a we see that E20 control flies carrying *Wolbachia* have the highest mortality rate at the 4th day and the highest cumulative mortality up to the 8th day. If we repeat the survival analysis starting from day 4 instead than from the day 3 then "Wolbachia" and "Wolbachia" x "Treatment" are not significant any more (p-value respectively: 0.2600 and 0.2500). Therefore we can conclude that as in the first experiment no protective effect of *Wolbachia* is observed.

"Sex" is significant for E20 and Eg57 flies. Interestingly in both cases infected males suffered a

lower mortality than infected females. A similar pattern was found in Tinsley et al. (2006), although only in the latest phase of infection. Indeed it has been shown that mated females suffer reduced infection defence (Short et al. 2012). As the females we used were not virgins, this could possibly explain our findings.

4.3.2 Effect of trans-generational immune priming

4.3.2.1 First infection experiment

The mean spore concentration in the oil/spores suspension was 1.1 10^8 spore/ml with a standard deviation of 0.6 10^8 spore/ml (10 independent measurements were performed). For the parental infection the mean spore density on the ink-jet transparency film was 5,457 spores/mm^2 with a standard deviation of 2,762 spores/mm^2 (8 independent measurements were performed). For the offspring infection the mean spore density on the ink-jet transparency film was 1,735 spores/mm^2 with a standard deviation of 558 spores/mm^2 (8 independent measurements were performed). No vial was discarded due to contamination.

4.3.2.1.1 Mortality three days after treatment

There was no difference in mortality among infected and control flies (figure 40, table 57).

	Treatment	Parents	Sex
p-value	0.5318	0.5193	0.0741

Table 57 P-values of model covariates.

In total the percentage of dead flies was 8.02%.

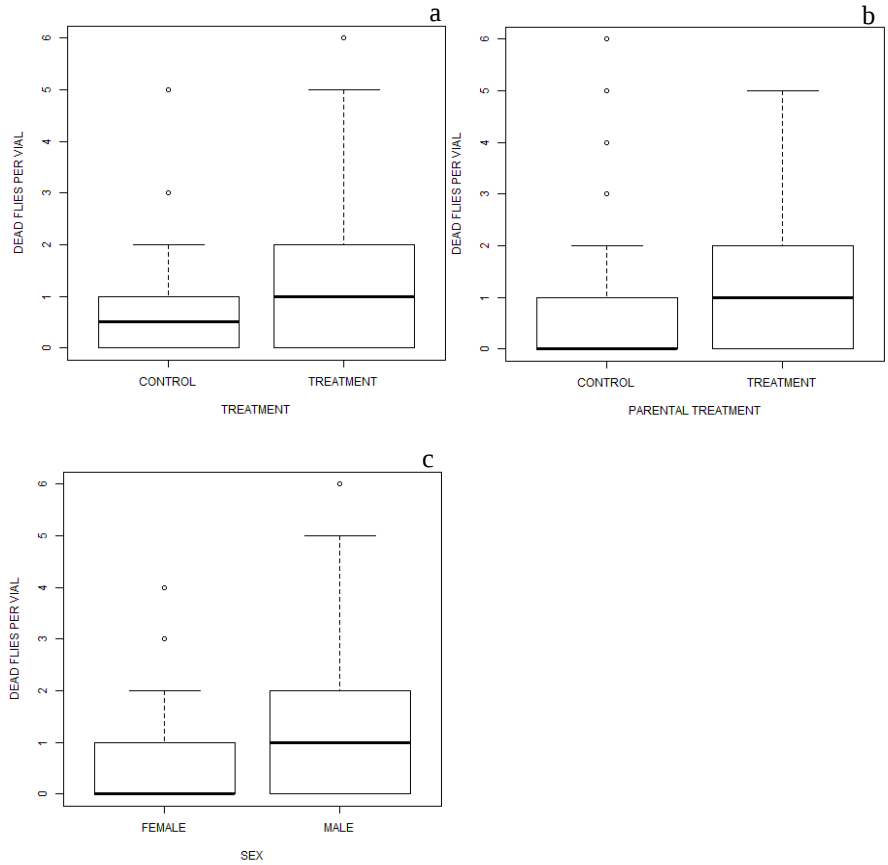

Figure 40 (a) Box plot representing the number of dead flies per vial during the first three days after treatment for control and infected flies. (b) Box plot representing the number of dead flies per vial during the first three days after treatment for flies from infected and control parents. (c) Box plot representing the number of dead flies per vial during the first three days after treatment for male and female flies.

4.3.2.1.1.1 Conclusions

"Treatment" was not significant at this time point, meaning that flies did not die as a consequence of the infection. Also "Parents" was not significant, meaning that the infection state of the parents did not have an effect on fly vigour. Finally, "Sex" was marginally significant with males exhibiting higher mortality than females.

4.3.2.1.2 Mortality six days after treatment and later

4.3.2.1.2.1 Linear model

There are two best models. In the first one "log time" and "time" are retained, while in the second "log time" and "time squared". As the first model has a slightly higher likelihood, the results from

this model are shown (table 58). The two models give anyway almost identical p-values for all covariates.

	Time	Log Time	Treatment
p-value	1.52e-15	< 2e-16	< 2e-16

Table 58 P-values of the significant covariates.

4.3.2.1.2.2 Kaplan-Meier survival curves

The Kaplan-Meier survival curve for each flies group is shown in figure 41. Infected flies suffered a higher mortality than control ones.

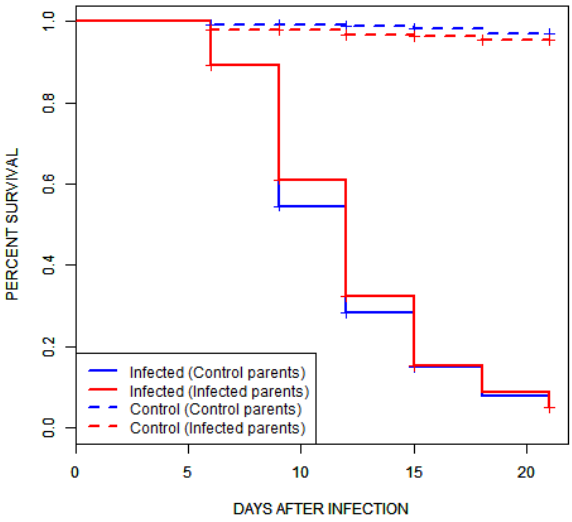

Figure 41 Kaplan-Meier plot representing survival curves for all flies groups. Full lines represent infected flies, while dashed lines represent control flies; red lines represents flies from infected parents, while blue lines represent flies from control parents (see legend).

4.3.2.1.2.3 Cox hazard model

"Treatment" was significant (p-value = 0.00).

4.3.2.1.2.4 Conclusions

These results suggest that there is no trans-generational immune priming in the study system. This is in agreement with a previous study that did not find evidence of trans-generational immune priming in *D. melanogaster* exposed to bacteria (Linder and Promislow 2009). No immune priming was found also in ants exposed to *B. bassiana* (Reber and Chapuisat 2012).

4.3.2.2 Second infection experiment

The mean spore concentration in the oil/spore suspension was 1.1 10^8 spore/ml with a standard deviation of 0.6 10^8 spores/ml (10 independent measurements were performed). Mean spore density on ink-jet paper was not recorded for parents and offspring in this experiment and is assumed to be similar to the values reported for the first experiment. No vial was discarded due to contamination.

4.3.2.2.1 Mortality three days after treatment

Control flies died more than infected ones (figure 42, table 59).

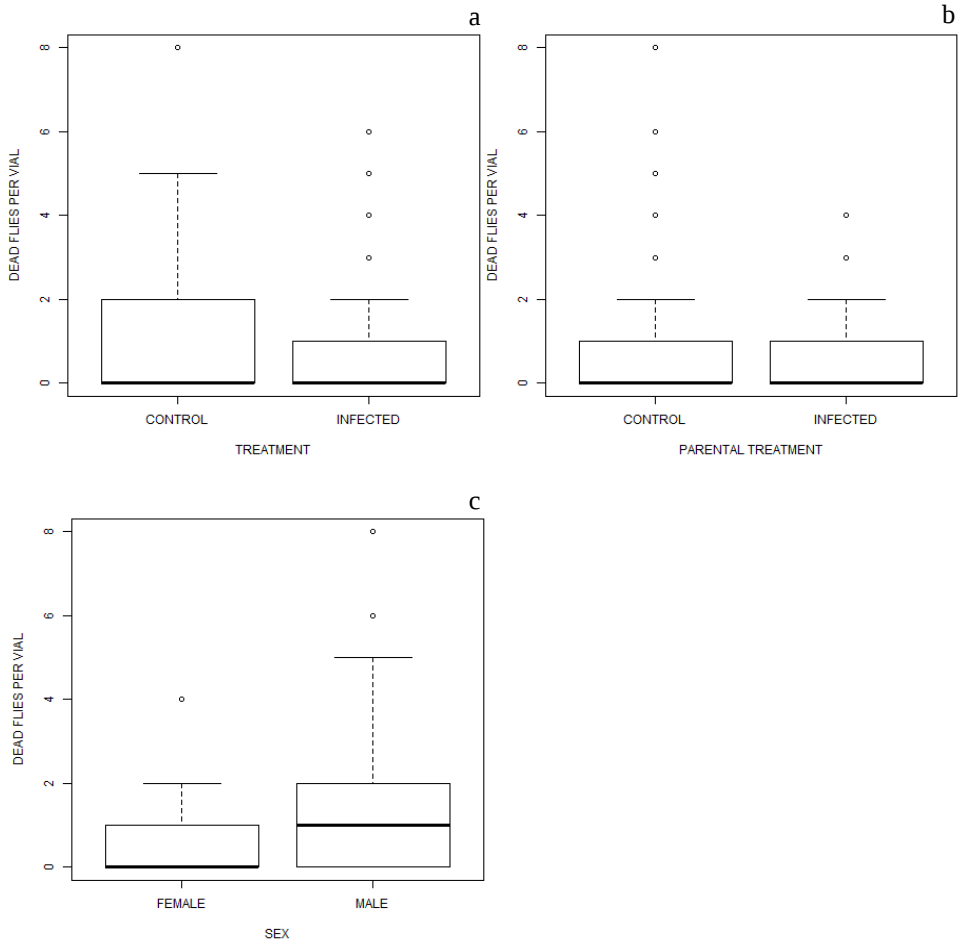

Figure 42 (a) Box plot representing the number of dead flies per vial during the first three days after treatment for control and infected flies. (b) Box plot representing the number of dead flies per vial during the first three days after treatment for flies from infected and control parents. (c) Box plot representing the number of dead flies per vial during the first three days after treatment for male and female flies.

Flies from control parents died more than flies from infected parents and male flies more than female ones (figure 42, table 59). In total the percentage of dead flies was 5.61%.

	Treatment	Parents	Sex
p-value	0.01211	0.00666	6.77e-05

Table 59 P-values of the significant covariates.

4.3.2.2.1.1 Conclusions

Control flies suffered higher mortality than treated ones, possibly due to the effect of the oil (see above). More interestingly, flies whose parents were exposed to the parasite suffered a lower mortality than the others. The same pattern was visible at six days after infection (p-value = 0.00454). This possibly points to an effect of priming on fly vigour: flies whose parents were infected are more able to survive a stress situation, but do not show increased survival against the same pathogen. This hypothesis is interesting and deserves further investigation. Finally “Sex” was significant with males showing higher mortality than females, as we have seen above.

4.3.2.2.2 Mortality from three days after treatment and later

4.3.2.2.2.1 Linear model

All time covariates were retained and significant (table 60). “Treatment” was significant.

	Time	Log Time	Time squared	Treatment
p-value	2.96e-05	3.04e-09	0.00103	< 2e-16

Table 60 P-values of the significant covariates.

4.3.2.2.2.2 Kaplan-Meier survival curves

The Kaplan-Meier survival curve for each flies group is shown in figure 43. Infected flies died more than control ones.

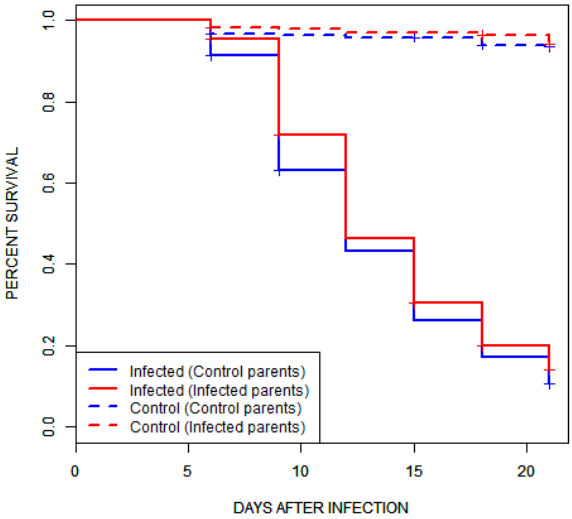

Figure 43 Kaplan-Meier plot representing survival curves for all flies groups. Full lines represent infected flies, while dashed lines represent control flies; red lines represents flies from infected parents, while blue lines represent flies from control parents (see legend).

4.3.2.2.2.3 Cox hazard model

"Treatment" was significant (p-value = 0.00).

4.3.2.2.2.4 Conclusions

Again we do not see any evidence of immune priming in the study system. These results do not support the presence of trans-generational immune priming in *D. melanogaster* exposed to *B. bassiana*.

5 DISCUSSION

5.1 ASSESSMENT OF SURVIVAL TO *B. BASSIANA* IN *D. MEALANOGASTER* POPULATIONS

Variation in susceptibility to a generalist parasite can reflect difference in life-history strategy and/or in immune investment between host populations (McDade 2003; Cornet et al. 2009; Horrocks et al. 2012). There is some evidence for an effect of latitude on host immune competence so that the higher species richness at tropics selects for increased immune investment (Schemske 2009). Indeed Tinsley and co-workers found lower susceptibility to the entomopathogenic fungus *Beauveria bassiana* in tropical *Drosophila melanogaster* populations (Tinsley et al. 2006).

Here we assessed susceptibility to *B. bassiana* in two tropical and two temperate *D. melanogaster* populations. We performed three independent infection experiments with two different parasite strains, but were not able to detect a significant difference in susceptibility among host populations. Furthermore, temperate populations performed better than tropical ones, showing lower mortality both in infection and control treatments. This is likely due to their higher general vigour. A possible explanation is that the inbred lines used to build the African and Asian out-crossed populations were kept in the lab for longer than the other lines and have possibly adapted to lab conditions. Alternatively, the difference could be due to the environment. Indeed, although flies were kept at 25° C during the experimental procedure, they were reared at 23° C and in temperate-like climatic conditions. Therefore European and American flies could be better acclimated and as a consequence more fit.

The absence of variation in parasite susceptibility among host populations could be due to several reasons. In a first place we used different populations and parasite strains as in Tinsley et al. (2006). Furthermore we worked with *D. melanogaster* out-crossed populations instead of F1 crosses. The use of out-crossed populations, although a closer approximation of natural conditions, introduces more variance in our measurements. In fact each individual is in principle genetically different from the others due to recombination, while in F1 crosses all offspring are virtually identical. However, despite the increased variance, we would expect a trend of lower susceptibility in tropical populations. As this is not the case, we are confident that no variance in susceptibility is present in our system. Another difference is that in Tinsley's experiment control flies were kept on food containing the antifungal agent Nipagin, while in our case both infected and control flies were reared on Nipagin-free food. The reason for our choice was to assure homogeneity among

experimental groups (see Materials and Methods). It is possible that the variability in mortality between host genotypes observed in Tinsley et al. (2006) reflects, at least partially, a difference in vigour among the *D. melanogaster* genotypes used.

We used two fungal strains coming from different geographic regions, Malaysia and France, and isolated from distinct host orders, Diptera and Coleoptera. Naively we expected the tropical strain to be more virulent against the tropical host populations and the temperate one against the temperate populations (i.e. local adaptation). In contrast with these expectations, we could not find evidence of local adaptation in our experiments.

A possible explanation is that *B. bassiana*, as a generalist pathogen, is not likely to co-evolve with any host species or population (Kawecki 1998). On the other hand, local adaptation was not found even in the case of *D. melanogaster* and its specific parasitoid *Asobara tabida* (Kraaijeveld, 1999) and, to our knowledge, has not been reported for any *D. melanogaster* – parasite combination. The absence of such reports is possibly due to the scarce information available about *D. melanogaster* ecology and to the difficulty to detect local adaptation (see Introduction). On the other hand, there is evidence of co-evolution between *D. melanogaster* and its parasite the sigma virus (Wilfert, 2013). This supports the hypothesis that local adaptation is likely to exist between *D. melanogaster* and some of its specific parasites in nature. Local adaptation to a-biotic environment has been found in *D. melanogaster* populations, for example in terms of correlation between local climate and ability to enter in diapause (Williams 2009) or between altitude and dissecation resistance (Parkash 2008). While latitude alone does not appear to be a good predictor of host immune competence in our study system, other environmental variables, such us temperature, humidity or a direct measure of parasite species richness, could be more informative. For example, fly populations coming from locations with a rich bacterial community have been found to be less susceptible to the bacteria *Lactococcus lactis* (Corby-Harris and Promislow 2008). Although ecology is important to understand host-parasite interaction (Schulenburg et al. 2009), we know little about *D. melanogaster* in the field. Including more detailed ecological information would increase our precision in testing the effect of the environment on host immune investment and other life history traits.

5.2 *D. MELANOGASTER* TRANSCRIPTIONAL RESPONSE TO *B. BASSIANA*

The role of host transcriptional response in determining the out-come of an infection has been

highlighted in several studies (Apidianakis et al. 2005; Lovegrove et al. 2006; Okado et al. 2009; Polesani et al. 2010; Moscou et al. 2011; Lockyer et al. 2012). Especially early transcriptional response seems to distinguish susceptible from resistant host genotypes (Okado et al. 2009; Lockyer et al. 2012). Here we characterized transcriptional response to *B. bassiana* in four out-cross *D. melanogaster* populations at two time points, 8 and 24 hours after infection, both by micro-array (8 hours) and RNA sequencing (8 and 24 hours).

At the earliest time point few genes were induced. While we were not really able to find a meaningful pattern out of the micro-array data, clearer results emerge from the RNA sequencing. The difference was possibly due to the higher power and lower noise of RNA sequencing, that appears to be a more suitable approach to identify small changes in gene expression profile (Wang et al. 2009).

Among the few genes that showed up at the earliest time point in the RNA sequencing analysis, we found several members of the cytochrome P450 (Cyp450) family. These genes code for enzymes that are important for drugs and toxic compounds detoxification. Other induced genes code for enzymes that further metabolise toxic substances and for transporters that could be involved in excretion. These results suggest a central importance of malpighian tubules, an excretory organ responsible for detoxification in insects (Yang et al. 2007) and recently proposed to play a role in immunity (Davies et al. 2012), in early response to fungal infection.

One cytochrome P450 gene, *Cyp6d2*, was significantly induced in the Asian and European populations at 8 hours after infection and in all populations at the latest time point. *Cyp6d2* is specifically expressed in *D. melanogaster* tracheal system (Chung et al. 2009), a hot-spot for fungal penetration (Pekrul and Grula 1979; Sahayaraj et al. 2013). Therefore *Cyp6d2* could be involved in detoxification of fungal toxins produced during cuticle penetration and possibly trigger the later defence response.

Indeed *B. bassiana* is known to produce toxins upon infection (see Introduction) and it has been shown that *B. bassiana* infection affects detoxifying enzyme activity in the insect *Eurygaster integriceps* (Zibaee et al. 2009). Cyp450s and other detoxification genes have been reported to degrade fungal and plant toxins in insects (Serebrov et al. 2006; Niu et al. 2008; Després et al. 2007).

Twenty four hours following infection we identified around 3,000 induced genes, considerably more than in previous studies (De Gregorio et al. 2001; Roxstrom-Lindquist et al. 2004). This difference is likely due to the superior power of the RNA sequencing technology and to the higher number of replicates especially in the "treatment analysis". These results indicate that a strong transcriptional response to *B. bassiana* is mounted between 8 and 24 hours after infection in *D. melanogaster.* In the following we will focus on the transcriptional response at 24 hours after infection.

The gene ontology categories (GOs) enriched in genes up-regulated upon infection are mainly connected with response to stimulus or regulation of some biological process, pointing to the importance of sensing and mounting a defence response to the pathogen. On the other hand the GOs enriched in genes down-regulated upon infection have to do mainly with translation, metabolism, biosynthesis and reproduction, most likely reflecting the decrease in gene expression of not essential functions in order to save resources for the defence response.

Indeed, metabolism reduction has been observed in *D. melanogaster* upon viral infection (Arnold et al. 2013) and it has been shown that the activation of the Toll pathway in the fat body at the same time induces immunity and reduces nutrient storage and growth rate by acting on the insulin signalling (DiAngelo et al. 2009). On the other hand, it is possible that the parasite itself suppresses host translation machinery in order to impair the defence response (Mohr and Sonenberg 2012). Further research is needed in order to evaluate the importance of these two factors in the *D. melanogaster – B. bassiana* system.

Notably, only a small minority of the induced genes were directly connected with immunity, while metabolism and stress response were more represented. Therefore, considering genes involved in stress response (Davies et al. 2012) and metabolism (Chambers et al. 2012) besides immunity genes can help us to better understand host response to parasites. On the other hand, in De Gregorio et al. (2001) and Roxstrom-Lindquist et al. (2004) immune GOs were the most enriched categories in induced genes.

A possible reason for this difference is that we looked at an earlier time point. In fact, although we choose 24 hours after infection, similarly to De Gregorio et al. (2001) and Roxstrom-Lindquist et al. (2004), we used a different infection protocol. While in these studies flies were infected by shaking them in a Petri dish with a sporulating culture of *B. bassiana*, we applied the more natural and less

traumatic approach from Tinsley et al. (2006). As our method allows flies to get in contact with fungal spores at the contaminated vial walls, it likely requires more time for infection to take place and introduces a delay in comparison with other studies. However, our results are of great interest, as they depict the early, and less characterised, transcriptional response to fungal infection in *D. melanogaster*.

Finally, the discrepancy could be due to the use of out-crossed populations instead of inbred lines. Indeed it has been shown that inbreeding by environmental interaction can affect gene expression in *D. melanogaster* (Kristensen et al. 2006) and this effect is likely to be stronger in the case of infection, as inbreeding has been reported to increase susceptibility to parasites (Luong et al. 2007; Ilmonen et al. 2008). This is the first study, to our knowledge, to assess transcriptional response to infection in *D. melanogaster* out-crossed populations. Therefore, studies directly comparing inbred lines and out-crossed populations are required to estimate the effect of inbreeding on genes induced following infection in *D. melanogaster*.

When we compare response to infection among populations we see that there is quite a big variation in the number of induced genes: from around 1,300 in the African population to around 200 in the Asian one. Also the fold change of induced genes varies, with the Asian population showing on average the highest values and the African one the lowest.

One possible explanation is the higher level of genetic diversity in the African population (Duchen et al. 2013). As gene expression level is dependent on genetic variation (Gilad et al. 2008), it is likely that more variability in gene expression and transcriptional response is present in the African population. This would explain the higher number of induced genes and the lower fold changes, if different genes are induced in different genotypes. Indeed, variability in gene expression has been found to be slightly higher in an African compared to a European *D. melanogaster* population both for male (Hutter et al. 2008) and female flies (Müller et al. 2011).

A crucial result is that despite the diversity of transcriptional response, all populations showed similar susceptibility to *B. bassiana*. As we used flies from different generations of the out-crossed populations for the survival assays and the RNA sequencing, and additionally we pooled the pairs of replicate populations few generations before performing the second study, it could be questioned if the results from the two analyses can actually be compared.

Despite these issues, as in each of the survival assays a distinct generation of the out-crossed populations was used and we did not find difference in susceptibility to *B. bassiana*, we are confident in relating transcriptional and survival data.

Our results are interesting and show that not always the number of induced genes is a good predictor of the ability to survive infection. Polesani et al. (2010) reported a higher number of genes induced upon infection in a resistant grapevine species than in a susceptible one. On the other hand, Langevin et al. (2012) found a stronger transcriptional response to infection in a susceptible rainbow trout genotype than in a resistant one. Therefore, no clear expectation exists on how transcriptional response strength correlates with susceptibility level and the time point after infection is likely to play an important role.

A possible reason why a different transcriptional response and the same susceptibility level to *B. bassiana* are observed is that *D. melanogaster* populations rely on distinct defence mechanisms. One example is resistance and tolerance (see Introduction). Genetic variability can affect the respective level of resistance and tolerance in host populations (Råberg et al. 2007; Ayres et al. 2008) and influence transcriptional response (Glass et al. 2012; Tai et al. 2013). Further work is required to assess variability in resistance and tolerance to fungal infection between *D. melanogaster* populations and its effect on transcriptional response to parasites.

5.2.1 Genes induced upon infection

Many genes of the Toll pathway and only few genes of the Imd pathway are induced in our study. This was expected, as Toll is responsive to fungi and Gram-positive bacteria, while Imd to Gram-negative bacteria (Lemaitre and Hoffmann 2007).

Several serine proteases and serpins genes, most of them not reported in De Gregorio et al. (2001) nor in Roxstrom-Lindquist et al. (2004), were induced. Serine proteases and serpins have regulatory functions and some of them are known to be involved in not yet well characterised cascades up-stream the Toll signalling pathway and the melanization and coagulation defence responses (Lemaitre and Hoffmann 2007; Tang 2009). Therefore the identification of new putative immune-related proteins of these families is important. Of special interest appears the gene CG9649 that is induced in all populations and in De Gregorio et al. (2001), although its function in immunity is not yet known.

Also genes involved in phagocytosis, melanization and coagulation defence responses were found. *Pdh* and *InaC*, which are required for phagocytosis (Stroschein-Stevenson et al. 2006), were induced in all populations. As their role in this process is not yet characterised, they are good candidates for further research.

Many members of the Cyp450 family were induced upon infection. This suggests that metabolism of toxic compounds is of importance in the defence response both at 8 and at 24 hours after infection. The gene *Cyp6d2* was induced in all populations and is discussed above. Another gene that attracted our attention was *Cyp6g1*. This gene is responsive to DDT (Daborn *et al.* 2002; Le Goff *et al.* 2003; Daborn et al. 2007; Schmidt et al. 2010) and mercury ion (Rand et al. 2012) and was induced in all populations in the present study and in De Gregorio et al. (2001). The wide chemical responsiveness of *Cyp6g1* and of other Cyp450s in *D. melanogaster* is reviewed in Giraudo et al. (2010) and could reflect their ability to detoxify a broad range of xenobiotics.

The genes *Urate oxidase*, *Obp99b* and *pale* were induced in all populations and in both De Gregorio et al. (2001) and Roxstrom-Lindquist et al. (2004). *Pale* is the only gene directly involved in immunity, as it codes for an enzyme required in the first phases of the melanization defence response (De Gregorio et al. 2001).

Obp99b is known to take part in autophagic cell death (Gorski et al. 2003). Recent studies have highlighted the importance of autophagy in *Drosophila* response to intracellular bacteria and viruses (Schnabel et al. 1991; Zirin and Perrimon 2010; Levine et al. 2011). However, its role in anti-fungal immunity is not defined yet.

Urate oxidase codes for an enzyme active in the malpighian tubules where it converts uric acid into allantonin and it was found to be strongly induced in *D. melanogaster* upon *Listeria monocytogenes* infection (Chambers et al. 2012). However, the role of *Urate oxidase*, uric acid and allontonin in *D. melanogaster* immunity is not yet clear and deserves further investigation.

Genes induced in all populations show higher fold change compared to genes induced only in one population. This pattern suggests that looking at genes induced in multiple populations upon the same stress allows to identify the strongly responsive ones. An alternative explanation is that, due to power considerations, genes that have a higher fold change are more likely to be significantly induced in all populations, while genes that have a lower fold change tend to be significant only in

one population and border-line significant in the others. We found that genes privately induced in one population are far from significance in the others (data not shown). Therefore we are confident that these genes are induced in a population specific manner.

In conclusion we found a relatively low percentage of induced genes directly involved in immunity in comparison with De Gregorio et al. (2001) and Roxstrom-Lindquist et al. (2004). This likely depends on the earlier stage after infection we investigated. Genes induced only in this study, especially the *Cyp450*s at 8 and 24 hours and the serine proteases and serpins at 24 hours after infection, could be involved in triggering the later immune response and deserve further investigation. On the other hand, genes induced in more than one study are probably generally important in transcriptional response to infection and should also be subject of further research. This is especially true if their role in immunity is not yet well understood, as in the case of *Urate oxidase* and *Obp99b* (see above).

5.2.2 Population genomics results

Commonly induced genes appear to have higher coding region conservation, as indicated by lower Ka/Ks ratio, than privately induced ones, with the possible exception of American privately induced genes. This would suggest that the generality and the level of induction of a gene upon stress/infection is a measure of its biological importance and therefore correlates with the sequence conservation of the protein it codes for. Nuzhdin et al. (2004) found that gene expression divergence and non synonimous substitution rate among *D. melanogaster* and *D. simulans* are correlated. This supports the idea that selection acts in a concerted manner on protein sequence and gene expression level (Lemos et al. 2005).

5.3 EFFECT OF *WOLBACHIA* AND PARENTAL PARASITE EXPOSURE ON *D. MELANOGASTER* SURVIVAL TO *B. BASSIANA*

The outcome of host-parasite interaction not only depends on the genotype of the two interacting partners. Not taking into account other factors, such host endosymbionts and gut microbiome (Haine 2008; Koch and Schmid-Hempel 2012) and trans-generational transfer of immunity (Sadd and Schmid-Hempel 2007), can obscure the origin of host variation in susceptibility. Here we assessed both of these factors in the *D. melanogaster – B. bassiana* system.

We did not find a protective effect of *Wolbachia pipientis* in *D. melanogaster* infected by *B. bassiana*. Our report adds to similar ones showing the same pattern in other host-parasite systems (Longdon et al. 2012; Rottschaefer and Lazzaro 2012). However, this is at odds with a previous study showing the opposite result (Panteleev et al. 2007). As discussed above, the difference could be due to the variation in protective ability among different *W. pipientis* strains (Osborne et al. 2009). The mechanism by which *W. pipientis* mediates protection is not known, although it has been shown that *Wolbachia* density in the head, the gut and the malpighian tubules correlates with antiviral protection in *D. simulans* and that strains present at low density do not defend against viruses (Osborne et al. 2012). It would be interesting to test if a low *Wolbachia* density in the *D. melanogaster* lines used in our experiments could explain the absence of a protective effect.

Wolbachia presence increased mortality of flies from the E20 line during the first three days in both experiments. As at this time point the effect of *B. bassiana* was not yet visible and the major cause of death was sticking to the vial walls or soaking in the oil, we conclude that the ability to survive was dependent on flies' general vigour and that *Wolbachia* reduced it in the E20 line.

Indeed, negative fitness effects of *W. pipientis* have been reported (Fleury et al. 2000; Wang et al. 2012). A similar pattern is present for the *D. melanogaster* Eg25 line in the first experiment, but not in the second one. A possible explanation is the lower mortality of the Eg25 line in comparison with the other two lines, especially in the second experiment. This could make the *Wolbachia* effect on flies' vigour, if present, more difficult to detect.

On the other hand, the antibiotic treatment had a negative effect on flies from the Eg57 line. This line was naturally *Wolbachia* free and was used as a control in our study. In both experiments we found a significantly higher mortality during the first three days in Eg57 flies that were exposed to tetracycline. As the discrepancy appeared only at this time point, we conclude that it did not have to do with susceptibility to the parasite, but that it reflected unequal fly vigour. A possible explanation is that by antibiotic treatment another symbiont or gut microbiome was removed. A negative effect of depriving *D. melanogaster* of its gut microbiome has indeed been reported (Ridley et al. 2012). It should be noted that we exposed tetracycline treated flies to food from their untreated counterpart for two days before performing the experiments. However, it is possible that this procedure was not enough to restore a normal gut microbiome.

If indeed a negative effect of tetracycline on fly vigour exists, it should affect also flies from the

E20 and Eg25 lines. The fact that E20 and Eg25 flies supplied with the antibiotic exhibited higher vigour could be explained if the positive effect of removing Wolbachia outweighed the negative one of tetracycline exposure. However, given the difference in host genotype, endosymbiont and gut microbiota among *D. melanogaster* lines, it is difficult to draw conclusions and more research is needed to distinguish the effect of each factor and of their interactions.

An interesting point in our study is that *W. pipientis*, and possibly fly gut microbiota, affected, if not susceptibility to *B. bassiana*, *D. melanogaster* vigour. It can be questioned if the measure we used for vigour, namely the number of flies that died sticking to vial walls or soaking in the oil, makes sense form a biological perspective. We should probably speak of "ability to cope with abiotic stress" instead of "vigour". In the first three days of the experiments flies had to cope with change in temperature, oily transparency film and absence of preservatives in the food. This was likely a stressful condition.

It has been shown that there is a genetic correlation for tolerance to different environmental stress and that stress tolerance is negatively associated with metabolic rate in *D. melanogaster* (Hoffmann and Parsons 1989). As *Wolbachia* can potentially affect the metabolism of its insect host (Brownlie et al. 2009; Evans et al. 2009), it would be interesting to investigate if it increases metabolic rate in the *D. melanogaster* lines used. It should also be tested if our result could be replicated when a different type of stress is applied. However, no effect of *Wolbachia* on heat or nutritional tolerance in *D. melanogaster* has been identified so far (Harcombe and Hoffmann 2004).

Finally, in the second experiment we found that infected males from the E20 and Eg57 lines died significantly later than infected females from the respective lines. This result could be explained by a report showing that mated females suffer reduction in immune activity in *D. melanogaster* due to male seminal fluid components (Short et al. 2012).

We did not observe trans-generational immune priming in *D. melanogaster* infected by *B. bassiana*. Similar results were found in other study systems (Linder and Promislow 2009; Reber and Chapuisat 2012). Interestingly, in the second experiment, flies whose parents were exposed to *B. bassiana* exhibited a lower mortality during the first six days. However, this trend was not significant overall. A possible explanation is that flies whose parents were exposed to the parasite had an increased ability to cope with stress. We did not observe the same pattern in the first experiment, possibly because a different *D. melanogaster* out-crossed population was used.

It is possible that we were not able to detect trans-generational immune priming because we exposed the offspring to a too high spore density and that this masked the effect of priming. A second hypothesis is that we selected eggs laid too early after parasite exposure. It has been shown that in the mealworm beetle *Tenebrio mollitor* immune challenged females provide their eggs with antimicrobial activity starting from the fourth day after immune challenge (Zanchi et al. 2012). As we collected eggs produced during the first two days after priming, it could have been too early to see an effect.

A third possibility is that we used a too high spore density when priming the parents. In the first experiment, for which these value were recorded, parents were exposed to a higher spore density than offspring. Conversely, in other studies working on the same subject, parents were challenged with a sub-lethal dose or with heat-killed parasites (Roth et al. 2010; Reber and Chapuisat 2012). Indeed, it has been shown that *D. melanogaster* primed with heat-killed *B. bassiana* spores suffers reduced mortality if subsequently exposed to the living parasite (Pham et al. 2007). Therefore, it would be interesting to assess if protection acquired in this way could persist through generations.

5.4 OUTLOOK

We did not find an effect of latitude on *D. melanogaster* susceptibility to *B. bassiana* and speculate that other environmental factors, such as local parasite species richness, could better predict host immune competence. To test this hypothesis, *D. melanogaster* populations for which ecological information is available should be used in future infection experiments. This will help to understand how ecology shapes immunity in *D. melanogaster* (Schulenburg 2009).

A drawback of our infection protocol is the low precision in the number of spores to which flies are exposed, as the use of an airbrush does not allow an exact quantification. New techniques that ensure a better determination of infective dose are now available (Farenhorst and Knols 2010) and should be used in future experiments.

During the experiment we reared flies on standard media without the anti-fungal agent Nipagin and the antimicrobial compound propanoic acid. This was done in order to avoid any possible effect on the infection procedure. However, in the absence of preservatives contamination from commensal bacteria and fungi occurred, affecting fly mortality and causing some vials to be discarded (see Material and Methods). As the effect of Nipagin and propanoic acid on *B. bassiana* has not been

tested, it should be assessed if their use has indeed negative consequences on the fungus, justifying the choice of preservatives-free food. Alternatively, flies could be reared on agar molasses plates, as we observed no contamination in the absence of preservatives when this media was used. Tests should be performed in order to define the best and more convenient infection protocol.

We found a different set of genes induced after *B. bassiana* infection than in De Gregorio et al. (2001) and Roxstrom-Lindquist et al. (2004). The most likely reason is that we assessed transcriptional response at an earlier stage, when fungal infection is just perceived, xenobiotics compounds are recognised and detoxified, and immune response begins to be mounted. A big difference is present also among the two time points examined in our study. While at 8 hours only few genes, mainly involved in detoxification, were induced, a strong response is observed at 24 hours after infection. As few hours can dramatically change the set of induced genes, further experiments should assess transcriptional response at multiple time points in order to better characterise the timing of defence transcriptional response. The development of RNA sequencing technology will make this approach feasible in the near future.

We identified genes induced in all host populations and in De Gregorio et al. (2001) and/or Roxstrom-Lindquist et al. (2004). As the role of most of these genes in defence response is not yet characterised, we propose to assess susceptibility and immune response to *B. bassiana* n *D. melanogaster* mutation lines, as in Jin et al. (2008). Future experiments should also determine change in protein level for some candidate genes following infection.

We found higher protein conservation at common than at private genes. However, this analysis was performed only on a small subset of genes and should be repeated on the full data. Further analysis should be performed to determine the level of genetic variability at common and private genes both for coding and regulatory regions, as there is evidence of regulatory evolution for gene expression (Fraser et al. 2010, Shibata et al. 2010).

As the *B. bassiana* genome is now available (Xiao et al. 2012), we could map the reads from the RNA sequencing to identify parasite genes differently expressed between 8 and 24 hours after infection. This will also allow to assess the relationship among host and parasite expression profiles in different *D. melanogaster* populations.

We did not find an effect of *Wolbachia* on *D. melanogaster* susceptibility to fungal infection.

However, *Wolbachia* affected fly fitness in one *D. melanogaster* line. On the other hand, we reported an effect of the tetracycline treatment on general fly vigour in a *Wolbachia*-free *D. melanogaster* line. This could be a consequence of the loss of important microbiome components. Future experiments should aim to characterise the microbiome composition of *D. melanogaster* lines and its effect on fly fitness and susceptibility to parasites, as with next-generation sequencing technology this analysis is now feasible and practical (Fink et al. 2013).

We did not find evidence of trans-generational immune priming in *D. melanogaster* infected with *B. bassiana*. However, it would be interesting to expose the parents to heat-killed spores, as in Pham et al. (2007), or to secreted fungal compounds and to assess if trans-generational priming is present in these cases. As in the mealworm beetle *Tenebrio molitor* the level of protection conferred to offspring has been found to depend on the time after parasite exposure (Zanchi et al. 2012), future experiments should be performed to test if this is the case also in our system.

5.5 RELEVANCE OF THE PRESENT WORK AND CONCLUSION

The present work addresses several aspects of host-parsite interactions. We first asked if susceptibility to the fungal pathogen *B. bassiana* varies among *D. melanogaster* populations and if tropical populations exhibit lower mortality than temperate ones. This question, prompted from the work of Tinsley et al. (2006), is of central interest in ecological immunology, the study of how immunity is shaped by ecological factors (Schulenburg et al. 2009). We found no variation in susceptibility among host populations and no general effect of latitude on host immune competence. We discuss other factors that could determine immune investment and that should be investigated in future work.

We looked for genotype x genotype interaction and local adaptation by assessing two parasite strains of different geographic origin. There is no evidence of genotype x genotype interaction and of parasite adaptation to hosts coming from close geographical regions. We hypothesize that the geographical scale assessed may not allow to identify local adaptation (Kaltz and Shykoff 1998; Gandon 2002; Kawecki and Ebert 2004; Vos et al. 2009). Furthermore, we discuss that *B. bassiana*, as a generalist pathogen, is not likely to engage in co-evolutionary arms race with its hosts (Kawecki 1998).

As change in gene expression profile following parasite exposure depends on the genotype of the

interacting partners (Riddel et al. 2009), we assessed transcriptional response to *B. bassiana* in four *D. melanogaster* populations and found different numbers of induced genes. We discuss that, as variation in gene expression is dependent on genetic variability (Gilad et al. 2008), the higher number of induced genes in the African population may be due to its higher level of genetic diversity (Duchen et al. 2013).

Time is important when looking at transcriptional response to infection and early response has been found to be a predictor of parasite susceptibility in *D. melanogaster* (Okado et al. 2009). We found that, while *D. melanogaster* expression profile is almost no affected by *B. bassiana* after 8 hours, a strong response is mounted 24 hours following infection. We identified early induced genes, mainly Cyp450s, and discuss their possible role in triggering later immune response.

Gene ontology categories enriched in genes up-regulated after infection are related to stress and regulation of biological processes, while categories enriched in genes down-regulated following infection are connected with translation, biosynthesis and reproduction. We discuss that, as metabolism reduction has been reported to accompany immune induction in *D. melanogaster* (DiAngelo et al. 2009), a trade-off between mounting a defence response and executing biosynthetic/reproduction processes is likely to exist.

The genes *Urate oxidase, Obp99b* and *pale* are induced in all populations in our study and in both De Gregorio et al. (2001) and Roxstrom-Lindquist et al. (2004). Therefore these genes appear to play a central role in defence response to fungi. We discuss the importance of better characterising *Urate oxidase* and *Obp99b,* as their function in immune defence is not yet clear.

As there is evidence that selection acts in a concerted manner on protein sequence and gene expression level (Nuzhdin et al. 2004; Lemos et al. 2005), we investigated protein conservation in genes commonly induced in all populations and found a pattern of purifying selection. As only a sub-set of private and common genes was assessed, we discuss the importance of repeating this analysis with the full data-set.

We found no protective effect of *W. pipientis* in *D. melanogaster* infected by *B. bassiana.* Our result is in agreement with studies performed in different host-parasite systems (Longdon et al. 2012; Rottschaefer and Lazzaro 2012). We report a negative effect of *Wolbachia* on fly vigour in one *D. melanogaster* inbreed line. We discuss this result in relation to other studies reporting detrimental

fitness effects of *Wolbachia* (Fleury et al. 2000; Wang et al. 2012) and propose a connection with its ability to influence host metabolism (Brownlie et al. 2009; Evans et al. 2009).

Trans-generational immune priming does not appear to play a role in *D. melanogaster* infected by *B. bassiana*. This result is in agreement with previous work in other host-parasite systems (Linder and Promislow 2009; Reber and Chapuisat 2012). We discuss the opportunity to investigate immune competence of offspring produced at different time points after parasite-exposure, as it has been reported that antimicrobial activity provided by *Tenebrio molitor* immune challenged females varies with time after immune challenge (Zanchi et al. 2012).

6 APPENDIX

6.1 Standard fly media preparation

Dissolve 52 g Kobe Agar in 2500 ml Water (Roth) by autoclaving 5 min. at 110°C. In the meantime add to 8500 ml of purified water:

500 ml Golden Syrup (Grafschafter),

500 g Bio -Polenta (Neuform),

500 g Dry yeast (Uniferm).

Boil, let cool down to 60 °C and then add:

60 ml propanoic acid (Roth),

16 g Nipagin (Geyer) in 150 ml Ethanol (Roth),

Finally add the dissolved agar.

Distribute the food in vials or bottles (VWR or K-TK) and let dry at room temperature over-night.

The next day close the vials/bottles with anti-mites stopper and store at 8°C.

6.2 Lines and crosses to build the out-crossed populations

In table 1 the inbreed lines used in the crosses to build the out-crossed populations are reported. Reciprocal crosses are performed (see main text).

AFRICA	AMERICA	ASIA	EUROPA
A 84F * A95 M	RAL 303 F * RAL 324 M	KL1 F * KL2 F	E1 F * E2 M
A 95 F * A84 F	RAL 324 F * RAL 303 M	KL2 F * KL1 F	E2 F * E1 M
A 145 F * A157 M	RAL 380 F * RAL 391 M	KL6 F * KL7 F	E11 F * E12 M
A 157 F * A145 M	RAL 391 F * RAL 380 M	KL7 F * KL6 F	E12 F * E11 M
A 184 F * A191 M	RAL 427 F * RAL 437 M	KL8 F * KL10 F	E13 F * E14 M
A 191 F * A184 M	RAL 437 F * RAL 427 M	KL10 F * KL8 F	E14 F * E13 M
A 229 F * A377 M	RAL 555 F * RAL 732 M	KL11 F * KL12 F	E15 F * E16 M
A 377 F * A229 M	RAL 732 F * RAL 555 M	KL12 F * KL11 F	E16 F * E15 M
A 384 F * A398 M	RAL 774 F * RAL 820 M	KL19 F * KL20 F	E17 F * E18 M
A 398 F * A384 M	RAL 820 F * RAL 774 M	KL20 F * KL19 F	E18 F * E17 M
	RAL 852 F * RAL 705 M	KL21 F * KL22 F	E19 F * E20 M
	RAL 705 F * RAL 852 M	KL22 F * KL21 F	E20 F * E19 M

Table 1 Lines used in the crosses to build the out-crossed populations. “M” stays for male and “F” for females.

6.3 Microarray protocols

These protocols come with minor modifications from a previous one written by Prof. Baines in 2007 and are based on the ones provided by the Drosophila Resource Centre (DGRC) (http://dgrc.cgb.indiana.edu) and Corning/Promega (http://www.corning.com). A list of the kits used and the composition of the Pre-hybridization and Washing Solutions are reported at the end.

6.3.1 RNA extraction protocol

This protocol was used for RNA extraction form frozen flies.

1. Collect all the frozen flies in one Eppendorf tube, add 600 µl Trizol and grind the flies completely.
2. Add 400 µl Trizol; mix by inverting and incubate at room temperature for 5 minutes.
3. Centrifuge 12,000 rcf at 4°C for 10 minutes, transfer the supernatant to a clean tube.
4. Add 200 µl chloroform, mix well by shaking the tubes vigorously for 15 seconds by hand, incubate at room temperature for 3 minutes.
5. Centrifuge 12,000 rcf at 4°C for 10 minutes, transfer the aqueous (upper) phase to a clean tube.
6. Add 500 µl isopropanol, mix thoroughly and incubate at room temperature for exactly 10 minutes.
7. Centrifuge 12,000 rcf at 4° C for 10 minutes, remove supernatant (a clearly-visible white pellet should remain).
8. Wash the pellet with 1 ml 75% ethanol prepared with RNAse-free water.
9. Centrifuge 12,000 rcf at 4° C for 10 minutes.
10. The sample can now be stored at -20° C overnight (up to 3-4 weeks).
11. Remove the ethanol completely and dry 5-10 minutes under the hood. Do not over-dry or samples may be difficult to resuspend.
12. Resuspend the pellet in 30 µl of RNAse-free water. Dissolving may be aided by several tapping + brief centrifugation's and/or heating at 37°-55° until dissolved.
13. Assess RNA concentration with Nanodrop after vortexing and brief centrifugation.
14. (Run 1 µl on a gel, rRna bands should be visible).
15. You should be left with 29 (or 28) µl of sample at a concentration of 4-5 µg/µl. This is enough for two hybridizations.
16. Proceed directly to *First-strand cDNA Synthesis.*

6.3.2 First-strand cDNA Synthesis

This protocol was used to synthetise first-strand cDNA from RNA.

1. Take two tubes and label them: 555 and 647, each will be used for a different dye. Add to the tubes in this order:

a) DEPC treated water; volume = 18 µl - X µl – 2µl,

b) Hexomere random primers (2.5 µg/µl); volume = 2 µl,

c) Purified RNA; volume = X µl containing 25-30 µg of RNA.

2. Incubate at 70°C for 5 minutes, quick chill on ice for 1 minute and briefly centrifuge again.
3. Add the following to each tube in this order.

a) 5 x First-Strand Buffer Volume = 6 µl

b) 0.1 DTT Volume = 1.5 µl

c) dNTP mix Volume = 1.5 µl

d) RnaseOUT (40U/uL) Volume = 1 µl

e) SuperScriptTM III RT (400U/µl) Volume = 2 µl

Final Volume = 30 µl

4. Mix gently and collect the contents of each tube by briefly centrifugation. Incubate at 46°C for 3 hours.
5. Proceed directly to *Hydrolysis and Neutralization.*

6.3.3 Hydrolysis and Neutralization

This protocol was used to degrade the original RNA.

1. Add 15 µl of 1 N NaOH to each reaction tube, mix thoroughly.
2. Incubate at 70 °C for 10 minutes.
3. Add 15 µl of 1N HCl immediately after the 10 minutes incubation to neutralize the pH, mix gently and centrifuge briefly.
4. Proceed directly to *Purifying First-Strand cDNA.*

6.3.4 Purifying First-Strand cDNA

This protocol was used to remove unincorporated dNTPs by ethanol precipitation.

1. Add 24 µl 3 M Sodium Acetate, pH 5.2 and 2 µl glycogen.
2. Add 360 µl ice-cold 100% ethanol and mix by vortexing.

3. Place at -20° C for at least 1 hour, sample can be incubated over-night.
4. Centrifuge at 14,000 rcf at 4° C for 20 minutes. Carefully remove and discard the supernatant; use pipette, not trash the supernatant because the pellet is not fixed to the bottom.
5. Wash the pellet with 1 ml 75% ethanol and centrifuge at 14,000 at 4°C for 2 minutes. Carefully remove and discard the supernatant; use pipette, not trash the supernatant because the pellet is not fixed to the bottom.
6. Centrifuge at 14,000 rcm at 4° C for 1 minute more and remove again the supernatant with the pipette.
7. Dry the sample in the hood for 10-15 minutes, sample will turn from white to viscous (glass-like) when ready. Avoid over drying, as it will be harder to resuspend the samples.
8. Resuspend the samples in 5 µl of 2 X Coupling Buffer, vortex thoroughly to resuspend and centrifuge quickly.
9. Proceed directly to *Labelling with Fluorescent Dye.*

6.3.5 Labelling with fluorescent Dye

This protocol was used to label the amino-modified cDNA with the Alexa Fluor dyes. The dyes or the already labelled cDNA were not exposed to direct sun or overhead light

1. Remove the Alexa Fluor dye vials from -20°C storage.
2. Add 2 µl of DMSO and 3 µL DEPC-treated H2O directly in each dye vial (2 µl) and mix thoroughly. Add it to the sample tube to get a total volume of 10 µl.
3. Mix samples by vortexing, centrifuge briefly and incubate at room temperature in the dark for 1-3 hours.
4. While waiting prepare a fresh Prehybridization solution and incubate it at 42 °C.
5. Proceed directly to *Purification of Labeled cDNA.*

6.3.6 Purifying Labelled cDNA

This protocol was used to purify the labelled cDNA.

1. Add 700 µl of Binding Buffer to the reaction tubes containing the labeled cDNA from Coupling. Vortex briefly and centrifuge shortly to mix.
2. Each Low-Elution Volume Spin Cartridge is pre-inserted into a collection tube. For multiple reactions, clearly label each collection tube and then load the cDNA/Binding Buffer solution directly onto the Spin Cartridge.
3. Centrifuge at 3,300 rcm for 1 minute. Remove the collection tube and discard the flow-through.
4. Place the Spin Cartridge in the same collection tube and add 600 µl of wash Buffer to the column.

5. Centrifuge at maximum speed for 30 seconds. Remove the collection tube and discard the flow-through.
6. Place the Spin Cartridge in the same collection tube and centrifuge at maximum speed for 30 seconds to remove any residual Wash Buffer. Remove the collection tube and discard it.
7. Place the Spin Cartridge onto a new amber collection tube and label it.
8. Add 20 µl of DEPC-treated water to the centre of the Spin Cartridge and incubate at room temperature for 1 minute.
9. Centrifuge at maximum speed for 1 minute to collect the purified cDNA. The eluate contains your purified cDNA.
10. Vacuum centrifuge for 30 minutes.

6.3.7 Prehybridization

This protocol was used to prepare the arrays for the hybridization.

1. Warm Prehybridization solution to 42° C.
2. Immerse arrays in Prehybridization Solution and incubate at 42° C for 45-60 minutes.
3. Transfer prehybridized arrays to Wash Solution 3 and incubate at ambient temperature (22° C to 25 ° C) for 5 minutes.
4. Repeat step 3.
5. Transfer arrays to a centrifuge tube filled with RNAse-free water at ambient temperature for 30 seconds.
6. Dry arrays by centrifugation at 2,500 rcf for 2 minutes. Keep arrays in a dust free environment while completing the preparation of the hybridization solution. Label array at the bar code and write down the bar code of each array.

6.3.8 Hybridization

This protocol was followed to hybridize the labelled cDNAs on the array.

1. Prepare Hybridization solution. The following volumes are enough to resuspend one sample:

Nuclease free water: 8,4 µl

Blocking agent 100 x : 1,1 µl

2x Hi-RPM Hybridization Buffer : 55 µl

2 Resuspend each of the two samples to be competitively hybridized in 40 µl of hybridization solution; let them 3 minutes at room temperature, then vortex and briefly centrifuge.

3. Incubate the labelled cDNA solution at 95° C for 3 minutes, protecting samples from light and then let them 1 minute on ice.
4. Centrifuge the cDNA at 13,500 rcf for 2 minutes to collect condensation.
5. Transfer the two samples to be competitively hybridized in one tube (one in other, total

volume around 80 µl), vortex and briefly centrifuge.

6. Place array in Corning Hybridization Chamber (make sure to fill the two moisturising wells with 10 µl H2O each). Pipet the label cDNA gently up and down and then transfer onto the surface of the printed side of the slide. Carefully place the cover glass on the array. Avoid trapping air bubbles between the array and the cover glass. Small air bubbles that do form usually dissipate during hybridisation. Assemble the chamber. Incubate the chamber-array at 42° C for 16-18 hours in a water bath.

6.3.9 Post-Hybridization Washes

These washes were performed after hybridization to prevent unspecific signal. Arrays were not allowed to dry out between washes, as this would have irreversibly increased background levels. Multiple containers were utilized to perform the washes in the most efficient manner. All containers and the volumes of washing solutions were prepared before starting the procedure.

Containers required:

-Wash Solution 1: 2

-Wash Solution 2: 2

-Wash Solution 3: 5

-Wash Solution 4: 1

1. Disassemble the hybridization chambers.

2. Immerse arrays in Wash Solution 1 at 42° C (immerse cuvette in the 42° C bath 1 hour before starting) until the coverslip moves freely away from the slide (around 3-4 minutes).

3. Transfer arrays to fresh Wash Solution 1 at 42° C for 5 minutes.

4. Transfer arrays to Wash Solution 2 at room temperature for 5 minutes.

5. Repeat step 4.

6. Transfer arrays to Wash Solution 3 at room temperature for 1 minute.

7. Repeat Step 6 four times.

8. Rinse arrays in Wash Solution 4 for 10 seconds.

9. Dry arrays by centrifugation at 1,600 rcf for 2 minutes.

10. Store arrays in light-proof container until ready to scan.

6.3.10 Commercial kits used

The following materials are supplied with the Core Module from Invitrogen (Cat. Number L1014-02):

1. Random hexamers

2. dNTP mix, including amino-modified nucleotides
3. 5x First-Strand Buffer
4. RnaseOUT
5. SuperScriptTM Reverse transcriptase (RT)
6. 0.1 M DTT
7. DEPC treated H2O
8. 2x Coupling Buffer
9. DMSO

The following materials are supplied with the Purification Module from Invitrogen (Cat. Nummer 45-0042):

21. S.N.A.P. column(s) and clear collection tube(s)
22. Amber collection tube(s)
23. Loading Buffer with isopropanol added
24. Wash Buffer with ethanol added.

The following materials are supplied with the Alexa Flour 555 and Alexa Fluor 647 Reactiv Dye DecaPacks from Invitrogen (Cat. Number A-32755):

25. Alexa Fluor 555
26. Alexa Fluor 647

2x Hi-RPM Hybridization Buffer is from Agilent (Cat. Number: 5190-0403)

The following material is supplies with the Pronto! universal microarray hybridisation kit from Corning (Cat. Number: 40026):

Blocking agent 100x

6.3.11 Prehybridization and washing solutions

Solutions were prepared following the UltraGAPS Coated slides Instruction Manual, Corning Life Science (http://www.corning.com). For all solution double-distilled water (ddH2O) was used.

-Prehybridization Solution: 5 x SSC, 0.1% SDS and 0.1 mg/ml BSA

-Wash Solution 1: 2 x SSC, 0.1% SDS

-Wash Solution 2: 0.1 x SSC, 0.1% SDS

-Wash Solution 3: 0.1 x SSC

-Wash Solution 4: 0.01 x SSC

6.4 Micro-array hybridisation scheme

COMPARISON	SAMPLE 1	SAMPLE 2	COMPARISON	SAMPLE 1	SAMPLE 2
1	Africa 1 I	Africa 1 C	13	Africa 2 I	Africa 2 C
2	America 1 I	America 1 C	14	America 2 I	America 2 C
3	Asia 1 I	Asia 1 C	15	Asia 2 I	Asia 2 C
4	Europe 1 I	Europe 1 C	16	Europe 2 I	Europe 2 C
5	Africa 1 I	America 1 I	17	Africa 2 I	America 2 I
6	America 1 I	Asia 1 I	18	America 2 I	Asia 2 I
7	Asia 1 I	Europe 1 I	19	Asia 2 I	Europe 2 I
8	Europe 1 I	Africa 1 I	20	Europe 2 I	Africa 2 I
9	Africa 1 C	America 1 C	21	Africa 2 C	America 2 C
10	America 1 C	Asia 1 C	22	America 2 C	Asia 2 C
11	Asia 1 C	Europe 1 C	23	Asia 2 C	Europe 2 C
12	Europe 1 C	Africa 1 C	24	Europe 2 C	Africa 2 C

Table 2 The table reports the comparisons among all population and treatment combinations. “I” stands for infected and “C” for controls. For each comparison two hybridisations (dye swaps) were performed.

6.5 Fly vials used for RNA sequencing

VIAL	POPULATION	TREATMENT	VIAL	POPULATION	TREATMENT	VIAL	POPULATION	TREATMENT
1	AF	C	25	AM	I08	49	AS	I24
2	AF	C	26	AM	I08	50	AS	I24
3	AF	C	27	AM	I08	51	AS	I24
4	AF	C	28	AM	I08	52	AS	I24
5	AF	C	29	AM	I08	53	AS	I24
6	AF	C	30	AM	I08	54	AS	I24
7	AF	I08	31	AM	I24	55	EU	C
8	AF	I08	32	AM	I24	56	EU	C
9	AF	I08	33	AM	I24	57	EU	C
10	AF	I08	34	AM	I24	58	EU	C
11	AF	I08	35	AM	I24	59	EU	C
12	AF	I08	36	AM	I24	60	EU	C
13	AF	I24	37	AS	C	61	EU	I08
14	AF	I24	38	AS	C	62	EU	I08
15	AF	I24	39	AS	C	63	EU	I08
16	AF	I24	40	AS	C	64	EU	I08
17	AF	I24	41	AS	C	65	EU	I08
18	AF	I24	42	AS	C	66	EU	I08
19	AM	C	43	AS	I08	67	EU	I24
20	AM	C	44	AS	I08	68	EU	I24
21	AM	C	45	AS	I08	69	EU	I24
22	AM	C	46	AS	I08	70	EU	I24
23	AM	C	47	AS	I08	71	EU	I24
24	AM	C	48	AS	I08	72	EU	I24

Table 3 Scheme indicating the number of vials for each population – treatment combination. "AF" stands for Africa, "AM" for America,"AS" for Asia and "EU" for Europe. "C" stands for control, "I08" for 8 hours infection and "I24" for 24 hours infection.

6.6 PCR conditions

NAME	SEQUENCE	Tm
28sF3633	TAC CGT GAG GGA AAG TTG AAA	58 °C
28sR4076	AGA CTC CTT GGT CCG TGT TT	58 °C
Wsp81F	TGG TCC AAT AAG TGA TGA AGA AAC	55 °C
Wsp691R	AAA AAT TAA ACG CTA CTC CA	55 °C

Table 4 Primers used to amplify the *Wsp* and the *28S* gene.

CYCLES	TEMPERATURE	TIME
1x	95° C	5 m
30 X	95°C	20 s
	X° C	1 m
	72° C	Y s
1 x	72° C	10 m

Table 5 PCR instructions for PCR reactions; “s” stands for seconds and “m” for minutes, X is 57° C for *Wsp* and 57° C for *28S*; Y is 38 s for *Wsp* and 45 s for *28S*.

COMPONENT	VOLUME
H2O	16.12 µl
10x PCR Buffer	2.5 µl
Mg 50 mM	1 µl
dNTPs (12.5 mM)	0.25 µl
Taq	0.13 µl
5’ primer (10 mM)	2 µl
3’ primer (10 mM)	2 µl
Genomic DNA (500ng/µl)	1 µl
Total	25 µl

Table 6 Components of PCR reactions.

6.7 Gene Ontology analysis at 8 hours after infection

8 HOURS INFECTION AMERICA (FUNCTION)			
GO Term	Description	p-value	FDR q-value
GO:0016491	oxidoreductase activity	1.40E-04	3.40E-01
CONTROL FLIES AMERICA (PROCESS)			
GO Term	Description	p-value	FDR q-value
GO:0045103	intermediate filament-based process	2.77E-04	1.00E+00
GO:0045104	intermediate filament cytoskeleton organization	2.77E-04	7.35E-01
GO:0060052	neurofilament cytoskeleton organization	2.77E-04	4.90E-01
GO:0046684	response to pyrethroid	2.77E-04	3.67E-01
CONTROL FLIES AMERICA (FUNCTION)			
GO Term	Description	p-value	FDR q-value
GO:0005248	voltage-gated sodium channel activity	8.31E-04	1.00E+00
CONTROL FLIES AMERICA (COMPONENT)			
GO Term	Description	p-value	FDR q-value
GO:0034706	sodium channel complex	5.54E-04	4.86E-01
GO:0001518	voltage-gated sodium channel complex	5.54E-04	2.43E-01
8 HOURS INFECTION ASIA (PROCESS)			

GO Term	Description	p-value	FDR q-value
GO:1901563	response to camptothecin	3.69E-04	1.00E+00
CONTROL EUROPE (FUNCTION)			
GO Term	Description	p-value	FDR q-value
GO:0008480	sarcosine dehydrogenase activity	1.85E-04	4.48E-01
GO:0046997	oxidoreductase activity, acting on the CH-NH group of donors, flavin as acceptor	1.85E-04	2.24E-01
GO:0004047	aminomethyltransferase activity	7.39E-04	5.98E-01

Table 7 Gene ontology categories enriched in induced genes. "PROCESS" stays for ontology categories related to biological process, "FUNCTION" for ontology categories related to molecular function and "COMPONENT" for ontology categories related to cellular and extra-cellular component. For each category p-value and false discovery rate corrected q-value are reported.

6.8 Gene Ontology analysis at 24 hours after infection

RESPONSE TO STIMULUS OR SUBSTANCE			
GO Term	Description	p-value	FDR q-value
GO:0050896	response to stimulus	2.40E-22	1.27E-18
GO:0009605	response to external stimulus	2.59E-17	1.96E-14
GO:0042221	response to chemical stimulus	3.33E-17	2.21E-14
GO:0048583	regulation of response to stimulus	1.16E-11	2.55E-09
GO:0048585	negative regulation of response to stimulus	6.16E-009	6.80E-007
GO:0010033	response to organic substance	1.68E-08	1.59E-06
GO:0014070	response to organic cyclic compound	3.92E-07	2.60E-05
GO:0097305	response to alcohol	7.25E-07	4.00E-05
GO:0051716	cellular response to stimulus	1.10E-006	5.94E-005
GO:0009628	response to abiotic stimulus	1.58E-05	6.20E-04
GO:1901700	response to oxygen-containing compound	1.58E-05	6.17E-04
GO:0009719	response to endogenous stimulus	2.74E-004	6.85E-003
GO:0045471	response to ethanol	3.90E-05	1.33E-03
GO:0009607	response to biotic stimulus	2.68E-004	6.77E-003
GO:0017085	response to insecticide	1.39E-004	4.03E-003
GO:0001964	startle response	9.21E-004	1.80E-002
IMMUNITY			
GO Term	Description	p-value	FDR q-value
GO:0019730	antimicrobial humoral response	9.17E-05	2.78E-03
GO:0051707	response to other organism	2.11E-04	5.72E-03
GO:0009636	response to toxic substance	4.23E-04	9.43E-03
GO:0008063	Toll signaling pathway	7.15E-04	1.44E-02
GO:0006955	immune response	4.28E-04	9.50E-03
CIRCADIAN			
GO Term	Description	p-value	FDR q-value
GO:0007623	circadian rhythm	1.48E-10	2.71E-08
GO:0048512	circadian behavior	4.35E-09	5.12E-07
GO:0022410	circadian sleep/wake cycle process	1.63E-04	4.58E-03
TRACHEAL SYSTEM			
GO Term	Description	p-value	FDR q-value
GO:0007424	open tracheal system development	5.37E-05	1.75E-03
GO:0035152	regulation of tube architecture, open tracheal system	2.19E-04	5.85E-03

Table 8 Subset of gene ontology categories enriched in genes up-regulated after infection in the African population. For each category p-value and false discovery rate corrected q-value are reported.

GO Term	Description	p-value	FDR q-value
GO:0022904	respiratory electron transport chain	7.20E-014	3.82E-010
GO:0022900	electron transport chain	2.46E-013	6.52E-010
GO:0044710	single-organism metabolic process	2.51E-012	4.44E-009
GO:0006091	generation of precursor metabolites and energy	5.69E-011	7.55E-008
GO:0032504	multicellular organism reproduction	9.34E-011	9.90E-008
GO:0000003	reproduction	2.49E-010	2.20E-007
GO:0055114	oxidation-reduction process	1.34E-008	1.02E-005
GO:0006123	mitochondrial electron transport, cytochrome c to oxygen	2.44E-008	1.62E-005
GO:0006120	mitochondrial electron transport, NADH to ubiquinone	2.73E-008	1.61E-005
GO:0015985	energy coupled proton transport, down electrochemical gradient	8.28E-007	4.39E-004
GO:0015986	ATP synthesis coupled proton transport	8.28E-007	3.99E-004
GO:0006754	ATP biosynthetic process	3.97E-006	1.75E-003
GO:0009206	purine ribonucleoside triphosphate biosynthetic process	1.37E-005	5.58E-003
GO:0009145	purine nucleoside triphosphate biosynthetic process	1.37E-005	5.18E-003
GO:0009201	ribonucleoside triphosphate biosynthetic process	1.80E-005	6.35E-003
GO:0009142	nucleoside triphosphate biosynthetic process	1.80E-005	5.95E-003
GO:0046034	ATP metabolic process	2.98E-005	9.31E-003

GO:0035092	sperm chromatin condensation	3.38E-005	9.95E-003
GO:1901566	organonitrogen compound biosynthetic process	3.71E-005	1.03E-002
GO:0044281	small molecule metabolic process	9.60E-005	2.54E-002
GO:0046390	ribose phosphate biosynthetic process	2.03E-004	5.14E-002
GO:0009260	ribonucleotide biosynthetic process	2.03E-004	4.90E-002
GO:1901659	glycosyl compound biosynthetic process	2.79E-004	6.43E-002
GO:0042455	ribonucleoside biosynthetic process	2.79E-004	6.16E-002
GO:0009163	nucleoside biosynthetic process	2.79E-004	5.91E-002
GO:0006818	hydrogen transport	5.89E-004	1.20E-001
GO:0015992	proton transport	5.89E-004	1.16E-001
GO:0009152	purine ribonucleotide biosynthetic process	5.89E-004	1.12E-001
GO:0042451	purine nucleoside biosynthetic process	7.29E-004	1.33E-001
GO:0046129	purine ribonucleoside biosynthetic process	7.29E-004	1.29E-001
GO:0006164	purine nucleotide biosynthetic process	7.39E-004	1.26E-001
GO:0050953	sensory perception of light stimulus	8.39E-004	1.39E-001
GO:0007601	visual perception	8.39E-004	1.35E-001
GO:0009165	nucleotide biosynthetic process	9.07E-004	1.41E-001

Table 9 Gene ontology categories enriched in genes down-regulated after infection in the African population. For each category p-value and false discovery rate corrected q-value are reported.

GO Term	Description	p-value	FDR q-value
GO:0044710	single-organism metabolic process	3.46E-16	1.84E-12
GO:0055114	oxidation-reduction process	3.50E-11	9.29E-08
GO:0044281	small molecule metabolic process	3.62E-11	6.39E-08
GO:0043436	oxoacid metabolic process	3.47E-08	4.60E-05
GO:0006082	organic acid metabolic process	3.47E-08	3.68E-05
GO:0019752	carboxylic acid metabolic process	9.33E-08	8.25E-05
GO:0009069	serine family amino acid metabolic process	1.82E-07	1.38E-04
GO:0008152	metabolic process	2.64E-07	1.75E-04
GO:1901605	alpha-amino acid metabolic process	2.27E-06	1.34E-03
GO:1901566	organonitrogen compound biosynthetic process	2.85E-06	1.51E-03
GO:0044712	single-organism catabolic process	3.20E-06	1.54E-03
GO:0044282	small molecule catabolic process	3.20E-06	1.41E-03
GO:0016054	organic acid catabolic process	4.68E-06	1.91E-03
GO:0046395	carboxylic acid catabolic process	4.68E-06	1.77E-03
GO:0015985	energy coupled proton transport, down electrochemical gradient	5.64E-06	2.00E-03
GO:0015986	ATP synthesis coupled proton transport	5.64E-06	1.87E-03
GO:0006544	glycine metabolic process	1.02E-05	3.17E-03
GO:0006754	ATP biosynthetic process	1.65E-05	4.86E-03
GO:0044711	single-organism biosynthetic process	1.66E-05	4.63E-03
GO:0009152	purine ribonucleotide biosynthetic process	2.94E-05	7.81E-03
GO:0006164	purine nucleotide biosynthetic process	3.67E-05	9.27E-03
GO:0009206	purine ribonucleoside triphosphate biosynthetic process	3.93E-05	9.46E-03
GO:0009145	purine nucleoside triphosphate biosynthetic process	3.93E-05	9.05E-03
GO:1901564	organonitrogen compound metabolic process	4.06E-05	8.97E-03
GO:0019395	fatty acid oxidation	4.62E-05	9.80E-03
GO:0009201	ribonucleoside triphosphate biosynthetic process	4.76E-05	9.71E-03
GO:0009142	nucleoside triphosphate biosynthetic process	4.76E-05	9.35E-03
GO:0046390	ribose phosphate biosynthetic process	5.03E-05	9.53E-03
GO:0009260	ribonucleotide biosynthetic process	5.03E-05	9.20E-03
GO:0034440	lipid oxidation	6.23E-05	1.10E-02
GO:0046034	ATP metabolic process	6.83E-05	1.17E-02
GO:1901576	organic substance biosynthetic process	9.45E-05	1.57E-02
GO:0072329	monocarboxylic acid catabolic process	1.06E-04	1.70E-02
GO:0072522	purine-containing compound biosynthetic process	1.18E-04	1.83E-02
GO:1901137	carbohydrate derivative biosynthetic process	1.82E-04	2.76E-02
GO:0006546	glycine catabolic process	1.84E-04	2.71E-02
GO:0006119	oxidative phosphorylation	1.84E-04	2.64E-02
GO:0009071	serine family amino acid catabolic process	1.84E-04	2.57E-02
GO:0001659	temperature homeostasis	2.31E-04	3.14E-02
GO:0060086	circadian temperature homeostasis	2.31E-04	3.06E-02
GO:0009058	biosynthetic process	2.33E-04	3.01E-02
GO:1901607	alpha-amino acid biosynthetic process	2.58E-04	3.26E-02
GO:0006091	generation of precursor metabolites and energy	3.04E-04	3.75E-02
GO:0032787	monocarboxylic acid metabolic process	3.06E-04	3.69E-02
GO:0044283	small molecule biosynthetic process	3.64E-04	4.29E-02
GO:0007618	mating	3.85E-04	4.44E-02
GO:0009416	response to light stimulus	4.09E-04	4.62E-02
GO:0022904	respiratory electron transport chain	4.13E-04	4.56E-02
GO:0009165	nucleotide biosynthetic process	5.66E-04	6.12E-02
GO:0022900	electron transport chain	5.73E-04	6.08E-02

GO:2000678	negative regulation of transcription regulatory region DNA binding	6.86E-04	7.13E-02
GO:2000677	regulation of transcription regulatory region DNA binding	6.86E-04	6.99E-02
GO:0009066	aspartate family amino acid metabolic process	6.91E-04	6.91E-02
GO:0000096	sulfur amino acid metabolic process	6.91E-04	6.78E-02
GO:0042451	purine nucleoside biosynthetic process	7.07E-04	6.82E-02
GO:0046129	purine ribonucleoside biosynthetic process	7.07E-04	6.70E-02
GO:0090407	organophosphate biosynthetic process	7.20E-04	6.70E-02
GO:0044249	cellular biosynthetic process	7.63E-04	6.97E-02
GO:1901293	nucleoside phosphate biosynthetic process	8.70E-04	7.82E-02
GO:0006635	fatty acid beta-oxidation	8.88E-04	7.85E-02
GO:0044703	multi-organism reproductive process	8.88E-04	7.72E-02
GO:0017085	response to insecticide	8.88E-04	7.60E-02
GO:0008652	cellular amino acid biosynthetic process	9.42E-04	7.93E-02

Table 10 Gene ontology categories enriched in genes down-regulated after infection in the European population.

6.9 Immunity related induced genes

SERINE PEPTIDASE ACTIVITY (GO:0008236)								
GENE	SYMBOL	NAME	T	AF	AM	AS	EU	ALSO IN
CG31205	CG31205		0.99	1.14	1.16		0.85	
CG5909	CG5909		0.87	0.92	1.1	0.8		DeGreg01 & Roxs04
CG6048	CG6048		0.73	0.69		1.04	0.88	
CG9649	CG9649		0.61	0.73	0.52	0.62	0.55	DeGreg01
CG6580	*Jon65Aii*	*Jonah 65Aii*	0.61	0.61		0.69	0.7	
CG9372	CG9372		0.51	0.8	0.38		0.43	Roxs04
CG33329	*Sp212*	*Serine-peptidase 212*	0.48	0.55	0.57		0.48	
CG1304	CG1304		0.46	0.62			0.63	
CG9631	CG9631		0.38	0.39	0.54			DeGreg01
CG31326	CG31326		0.35	0.41	0.41			
CG10772	*Fur1*	*Furin 1*	0.32	0.35				
CG2045	*Ser7*	*Ser7*	0.32	0.31				DeGreg01 & Roxs04
CG18493	CG18493		0.28	0.4				
CG31199	CG31199		0.27	0.43				
CG3066	*Sp7*	*Serine protease 7*	0.27					DeGreg01 & Roxs04
CG17242	CG17242		0.26				0.52	
CG18211	*βTry*	*β Trypsin*	0.24	0.28				
CG2071	*Ser6*	*Serine protease 6*	0.24					
CG31200	CG31200		0.23	0.44	0.35			
CG5896	*grass*	*Gram-positive Specific Serine protease*	0.23	0.3				
CG42280	*ome*	*omega*	0.23	0.28				
CG18734	*Fur2*	*Furin 2*	0.22	0.3				
CG31267	CG31267		0.22					
CG31217	*modSP*	*modular serine protease*	0.2					
CG17475	CG17475		0.19					
CG11841	CG11841		0.18	0.27	0.32			DeGreg01 & Roxs04
CG4821	*Tequila*	*Tequila*	0.18	0.23				
CG11066	*scaf*	*scarface*	0.17	0.31				
CG32483	CG32483		0.17					
CG6438	*amon*	*amontillado*	0.15	0.24				
CG34043	CG34043		0.15		0.43			
CG10586	*Sems*	*Seminase*	-0.13					

CG3734	CG3734		-0.14					
CG7532	*l(2)34Fc*	*lethal (2) 34Fc*	-0.15					DeGreg01
CG4477	CG4477		-0.16					
CG11037	CG11037		-0.17	-0.24				
CG4271	CG4271		-0.2					
CG16996	*Phae1*	*Phaedra 1*	-0.22	-0.27				
CG18030	*Jon99Fi*	*Jonah 99Fi*	-0.23	-0.33				DeGreg01
CG32382	*sphinx2*	*sphinx2*	-0.24					
CG5390	CG5390		-0.25	-0.25			-0.29	
CG9672	CG9672		-0.25					DeGreg01
CG32383	*sphinx1*	*sphinx1*	-0.25					
CG10477	CG10477		-0.26	-0.36			-0.27	
CG12558	CG12558		-0.26					
CG12350	*λ Try*	*λ Try*	-0.27		-0.46			
CG12386	*ηTry*	*η Trypsin*	-0.27					
CG33276	CG33276		-0.31					
CG7754	*ιTry*	*ι Trypsin*	-0.33	-0.41	-0.45			
CG8579	*Jon44E*	*Jonah 44E*	-0.33		-0.57			DeGreg01
CG10041	CG10041		-0.34	-0.42				
CG11664	CG11664		-0.37					
CG12385	*θTry*	*θTrypsin*	-0.38					DeGreg01
CG8871	*Jon25Biii*	*Jonah 25Biii*	-0.39					DeGreg01 & Roxs04
CG6298	*Jon74E*	*Jonah 74E*	-0.45					Roxs04
CG10475	*Jon65Ai*	*Jonah 65Ai*	-0.53	-0.45				DeGreg01
CG4812	*Ser8*	*Ser8*	-0.72	-0.63	-0.88			DeGreg01
CG9564	*Try29F*	*Trypsin 29F*	-0.78	-1.08		-0.95	-0.79	
CG18179	CG18179		-0.87					DeGreg01
CG3739	CG3739		-1.18	-1.15		-1.31	-1.44	
CG11842	CG11842			0.45				DeGreg01 & Roxs04
CG18180	CG18180			0.38				DeGreg01
CG12351	*δ Try*	*δ Trypsin*		0.27				DeGreg01
CG30028	*γTry*	*γTrypsin*		0.25				
CG30025	CG30025			0.24				
CG30031	CG30031			0.24				
CG18444	*αTry*	*α Trypsin*		0.24				
CG5246	CG5246					-0.56		Roxs04

Table 11 Genes coding for serine proteases induced in one or more populations and/or in the treatment analysis are shown in the table. When a gene is significant the log2 fold change in expression is shown. All genes are member of the gene ontology "serine peptidase activity" (GO:0008236) as reported in Flybase.

SERINE-TYPE ENDOPEPTIDASE INHIBITOR ACTIVITY (GO:0004867)								
GENE	SYMBOL	NAME	T	AF	AM	AS	EU	ALSO IN
CG1857	*nec*	*necrotic*	0.3	0.33	0.37			DeGreg01 & Roxs04
CG7219	*Spn28Dc*	*Serpin 28Dc*	0.26		0.44			DeGreg01 & Roxs04
CG18525	*Spn88Ea*	*Serpin 88Ea*	0.16					DeGreg01 & Roxs04
CG10913	*Spn55B*	*Serpin 55B*	-0.18					
CG8342	*Kaz-m1*	*Kazal-type protease inhibitor m1*	-0.21					

CG42472	*Sfp33A1*	*Seminal fluid protein 33A1*	-0.22	-0.37				
CG10031	CG10031		-0.22					
CG16712	CG16712		-0.22					Roxs04
CG42459	*Sfp23F*	*Seminal fluid protein 23F*	-0.22					
CG6953	*fat-spondin*	*fat-spondin*	-0.23	-0.29			-0.33	DeGreg01
CG6289	*Spn77Bc*	*Serpin 77Bc*	-0.24		-0.59			
CG42461	*Sfp24Ba*	*Seminal fluid protein 24Ba*	-0.27					
CG31777	CG31777		-0.28					
CG44008	CG44008		-0.32				-0.35	
CG3604	CG3604		-0.33					DeGreg01 & Roxs04
CG31704	CG31704		-0.34	-0.37		-0.54		
CG6663	*Spn77Bb*	*Serpin 77Bb*	-0.34		-0.7		-0.27	DeGreg01
CG1262	*Acp62F*	*Accessory gland protein 62F*	-0.36	-0.31		-0.5		
CG16704	CG16704		-0.41	-0.6			-0.52	Roxs04
CG42467	CG42467		-0.46					
CG31515	CG31515		-0.69	-0.6		-0.79	-0.69	

Table 12 Genes coding for Serpins induced in one or more populations and/or in the treatment analysis are shown in the table. When a gene is significant the log2 fold change in expression is shown. All genes are member of the gene ontology "Serine-type endopeptidase inhibitor activity" (GO:0004867) as reported in Flybase.

PHAGOCYTOSIS (GO:0006909)								
GENE	SYMBOL	NAME	T	AF	AM	AS	EU	ALSO IN
CG4899	*Pdh*	*Photoreceptor dehydrogenase*	0.76	0.96	0.54	0.72	0.77	
CG6518	*inaC*	*inactivation no afterpotential C*	0.62	0.63	0.63	0.56	0.69	
CG2993	CG2993		0.32					
CG5599	CG5599		0.31	0.47				
CG5166	*Atx2*	*Ataxin-2*	0.3	0.31				
CG16791	CG16791		0.27	0.36				
CG7068	*Tep3*	*Thioester-containing protein 3*	0.25	0.37				
CG4027	*Act5C*	*Actin 5C*	0.25	0.33				
CG2244	*MTA1-like*	*MTA1-like*	0.25	0.28				
CG10539	*S6k*	*RPS6-p70-protein kinase*	0.23	0.28				
CG11527	*Tig*	*Tiggrin*	0.22	0.25				
CG5848	*cact*	*cactus*	0.21	0.23				DeGreg01 & Roxs04
CG7586	*Mcr*	*Macroglobulin complement-related*	0.2	0.28				
CG7052	*Tep2*	*Thioester-containing protein 2*	0.18					DeGreg01 & Roxs04
CG2028	*CkIα*	*Casein kinase Iα*	0.16	0.21				
CG3638	CG3638		0.16					
CG10233	*rtp*	*retinophilin*	0.16					
CG9351	*flfl*	*falafel*	0.15					
CG32697	*l(1)G0232*	*lethal (1) G0232*	0.15					
CG7398	*Trn*	*Transportin*	0.15					
CG4122	*svr*	*silver*	0.14	0.23				
CG7149	CG7149		0.14					
CG5215	*Zn72D*	*Zinc-finger protein at 72D*	0.14					
CG11804	*ced-6*	*ced-6*	-0.13					
CG3843	*RpL10Aa*	*Ribosomal protein L10Aa*	-0.14					

CG5826	*Prx3*	*Peroxiredoxin 3*	-0.15					
CG4780	*membrin*	*membrin*	-0.16					
CG3948	*ζ COP*	*ζ COP*	-0.17					
CG5861	CG5861		-0.18	-0.32				
CG7610	*ATPsyn-γ*	*ATP synthase-γ chain*	-0.2					
CG30427	CG30427		-0.2					
CG9527	CG9527		-0.21					DeGreg01
CG8189	*ATPsyn-b*	*ATP synthase subunit b*	-0.22					
CG32089	*Vha16-2*	*Vacuolar H+ ATPase 16kD subunit 2*	-0.23					
CG5853	CG5853		-0.44		-0.48	-0.52		
CG5178	*Act88F*	*Actin 88F*	-0.71	-0.68				DeGreg01
CG3494	CG3494			-0.23				

Table 13 Genes coding for proteins involved in the phagocytosis defence response that are induced in one or more populations and/or in the treatment analysis are shown in the table. When a gene is significant the log2 fold change in expression is shown. All genes are member of the gene ontology "Imd signalling pathway" (GO:0006909) as reported in Flybase.

IRON ION BINDING (GO:0005506)								
GENE	SYMBOL	NAME	T	AF	AM	AS	EU	ALSO IN
CG4373	*Cyp6d2*	*Cyp6d2*	1.06	0.88	1.09	1.12	1.15	
CG10118	*ple*	*pale*	0.58	0.61	0.59	0.6	0.51	Roxs04 & DeGreg01
CG10247	*Cyp6a21*	*Cyp6a21*	0.49	0.62	0.69		0.41	
CG9438	*Cyp6a2*	*Cytochrome P450-6a2*	0.49	0.52			0.35	
CG8859	*Cyp6g2*	*Cyp6g2*	0.47	0.46	0.64		0.42	
CG10241	*Cyp6a17*	*Cytochrome P450-6a17*	0.45	0.43	0.53		0.44	
CG8453	*Cyp6g1*	*Cyp6g1*	0.43	0.4	0.5	0.47	0.37	DeGreg01
CG10842	*Cyp4p1*	*Cytochrome P450-4p1*	0.4	0.46	0.63			
CG6910	*CG6910*		0.3	0.43				DeGreg01
CG2397	*Cyp6a13*	*Cyp6a13*	0.29				0.37	
CG9674	CG9674		0.29	0.27				
CG8733	*Cyp305a1*	*Cyp305a1*	0.24					
CG10245	*Cyp6a20*	*Cyp6a20*	0.22		0.47			
CG6342	*Irp-1B*	*Iron regulatory protein 1B*	0.22	0.26				
CG10843	*Cyp4p3*	*Cyp4p3*	0.22					Roxs04 & DeGreg01
CG11715	*Cyp4g15*	*Cyp4g15*	0.22	0.45				
CG10246	*Cyp6a9*	*Cytochrome P450-6a9*	0.17		0.4			
CG10240	*Cyp6a22*	*Cyp6a22*	0.16					
CG11466	*Cyp9f2*	*Cyp9f2*	0.14					
CG6045	*CG6045*		0.12					
CG4349	*Fer3HCH*	*Ferritin 3 heavy chain homologue*	-0.16					
CG31022	*PH4α EFB*	*prolyl-4-hydroxylase-alpha EFB*	-0.17					
CG3466	*Cyp4d2*	*Cytochrome P450-4d2*	-0.17					
CG4335	CG4335		-0.17					
CG15539	CG15539		-0.18					
CG1998	CG1998		-0.18					
CG18233	CG18233		-0.18					
CG6042	*Cyp12a4*	Cyp12a4	-0.19				-0.29	

CG10833	*Cyp28d1*	Cyp28d1	-0.21					DeGreg01
CG5137	*Cyp312a1*	Cyp312a1	-0.22	-0.31				
CG1644	*Cyp6t1*	Cyp6t1	-0.25					
CG9964	*Cyp309a1*	*Cyp309a1*	-0.28				-0.53	
CG5493	CG5493		-0.28	-0.34			-0.37	DeGreg01
CG17970	*Cyp4ac2*	*Cyp4ac2*	-0.3					
CG18559	*Cyp309a2*	*Cyp309a2*	-0.3	-0.43			-0.4	
CG14032	*Cyp4ac1*	*Cyp4ac1*	-0.3	-0.38				DeGreg01
CG3050	*Cyp6d5*	*Cyp6d5*	-0.35			-0.58	-0.43	
CG17903	*Cyt-c-p*	*Cytochrome c proximal*	-0.36	-0.26			-0.53	
CG3360	*Cyp313a1*	*Cyp313a1*	-0.38			-0.74		
CG3540	*Cyp4d14*	*Cyp4d14*	-0.38	-0.36	-0.57	-0.65		DeGreg01
CG30489	*Cyp12d1-p*	*Cyp12d1-p*	-0.41			-0.7	-0.68	
CG33503	*Cyp12d1-d*	*Cyp12d1-d*	-0.42			-0.76	-0.64	
CG6730	*Cyp4d21*	*Cyp4d21*	-0.73	-0.43		-1.04	-0.85	
CG13263	*Cyt-c-d*	*Cytochrome c distal*		-0.23				
CG4769	*CG4769*						-0.27	
CG4105	*Cyp4e3*	*Cytochrome P450-4e3*					-0.88	

Table 14 Genes coding for iron ion binding proteins that are induced in one or more populations and/or in the treatment analysis are shown in the table. When a gene is significant the log2 fold change in expression is shown. All genes are member of the gene ontology "iron ion binding" (GO:0005506) as reported in Flybase.

LIPASE ACTIVITY (GO:0016298)								
GENE	SYMBOL	NAME	T	AF	AM	AS	EU	ALSO IN
CG31272	CG31272		0.51	0.64			0.43	
CG6675	CG6675		0.49	0.35	0.71	0.53		Roxs04&DeGreg01
CG4979	*sxe2*	*sex-specific enzyme 2*	0.39	0.48	0.69			
CG4267	CG4267		0.35	0.41	0.52			Roxs04&DeGreg01
CG4574	*Plc21C*	*Phospholipase C at 21C*	0.24	0.3				
CG17097	CG17097		0.23				0.33	
CG14034	CG14034		-0.16					
CG6113	*Lip4*	*Lipase 4*	-0.18					
CG10116	CG10116		-0.2					
CG1583	GIIIspla2		-0.24					
CG15533	CG15533		-0.3					
CG6295	CG6295		-0.34					
CG5966	CG5966		-0.41					
CG8093	CG8093		-0.43	-0.47	-0.45			
CG34447	CG34447		-0.48	-0.4		-0.61		
CG6271	CG6271		-0.5	-0.63	-0.67			
CG6277	CG6277		-0.56	-0.54	-0.77			
CG5932	*mag*	*magro*	-0.59	-0.63	-0.64			DeGreg01
CG31091	CG31091		-0.65					
CG17192	CG17192		-0.81					DeGreg01
CG42237	CG42237		-0.9	-0.88	-0.85	-0.9	-0.97	
CG5162	CG5162						0.3	

Table 15 Genes coding for proteins with lipase activity that are induced in one or more populations and/or in the treatment analysis are shown in the table. When a gene is significant the log2 fold change in expression is shown. All genes are member of the gene ontology "lipase activity" (GO:0016298) as reported in Flybase.

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Data analysis: R; microarray data analysis (BAGEL, R-maanova); survival analysis (R), RNAseq

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Research Interest
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Pubblications
1) Silvia Ghirotto, Stefano Mona, Andrea Benazzo, **Francesco Paparazzo**, David Caramelli, and Guido Barbujani. *Inferring Genealogical processes from Patterns of Bronze-Age and Modern DNA variation in Sardinia* , Mol Biol Evol. 2010 Apr; 27(4): 875-86.

2) **Francesco Paparazzo**, Stephan Hutter, Aurelien Tellier, Wolfgang Stephan, Stephan Hutter. *Global assessment of survival and transcriptional response to a fungal pathogen in* Drosophila melanogaster. (In preparation)

3) **Francesco Paparazzo**, Stephan Hutter, Aurelien Tellier, Wolfgang Stephan, Stephan Hutter. *Effects of Wolbachia and of trans-generational immune priming on survival to a fungal pathogen in* Drosophila melanogaster. (In preparation).

Presentations at conferences and summer schools
Paparazzo F., Aurelien T., Stephan W., Stephan H. Host-Parasite interaction in Drosophila melanogaster (oral presentation). 3rd Symposium of the German Research Association (DFG) Priority Program “Host-Parasite Coevolution – Rapid Reciprocal Adaptation and its Genetic Basis”(SPP 1339). 29th August to 2nd September 2013.

Paparazzo F., Aurelien T., Stephan W., Stephan H. Host-Parasite interaction in Drosophila melanogaster (poster presentation). 15th International conference of immunology. 22-27 August 2013.

Paparazzo F., Aurelien T., Stephan W., Stephan H. Host-Parasite interaction in Drosophila melanogaster (oral presentation). Winter School of the German Research Association (DFG) Priority Program “Host-Parasite Coevolution – Rapid Reciprocal Adaptation and its Genetic Basis”(SPP 1339). 27- 29 February 2012.

Paparazzo F., Aurelien T., Stephan W., Stephan H. Host-Parasite interaction in *Drosophila melanogaster* (poster presentation). Conference Jacques-Monod “Coevolutionary arms race between parasite virulence and host immune defense: challenge from state of the art research”. 3-7 September 2011.

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