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Gene Expression Patterns in Flea Vectors of *Yersinia pestis*

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ABSTRACT

Gene Expression Patterns in Flea Vectors

of *Yersinia Pestis*

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Plague bacteria (*Yersinia pestis*) are transmitted to susceptible mammals by fleas. At least 25 flea species found in North America have been identified as plague vectors. The most efficient flea vector is the Oriental rat flea *Xenopsylla cheopis*, while the cat flea *Ctenocephalides felis* is a poor vector. The factors that determine vector competence of different fleas are not known. The main obstacles that the bacteria must overcome in the flea gut are also unknown. Fleas' molecular responses to *Y. pestis* invading could be a determining factor to control the bacterial survival and growth. Good and poor vectors might have different gene expression patterns when they are infected with *Y. pestis*. To investigate this hypothesis, we constructed cDNA libraries of infected fleas (*X. cheopis* and *C. felis*) using Suppression Subtractive Hybridization at 1 and 2 days post-infection. The infection approaches were either hemocoel injection or oral infection. We measured expression of several of the genes using quantitative real-time PCR. The results indicated that changes in gene expression were modest. We observed that the route of infection (oral vs. hemocoel injection) had a strong effect on the genes that were upregulated, with hemocoel injection inducing more obvious immune-related genes than oral infection. We also saw that infected *X. cheopis* has different gene expression patterns than infected *C. felis*. Several of the genes from both species are predicted to be involved in production and removal of reactive oxygen species (ROS). Consistent with this observation, the levels of peroxide in *X. cheopis* midguts was higher following oral infection with *Y. pestis*, than in uninfected fleas, and *Y. pestis* grew differently in antioxidant-fed fleas, demonstrating that ROS production could be an important defense in fleas early after infection

Keywords: *Yersinia pestis,* fleas, vectors, gene expression, ROS

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INTRODUCTION

Plague

Plague (*Yersinia pestis*) is a severe, life-threatening infectious disease usually transmitted between rodents and humans by the bite of infected fleas. *Yersinia pestis*, a gram negative rod-shaped bacterium, discovered by bacteriologist Alexandre Yersin in 1894, is the causative pathogen of plague (Yersin A, 1894). In history, three plague pandemics occurred over the world. The first one, called Justinian plague, began in AD 541 in Egypt and moved to North Africa, Europe, and central and southern Asia and Arabia. From then until AD 700, 50% to 60% of the population died due to plague and also probably other epidemics (Perry RD, 1997). The second large disastrous plague pandemic, also known as the black death, began in 1346 and killed 17 to 28 million people in Europe, accounting for one-third of the European population at that time (Slack P, 1989). In 1855, the third pandemic began in China, swept across the world. The mortality rate was lower compared to previous two pandemics due to the development of medicine and public health (Perry RD, 1997). Plague cases are still being reported annually in different parts of the world (Inglesby TV, 2000).

Human plague has three primary types: bubonic, pneumonic and septicemic. Bubonic plague is the most common form of human plague, which is usually acquired following the bite of infected fleas, and rarely through contact with infected animals. After being deposited in the skin, the bacterium (*Y. pestis*) is spread via lymph systems to lymph nodes, where they multiply, cause lymphadenopathy, and form the painful bubo (Inglesby TV, 2000). Septicemic plague results from the direct invasion of *Y. pestis* to the bloodstream. Pneumonic plague may occur by the inhalation of infected droplets (primary pneumonic plague) or by spread of the bacteria to the lungs during untreated bubonic or septicemic plague (secondary pneumonic plague) (Inglesby

TV, 2000).

Yersinia pestis

Yersinia pestis is the causative bacterium of plague. As a vector-borne bacterium, *Y. pestis* must adapt to environments in two different hosts, fleas and mammals, for transmissible infections (Hinnebusch BJ, 2005). Two different subsets of *Y. pestis* genes are hypothesized to be expressed in these two different hosts. *Y. pestis* genes playing a crucial role in infecting fleas are defined as the transmission factors, while those important for infection in mammalian hosts are virulence factors (Hinnebusch BJ, 2005).

Y. pestis diverged from *Yersinia pseudotuberculosis*, which is a food- and water-borne enteric pathogen, within the last 1,500 to 20,000 years (Achtman M, 1999; Chain PS, 2004; Hinnebusch BJ, 2005). These two *Yersinia* species share a conserved chromosome with predicted ~75% identical proteins (Chain PS, 2004). The evolution went through at least three steps: horizontal gene transfers brought two *Y. pestis* specific plasmids, pPCP and pMT1, and the new function of the *hms* chromosomal genes was acquired (Chain PS, 2004; Hinnebusch BJ, 2005). The *hms* genes are chromosomal genes involved in synthesis of extracellular matrix. These genes are required for biofilm formation in vitro, and blockage of the proventriculus (Jackson S 1956, Hinnebusch BJ, 2005). Plasmid pPCP1 encodes the plasminogen activator, which helps the spread of *Y. pestis* to the lymph nodes from inoculation sites in mammal hosts (Sodeinde OA, 1992). Plasmid pMT1 encodes murine toxin (Ymt) with phospholipase D activity that enables survival of *Y. pestis* in the flea midgut (Hinnebusch BJ, 2002). The F1 capsule is also encoded by Plasmid pMT1, which prevents phygocytosis in mammal hosts. It is expressed at 37°C rather than 26°C (Du Y, 2002). A temperature and low calcium dependent plasmid pCD1, shared by *Y. pestis* and *Y. pseudotuberculosis,* encodes a type III secretion system, *Yersinis* outer proteins and

V antigen, which are required for yersiniae pathogenicity in humans (Perry RD, 1997). The emergence of *Y. pestis* as a deadly invasive pathogen coincided with the evolution of flea-borne transmission, as *Y. pseudotuberculosis* is not transmitted by fleas. In fact, *Y. pseudotuberculosis* is able to survive in flea midguts but is acutely toxic to fleas, and does not form biofilms in the proventriculus (Erickson DL, 2007). *Y. pestis* and *Y. pseudotuberculosis* both have the ability to replicate in macrophages (Pujol C, 2003).

Lipopolysaccharide (LPS) is an important pathogen-associated molecule of gram negative bacteria, which could be targeted by the innate immune systems of insects and mammals. *Y. pestis* has R (rough)-type LPS lacking O-antigen, while *Y. pseudotuberculosis* has full S (smooth)-type LPS (Prior JL, 2001; Knirel YA, 2006). Temperature shift induces changes of the structure and number of lipid A of *Y. pestis* and *Y. pseudotuberculosis*, regulated by the PhoP-PhoQ system (Rebeil R, 2004). When grown at 21°C, *Y. pestis* lipid A is predominantly hexa-acylated: four 3-OH-C14, one C12 and one C16:1 acyl groups. When *Y. pestis* is grown at 37°C, the lipid A is predominantly tetra-acylated (Rebeil R, 2004). Tetra-acylated lipids have the weak activity to stimulate the immune responses in human cells. Tetra-acylated *Y. pestis* lipid A inhibits the activation of Toll-like receptors (TLRs), such as TLR4, TLR2 and TLR9, to evade the inflammation responses via signaling pathways induced by LPS (Montminy SW, 2006; Telepnev MV, 2009).

Flea-borne plague transmission mechanisms

People have been studying the role of fleas in plague transmission for a long time. Early in 1897, Ogata suggested that fleas probably could transmit *Y. pestis* (Pollitzer R, 1954; Gage KL, 2005), and in 1914-1915, based on the physiology and structure of the flea gut, Bacot and Martin (1914) (Bacot AW, 1914) described the proventriculus blockage-regurgitation mechanism, which

is called biological transmission and is believed to be the dominant paradigm for flea-borne plague transmission.

According to this model, after fleas take the host blood meal containing *Y. pestis*, the organism grows, colonizes and accumulates in the midgut. And eventually the aggregates of *Y. pestis* will go to the proventriculus, which will be blocked to prevent any blood meals from flowing into the midgut. Blockage can occur as few as 5 days after infection, but usually peaks 2-3 weeks later (Mury CB, 2004). Blockage involves formation of a biofilm, which is defined as a dense association of bacteria cells coated with an extracellular matrix, often attached to a surface (Costerton JW. 1995; Jarrett CO, 2004). Biofilm is only produced at a lower temperature range 21-28°C, not at 37°C, which matches the fact that *Y. pestis* only forms biofilm in fleas, not in mammalian bodies (Jones HA, 1999).

With no blood in the midgut, fleas will be starved and their attempts to feed will dramatically increase. When the esophagus is distended with blood, the blood meal will partially return back to the biting site in subsequent hosts. In this way, plague (*Y. pestis*) can be transmitted from one host to another. In 1915 (Bacot AW, 1915), Bacot made changes to this model. He found that fleas with partial blockage can transmit plague more effectively, even though part of blood meal can pass the proventriculus to the midgut to be digested. Regurgitation can still happen because of the non-continual pumping during feeding. The reason why the partially blocked fleas transmit plague more efficiently could be that once they have some blood for digestion in the midgut, there will be several proteins or enzymes secreted for digestion. And these secretions could increase the fleas attempt to feed and make fleas bite more frequently. This revised transmission model provided the explanation to the phenomenon that some of the flea species are good transmitters but cannot be completely blocked. It is also possible that soon

after infection, but before the blockage forms, rare mechanical transmission could occur. Fleas' mouthparts will be contaminated with viable *Y. pestis* when they suck infectious blood meals. Then, during the next bites, the bacteria that have survived on the surface of the mouthparts might be transferred (Hinnebusch BJ, 2005).

Recently, Eisen and colleagues have demonstrated early-phase transmission of unblocked *Oropsylla montana* fleas (Eisen RJ, 2006). It is a separate mode of biological (not mechanical) transmission that does not involve proventricular blockage. This transmission has been observed in several flea species, i.e., oriental rat flea *Xenopsylla cheopis* (Eisen RJ, 2007), prairie dog flea *O. hirsute* (Wilder AP, 2008b), prairie dog flea *O. tuberculata cynomuris* (Wilder AP, 2008b), cat flea *Ctenocephalides felis* (Eisen R.J, 2008a; Wilder AP, 2008a), and mouse flea *Aetheca wagneri* (Eisen R.J, 2008b). Nothing currently is known about the early-phase transmission mechanism, but it could be responsible for some rapidly spreading *Y. pestis* epizootics (Eisen RJ, 2006). During the early-phase transmission, regurgitation probably occurs in fleas. Early-phase transmission by unblocked *X. cheopis* is at peak efficiency 2-3 days post-infection.and is effective up to one week (Eisen RJ, 2007), which suggests that 1-2 days post-infection could be a "preparation time" for the later early-phase transmission. During this period, *Y. pestis* colonize in the flea digestive gut. *Y. pestis* needs to grow and survive in flea gut for the later transmission, implicating that flea-*Y. pestis* interaction in this period is the determinant factor for success or failure in plague transmission. Although several transmission factors and virulence factors of *Y. pestis* have been identified, no flea factors contributed to the transmission that has been identified. Identifying these factors may help tell the story from the other side.

The efficiency of *Y. pestis* infection of fleas is much lower than that of mammalian hosts. The LD_{50}, for mice is less than 10 colony forming units (Perry RD, 1997), while the ID_{50} for the

X.cheopis flea is about 5000 CFU (Lorange EA, 2005). Recently, several transmission factors of *Y. pestis* have been identified. Neither of these genes is important for infecting mammals, which showed that transmission factors of *Y. pestis* are different than the virulence factors.

Flea vectors

The insect order *Siphonaptera* includes about 2,500 species and subspecies of fleas (Lewis RE, 1998). Fleas are small wingless bloodsucking insects that feed on warm-blooded animals. Their bodies are small (1/16"), hard, polished, and covered with many hairs and short spines directed backward. Their compressed bodies (i.e., flattened side to side) allow them to easily move through hairs on hosts' bodies. The mouthpart of an adult flea contains stylets that are used to pierce the skin of the host animal for sucking blood from the host (Gage KL, 2004). Their legs are long and well adapted for jumping to facilitate host acquisition. Their jumping ability, laterally compressed bodies, well developed sense organs and haematophagous habit all contribute to transmission of plague (Gage KL, 2004).

Flea gut structure and environment is very important in bacterial growth. The flea digestive tract mainly contains the esophagus, proventriculus, midgut and hindgut (Hinnebusch BJ, 2005). Midgut is the main location for flea to digest and absorb the blood meal. There are a layer of epithelial cells covering the interior surface of the midgut.The proventriculus is a pear shaped valve linking the midgut and esophagus, with closely packed inward-directing spines lying on the interior. These spines are believed to be able to crush blood cells in blood meals with the help of a layer of acellular cuticle coat outside spines. Female *X. cheopis* has 450 spines, and male *X. cheopis* has 264 (Munshi DM. 1960). During digestion, the proventriculus can be closed tightly to prevent any blood meal leaking out from the midgut (Hinnebusch BJ, 2005).

Vector competence is defined as "the innate ability of a vector to acquire a pathogen and to

successfully transmit it to another susceptible host" (Scott CW, 2004). Until recently, at least 28 flea species that were found in North America have been identified as plague vectors, but experimentally, their vector competence varies greatly (Eisen RJ, 2009). The cat flea (*C. felis*) and the human flea (*Pulex irritans*) are inefficient vectors (Laudisoit A, 2007), while *X. cheopis* is an especially significant vector for plague. Generally, those which experience blockage are more efficient transmitters (Mury CB, 2004).

Insect immunity

Insect vectors of infectious diseases must defend themselves from possible damage from the pathogens that they carry. Insects rely mainly on the innate immune system to defend themselves, which is fast and transient (Lemaitre B, 2007). This defense system controls where and to what extent the pathogenic microbes can multiply. The insect innate immune system consists three defense lines. First, the columnar epithelial cells and peristalsis of the gut act as a physical barrier against colonization. Second are the humoral immune barriers including osmotic stress, digestive enzymes, lysozymes and two inducible components: the production of antimicrobial peptides (AMP) and the secretion of reactive oxygen species (ROS) and nitric oxide in the gut. Finally, the cellular immune system includes phagocytosis (Hillyer JF, 2003), nodule formation (Miller JS, 1994), encapsulation (Peters A, 1997) and melanization (Held KG, 2007) in the hemocoel (Lemaitre B, 2007).

When pathogenic bacteria invade, pattern-recognition molecules will interact with the pathogen-associated molecules (e.g. lipopolysaccharides in gram-negative bacteria and teichoic acids in gram-positive bacteria) to stimulate and initiate the insects' immune responses (Mury CB, 2004). Several pattern-recognition proteins in insects have been identified: beta-1,3-Glucan-recognition protein/gram-negative-bacteria-binding protein, C-type lectin,

hemolin and peptidoglycan-recognition protein (Mury CB, 2004). They are synthesized primarily in the fat body but also in hemocytes and other tissues. These recognition reactions trigger humoral and cellular responses (Mury CB, 2004).

Among these insect immune components, antimicrobial peptides (AMP) play an important role. AMPs are widespread and are found in plants, vertebrates and insects. An individual insect may produce about 10-15 AMPs with different antimicrobial spectra (Hoffmann JA, 1993; Laszlootvos JR, 2000). Typically these peptides are less than 10kD, hydrophobic, and can recognize the acidic phospholipids on the membrane of bacteria or other pathogens. Insects can express antimicrobial peptides in hemolymph as early as 2h after septic injury (Meister M, 1997). Five different families of AMP are found in *Drosophila*: cecropin, defensin, drosocin, diptericin and attacin, plus two additional antifungal peptides, drosomycin and metchnikowin (Bulet P, 1999). Recent research on the identification of the salivary gland transcriptome of the oriental rat flea *X. cheopis* resulted in the sequence of an AMP family defensin gene (Andersen OF, 2007). Two signaling pathways regulate the expression of AMPs: the Toll and immune deficiency (Imd) pathways, which control the transcription factor nuclear factor kappa B (NF-κB) homologs (Silverman N, 2008). In mosquitoes and the blood sucking fly *Stomoxyscalcitrans*, the midgut epithelium has been shown to secrete antimicrobial peptides to the gut lumen, induced by blood meals containing bacteria (Dimopoulos G.,1997; Lehane MJ, 1997). The proventriculus has also been shown to be a site for immune reactions in *Drosophila* (Tzou P, 2000) and Tsetse flies (Hao Z, 2003), with increased levels of antimicrobial peptides, nitric oxide, and reactive oxygen species compared with the midgut.

Whether this occurs in fleas is unknown. *Y. pestis* does not enter hemocoel or salivary gland, even not adhere the flea midgut epithelium. It stays in the flea digestive gut, although it has a

close phylogenic relationship to other enteric pathogens (Hinnebusch BJ, 1996). Also, fleas do not form chitinous peritrophic membranes around the bloodmeal, which is unlike other haematophagous insects like sandflies (*Phlebotomus papatasi)* (Blackburn K, 1988). These facts suggest that the flea digestive gut, especially the midgut, is the main location for flea-*Y. pestis* interactions. Although flea midgut PH value has been reported to be 6 to 7, we know little about the flea gut environment (Wigglesworth VB, 1972). About 30% ~ 50% of *X. cheopis* fleas clear their *Y .pestis* infection with 24h to 48h (Hinnebusch BJ, 2002) and only 50% of all infected *X. cheopis* fleas can transmit *Y. pestis* successfully (Burroughs AL, 1947). These facts implicated that the flea gut environment is a hostile place for the survival of *Y.pestis*.

NF-κB pathways are not usually induced in the *Drosophila* epithelia by most natural bacteria-contaminated food feeding. The phytopathogenic bacteria *Erwinia carotovora* is the exception (Basset A, 2000; 2003; Won JL, 2005). Yet in yeast and *E. coli* oral infected *Drosophila*, dual oxidase enzymes that can produce reactive oxygen species (ROS) and catalases that can remove excess ROS have been induced consecutively (Won JL, 2005). The gut Imd/AMP immunity is required to combat bacteria that are resistant to ROS, but not ROS-sensitive microbes (Won JL, 2005). Thus, the production of ROS can be considered a first-line defense that protects the epithelial cells from high numbers of bacteria and the secondary Imd response is only activated when the initial response fails to clear the infection.

ROS are natural byproducts of normal oxygen metabolism but can also be produced by enzymes such as NADPH oxidase in phagocytes and dual oxidases in epithelial cells. ROS include superoxide anion, hydroxyl radicals, and hydrogen peroxide, but these can also be converted to compounds with very high antimicrobial activities, such as hypochlorous acid (Weiss SJ, 1989) and hypothiocyanite (Carlsson J, 1984), by peroxidase enzymes in mammals.

ROS seem to be important in the microbial defense in insects (Phoebe T, 2002). Host cells will remove the excess ROS by some enzymes such as superoxide dismutase (SOD) and catalase to protect themselves from damage. A range of antioxidants, such as N-acetyl-cysteine, ascorbic acid and uric acid can reduce the oxidative stress in insect midgut and enhance the growth of some pathogens (Macleod ET, 2007). These results highlight the important role of gut ROS on pathogen infections of arthropod vectors.

Research approaches

Suppression Subtractive Hybridization is a PCR-based method that suppresses the amplification of common genes between target sample and control sample, and identifies genes specifically expressed in the target sample. SSH has been successfully used to identify the immune-induced genes in the dipterans *Anopheles gambiae* (Oduol F, 2000) and *Eristalis tenax* (Altincicek B, 2007a), the hemipteran *Rhodnius prolixus* (Ursic-Bedoya RJ, 2007), the apterygote insect *Thermobia domestica* (Altincicek B, 2007b) and the beetle *Tribolium castaneum* (Altincicek B, 2008).

Here, for the first time, I identified the genes up-regulated in *X. cheopis* infected with *Y. pestis*, either by oral feeding or by hemocoel injection, using suppression subtractive hybridization (SSH). The genes induced in the *Y. pestis* oral-infected cat flea *Ctenocephalides felis*, a poor plague vector were also identified. Genes encoding transcripts that involve in genetic information processing, immune defense effector molecules, stress associated proteins, metabolism, cell motility and migration, signaling, protease and reactive oxygen species defense have been identified, along with some genes with unknown functions. Four cDNA libraries were constructed by SSH, which were analyzed for the gene distributions comparing flea responses between different infection approaches, different post-infection time points and also different

plague vectors. Further investigation showed that *Y. pestis* infection induces ROS production in the *X. cheopis* midgut one day after oral infection. Interference of *X. cheopis*'s ROS production when antioxidant was added to the bloodmeal was also observed. This enhanced the initial growth of *Y .pestis.*

MATERIALS AND METHODS

Flea infections

Oriental rat fleas, *Xenopsylla cheopis* were reared in glass jars at 26°C and 80% relative humidity. They were fed on neonatal mice twice weekly. The flea bedding mixture consisted of 300g sand, 12g sheep blood, 30g mouse chow and 300mg antifungal (mehtyl p-hydroxy benzoate). Cat fleas (*C. felis*) were purchased from Professional Laboratory and Research Services Inc. (Corapeake, North Carolina). Oral infection with *Y. pestis* was performed by the means of previously described membrane feeder apparatus through mouse skins (Erickson DL, 2007; Hinnebusch BJ, 1996). 20 fleas were fed on 5 ml of heparinized human blood containing live *Y. pestis* strain KIM6+ with the final concentration ~1×10^8 colony-forming units (CFU) per ml. Prior to the infection, *Y. pestis* KIM6+ were grown in Brain-Heart Infusion (BHI) broth (FLUKA, Switzerland) overnight and pelleted by centrifugation, resuspended in 1ml of phosphate-buffered saline (PBS), and added to blood meals. The feeding lasted 1hr, and the blood was kept at 37°C during the feeding. After feeding, 1ml of blood was collected, diluted and plated on BHI agar plates containing Irgasan (5μg/ml). Plates were incubated at 30°C for 48 hr, and the CFU were counted to determine the *Y. pestis* concentration in blood meals. Another 20 fleas were fed under the same condition, on the same blood source but without *Y. pestis* added, as a control group. We collected fleas after feeding, put them on the laboratory chill table (BioQuip) and picked up the successfully fed ones by checking the presence of their distended guts full with blood meals under a dissecting microscope.

Hemocoel injection was performed by directly injecting *Y. pestis* strain KIM6+ with the final concentration ~1×108 CFU/ml into the flea hemocoel using UMP2 Microsyringe Injector and Micro4 Controller (World Precision Instruments, Inc.) and a 36-gauge needle. *Y. pestis* were

grown and the concentration was determined following the method described previously in the oral infection. The bacteria were pelleted by centrifugation, resuspended in 1 ml phosphate-buffered saline (PBS). For 20 fleas, 0.2ul of *Y. pestis* suspension was injected into the hemocoel of each flea, and sterile PBS into another 20 fleas as a control (Dzitoyeva S, 2001).

RNA extraction

After infection, fleas were kept at 21°C and 80% humidity for 1 or 2 days. Then fleas were collected and total RNA was isolated with TRIzol reagent according to manufacturer's instruction (Invitrogen). Total RNA was treated with TURBO^{TM} DNase (Ambion, Inc) by Incubated at 37°C for 30 min according to manufactures instruction and purified with RNA purification Mini Elute column kit (Qiagen). RNA integrity was checked by ethidium bromide TAE gel and quantity was determined by NanoDrop® ND-1000 UV-Vis Spectrophotometer (RIC, BYU LIFE SCIENCE).

Subtracted cDNA libraries construction

We performed Suppression Subtractive Hybridization to identify *X. cheopis* genes induced by *Y. pestis* oral infection (1 or 2 days post-infection) and hemocoel injection (1 day post-infection), and *C. felis* genes induced by *Y. pestis* oral infection (1 day post-infection). Suppression Subtractive Hybridization was performed by following the PCR-Select cDNA Subtraction Kit (Clontech) according to the protocol of the manufacture. dscDNA of infected fleas and uninfected fleas was synthesized by using SMART PCR cDNA Sythesis Kit (Clontech). With this kit we started the cDNA synthesis from 200ng of total RNA instead of 2μg of mRNA in the traditional subtractive hybridization method. We did reverse transcription with a modified oligo (dT) primer with a RsaI site in it. The MMLV Reverse Transcriptase provided in the kit had the terminal transferase activity to switch its template as needed, which also allowed it to

add a few additional nucleotides, primarily deoxycytidine, to the 3' end of the ss cDNA when the enzyme reached the 5’ end of the mRNA. And using this extended deoxycytidine stretch as a template, the reverse transcriptase added an oligo G sequence to the mRNA 5’ end. This oligo G sequence was base-pair to the deoxycytidine stretch in 3' end of the ss cDNA, and also had a RsaI site in it. Then the reverse transcriptase changed its template from cDNA to mRNA to produce the complete cDNA strand. So the ss cDNA have two RsaI sites on two ends respectively. Then we amplified cDNA with corresponding primers.

The next step was to optimize the PCR cycles for cDNA amplification by running different cycles and comparing PCR products in TAE gels. The optimization was to find out with how many PCR cycles, the exponential amplification of the cDNA was done, to avoid over amplification. We performed PCR in 18 cycles, 21 cylces, 24 cycles, 27 cycles and 30 cycles and observed all the products in a TAE gel. For the cycles in which the yield of PCR products stopped increasing, the optimal PCR cycle would be two less than that cycle number.

After amplifying cDNA in optimal cycles, we digested cDNA with RsaI at 37°C.for 1.5hr. After RsaI digestion, cDNA from infected fleas was divided into two groups and were ligated to two different adaptors respectively, called tester cDNA 1 and 2, while the cDNA from uninfected fleas was not digested or ligated and was called driver cDNA. In the first hybridization, tester cDNA 1 and 2 was hybridized with the excess driver cDNA at 68°C for 8 hr respectively. The concentration of driver cDNA was 15 times more than that of tester cDNAs. Then two batches of hybridization products (4μl for each) were mixed together and more excess driver cDNA (2μl) was added in the second hybridization.

After hybridization, PCR amplification was performed using primers specific for two adaptor sequences. Because tester cDNA had adaptor sequences and driver cDNA not, with

excess driver cDNA in the hybridization, tester cDNA representing the specific upregulated genes would bind to tester cDNA. Only dscDNA with two different adaptor sequences in two ends had been amplified exponentially. dscDNA only with one adaptor sequence in one end had been linearly amplified. dscDNA without any adaptor seqeneces and dsDNA with two same adaptor sequences in two ends to form self-linkage had not been amplified at all. Therefore in the final PCR products, the cDNA representing the specific upregulated genes in tester cDNA were dominant.

To further amplify the dominant PCR products, we ran 2nd PCR with the same set of primers specific for adaptor sequences in the 1st PCR, using the 1st PCR products as a template. To run a control for the hybridized product, we barely mixed driver cDNA and tester cDNA, which had not been hybridized with each other, and ran the same 1st and 2nd PCR with this mixture. The PCR products were checked and compared in TAE gels, and we checked to see if the unhybridized control PCR product was different from the hybridized one or not, to find out the hybridization was efficient or not. The different patterns for two PCR products in TAE gel meant that the hybridization was successful. And we moved to next step.

Transformation of the hybridized product cDNA to competent cells

For each SSH procedure, amplified cDNA fragments were cloned using the TOPO TA Cloning Kit (Invitrogen) according to the protocol of the manufacture. cDNA were inserted to the pCR II-TOPO vector and transformed to the TOP10 competent cells, grown on LB plates plus 30μg/ml of kanamycin overnight at 37°C. The cDNA inserts were amplified from each clone via colony PCR using TOPO forward and reverse primers that bind ~250 bases upstream and downstream from the insertion site. The conditions for the colony PCR were 1 cycle of initial cell breakage at 95°C for 5 min, then 34 cycles of DNA denaturation at 95°C for 30 sec,

annealing at 55°C for 30 sec and extension at 72°C for 4 min, and 1 cycle of final extension at 72°C for 7 min. The primer sequences were Topo forward 5' - TTA TGC TTC CGG CTC GTA TG - 3' and Topo reverse 5' - GTG CTG CAA GGC GAT TAA GT - 3'.

Sequencing of inserts and analysis

Excess primers and dNTPs from the colony PCR reactions were removed using EXO-SAP (exonuclease - shrimp alkaline phosphatase) treatment. In this reaction, Exonuclease I, Antarctic Alkaline Phosphatase and buffer were added to the colony PCR products that contained cDNA inserts. The mixture was incubated at 37°C for 20 min and then incubated at 80°C for 15 min to inactivate the enzymes.

After the clean-up reaction, BigDye terminator v.3.0 ready cycle sequencing reaction (Ambion) was performed to sequence the cDNA inserts. Cleaned PCR products and sequencing M13 primer were added to BigDye reaction mix, and then incubated at 75ºC for 3 min, followed by 44 cycles of denaturation at 95ºC for 10 sec, annealing at 55ºC for 20 sec and extension at 60ºC for 4 min. The primer sequences were M13 forward 5'- GTA AAA CGA CGG CCA GT -3' or M13 reverse 5'- CAG GAA ACA GCT ATG AC -3'.

The BigDye sequencing reactions were cleaned of excess dye using Sephadex G-50 (GE Healthcare) resin. We filled MultiScreen HV 96-well plates (Millipore MAHVN4550) with dry Sephadex G-50 and added 300µl of ddH2O to each well to swell the resin. After removing the excess water, we loaded each Big Dye reaction product and centrifuged at 2500 rpm for 2min, which eluted the purified sequencing product. The sequencing reactions were dried in speed vacuum at 60ºC prior to electrophoresis (DNASC, BYU).

The forward and reverse sequences were assembled and aligned using the GENEIOUS Pro v. 4.6.2 software package (Biomatters Ltd, New Zealand). The adaptor sequences linking at two

ends of the sequenced products were removed. The good quality sequences were searched by TBLASTX at an E-value cut off less or equal to 1 x 10^{-5}. Bacteria sequences and some new sequences with no homologues in public database at NCBI were not included in cDNA libraries.

Quantitative real-time RT-PCR

cDNA was synthesized from RNA samples of infected and uninfected fleas with SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) according to the protocol of the manufacture. 2μg of total RNA was used and we diluted the resulting cDNA by 20 fold and used the diluted cDNA in quantitative real-time RT-PCR. Quantitative real-time RT-PCR was performed with the mixture of 5ul of Power SYBR Green PCR master mix (PE Applied Biosysthems), 1ul of cDNA, 1ul of forward primer (5uM), 1ul of reverse primer (5uM) and 2 ul of ddH2O in a Light cycler 480 RT-PCR system (Roche Applied Science, Mannheim, Germany) equipped with light cycler 480 software. The thermal cycling program was set as 1 cycle of at 94°C for 3 min, then 40 cycles of at 95°C for 30 sec, at 60°C for 30 sec and at 72 °C for 1.5 min, and 1 cycle of final extension at 72°C for 7 min. The threshold cycles (Ct) were calculated automatically by light cycler 480 software. We used the $2^{-\Delta\Delta CT}$ method to calculate the expression changing folds of target genes (Livak KJ, 2001). *X. cheopis* clone XC-71 chromosomal gene L13 was used as a control gene. All the primers used in quantitative real-time PCR were designed with IDT SciTools (Integrated DNA Technologies, Coralville, IA). Table 6 shows the gene-specific primer sequences.

Midgut ROS level assay

To determine the change in ROS levels due to *Y. pestis* infection, we infected a group of *X. cheopis* fleas with *Y. pestis* as above. 24 hr after infection, the intestines of each flea group were rapidly hand-dissected in PBS containing aminotriazol (2mg/ml) under a dissecting microscope,

and then the dissected intestines were triturated in 100ul of PBS containing aminotriazol (2mg/ml). Midgut ROS level assay was performed with the PeroXOquant™ Quantitative Peroxide Assay Kits (PIERCE) to the triturated flea midguts according to the protocol of the manufacture. A standard curve was made with hydrogen peroxide (0, 200μM, 400μM, 600μM, 800μM, 1000μM). The change in absorbance of xylenol orange at 590nm was measured using the PE Packard Fusion Universal Microplate Reader (RIC, BYU LIFE SCIENCE). The data were expressed as the mean and standard deviations of three different experiments.

Antioxidant Treatment of fleas

To determine the effects of antioxidants on *Y. pestis* initial infection rates in *X. cheopis* midgut, we added antioxidant N-acetyl-cysteine to the flea blood meals. One hundred *X. cheopis* fleas were fed on heparinized human blood with N-acetyl-cysteine (1mM) as above. Another group of 100 *X. cheopis* fleas were orally fed on pure heparinized human blood (same blood source as the first group) as a control. After feeding, fleas were kept at 21°C and 80% humidity. 3 days later, we repeated the feeding. And another 3 days later, we orally infected fleas from each group with the same heparinized human blood containing *Y. pestis* KIM6+ with concentration $\sim 1\times10^8$ following the method described previously. .

Enumeration of *Y. pestis* in fleas

20 fleas were collected at each time points (0d, 1d, 2d and 3d) after infection from each group. Fleas were individually triturated with sterile glass sand in 1.5ml centrifuge tubes and 500ul of PBS was added to each tube and mixed well. 150ul solution from each tube was plated in Brain-Heart Infusion Agar or Terrific Broth plates with Irgasan (5μg/ml). Plates were incubated at 30°C for 48 hr and CFU per flea was counted. For plates with too many colonies to count, we diluted the original solution by 1:100 or 1:1000 and plated them until the CFU per flea

could be counted. Percentage of fleas infected and average *Y. pestis* colonization per flea in infected fleas were plotted in antioxidants treated group and the control group (Macleod ET, 2007).

RESULTS AND DISCUSSION

General Sequencing Results and Gene Distributions

We created four cDNA libraries from infected fleas that varied in the species of flea (*X. cheopis* vs. *C. felis*), the length of time after infection (1 or 2 days) and the infection method (oral feeding vs. hemocoel injection). The OR1 and OR2 libraries represented the upregulated *X. cheopis* genes induced by a bloodmeal containing *Y. pestis* KIM6+ 1 or 2 days (respectively) after oral infection. The HM1 library represented the upregulated *X. cheopis* genes 1 day following hemocoel injection, and the COR1 library represented the *C. felis* genes induced 1 day following oral infection.

For the OR1 library, 391 colonies were randomly chosen for sequencing which yielded 119 unique sequences that were of sufficient quality and had matches in the NCBI database (Table 1). Similarly, the OR2 library (Table 2) yielded 80 (of 343) unique sequences with database matches, the HM1 library (Table 3) gave 75 (of 312), and the COR1 library (Table 4) resulted in 93 cDNA sequences (of 282 colonies sequenced).

We classified genes in the libraries by their different predicted functions: genetic information processing, immune defense effector molecules, stress associated proteins, metabolism, cell motility and migration, signaling, protease, reactive oxygen species production and defense, and genes of unknown function. Gene distribution in the three *X. cheopis* libraries showed some similarities (Fig. 1) but also had several important differences. For instance, there were more genes for immune defense and effector molecules in HM1 (9.3%) than in OR1 (5.0%) and more for signaling pathways in HM1 (16.0%) than in OR1 (12.6%), which indicated that hemocoel injection could induce more direct and stronger immune responses in fleas than oral infection. Hemocoel in insects is a body cavity with hemolymph (blood) freely moving in it. A

specialized tissue, the fat body, whose function is similar to “liver”, is also in the hemocoel and stores lipids and produces most of the hemocoel proteins (James.LN, 2001). Therefore, this result reflects the fact that bacteria injected into the hemocoel bypass the midgut epithelia and face hemocoel immunity directly (Lemaitre B, 2007), and the hemocoel immunity to invading bacteria is stronger than the gut immunity. Conversely, the OR1 library had more genes for proteases (3.4% in OR1, 1.3% in HM1) and stress-associated proteins (3.4% in OR1, 1.3% in HM1) than HM1, which indicated that oral infection in which bacteria colonize in flea midgut may produce more direct stress to fleas.

Comparing the response to oral infection 1 day vs. 2 days after infection showed a greater abundance of proteases (1.3% in OR2, 3.4% in OR1), stress associated proteins (1.3% in OR2, 3.4% in OR1) and immune defense and effector molecules (3.8% in OR2, 5.0% in OR1) in OR2 compared to OR1. This indicates that the *X. cheopis* immune responses at 2 days post-infection are a little weaker than at 1 day post-infection. Thus it can be inferred that *Y. pestis* faces stronger initial immune-related stress in the flea guts, which could clear a significant portion of the bacteria, but later, those that remain will be able to adapt to the hostile environment and colonize for later transmission.

There were several genes found in more than one *X. cheopis* cDNA library. A gene encoding glycogenin was detected in HM1 and OR2; 4 genes encoding matrix metalloproteinase, vitellogenin, chromaffin granule amine transporter, and a conserved hypothetical protein with a chromatin organization modifier domain overlapped in HM1 and OR1; a gene encoding muscle myosin heavy chain was found in both OR1 and OR2 (Fig. 2).

Threre were also differences in gene distribution in the *C. felis* 1d post-oral infection cDNA library (COR1) compared with *X. cheopis* 1d post-oral infection cDNA library (OR1) (Fig. 3).

More specifically, genes encoding proteins functioning in genetic information processing (30.1% in COR1, 21% in OR1) and signaling (18.3% in COR1, 12.6% in OR1) appeared more in COR1 than OR1. On the other hand, more genes encoding proteins involved in immune defense effector molecules (2.2% in COR1, 5.0% in OR1) and cell motility and migration (in 2.2% COR1, 5.9% in OR1) showed up in OR1 than in COR1. These differences may be good targets for future functional studies to identify the genes that contribute to difference in vector competence for plague transmission.

Specific Genes Associated with Functional Categories

Genetic information processing

Proteins involving in gene transcription and protein biosynthesis were shown to be induced by bacteria infection in insects, either by oral feeding (Irving P, 2001) or by hemocoel injection (Ursic-Bedoya RJ, 2007). And we observed several eukaryotic initiation factors such as, eIF5C, eIF4γ in HM1 and eIF4e, eIF4AIII in COR1, elongation factor binding protein Eftud2 in OR2, arginine/serine-rich splicing factor in OR1, and zinc finger proteins in all four libraries. These genes' presence implied that the requirement of some immune-related genes' expression is acutely increasing.

Immune defense and effector molecules

Insects rely on multiple innate immunity reactions to defend from the invasion of pathogens. There is no research documented about flea immunity until now. We observed several transcripts of proteins with similar sequences to several pathogen recognition molecules, eg. beta-1,3-glucan recognition protein 1 in HM1, leukocyte receptor cluster (lrc) member in OR2, matrix metalloproteinase with putative peptidoglycan binding domain in both OR1 and HM1 and CD36 antigen in COR1, which indicates that immune responses could be initiated by the working of

these molecules in *X. cheopis*.

Vitellogenin is a major reproductive protein in insects as a yolk precursor，which can regulate insects social behaviors (Nelson CM, 2007) and protect them from oxidative stress (Seehuus SC, 2006). However, vitellogenin can also act as a pattern recognition molecule in opsonizing bacteria in fish (Li Z, 2008). We found vitellogenin genes in all three *X. cheopis* cDNA libraries but not in *C. felis* libraries. It might be suggested that vitellogenin proteins have specific immunity function in infected *X. cheopis* not in infected *C. felis.*

Ferritins are important intracellular iron storage molecules (Andrews SC, 1992), found in various organisms, including insects. The presence of ferritin in HM1 library suggests that it may have a role in protecting fleas from infection. Ferritins in *Drosophila melanogaster* (Georgieva T, 2002) and *Aedes aegypti* (Geiser DL, 2007) are induced by iron load, following blood meal intake. *A. aegypti* ferritin could also protect it from the oxidative stress by storing iron (Geiser DL, 2003). Other researchers have found that several vertebrate ferritins, such as human ferritin (Rogers JT, 1990), are involved in innate immune responses. Invertebrates including horseshoe crabs (Ong DS, 2005) and starfish (Beck G, 2002) also produce ferritin in response to infection, perhaps as an iron-withholding strategy. Although the specific signal that induces ferritin expression has not been determined in every case, it has been shown that LPS injected into *Tribolium castaneum* enhances ferritin expression (Altincicek B, 2008).

In COR1, there was a gene encoding CD36 antigen, which is a member of the class B scavenger receptor. It is involved in recognition of exposed phosphatidylserine on apoptotic cells and stimulation of macrophages (Fadok VA, 1998). It has also been shown to function as a pathogen recognition molecule by recognizing the modified low-density lipoproteins (Mukhopadhyay S, 2004).

Other immune defense effectors include a transcript with homolog GF23306 of *Drosophila ananassae* encoding a small lysozyme protein and a transcript with homology to a protein region of a serine protease inhibitor in HM1, two transcripts encoding palmitoyl-protein thioesterase (enzymes functioning in lysosomal degradation) in OR2, and a transcript encoding deoxyribonuclease I, responsible for DNA fragmentation during apoptosis (Apostolov EO, 2009).

Signaling proteins

In *Drosophila*, several signaling pathways that regulate the cellular responses (mainly induced in hemocytes) and humoral responses (mainly induced in fat body) have been identified (Marmaras VJ, 2009). Toll and Imd are two important NF-κB pathways in humoral responses to induce the release of antimicrobial peptides. The JAK/STAT pathways in the fat body enhance hemocyte immunity. Intracellular signaling pathways like Rho/MAPK and the ProPO activation system are documented to regulate cellular responses (Marmaras VJ, 2009).

There were one gene in HM1 and three genes OR2 predicted to be involved in the NF-κB pathways, but no NF-κB pathway genes appeared in OR1 and COR1. There were no antimicrobial peptide genes in any of the libraries. This suggests that in infected *X. cheopis* fleas the NF-κB pathway is induced, but that it occurs sooner by hemocoel injection than oral infection. At our experimental time points, the downstream products (antimicrobial peptides) have not been produced yet, but they might be produced later. Alternatively, other early defense mechanisms could control the bacteria enough so that the antimicrobial peptide production is not necessary.

We identified two serine protease genes in OR1 and another serine protease with similar sequence to prophenoloxidase activating factor in OR2 (Christensen BM, 2005). The proPO

cascade might be activated by invading bacteria (Marmaras VJ, 2009). Several serine protease perform step-limited proteolysis to convert inactive PO to activated PO, and then activated proPO catalyses the formation of quinones, which are reactive intermediates for melanization, nodulation and phagocytosis in several insects (Ling E, 2005； Mavrouli MD, 2005; Christensen BM, 2005). The presence of proPO activating factor and some serine protease implied that the proPO system might be activated in *X. cheopis* flea by oral infection.

Rho, a member of the monomeric G-protein family that also includes Rac and Cdc42, is an important intracellular molecule. Rho members are homologous to Ras (Marmaras VJ, 2009). Rho proteins have been shown that they could control phagocytosis and engulfment of apoptotic cells through the Ras/Rho/MAPs pathway in various model systems, such as the nematode *Caenorhabditis elegans* (Reddien PW, 2000). We identified several genes encoding Rho members and guanine nucleotide exchange factors in Rho signaling pathway in OR1, HM1 and COR1, but not in OR2. This might indicate that in fleas, Rho pathway was activated at 1d-post infection, and turned to be weak 2 days after infection.

We also observed a gene with homology to phospholipase A2 in OR2. Phospholipase A2 was induced in bacterial-challenged tobacco hornworms and was suggested to start the synthesis of eicosanoids (Tunaz H, 2003). Eicosanoids are oxygenated metabolites of certain fatty acids and they could regulate cellular immunity in bacterial-challenged insects (Marmaras VJ, 2009). Another transcript whose function could prepare phagocytic cells for action is one encoding the Src oncogene, identified in COR1. Src pathway was shown to be involved in phagocytosis in *Drosophila* (Jennifer SZ, 2008).

Stress- associated proteins

Heat shock responses are shown to be a component of the innate immunity in *C. elegans*

intestine (Mohri-Shiomi A, 2008), *T. castaneum* (Altincicek B, 2008), and in *Rhodnius prolixus* midgut (Ursic-Bedoya RJ, 2007). Here we observed some heat shock protein genes in OR1, OR2 and COR1, but not in HM1. This may indicate that hemocoel injection induced weaker or slower heat shock responses than oral infection approach. In HM1, although without heat shock protein, we identified a gene encoding LEA1 (late embryogenesis abundant) protein. This protein is associated with combating dehydration stress (Kikawada T, 2006).

Reactive oxygen species

In *Drosophila*, local production of ROS and AMP are two inducible defenses. The NF-κB/AMP system is shown to be a complementary defense to ROS immunity, which means only the ROS-resistant bacteria will trigger the later AMP synthesis in the gut (Ha EM 2005a; Ha EM, 2005b; Lemaitre B, 2007). In *Drosophila* larvae and adults, ROS is mainly produced by the NADPH oxidase enzyme dRoux in response to bacteria that contact the epithelial barrier (Ha EM 2005a). To keep the redox balance, enzymes to detoxify the reactive oxygen species are required (Ha EM, 2005b). Catalases, glutathione peroxidases, and thioredoxin peroxidases are examples of enzymes that can serve this purpose. In three SSH libraries, several genes were predicted to involve in either helping produce reactive oxygen species (eg, Dual oxidase maturation factor in OR1; cytochrome P450, cytochrome oxidase subunit I and uricase in OR2; peroxidasin in HM1) or detoxifying reactive oxygen species (eg, thioredoxin reductase in OR2) (Table 5). The presence of ROS defense genes in these 3 libraries indicated that *Y. pestis* infection of *X. cheopis* through two approaches induced ROS defenses. In OR2, there was one protein involving in ROS removal. This result probably indicated that at 2 days post-infection, fleas started removing excessive ROS. In COR1, several genes for ROS defense were also induced. In *C. felis* fleas, the ROS defense could also play an important role in the early flea-bacterial interaction.

Quantitative real-time PCR analysis of selected target genes

To determine the relative expression levels of some genes in immune-challenged *X. cheopis* fleas, we performed quantitative real-time PCR. We compared mRNA from uninfected fleas with mRNA from infected fleas (1d post-oral infection, 2d post-oral infection and 1d post-hemocoel injection). We examined genes encoding serine protease, phospholipase A2, thioredoxin reductase, cadherin-N, cytochrome P450 in fleas 2 days post-oral infection, genes encoding proline oxidase and glycogenin in fleas 1 day post-hemocoel injection, and genes encoding chilling induced protein and another serine protease in fleas 1 day post-oral infection (Fig.4). A ribosomal protein L13 gene was used as housekeeping gene and Table 6 showed all the primers we used. The real-time PCR results showed that nearly all of these genes were modestly upregulated in infected fleas (1.5-2.5x increase). This indicates that at early time points post-infection, fleas are not greatly affected by *Y. pestis*. When grown at 37°C and 21°C, *Y. pestis* has different numbers and structures of acyl groups of lipid A. The bacteria were kept at 37°C in human blood during the feeding and then they entered flea guts where the ambient temperature was lower. Therefore, *Y. pestis* would increase the number of lipid A acyl groups after entering fleas, which could probably futher protect them from the humoral immnity in flea guts (Rebeil R, 2004). The reason why *Y. pestis* did not induce strong immune responses in fleas early after oral infection is probably because they had not finished the change of their lipid A at the very early time points after infection and the lipid A had less number of acyl groups. So when *Y. pestis* first appeared in flea guts, their lipid A could not be well recognized by the pattern- recognition molecules in fleas.

Recently, Dreher-Lesnick SM *et al.* analyzed the *Rickettsia typhi*-infected and uninfected cat flea *C. felis* midgut cDNA libraries, and their real-time PCR showed that *R. typhi* infection

had similarly small effects on the defense response genes (Dreher-Lesnick SM, 2009). *R. typhi* is a pathogen transmitted by *C. felis* fleas, causing murine typhus. It could also be that the greatest changes in gene expression occur in cells directly exposed to the bacteria (ie. midgut epithelial cells in oral infection and hemocytes in systemic injection) and these gene expression changes are diluted when we isolate RNA from whole fleas.

Y. pestis elicits ROS production in _X. cheopis_ midguts

To further investigate ROS defense of *X. cheopis* to *Y. pestis* oral infection, we orally infected *X. cheopis*, dissected their midguts after 24 hours, and performed whole-gut ROS assays using the PeroxiQuant ROS detection kit (Pierce). We compared the hydrogen peroxide level in midguts infected fleas with that in fleas which were fed on the same blood source without *Y. pestis* (Fig. 5). The level of ROS in infected *X. cheopis* fleas' midgut was higher than in the uninfected fleas' midgut, suggesting that *Y. pestis* could induce ROS production in *X. cheopis* midgut at 1day after oral infection, and producing ROS for higher oxidative stress in flea midgut may play an important role in defending *Y. pestis* oral infection in fleas at 1 day post-infection.

To further investigate the role of ROS in defense against *Y. pestis*, we treated fleas with the antioxidant N-acetyl-cysteine prior to infection. Our results showed that the antioxidant feeding increased the *X. cheopis* flea infection rate over a three-day period. It also increased the average *Y. pestis* colonization per flea (Fig.6 and 7). This result verified our hypothesis that ROS defense contributes to the flea-*Y. pestis* interaction responses.

CONCLUSION

To our knowledge, there are little known about flea molecular responses to *Y. pestis* infection during the plague transmission, although many studies about the *Y. pestis* gene expressions have been done. My research, for the first time, provided a sight of the whole flea gene expression pattern at the very beginning time after *Y. pestis* infection, which is very important for understanding flea-*Y. pestis* interactions during plague transmission.

This research constructed four subtracted cDNA libraries, representing genes whose expression induced by *Y. pestis* KIM+ infection through different infection approaches (oral feeding or hemocoel injection), at different time points post-infection (1d or 2d), and in different flea vectors (*X. cheopis* rat flea or *C. felis* cat flea). General gene distribution according to function classification in four cDNA libraries showed some similarity. For specific libraries, genes proportion different indicated that hemocoel injection elicited a little stronger immune-related responses than oral infection. In orally infected *X. cheopis* fleas, immune responses turned weaker at 2d post-infection compared to 1d. In *X. cheopis* fleas and *C. felis* fleas, for some gene categories, there are some differences in gene proportion. This difference could be the foundation for further analysis about the plague vector competence. The presence of the ROS related genes in cDNA libraries and the midgut ROS assay showed that the ROS immunity played an important role in flea responses to *Y. pestis* infection.

Several factors need to be considered in this study. The transcription level can not completely represent the protein expression level. Some mRNAs probably have not been translated to proteins at the time of the cDNA library construction. Also there were a set of genes whose function are unknown at this time, so the distribution of genes in the cDNA libraries will be changed and improved after those genes' function have been identified.

There are still more research based on this study could be done. Real-time PCR could be used to identify more interesting gene expressions. To identify the proPO system's role in defending *Y. pestis* infection, we can knock-down the related genes appearing in cDNA libraries using RNA interference technology. Also, we can clone some immune effector genes and purify proteins corresponding to them to check their antibacterial properties *in vitro*. To compare immune-related responses in *X. cheopis* and *C. felis*, the same ROS midgut assay can be done on *C. felis* under the same lab condition.

Early-phase transmission is a relatively newly found plague transmission mechanism, which could be the mechanism of some rapidly spreading plague epizootics. Nothing is known about this mechanism on the molecular biology level. Research regarding the molecular mechanisms that control the *Y. pestis* transmission cycle is valuable, although it is hampered by a lack of sequence information and gene expression data from infected fleas. This kind of research could contribute to overcoming the lack of flea genomic gene sequencing, and provide new targets for plague control, or to help make more effective insecticides, producing transgenic fleas or to make transmission-blocking vaccines.

Table 1. Upregulated genes in infected *X. cheopis* fleas (OR1) 1 day following oral infection

Homolog accession no.	Annotation(Organism)	E value
	Genetic information processing (25)	
gb|ABM55416.1|	ribosomal protein S15A [*Xenopsylla cheopis*]	2.00E-20
emb|CAJ17245.1|	ribosomal protein L7Ae [*Scarabaeus laticollis*]	1.00E-32
ref|NP_001011604.2|	ribosomal protein S8 [*Apis mellifera*]	8.00E-40
ref|XP_973872.2|	pitchoune CG6375-PB [*Tribolium castaneum*]	6.00E-26
ref|XP_971062.1|	Alanyl-tRNA synthetase domain containing 1 [*Tribolium castaneum*]	2.00E-19
gb|AAR91689.1|	zinc finger protein 118 [*Mus musculus*]	4.00E-29
ref|NP_001037117.1|	zinc finger protein [*Bombyx mori*]	2.00E-50
ref|XP_001377456.1|	novel KRAB box and zinc finger, C2H2 type domain containing protein [*Monodelphis domestica*]	4.00E-20
ref|XP_001606059.1|	CG32778-PA [*Nasonia vitripennis*]	1.00E-09
gb|AAC16612.1|	transposase [*Drosophila simulans*]	3.00E-15
ref|NP_001137637.1|	CAP, isoform L [*Drosophila melanogaster*]	6.00E-08
ref|XP_002431316.1|	ssm4 protein, putative [*Pediculus humanus corporis*]	2.00E-18
ref|XP_001662952.1|	bifunctional purine biosynthesis protein [*Aedes aegypti*]	4.00E-96
ref|XP_967079.2|	ubiquitin-protein ligase [*Tribolium castaneum*]	1.00E-46
ref|XP_396057.3|	poly A binding protein, cytoplasmic 1 isoform 1 [*Apis mellifera*]	6.00E-41
ref|XP_002426878.1|	arginine/serine-rich splicing factor, putative [*Pediculus humanus corporis*]	5.00E-08
ref|XP_001850562.1|	U2 small nuclear ribonucleoprotein [*Culex quinquefasciatus*]	1.00E-22
ref|XP_969617.2|	similar to CG6694 CG6694-PA [*Tribolium castaneum*]	8.00E-08
ref|XP_002105216.1|	GD21365 [*Drosophila simulans*]	7.00E-57
ref|NP_001154946.1|	structural maintenance of chromosomes 6 [*Nasonia vitripennis*]	4.00E-18
ref|XP_971697.2|	conserved hypothetical protein [*Tribolium castaneum*]	4.00E-09
ref|XP_970801.2|	fusilli CG8205-PD [*Tribolium castaneum*]	2.00E-53
ref|XP_308051.4|	AGAP002144-PA [*Anopheles gambiae str. PEST*]	8.00E-13

ref|XP_393186.1|	CG6322-PA [*Apis mellifera*]	6.00E-30
dbj|BAC57906.1|	reverse transcriptase [*Anopheles gambiae*]	6.00E-22
	Metabolism (25)	
ref|XP_001657506.1|	hypothetical protein AaeL_AAEL006126 [*Aedes aegypti*]	5.00E-34
ref|XP_001662568.1|	short-chain dehydrogenase [*Aedes aegypti*]	8.00E-41
ref|XP_001662544.1|	hydroxybutyrate dehydrogenase [*Aedes aegypti*]	3.00E-09
ref|XP_001650538.1|	gh regulated tbc protein-1 [*Aedes aegypti*]	1.00E-12
ref|NP_001040301.1|	glycosyl-phosphatidyl-inositol-anchored protein [*Bombyx mori*]	2.00E-15
ref|XP_001654194.1|	pnuts protein [*Aedes aegypti*]	4.00E-09
ref|XP_394380.3|	CG32138-PB, isoform B isoform 1 [*Apis mellifera*]	6.00E-89
ref|XP_001849901.1|	plekhh1 [*Culex quinquefasciatus*]	4.00E-09
ref|XP_001606553.1|	armadillo repeat containing 8 [*Nasonia vitripennis*]	1.00E-09
ref|XP_001654032.1|	ubiquitin-conjugating enzyme E2 i [*Aedes aegypti*]	2.00E-46
ref|XP_967064.1|	ran-binding protein [*Tribolium castaneum*]	2.00E-16
ref|XP_001846020.1|	WDVCF 1 [*Culex quinquefasciatus*]	7.00E-09
ref|XP_002427008.1|	ATP synthase subunit alpha, putative [*Pediculus humanus corporis*]	4.00E-46
ref|XP_966693.2|	vacuolar ATP synthase subunit h isoform 1 [*Tribolium castaneum*]	5.00E-23
ref|XP_967555.2|	similar to rCG61344 [*Tribolium castaneum*]	2.00E-14
ref|XP_002422771.1|	Tob1 protein, putative [*Pediculus humanus corporis*]	1.00E-28
dbj|BAD26682.1|	heterotrimeric guanine nucleotide binding protein gamma subunit-like protein [*Plutella xylostella*]	1.00E-25
ref|XP_392652.2|	Abl tyrosine kinase CG4032-PA [*Apis mellifera*]	8.00E-16
ref|XP_001657344.1|	vacuolar proton atpases [*Aedes aegypti*]	9.00E-33
ref|XP_002053640.1|	GJ23240 [*Drosophila virilis*]	6.00E-14
ref|XP_968366.1|	adenosylhomocysteinase [*Tribolium castaneum*]	1.00E-25
ref|XP_966486.1|	ADAM metalloprotease, partial [*Tribolium castaneum*]	7.00E-59
ref|XP_001662952.1|	bifunctional purine biosynthesis protein [*Aedes aegypti*]	6.00E-70
ref|XP_968571.2|	ornithine decarboxylase [*Tribolium castaneum*]	2.00E-45

ref|XP_967401.1|	GA21392-PA [*Tribolium castaneum*]	2.00E-13
	Cell Motility and Migration (7)	
ref|XP_002083321.1|	GD13413 [*Drosophila simulans*]	3.00E-30
ref|XP_001813596.1|	Myosin heavy chain CG17927-PF isoform 3 [*Tribolium castaneum*]	2.00E-104
dbj|BAG30740.1|	muscle myosin heavy chain [*Papilio xuthus*]	2.00E-96
ref|XP_002066324.1|	GK18232 [*Drosophila willistoni*]	8.00E-37
ref|XP_001949443.1|	fimbrin/plastin [*Acyrthosiphon pisum*]	2.00E-110
ref|XP_002011295.1|	GI16449 [*Drosophila mojavensis*]	2.00E-08
ref|XP_001120828.1|	similar to beta-tubulin cofactor C [*Apis mellifera*]	4.00E-22
	Signaling(15)	
ref|XP_001989328.1|	GH11666 [*Drosophila grimshawi*]	2.00E-64
dbj|BAE00010.1|	Lipophorin receptor [*Rhyparobia maderae*]	8.00E-70
ref|XP_001847206.1|	phospholipid scramblase 1 [*Culex quinquefasciatus*]	6.00E-35
ref|XP_002132955.1|	GA26108 [*Drosophila pseudoobscura pseudoobscura*]	6.00E-08
ref|XP_973579.1|	receptor for activated protein kinase C-like [*Tribolium castaneum*]	8.00E-40
ref|XP_002613080.1|	hypothetical protein BRAFLDRAFT_89962 [*Branchiostoma floridae*]	1.00E-17
ref|XP_001600608.1|	similar to kinesin light chain 1 and [*Nasonia vitripennis*]	1.00E-65
ref|XP_002423662.1|	FERM, RhoGEF and pleckstrin domain-containing protein, putative [*Pediculus humanus corporis*]	4.00E-39
ref|XP_001900327.1|	Senescence-associated protein [*Brugia malayi*]	2.00E-16
ref|XP_001846841.1|	conserved hypothetical protein [*Culex quinquefasciatus*]	4.00E-39
ref|XP_966998.2|	GA20259-PA [*Tribolium castaneum*]	7.00E-27
ref|XP_393491.3|	CG11148-PA, isoform A isoform 1 [*Apis mellifera*]	8.00E-11
ref|XP_002063498.1|	GK21943 [*Drosophila willistoni*]	3.00E-07
ref|XP_393548.3|	CG33249-PA [*Apis mellifera*]	4.00E-42
ref|XP_972946.1|	Protein CREG1 precursor [*Tribolium castaneum*]	7.00E-09
	Membrane transporter proteins (9)	
ref|XP_001846062.1|	tumor endothelial marker 7 [*Culex quinquefasciatus*]	9.00E-28

ref|XP_966566.2|	nuclear oncoprotein skia [*Tribolium castaneum*]	1.00E-34
ref|XP_001942534.1|	chromaffin granule amine transporter [*Acyrthosiphon pisum*]	6.00E-29
gb|ACU31000.1|	U1 small nuclear ribonucleoprotein A [*Culex quinquefasciatus*]	3.00E-37
ref|XP_973263.1|	importin subunit beta [*Tribolium castaneum*]	6.00E-23
ref|XP_002059256.1|	GJ16295 [*Drosophila virilis*]	7.00E-09
ref|XP_972827.2|	laminin A chain, putative [*Tribolium castaneum*]	5.00E-22
ref|XP_001607729.1|	prominin (prom) protein [*Nasonia vitripennis*]	2.00E-49
ref|XP_001653012.1|	hypothetical protein AaeL_AAEL001299 [*Aedes aegypti*]	4.00E-34
	Immune defense effector molecules (6)	
dbj|BAA22791.1|	vitellogenin [*Athalia rosae*]	2.00E-20
gb|AAV31932.1|	vitellogenin C [*Toxorhynchites amboinensis*]	2.00E-22
gb|AAF82131.1|AF281078_1	vitellogenin 1 [*Anopheles gambiae*]	1.00E-39
ref|XP_969495.1|	matrix metalloproteinase [*Tribolium castaneum*]	8.00E-10
ref|XP_973587.1|	deoxyribonuclease I [*Tribolium castaneum*]	2.00E-36
ref|XP_320813.4|	AGAP011696-PA [*Anopheles gambiae str. PEST*]	1.00E-40
	Stress-associate protein (4)	
ref|XP_971024.1|	chilling-inducible protein, putative [*Tribolium castaneum*]	4.00E-33
gb|ACO57617.1|	heat shock protein 90 [*Pteromalus puparum*]	8.00E-11
gb|AAV91360.1|	heat shock protein 1 [*Lonomia obliqua*]	2.00E-46
ref|XP_001654428.1|	heat shock transcription factor (hsf) [*Aedes aegypti*]	1.00E-43
	Proteinase (4)	
ref|XP_693540.1|	trypsinogen 7 [*Danio rerio*]	4.00E-09
ref|XP_001650897.1|	mitochondrial processing peptidase beta subunit [*Aedes aegypti*]	1.00E-80
ref|XP_001863592.1|	mitochondrial processing peptidase beta subunit [*Culex quinquefasciatus*]	2.00E-158
ref|NP_001155060.1|	serine protease homolog 21 [*Nasonia vitripennis*]	6.00E-26
	Reactive Oxygen Species Defense (2)	
ref|XP_001663120.1|	NADH-ubiquinone oxidoreductase 42 kda subunit [*Aedes aegypti*]	2.00E-43
ref|XP_001658450.1|	hypothetical protein AaeL_AAEL007562 [*Aedes aegypti*]	7.00E-17

	Unknown (22)	
ref|XP_001603111.1|	red protein (ik factor) (cytokine ik) [*Nasonia vitripennis*]	4.00E-05
ref|XP_001656716.1|	ecotropic viral integration site [*Aedes aegypti*]	7.00E-46
ref|XP_309553.4|	AGAP011096-PA [*Anopheles gambiae str. PEST*]	2.00E-33
ref|XP_001846875.1|	conserved hypothetical protein [*Culex quinquefasciatus*]	1.00E-17
ref|XP_001849187.1|	conserved hypothetical protein [*Culex quinquefasciatus*]	8.00E-10
ref|XP_624126.2|	CG9056-PA [*Apis mellifera*]	3.00E-16
ref|XP_002426743.1|	hypothetical protein Phum_PHUM269490 [*Pediculus humanus corporis*]	4.00E-15
|XP_001600591.1|	CG17652-PA [*Nasonia vitripennis*]	3.00E-42
ref|XP_971716.1|	CG5274 CG5274-PA [*Tribolium castaneum*]	8.00E-15
ref|XP_624126.2|	CG9056-PA [*Apis mellifera*]	3.00E-16
ref|XP_001606807.1|	conserved hypothetical protein [*Nasonia vitripennis*]	1.00E-19
ref|XP_001849187.1|	conserved hypothetical protein [*Culex quinquefasciatus*]	5.00E-09
ref|XP_625204.1|	CCR4-NOT transcription complex subunit 2 (CCR4-associated factor 2) [*Apis mellifera*]	3.00E-27
ref|XP_001604891.1|	ENSANGP00000015396 [*Nasonia vitripennis*]	7.00E-17
ref|XP_974879.1|	ebna2 binding protein P100 [*Tribolium castaneum*]	1.00E-40
ref|XP_002425520.1|	hypothetical protein Phum_PHUM205220 [*Pediculus humanus corporis*]	3.00E-27
ref|XP_002426744.1|	conserved hypothetical protein [*Pediculus humanus corporis*]	2.00E-18
emb|CAA19735.1|	EG:73D1.1 [*Drosophila melanogaster*]	1.00E-48
ref|XP_001120146.1|	CG8320-PA [*Apis mellifera*]	2.00E-40
ref|XP_396656.1|	SRY interacting protein 1 CG10939-PA [*Apis mellifera*]	6.00E-31
gb|ABB47755.2|	expressed protein [*Oryza sativa* (japonica cultivar-group)]	1.00E-15
ref|XP_001120614.1|	lethal (2) k07433 CG33130-PA, isoform A [*Apis mellifera*]	5.00E-10

Table 2. Upregulated genes in infected *X. cheopis* fleas (OR2) 2 days following oral infection

Homolog accession no.	Annotation(Organism)	E value
	Genetic information processing (16)	
gb|AAC04625.1|	ribosomal protein S2 [*Rattus norvegicus*]	7.00E-17
ref|XP_973749.2|	AGAP005884-PA, partial [*Tribolium castaneum*]	4.00E-42
ACA50099	reverse transcriptase-like protein [*Ectropis obliqua*]	8.00E-06
XP_001949782.1	Probable RNA-directed DNA polymerase fromvtransposon X-element	2.00E-10
gb|AAA28508.1|	putative reverse transcriptase [*Drosophila melanogaster*]	6.00E-55
XP_974870.1	mindbomb homolog 1 [*Tribolium castaneum*]	1.00E-135
XP_001841734	Eftud2 protein [*Culex quinquefasciatus*]	5.00E-06
gb|ABC41931.1	transposase *[Sitodiplosis mosellana*]	4.00E-17
XP_972095.2	rap55 [*Tribolium castaneum*]	3.00E-41
XP_001945840.1	pol-like protein [*Acyrthosiphon pisum*]	1.00E-54
XP_001360421	GA18903 [*Drosophila pseudoobscura pseudoobscura*]	2.00E-22
ref|XP_001663329.1|	hypothetical protein AaeL_AAEL013131 [*Aedes aegypti*]	4.00E-15
XP_001660176.1	zinc finger protein [*Aedes aegypti*]	2.00E-24
ref|XP_001604610.1|	ENSANGP00000021856 [*Nasonia vitripennis*]	1.00E-47
ref|XP_001845555.1|	conserved hypothetical protein [*Culex quinquefasciatus*]	1.00E-27
XP_001810193	putative peptidyl-prolyl cis-trans isomerase E [*Tribolium castaneum*]	6.00E-31
	Metabolism (24)	
XP_001863621.1	conserved hypothetical protein [*Culex quinquefasciatus*]	8.00E-23
ref|XP_001848733.1|	conserved hypothetical protein [*Culex quinquefasciatus*]	3.00E-81
ref|XP_968195.1|	CG9205 CG9205-PA [*Tribolium castaneum*]	5.00E-42
XP_001867085	sarcosine dehydrogenase, mitochondrial [*Culex quinquefasciatus*]	1.00E-102
ref|XP_970031.1|	dihydroorotate dehydrogenase, mitochondrial [*Tribolium castaneum*]	1.00E-84
XP_001863548.1	tyrosine-protein phosphatase Lar [*Culex quinquefasciatus*]	5.00E-132
XP_392375.3	dual specificity phosphatase 10 [*Apis mellifera*]	7.00E-38

XP_001863038.1	uridine cytidine kinase i [*Culex quinquefasciatus*]	3.00E-12
XP_968029.2|	similar to glycogenin [*Tribolium castaneum*].	2.00E-95
XP_001655989.1	radical sam proteins [*Aedes aegypti*]	8.00E-11
gb|ACH56921.1|	vacuolar H+-ATPase V0 sector subunits c/c' [*Simulium vittatum*]	2.00E-12
XP_001849275.1	ATP synthase alpha subunit vacuolar [*Culex quinquefasciatus*]	7.00E-64
EDW72138.1	GK10328 [*Drosophila willistoni*]	2.00E-21
NP_001153410	aldo-keto reductase-like [*Nasonia vitripennis*]	2.00E-55
ref|XP_001945966.1|	CG15117 CG15117-PA [*Acyrthosiphon pisum*]	2.00E-25
XP_001944161.1	similar to Gag protein, partial [*Acyrthosiphon pisum*]	7.00E-16
CAD29637	putative 5-oxoprolinase [*Anopheles gambiae*]	2.00E-167
XP_001664249	1-acylglycerol-3-phosphate acyltransferase [*Aedes aegypti*]	7.00E-28
XP_001601621	gh regulated tbc protein-1 [*Nasonia vitripennis*]	5.00E-62
XP_001600219.1	pyruvate carboxylase [*Nasonia vitripennis*]	1.00E-24
ref|XP_975030.2|	importin alpha [*Tribolium castaneum*]	8.00E-28
ref|XP_002423886.1|	conserved hypothetical protein [*Pediculus humanus corporis*]	5.00E-62
XP_002052710.1	GJ20220 [*Drosophila virilis*]	3.00E-57
XP_973672.1	CD98hc amino acid transporter protein [*Tribolium castaneum*]	3.00E-08
	Cell Motility and Migration (2)	
gb|ABO33165.1	alpha tubulin [*Mesenchytraeus solifugus*]	9.00E-108
dbj|BAG30740.1|	muscle myosin heavy chain [*Papilio xuthus*]	6.00E-122
	Signaling (10)	
XP_966383	serine/threonine protein kinase death domain protein[*Tribolium castaneum*]	1.00E-08
XP_001658496.1	toll [*Aedes aegypti*]	5.00E-07
XP_971982	AGAP000407-PA [*Tribolium castaneum*]	8.00E-106
XP_002427459	RING finger protein, putative [*Pediculus humanus corporis*]	9.00E-38
XP_970287.1	RING finger protein 11 (Sid 1669) [*Tribolium castaneum*]	9.00E-10
XP_002423598.1	protein TFG, putative [*Pediculus humanus corporis*]	2.00E-39

XP_001851278.1	cornichon protein [*Culex quinquefasciatus*]	2.00E-68
XP_969684.1	cell division control protein [*Tribolium castaneum*]	1.00E-178
XP_971204.1	phospholipase A2, group VI (cytosolic,calcium-independent) [*Tribolium castaneum*]	4.00E-99
XP_319370.4	AGAP010192-PA [*Anopheles gambiae str. PEST*]	3.00E-74
	Membrane transporter proteins (8)	
ref|XP_001813291.1|	predicted protein [*Tribolium castaneum*]	2.00E-59
ref|XP_969878.1|	Na[+]-dependent inorganic phosphate cotransporter CG10207-PA [*Tribolium castaneum*]	2.00E-74
NP_525116	Na[+]-dependent inorganic phosphate cotransporter [*Drosophila melanogaster*]	3.00E-79
ref|XP_396067.2|	CG31637-PA isoform 3 [*Apis mellifera*]	3.00E-62
XP_001369997	SNARE protein Ykt6 [*Monodelphis domestica*].	1.00E-09
XP_001861581	type II transmembrane protein [*Culex quinquefasciatus*].	2.00E-29
XP_001652281	sorting nexin [*Aedes aegypti*]	3.00E-09
XP_972854.2	lysosomal-associated transmembrane protein [*Tribolium castaneum*]	2.00E-12
	Immune defense effector molecules (3)	
NP_071947.1	palmitoyl-protein thioesterase [*Rattus norvegicus*]	5.00E-26
gb|EDL80359.1|	palmitoyl-protein thioesterase 1, isoform CRA_a [*Rattus norvegicus*]	5.00E-26
XP_972554.2	leukocyte receptor cluster (lrc) member [*Tribolium castaneum*]	3.00E-27
	Stress Associated Proteins (1)	
gb|ABM88156.1|	heat shock cognate 70 [*Plodia interpunctella*]	1.00E-34
	Proteinase (2)	
XP_001947171.1	Endoplasmic reticulum metallopeptidase 1 (Felix-ina) [*Acyrthosiphon pisum*]	6.00E-57
XP_001655706.1	serine protease [*Aedes aegypti*]	2.00E-47
	Reactive Oxygen Species defence (5)	
ref|XP_001851125.1|	adrenodoxin [*Culex quinquefasciatus*]	3.00E-36

XP_001845096.1	uricase [*Culex quinquefasciatus*]	3.00E-23
gb|AAF01930.1|AF150910_1	cytochrome oxidase subunit I [*Greya solenobiella*]	6.00E-84
XP_001662666.1	thioredoxin reductase [*Aedes aegypti*]	1.00E-69
emb|CAQ57675.1|	cytochrome P450 [*Nilaparvata lugens*]	5.00E-76
	Unknown Proteins (9)	
XP_975221	chemokine-like factor superfamily 4 [*Tribolium castaneum*]	9.00E-34
XP_970527	CG3760 CG3760-PB [*Tribolium castaneum*]	2.00E-13
ref|XP_001843990.1|	conserved hypothetical protein [*Culex quinquefasciatus*]	2.00E-26
ref|XP_001604348.1|	conserved hypothetical protein [*Nasonia vitripennis*]	2.00E-16
XP_001653927.1	hypothetical protein AaeL_AAEL009672 [*Aedes aegypti*]	7.00E-92
XP_002135891.1	GA22538 [*Drosophila pseudoobscura pseudoobscura*]	9.00E-24
XP_001866062.1	conserved hypothetical protein [*Culex quinquefasciatus*]	2.00E-11
XP_972996.1	CG16798 CG16798-PA [*Tribolium castaneum*]	4.00E-38
ref|NP_001153390.1|	BTB (POZ) domain containing 2-like [*Nasonia vitripennis*]	3.00E-27

Table 3. Upregulated genes in infected *X. cheopis* fleas (HM1) 1 day following hemocoel infection

Homolog accession no.	Annotation(Organism)	E value
	Genetic information processing (18)	
emb|CAJ17282.1|	ribosomal protein L15e [*Platystomos albinus*]	2.00E-33
gb|ABM55440.1|	ribosomal protein S25 [*Xenopsylla cheopis*]	2.00E-08
ref|XP_001656366.1|	elongase, putative [*Aedes aegypti*]	2.00E-49
ref|XP_001649593.1|	DNA primase large subunit [*Aedes aegypti*]	1.00E-20
ref|XP_001845400.1|	ribonucleoprotein [*Culex quinquefasciatus*]	2.00E-05
ref|XP_395256.3|	eukaryotic initiation factor 5C CG2922-PG, isoform G [*Apis mellifera*]	3.00E-64
ref|XP_394020.3|	topoisomerase 1-binding RING finger [*Apis mellifera*]	3.00E-19
ref|XP_002433761.1|	lysyl-tRNA synthetase, putative [*Ixodes scapularis*]	4.00E-11
ref|XP_001663284.1|	adenylyl cyclase-associated protein [*Aedes aegypti*]	4.00E-09
ref|XP_001658715.1|	eukaryotic translation initiation factor 4 gamma [*Aedes aegypti*]	3.00E-37
ref|XP_001120264.1|	single-strand recognition protein (SSRP),(Chorion-factor 5) [*Apis mellifera*]	2.00E-50
ref|XP_971697.2|	conserved hypothetical protein [*Tribolium castaneum*]	8.00E-10
ref|XP_395830.2|	CCR4-NOT transcription complex, subunit 1 isoform a [*Apis mellifera*]	4.00E-42
ref|XP_001661809.1|	tyrosyl-dna phosphodiesterase [*Aedes aegypti*]	3.00E-25
gb|ACJ50597.1|	zinc finger protein [*Lutzomyia shannoni*]	2.00E-50
ref|XP_001661051.1|	myst histone acetyltransferase [*Aedes aegypti*]	2.00E-14
ref|XP_972555.2|	similar to Midasin (MIDAS-containing protein)[*Tribolium castaneum*]	6.00E-13
ref|XP_392695.2|	Polycomb protein Suz12 (Suppressor of zeste 12 protein homolog) [*Apis mellifera*]	2.00E-46
	Metabolism (18)	
ref|XP_973726.1|	similar to inter-alpha (globulin) inhibitor H4 (plasma Kallikrein-sensitive glycoprotein) [*Tribolium castaneum*]	5.00E-24
ref|XP_397605.3|	CG8789-PA, isoform A [*Apis mellifera*]	2.00E-06
ref|XP_001952558.1|	hypothetical protein, partial [*Acyrthosiphon pisum*]	4.00E-21

ref|YP_001798458.1|	ATP synthase F0 subunit 6 [*Trigoniophthalmus alternatus*]	4.00E-23
ref|XP_557068.3|	AGAP006395-PA [*Anopheles gambiae str. PEST*]	7.00E-06
ref|XP_001601938.1|	tyrosine-protein kinase btk29a [*Nasonia vitripennis*]	1.00E-119
ref|XP_001866801.1|	probable ER retained protein [*Culex quinquefasciatus*]	5.00E-08
ref|NP_001034008.1|	nuclear fallout, isoform E [*Drosophila melanogaster*]	3.00E-10
ref|XP_967467.2|	Shaker cognate l CG9262-PB [*Tribolium castaneum*]	6.00E-16
ref|XP_002428402.1|	Ran-binding protein, putative [*Pediculus humanus corporis*]	3.00E-12
ref|XP_968029.2|	glycogenin [*Tribolium castaneum*]	3.00E-14
ref|XP_001948292.1|	Thymus high mobility group box protein TOX [*Acyrthosiphon pisum*]	2.00E-29
ref|XP_001842602.1|	bicoid-interacting protein 3 [*Culex quinquefasciatus*]	2.00E-07
ref|XP_002427742.1|	luciferase, putative [*Pediculus humanus corporis*]	2.00E-28
ref|XP_001844183.1|	pyruvate carboxylase, mitochondrial [*Culex quinquefasciatus*]	9.00E-38
ref|XP_623111.2|	Clathrin heavy chain CG9012-PA, isoform A isoform 1 [*Apis mellifera*]	2.00E-111
ref|XP_001653374.1|	Dual specificity tyrosine-phosphorylation-regulated kinase [*Aedes aegypti*]	3.00E-05
ref|XP_002429604.1|	insulin receptor substrate-1, putative [*Pediculus humanus corporis*]	1.00E-15
	Cell motility and migration (6)	
ref|XP_973767.2|	kakapo [*Tribolium castaneum*]	3.00E-49
ref|XP_001606978.1|	actin [*Nasonia vitripennis*]	1.00E-14
ref|XP_001809722.1|	Muscle-specific protein 300 CG33715-PD [*Tribolium castaneum*]	6.00E-08
ref|XP_974183.2|	zipper CG15792-PD [*Tribolium castaneum*]	1.00E-31
ref|XP_972336.2|	inter-alpha-trypsin inhibitor family heavy chain-related protein [*Tribolium castaneum*]	8.00E-27
ref|XP_970266.1|	rhomboid [*Tribolium castaneum*]	2.00E-15
	Signaling (12)	
ref|XP_002424845.1|	hypothetical protein Phum_PHUM156460 [*Pediculus humanus corporis*]	4.00E-11
ref|XP_002427385.1|	proline oxidase, putative [*Pediculus humanus corporis*]	5.00E-33
ref|XP_002063487.1|	GK21935 [*Drosophila willistoni*]	6.00E-58

ref|XP_001991713.1|	GH12805 [*Drosophila grimshawi*]	2.00E-09
ref|XP_002062049.1|	GK16864 [*Drosophila willistoni*]	7.00E-27
ref|XP_967045.2|	CG30084 CG30084-PF [*Tribolium castaneum*]	5.00E-20
ref|XP_309330.4|	AGAP011322-PA [*Anopheles gambiae str. PEST*]	2.00E-31
ref|XP_320202.4|	AGAP012354-PA [*Anopheles gambiae str. PEST*]	1.00E-16
ref|XP_002404066.1|	metaxin, putative [*Ixodes scapularis*]	7.00E-19
ref|XP_001664011.1|	guanine-nucleotide exchange factor c3g [*Aedes aegypti*]	2.00E-12
ref|XP_002068611.1|	GK20329 [*Drosophila willistoni*]	2.00E-60
ref|XP_002423662.1|	FERM, RhoGEF and pleckstrin domain-containing protein, putative [*Pediculus humanus corporis*]	2.00E-45
	Membrane transporter Proteins (4)	
ref|XP_975293.1|	importin alpha 1a [*Tribolium castaneum*]	1.00E-23
ref|XP_623691.1|	Spectrin alpha chain [*Apis mellifera*]	1.00E-34
ref|XP_001942534.1|	chromaffin granule amine transporter [*Acyrthosiphon pisum*]	2.00E-75
ref|XP_972510.2|	Uncharacterized MFS-type transporter C19orf28	4.00E-12
	Immune defense and effector molecules (7)	
dbj|BAA22791.1|	vitellogenin [*Athalia rosae*]	6.00E-18
gb|ABD97990.1|	vitellogenin 1 [*Anopheles stephensi*]	1.00E-52
ref|XP_002432696.1|	secreted ferritin G subunit precursor, putative [*Pediculus humanus corporis*]	3.00E-40
gb|ACI32825.1|	beta-1,3-glucan recognition protein 1 [*Helicoverpa armigera*]	9.00E-09
ref|XP_001964664.1|	GF23306 [*Drosophila ananassae*]	4.00E-43
ref|XP_394721.3|	axotactin CG18296-PA [*Apis mellifera*]	1.00E-40
ref|XP_969495.1|	matrix metalloproteinase [*Tribolium castaneum*]	7.00E-15
	Stress Associated Proteins (1)	
ref|NP_001024042.1|	plant Late Embryo Abundant (LEA) related family member (lea-1) [*Caenorhabditis elegans*]	3.00E-21

	Proteinase (1)	
gb|ACI67243.1|	Complement factor D precursor [*Salmo salar*]	2.00E-08
	Reactive Oxygen Species (1)	
ref|XP_968570.1|	PREDICTED: similar to peroxidasin [*Tribolium castaneum*]	1.00E-79
	Unknown Proteins (7)	
ref|XP_001944392.1|	CG32138 CG32138-PB [*Acyrthosiphon pisum*]	7.00E-84
ref|XP_001950180.1|	nuclear fallout, isoform E [*Drosophila melanogaster*]	7.00E-31
ref|XP_002429568.1|	coiled-coil domain-containing protein, putative [*Pediculus humanus corporis*]	1.00E-09
ref|XP_001605599.1|	HLA-B associated transcript-2 [*Nasonia vitripennis*]	5.00E-06
ref|XP_396843.3|	CG32542-PA [*Apis mellifera*]	1.00E-68
ref|NP_001155511.1|	hypothetical protein LOC100162067 [*Acyrthosiphon pisum*]	6.00E-21
ref|XP_002067503.1|	GK16158 [*Drosophila willistoni*]	3.00E-12

Table 4. Upregulated genes in infected *C. felis* fleas (COR1) 1 day following oral infection

Homolog accession no.	Annotation(Organism)	E value
	Genetic information processing (28)	
gb|ACY71296.1|	ribosomal protein L13A [*Chrysomela tremulae*]	3.00E-42
ref|NP_001129593.1|	ribosomal protein S3A [*Acyrthosiphon pisum*]	2.00E-18
ref|XP_968064.1|	PREDICTED: similar to S3Ae ribosomal protein [*Tribolium castaneum*]	6.00E-35
emb|CAJ17226.1|	ribosomal protein L3e [*Scarabaeus laticollis*]	2.00E-63
gb|AAV91393.1|	ribosomal protein 21 [*Lonomia obliqua*]	5.00E-18
ref|XP_001851443.1|	elongation factor Tu [*Culex quinquefasciatus*]	1.00E-79
gb|ACO15550.1|	Eukaryotic initiation factor 4A-III [*Caligus clemensi*]	1.00E-05
ref|XP_001655944.1|	hypothetical protein AaeL_AAEL002763 [*Aedes aegypti*]	6.00E-17
ref|XP_310701.4|	AGAP000399-PA [*Anopheles gambiae str. PEST*]	4.00E-56
ref|XP_395115.3|	CG31992-PA, isoform A, partial [*Apis mellifera*]	3.00E-26
ref|NP_001037374.1|	chorion specific C/EBP [*Bombyx mori*]	3.00E-22
ref|NP_001164154.1|	eukaryotic initiation factor 4E [*Tribolium castaneum*]	1.00E-21
ref|XP_002165214.1|	predicted protein [*Hydra magnipapillata*]	1.00E-15
ref|XP_393329.2|	CG5732-PA [*Apis mellifera*]	1.00E-17
ref|XP_001600475.1|	hypothetical protein [*Nasonia vitripennis*]	6.00E-45
ref|XP_001122313.1|	CG9143-PA [*Apis mellifera*]	2.00E-42
gb|EFA00140.1|	hypothetical protein TcasGA2_TC002961 [*Tribolium castaneum*]	8.00E-126
ref|XP_969772.2|	eukaryotic translation initiation factor 4 gamma, 2 [*Tribolium castaneum*]	4.00E-55
ref|NP_001037444.1|	transcription factor E74 [*Bombyx mori*]	3.00E-27
ref|XP_001647950.1|	werner helicase interacting protein [*Aedes aegypti*]	3.00E-14
ref|XP_002423481.1|	LIM-only protein, putative [*Pediculus humanus corporis*]	1.00E-06
ref|XP_001859676.1|	mixed-lineage leukemia protein [*Culex quinquefasciatus*]	1.00E-11
ref|XP_001988769.1|	GH11346 [*Drosophila grimshawi*]	6.00E-09
ref|XP_002036926.1|	GM12402 [*Drosophila sechellia*]	8.00E-24

ref|XP_002423669.1|	rbm25 protein, putative [*Pediculus humanus corporis*]	8.00E-63
ref|XP_002423654.1|	zinc finger protein Kr18, putative [*Pediculus humanus corporis*]	6.00E-27
ref|XP_313154.4|	AGAP004238-PA [*Anopheles gambiae str. PEST*]	5.00E-31
gb|EFA11670.1|	hypothetical protein TcasGA2_TC008534 [*Tribolium castaneum*]	1.00E-16
	Metabolism (23)	
ref|XP_002047385.1|	GJ11957 [*Drosophila virilis*]	2.00E-59
gb|EFA02011.1|	hypothetical protein TcasGA2_TC007633 [*Tribolium castaneum*]	2.00E-25
gb|ACO09849.1|	Serine/threonine-protein phosphatase 6 [*Osmerus mordax*]	2.00E-63
ref|XP_002155361.1|	GL15175, partial [*Hydra magnipapillata*]	2.00E-21
gb|ABR27955.1|	vacuolar ATP synthase 16 kDa proteolipid subunit [*Triatoma infestans*]	1.00E-11
ref|XP_001844128.1|	endoplasmin [*Culex quinquefasciatus*]	5.00E-85
ref|XP_001663146.1|	n-acetylgalactosaminyltransferase [*Aedes aegypti*]	5.00E-59
ref|XP_001850806.1|	phosphoribosylamine-glycine ligase [*Culex quinquefasciatus*]	8.00E-27
ref|XP_001862416.1|	serine hydroxymethyltransferase [*Culex quinquefasciatus*]	2.00E-56
gb|AAT01075.1|	glyceraldehyde 3-phosphate dehydrogenase [*Homalodisca vitripennis*]	5.00E-85
ref|XP_968505.1|	karyopherin alpha 6 [*Tribolium castaneum*]	9.00E-100
ref|XP_001847611.1|	succinate dehydrogenase flavoprotein subunit, mitochondrial [*Culex quinquefasciatus*]	2.00E-42
ref|XP_001812780.1|	phospholipase c beta isoform 2 [*Tribolium castaneum*]	6.00E-51
ref|XP_001658186.1|	s-adenosylmethionine decarboxylase [*Aedes aegypti*]	2.00E-27
ref|XP_002072894.1|	GK13848 [*Drosophila willistoni*]	5.00E-95
gb|EFA11212.1|	hypothetical protein TcasGA2_TC005176 [*Tribolium castaneum*]	6.00E-74
gb|EEZ97285.1|	hypothetical protein TcasGA2_TC011089 [*Tribolium castaneum*]	2.00E-24
ref|XP_001656514.1|	inorganic pyrophosphatase [*Aedes aegypti*]	3.00E-51
ref|XP_002163391.1|	predicted protein, partial [*Hydra magnipapillata*]	4.00E-39
ref|XP_002431431.1|	Karyogamy protein KAR4, putative [*Pediculus humanus corporis*]	3.00E-39
ref|XP_392624.3|	CG30426-PA [*Apis mellifera*]	4.00E-46
gb|EEQ46379.1|	hypothetical protein CAWG_04728 [*Candida albicans WO-1*]	2.00E-05

ref|XP_001862774.1|	sphingomyelin phosphodiesterase [*Culex quinquefasciatus*]	4.00E-38
	Cell motility and migration (2)	
gb|ACU29543.1|	alpha-tubulin [*Liposcelis bostrychophila*]	6.00E-09
ref|XP_001848911.1|	titin [*Culex quinquefasciatus*]	2.00E-05
	signaling (17)	
ref|XP_391884.3|	Rho GTPase activating protein 21 isoform 1 [*Apis mellifera*]	5.00E-12
ref|XP_001599458.1|	CG32156-PC [*Nasonia vitripennis*]	4.00E-30
ref|XP_969578.2|	AGAP007137-PB [*Tribolium castaneum*]	3.00E-74
ref|XP_001198716.1|	RAB14, member RAS oncogene family, partial [*Strongylocentrotus purpuratus*]	2.00E-21
ref|XP_002399506.1|	lumican, putative [*Ixodes scapularis*]	8.00E-21
ref|XP_396043.2|	Src oncogene at 42A CG7873-PA, isoform A isoform 1 [*Apis mellifera*]	6.00E-46
ref|XP_001659196.1|	protein disulfide isomerase [*Aedes aegypti*]	2.00E-19
gb|EFA10728.1|	BMP and activin membrane-bound inhibitor-like [*Tribolium castaneum*]	7.00E-22
ref|XP_973720.1|	Suppressor of Cytokine Signaling at 16D CG8146-PA [*Tribolium castaneum*]	3.00E-21
ref|XP_002429582.1|	RalBP1-associated Eps domain-containing protein, putative [*Pediculus humanus corporis*]	9.00E-14
ref|XP_001659196.1|	protein disulfide isomerase [*Aedes aegypti*]	2.00E-19
ref|XP_001607156.1|	Argonaute-2 [*Nasonia vitripennis*]	7.00E-09
ref|NP_001128420.1|	protein kinase C [*Apis mellifera*]	1.00E-27
ref|XP_397118.3|	CG8557-PA, isoform A [*Apis mellifera*]	3.00E-35
ref|XP_001119958.1|	p21/Cdc42/Rac1-activated kinase 1 [*Apis mellifera*]	8.00E-29
ref|XP_396577.2|	serine/threonine protein kinase TAO1 [*Apis mellifera*]	7.00E-22
ref|XP_624051.1|	Pak3 CG14895-PB, isoform B isoform 2 [*Apis mellifera*]	7.00E-47
	Membrane transporter Proteins (5)	
ref|XP_002429229.1|	moesin/ezrin/radixin, putative [*Pediculus humanus corporis*]	9.00E-63
ref|XP_968505.1|	karyopherin alpha 6 [*Tribolium castaneum*]	9.00E-100
ref|XP_001656872.1|	ATP-dependent bile acid permease [*Aedes aegypti*]	4.00E-16

ref|NP_001157315.1|	longitudinals lacking isoform 6 [*Tribolium castaneum*]	1.00E-18
ref|XP_001654894.1|	hypothetical protein AaeL_AAEL010765 [*Aedes aegypti*]	8.00E-37
	Immune defense and effector molecules (2)	
ref|NP_608649.2|	apoptosis inducing factor, isoform A [*Drosophila melanogaster*]	1.00E-38
ref|XP_001653225.1|	cd36 antigen [*Aedes aegypti*]	7.00E-42
	Stress Associated Proteins (3)	
gb|ADA61011.1|	90 kDa heat shock protein [*Hepialus pui*]	1.00E-20
ref|XP_001662370.1|	fk506 binding protein [*Aedes aegypti*]	2.00E-35
ref|NP_001040292.1|	DnaJ/hsp40 homolog subfamily A member 1 [*Bombyx mori*]	3.00E-19
	Proteinase (2)	
ref|XP_968871.2|	protease m1 zinc metalloprotease [*Tribolium castaneum*]	1.00E-13
ref|XP_554420.3|	AGAP004023-PA [*Anopheles gambiae str. PEST*]	3.00E-17
	Reactive Oxygen Species (3)	
ref|XP_001648452.1|	peroxiredoxins, prx-1, prx-2, prx-3 [*Aedes aegypti*]	1.00E-64
gb|AAY34441.1|	adrenodoxin reductase [*Anopheles gambiae*]	3.00E-11
ref|XP_001947632.1|	NADH-ubiquinone oxidoreductase fe-s protein 2 (ndufs2) [*Acyrthosiphon pisum*]	1.00E-26
	Unknown Proteins (8)	
ref|XP_001650606.1|	hypothetical protein AaeL_AAEL005262 [*Aedes aegypti*]	6.00E-27
ref|XP_001054809.1|	hypothetical protein [*Rattus norvegicus*]	3.00E-09
ref|XP_001606493.1|	conserved hypothetical protein [*Nasonia vitripennis*]	4.00E-15
gb|EFA10124.1|	hypothetical protein TcasGA2_TC012304 [*Tribolium castaneum*]	1.00E-33
ref|XP_002134262.1|	GA22930 [*Drosophila pseudoobscura pseudoobscura*]	1.00E-21
ref|XP_001654169.1|	hypothetical protein AaeL_AAEL001875 [*Aedes aegypti*]	3.00E-64
ref|XP_002003460.1|	GI22420 [*Drosophila mojavensis*]	1.00E-24
gb|EFA01815.1|	hypothetical protein TcasGA2_TC007417 [*Tribolium castaneum*]	4.00E-09

Table 5. Genes involved in ROS production or removal

HM1

XP_968570.1	PREDICTED: similar to peroxidasin [*Tribolium castaneum*]	Production

OR1

XP_001663120.1	NADH-ubiquinone oxidoreductase 42 kda subunit [*Aedes aegypti*]	Production
XP_001658450.1	hypothetical protein AaeL_AAEL007562 [*Aedes aegypti*] Dual oxidase maturation factor	Production

OR2

XP_001851125.1	adrenodoxin [*Culex quinquefasciatus*]	Production
CAQ57675.1	cytochrome P450 [*Nilaparvata lugens*]	Production
XP_001845096.1	uricase [*Culex quinquefasciatus*]	Production
gb|AAF01930.1|AF150910_1	cytochrome oxidase subunit I [*Greya solenobiella*]	Production
XP_001662666.1	thioredoxin reductase [*Aedes aegypti*]	Removal

Table 6. *X. cheopis* gene-specific primers used in quantitative real-time PCR analysis.

Gene	Primer forward 5' to 3'	Primer reverse 5' to 3'	Product length(bp)
OR2-serine protease	TTT CTG CAA GTT CAA CTG GTG CCC	AGA TAC GTG CTG GTG AAT GGG ACA	162
OR2-phospholipase A2, group VI	ATG GAG GCA TCA ACA CCA GGC TAT	AAC ATG TAA AGC AGT GCG GCC TTC	192
OR2-thioredoxin reductase	GGT ATT CCT TGA CGG AAG CTG TGC AA	CGG CCG CCA ACA GCA ATA ACA ATA	199
OR2-cadherin-N	TAG CTC CAC ATG TGC ACT CAG CTA	CAT GGT CGG GAT TGA CGA ATG CTT	161
OR2-cytochrome P450 4C1	GCG CCC AGG AAG ATG AGA ATA ACT	TCG ATT TGC GCT CTG CTA TCA CCT	195
HM1-proline oxidase	CGG CAA CAA GTC CAT GCC ACA ATA	TCC TCT CGC AAG TAG CTT CGT TCA	113
HM1-glycogenin	GGA GTT GCG AGG ACC ACC AAT TTA	GAC GCT TGC GAC AAA CGA CTC ATA	102
OR1- serine protease homolog 21	ACT TAT GCA GTG GCA ACA ACT CCG	TAT ATC CGC AAC CTG GTG GAT GCT	186
XC-71 ribosomal protein L13	AAA GCC CGT GTC GTT ACT GAG GAT	ACA TCA TCT GGA TTC TCA GCC GCA	134

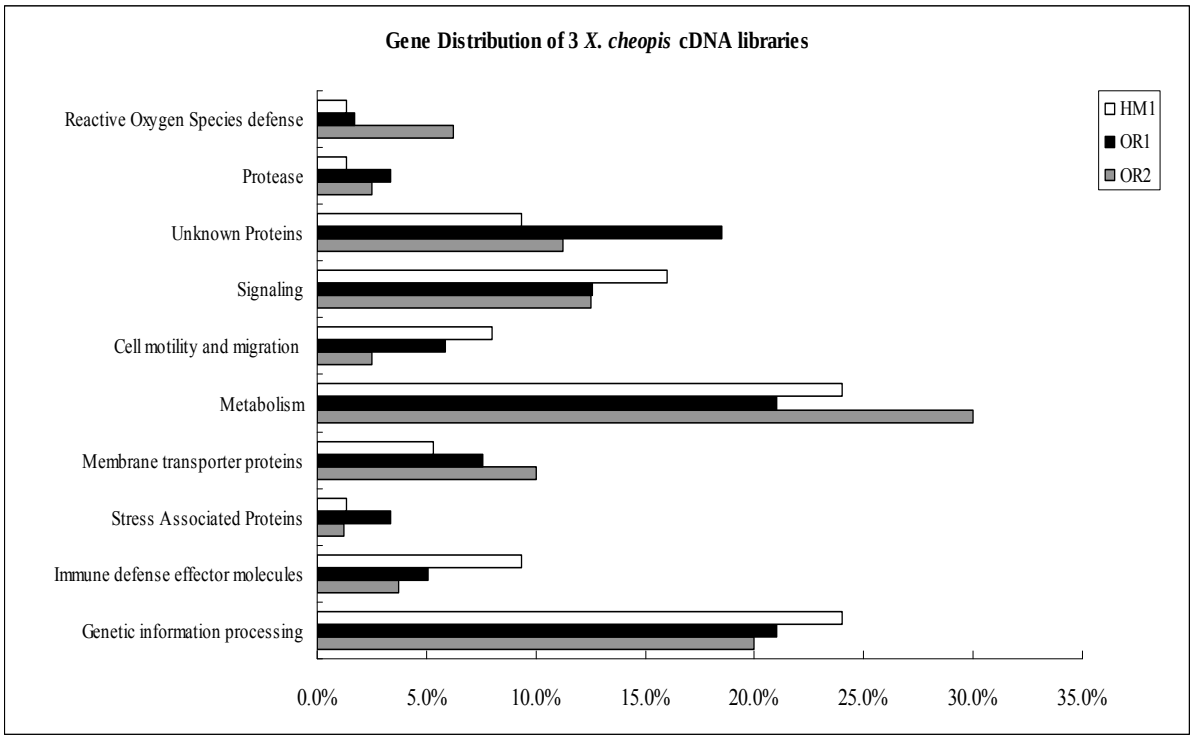

Figure 1. Gene distribution of *X. cheopis* cDNA liraries according to predicted functions. OR1: 1d post-oral infected *X. cheopis* cDNA library, OR2: 2d post-oral infected *X. cheopis* cDNA library, HM1: 1d post-hemocoel injected *X. cheopis* cDNA library The X axis represents the percentage of each function category out of the whole cDNA library.

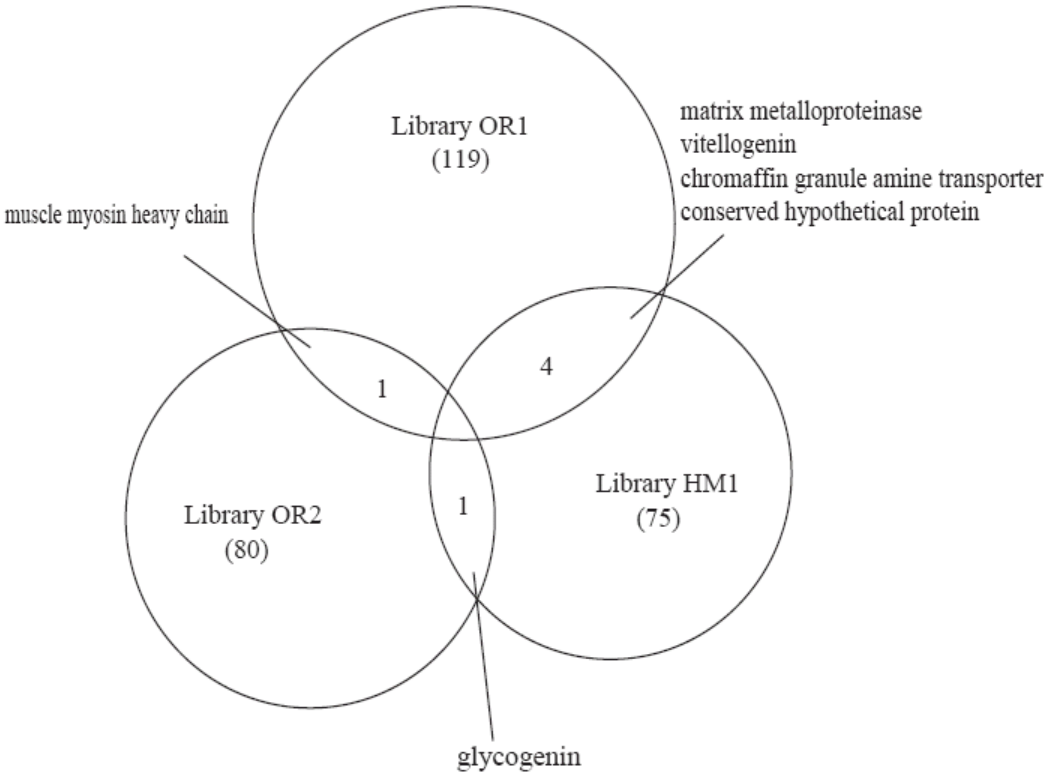

Figure 2. Overlapping genes between *X. cheopis* cDNA libraries.

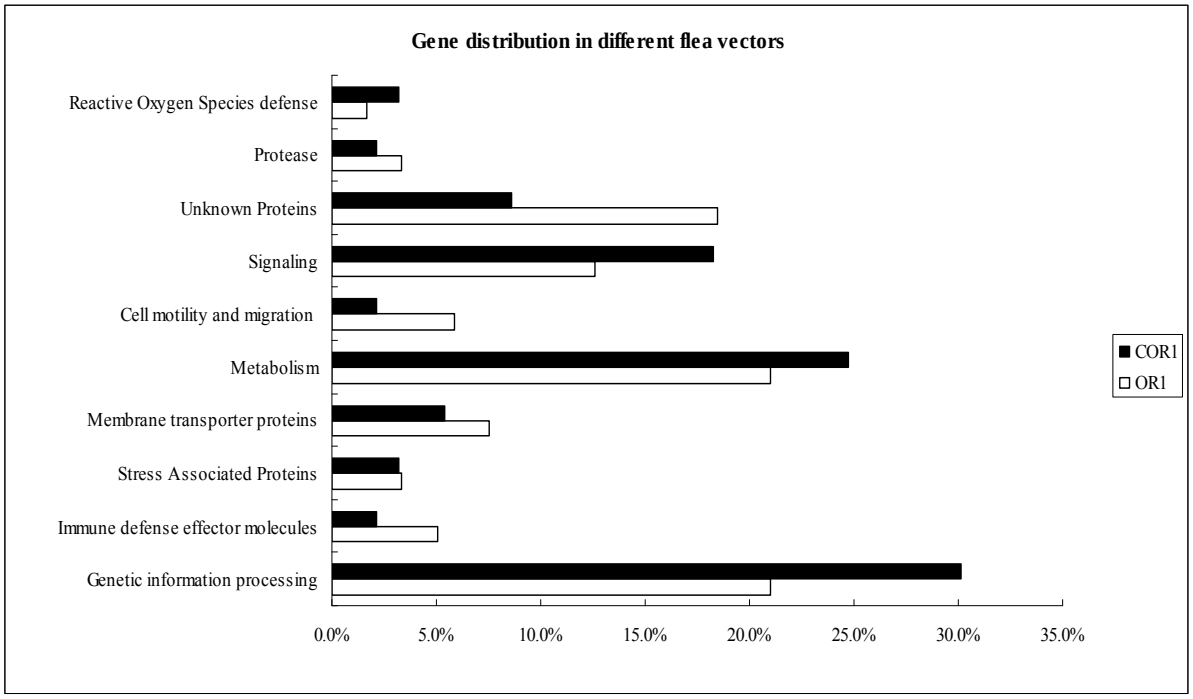

Figure 3. Gene distribution in 1d post-oral infected *X. cheopis* cDNA library (OR1) and 1d post-oral infected *C. felis* cDNA(COR1) library according to predicted functions. The X axis represents the percentage of each function category out of the whole cDNA library.

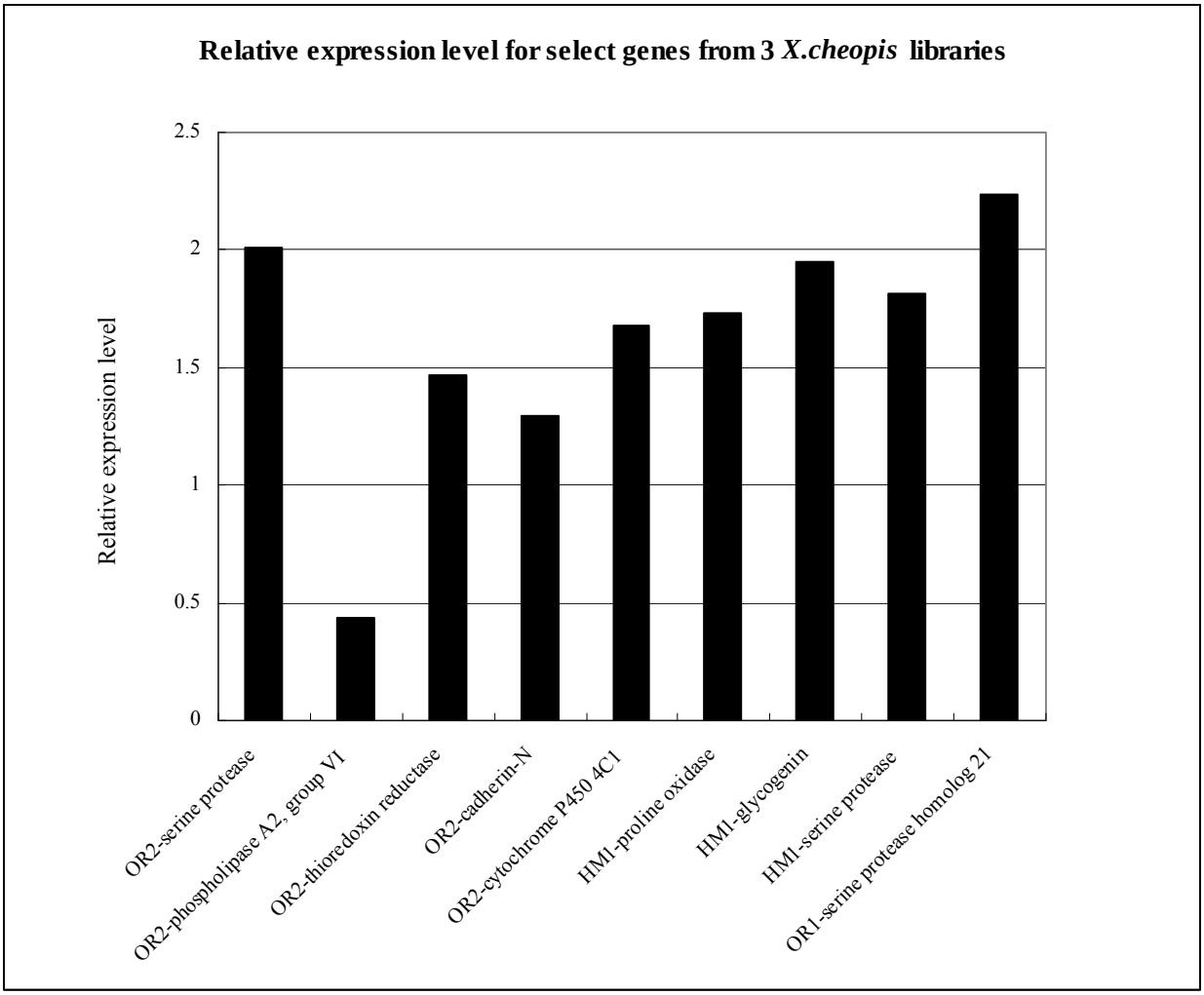

Figure 4. Real-time quantitative PCR analysis of the relative expression levels for selected genes from infected *X. cheopis* cDNA libraries.

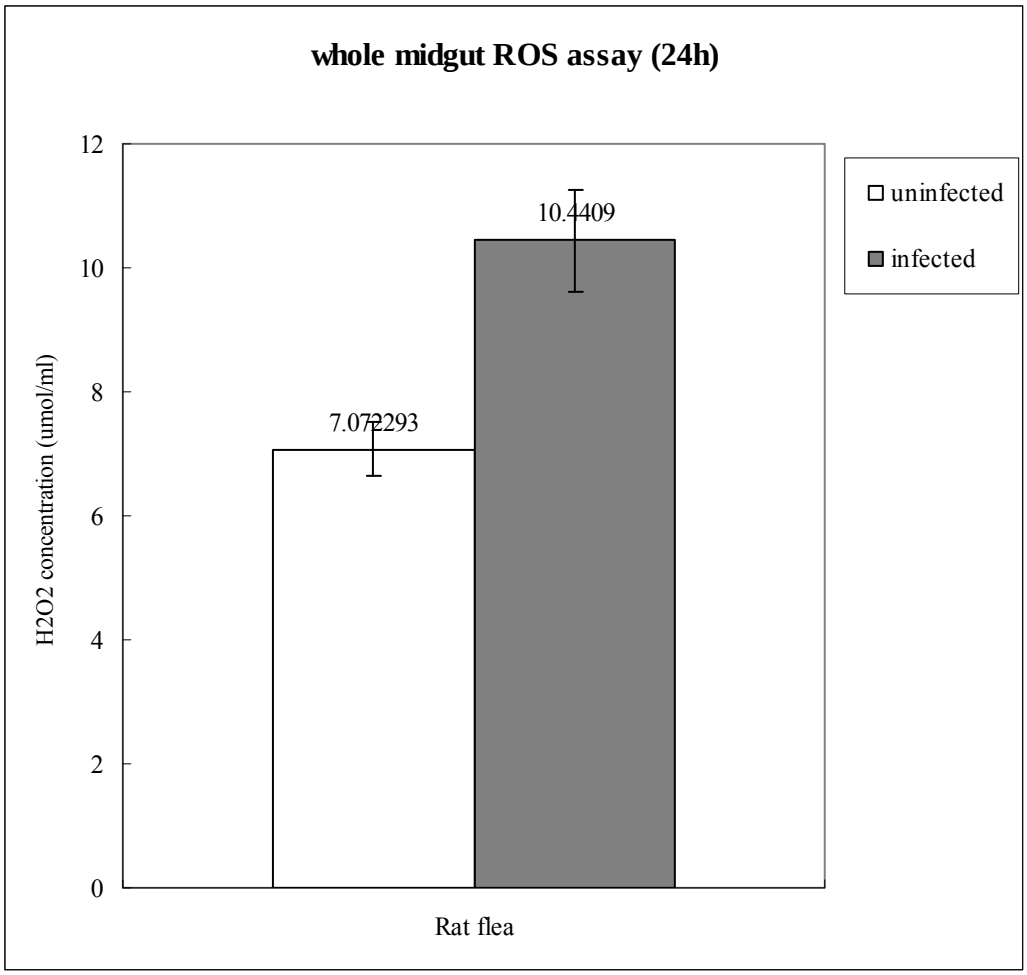

Figure 5. ROS level in 24 h post-oral uninfected and infected *X. cheopis* midguts. P <0.001.

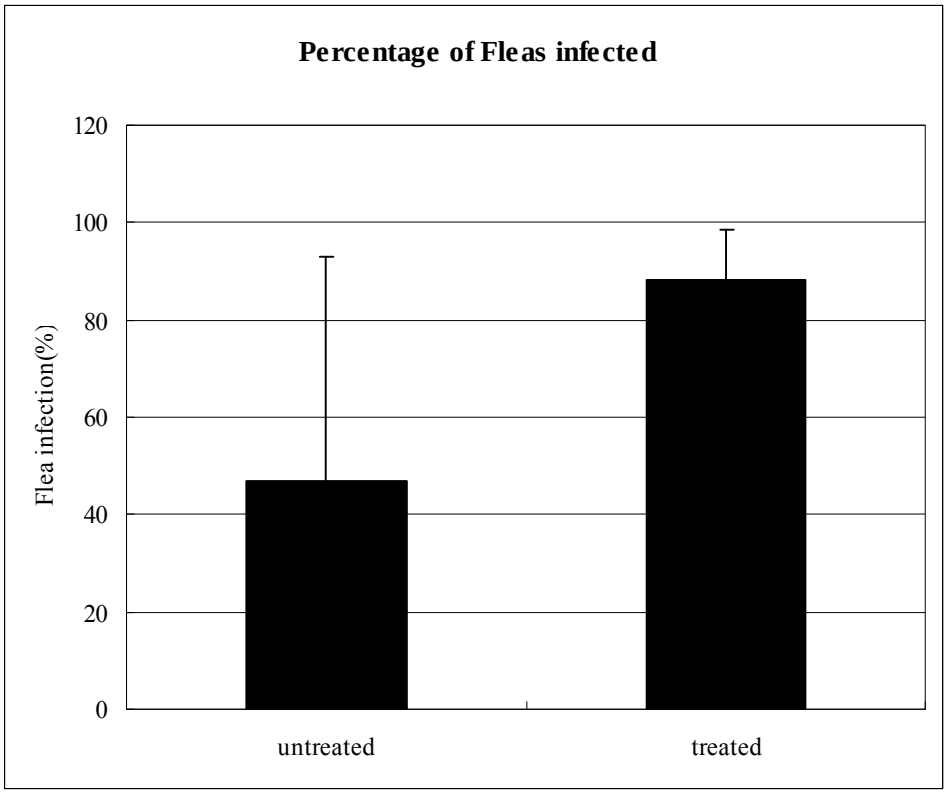

Figure 6. *X. cheopis* flea infection rate in antioxidant feeding group (treated) and the control group (untreated) in first 3-day period after *Y. pestis* infection. The antioxidant feeding increased the *X. cheopis* flea infection rate from 46.7% to 88.3%. P = 0.103.

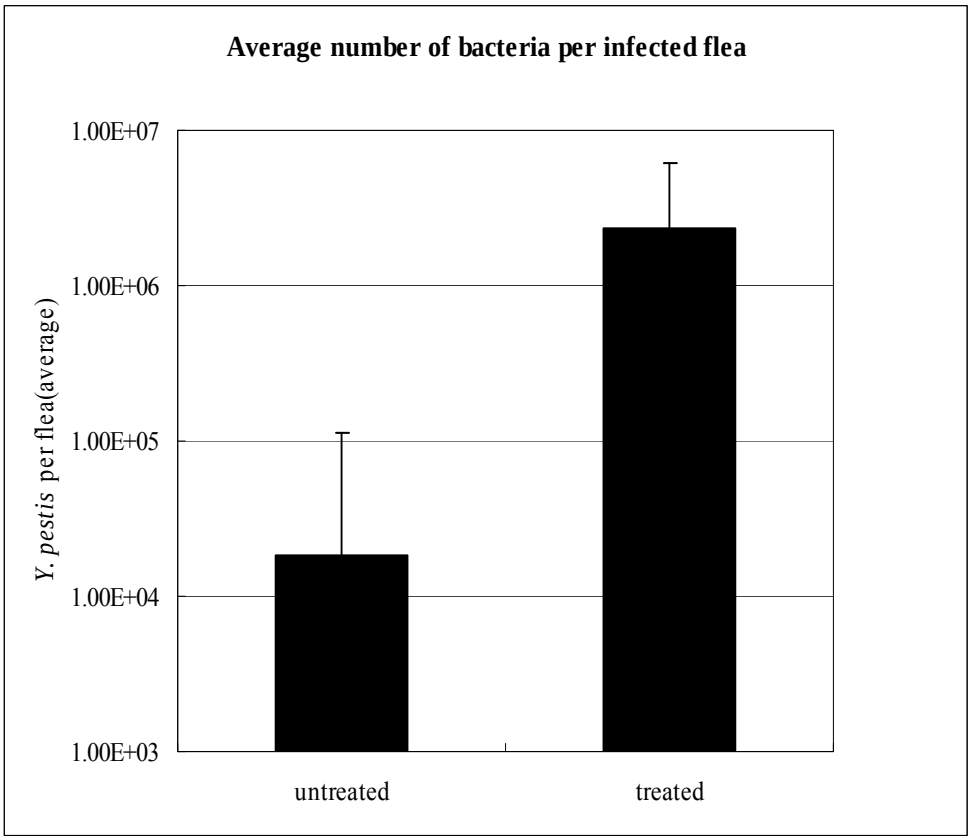

Figure 7. Average *Y. pestis* colonization per flea in antioxidant feeding group (treated) and the control group (untreated) in first 3-day period after *Y. pestis* infection. The antioxidant feeding increased the average *Y. pestis* colonization per flea from 1.82E+04 to 2.32E+06. P = 0.083.

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