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Abstract
The recent resurgence of the common bed bug (Cimex lectularius L.) infestations worldwide has created a need for renewed research on biology, behavior, population genetics and management practices. Humans serve as exclusive hosts to bed bugs in urban environments. Since a majority of humans consume Ibuprofen (as pain medication) and caffeine (in coffee and other soft drinks) so bed bugs subsequently acquire Ibuprofen and caffeine through blood feeding. However, the effect of these chemicals at genetic level in bed bug is unknown. Therefore, this research was conducted to determine differential gene expression in bed bugs using RNA-Seq analysis at dosages of 200 ppm Ibuprofen and 40 ppm caffeine incorporated into reconstituted human blood and compared against the control. Total RNA was extracted from a single bed bug per replication per treatment and sequenced. Read counts obtained were analyzed using Bioconductor software programs to identify differentially expressed genes, which were then searched against the non-redundant (nr) protein database of National Center for Biotechnology Information (NCBI). Data on comparison of differentially expressed genes between control and Ibuprofen treatments revealed that 659 genes were significantly differentially regulated and 95% of them returned BLAST hits. Heat stress proteins were among the top significantly differentially down regulated genes. Comparison of the control vs caffeine treatments revealed that 2,161 genes were significantly differently regulated (Padj <0.05). Heat shock proteins were among the top ten down regulated genes in both treatments. Finally, using RNAi to identify the exact function of these highly differentially expressed genes and regulating these genes may offer potential for managing bed bug populations.

Keywords: Cimex lectularius; Bed bugs; Ibuprofen caffeine; Differential gene expression; Next RNA sequencing

Introduction
Bed bugs (Cimex lectularius L., Hemiptera: Cimicidae) are intermittent ectoparasitiges. They spend most of their time in their harborage of cracks and crevices of structures and leave only to feed. They feed mainly on the blood of mammals and birds, such as bats, humans and fowls. Bed bug nymphs and adults of both sexes require a blood meal for survival, growth and reproduction. Of the 92 described Cimicidae species, only three of them (C. lectularius, C. hemipterus F. and Leptocimex boueti Brumpt) attack humans, and they have been closely associated with humans since recorded history [1,2]. The recent resurgence of the common bed bug infestations worldwide has created a need for renewed interest in conducting new research on biology, behavior, population genetics and management practices. Humans consume Ibuprofen to relieve headache, minor aches, and pains. Similarly, humans use coffee and other soft drinks containing caffeine. Bed bugs-potentially procure Ibuprofen and caffeine from the blood stream while feeding on humans [1-3]. The biological impacts of these chemicals on bed bugs were recently determined by Narain and Kamble [4]. This research is the next phase in exploring the effects of Ibuprofen and caffeine on differential gene expression in bed bugs.

Ibuprofen (iso-butyl-propanoic-phenolic acid) is a non-steroidal anti-inflammatory drug and it is used for reducing fever and pain from headache, toothache, back pain, arthritis, menstrual cramps, or minor injury, Ibuprofen is the active ingredient in more than 50 brand names and generic medications, such as Advil, Motrin that humans consume [5,6].

Caffeine (1,3,7-trimethylxanthine) is a bitter white crystalline alkaloid that acts as a central nervous system stimulant. Caffeine is the most widely consumed addictive substance in the world at the approximate rate of 70 to 76 mg/person/day. The caffeine consumption in USA was estimated at 196 to 238 mg daily [7], and major sources are coffee, chocolate, tea and some soft drinks. The caffeine amount in food products depends on serving size, type of product and preparation method [8-10].

Molecular research on modulation of gene expression and functional genomics associated with insecticide resistance has contributed to the in-depth understanding of resistance evolution in insects [11]. Insects have numerous insecticide detoxification genes. Strode et al. [12] reported 235 cytochrome P450, glutathione transferase and carboxy/cholinesterase genes in Aedes aegypti L. which is an increase of 58% and 36% compared with Drosophila melanogaster Meigen and Anopheles gambiae Giles, respectively. The human body louse, Pediculus humanus humanus L. has 37 cytochrome P450, 13 glutathione-S-transferase and 17 esterase genes which are approximately half the number found in D. melanogaster and A. gambiae [13]. A subset of these gene families is associated with xenobiotic detoxification. Ibuprofen and caffeine added to the blood would be identified by the bed bugs as xenobiotic which would result in an up regulation of these detoxification genes. Zhu et al. [14] identified 14 molecular markers which are associated with pyrethroid resistance in the bed bug. Some of these genes are involved in reducing or slowing down the amount of the toxin reaching the target sites and others are associated with increased metabolic detoxification and translocation by ATP-binding cassette (ABC) transporters. Mamidala et al. [15] used next-generation RNA sequencing technique to discover Differentially Expressed Genes (DEGs) between pesticide resistant and susceptible strains of C. lectularius and identified cuticular proteins, metabolic detoxification proteins and ABC transporters in their pool of DEGs.

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This research uses ribonucleic acid sequencing (RNA-Seq) analysis to determine differential gene expression in bed bugs fed with Ibuprofen and caffeine in reconstituted human blood. We hypothesize that there would be an up regulation in the detoxification genes in bed bugs after ingesting Ibuprofen and caffeine. This hypothesis was tested with the following objectives: 1) determine the differential gene expression in bed bugs 24 hr after ingesting Ibuprofen and caffeine; and 2) delineate the functions of the significantly differentially expressed genes.

Materials and Methods

Insects

The colony of bed bugs (Harlan strain) used throughout this study was established from live specimens obtained from the Department of Entomology, University of Minnesota, St. Paul, MN on March 2011. The Minnesota bed bug colony are descendants from the colony acquired from the Insect Control Research Center (Baltimore, MD) [16], with specimens collected from a natural infestation in Gainesville, FL prior to 1983. In our laboratory, the bed bugs were confined in 500 mL Van Water and Rogers (VWR) short wide-mouth glass jar (VWR International, Radnor, PA), containing 9.0 cm circular VWR filter papers (Radnor, PA) with four folds for harborage and substrate for eggs. The glass jars containing bed bugs were covered with organza fabric (JoAnn’s Fabric, Lincoln, NE) for ventilation and containment. All jars were maintained in a Percival Scientific environmental growth chamber (Perry, IA) at 23 ± 2°C, 55 ± 5% relative humidity, and a photoperiod of 12:12 (Light: Dark) [17]. The bed bugs were fed weekly with expired Reconstituted Human Blood (RHB) obtained from the Nebraska blood bank (Lincoln, NE).

Treatments

This study included three treatments: 1) Ibuprofen 200 ppm, 2) caffeine 40 ppm, and 3) control (untreated). Each treatment had three replications. Each concentration of Ibuprofen and caffeine was incorporated in the reconstituted human blood and ten adult female bed bugs per treatment/replication were allowed to feed for 45 minutes, as per the feeding experiment described in Narain and Kamble [4]. After the completion of feeding, bed bugs were placed in a growth chamber set at 23 ± 2°C, 55 ± 5% R. H., and 12:12 (Light: Dark) with light with and dark 10 AM for 24 hr. The 24 hr time interval was chosen to conduct the differential expression analysis in the bed bugs based on research of Mariniotti et al. [18] and Dana et al. [19] who reported that after a blood meal, genes expressed in A. gambiae begin to climb and reach their peak at 24 hr post feeding (greater than 100 fold for some genes), then begin to drop.

Individual bed bugs that fed to repletion were then removed and placed in 1.5 mL centrifuge tube, flash frozen in liquid nitrogen, and kept in -80°C freezer for RNA extraction.

RNA extraction and sequencing

Five extractions of total RNA per treatment (Ibuprofen, caffeine and control) from a single bed bug were done using RNasy Mini Kit (Qiagen, Valencia, CA, Cat. 74104) and treated with RNase-Free DNase (Qiagen, Valencia, CA, Cat. 79254) to eliminate the DNA contamination, according to the manufacturer’s instructions. The quality and quantity of RNA samples were evaluated on NanoDrop-l000 (Thermo Fisher Scientific, Pittsburgh, PA) and on 1% AMRESCO Formaldehyde-Free RNA Gel Kit N726-KIT (Qiagen, Valencia, CA). Nine RNA samples (3 treatments x 3 replications) were submitted to the University of Nebraska-Omaha Medical Center (UNMC) Next Generation Sequence (NGS) Core Facility for additional quality assessment of the total RNA samples on Agilent 2100 bioanalyzer (Agilent Technologies Inc. Santa Clara, CA) and sequencing on the Illumina HiSeq 2500 Sequencer (Illumina, Inc., San Diego, CA).

RNA sequencing data analysis

The differential expression was determined by the number of RNA reads that mapped to the transcript which was correlated with its abundance level. In RNA-Seq analysis, the expression signal of a transcript depends on the sequencing depth and expression levels of other transcripts [20,21]. RNA-Seq analysis was enabled by the reduced costs in rapid, high-throughput sequencing technology [22]. A typical statistical approach for differential expression (DE) detection uses Poisson or negative binomial distributions and normalization procedure to model the gene count data [23]. This study used Bioconductor packages DESeq and edgeR to conduct the differential gene expression analysis on the read counts from the RNA sequencing data.

RNA sequencing data analysis was conducted on the Holland Computing Center (HCC) at the University of Nebraska, Lincoln, NE. Programs and scripts used for the analyses included Sickle 1.33 [24], Bowtie 2.2.1.0 [25], TopHat 2.0.9 [26,27], HTSeq-count 0.6.1[28], R 3.0.1 [29], DESeq 1.2.8 [30], DESeq 2 1.4.4 [31], edgeR 3.4.2 [32], Blast 2.2.26 [33,34] and Blast2GO [35]. The bed bug reference genome and annotation (V 0.5.2, released November 2011) were used in this analysis and are available on the i5K Pilot Project at Baylor College of Medicine on Human Genome Sequencing Center website https://www.hgsc.bcm.edu/arthropods/bed-bug-genome-project.

The Bowtie2 software, bowtie2-builder and bowtie2-inspect were used to create and inspect an index of the bed bug genome. The raw RNA-seq reads were filtered using sickle which removed and trimmed the low quality (Q <20) sequence reads for better mapping. The filtered RNA-seq reads were mapped to the indexes of the bed bug genome using TopHat2. The file with mapped sequencing reads and the list of annotated genes were then input into HTSeq-count to determine the number reads that mapped to each gene and to generate the read counts data.

Analysis of the read counts obtained from the RNA sequences for the differentially expressed genes were done using DESeq and edgeR. For RNA sequencing data analysis, the approach used by the DESeq program was to take the read count data generated, after it was mapped to annotated genes then performed the statistical analysis to discover quantitative changes of expression levels between experimental groups based on the Fisher’s exact test [28,36]. The edgeR program implemented an exact statistical approach for multi-group experiments based on generalized linear models [32,37]. For both programs, adjustment for multiple testing was accomplished by the False Discovery Rate (FDR) procedure of Benjamini and Hochberg [38]. By using both programs to identify the significantly differentially expressed genes, one program corroborated the results obtained from the other.

The read counts data from the ethanol treatment were compared against the control (untreated treatment) using DESeq to identify gene differentially expressed. DESeq calculated the variance-mean dependence from the read count data and tested for differential gene expression using the negative binomial distribution. The analyses were repeated with edgeR which used empirical Bayes estimation and exact tests based on the negative binomial distribution to determine the differential expression. A significantly differentially expressed gene was
determined as having an adjusted p-value (padj) and false discovery rate (FDR) of less than 0.05 [39]. A comparison of the results from DESeq and edgeR were made and genes that were present in both outputs, and met the conditions for significance, were accepted as differentially expressed genes between the control (untreated) and the treatments.

**Blast2GO analysis**

The DEGs genes were compared against the nr database of the NCBI (ftp://ftp.ncbi.nlm.nih.gov/blast/db/) using the BLASTx algorithm. Blast2GO (http://www.blast2go.com/b2ghome) was used to assign functional groups to the predicted proteins from control (untreated) and the treatments and gene ontology associations were obtained with similarity (e < 10^-5) to proteins in the nr database. The gene function enrichment analyses were also conducted in Blast2GO, which applied the Fisher’s Exact Test to annotated sequences without consideration of the number of reads. Gene ontology categories were identified as significantly enriched at P < 0.05.

**Gene validation**

Two genes, cytochrome p450 9e2 (significantly up-regulated) and vitellogenin (significantly down-regulated) were selected for validation by using primer3 (http://bioinfo.ut.ee/primer3/, [41,42]. The primer sequences and efficiency test for bed bug genes used in qRTP-PCR expression validation are presented in Table 1. The primer sets with primer efficiency between 90-110% were used for qPCR. The quantitative real time polymerase chain reaction (qRT-PCR) was also conducted on same equipment by using the same kit (PN 4385372), according to the protocol in the manual.

**Results**

**Differential gene expression**

A summary of the data obtained from the mapping of the RNA sequences to the bed bug genome for the Ibuprofen, caffeine and control treatments is presented in Table 2. The Q score (Q < 20) was used to access the sequencing data and averages of 0.0388% (range 0.037 to 0.041) of the sequences were removed by sickle [24]. The overall alignment rate varied between 74.5% for Ibuprofen replication 1 and 79.5% for caffeine replication 2. The concordant pair alignment rate ranged from 66.9 to 72.0% (Table 2).

The read count data obtained after the RNA sequencing data were mapped to the bed bug genome and analyzed using Bioconductor programs DESeq and edgeR. The output MA-plot between control (untreated) vs Ibuprofen from DESeq (Figure 1) depicted the log2 fold change of the distribution of the differentially expressed genes. Blue and green lines identified the boundaries of one and two log2 fold change of the genes up or down regulated. The red points identified genes that were significantly differentially expressed (Padj < 0.05). When comparing the read count data generated from HTSeq-count for control and Ibuprofen, 670 genes were identified as significantly differentially expressed (Padj < 0.05) by the DESeq software, and 1,690 (FDR = 0.05) by edgeR (Table 3). The output from DESeq and edgeR indicated that 659 of the genes were common which included 344 significantly differentially up regulated and 315 significantly down regulated genes (Figure 2) The top five species

<table>
<thead>
<tr>
<th>Primer Sequence (5’ to 3’)</th>
<th>Efficiency test</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1241E_QRT1_LEFT</td>
<td>TCCCAAGTTTGACCAATGC</td>
</tr>
<tr>
<td>CYP1241E_QRT1_RIGHT</td>
<td>GAGACATGTGGACTGCTTT</td>
</tr>
<tr>
<td>VETO1309_QRT4_LEFT</td>
<td>ACCTACTTTCCGTGCTCAACA</td>
</tr>
<tr>
<td>VETO1309_QRT4_RIGHT</td>
<td>GTCTCTTTTGTGGCGGCA</td>
</tr>
</tbody>
</table>

**Table 1:** Primer sequences and efficiency test for bed bug genes used in qRTP-PCR expression validation.

<table>
<thead>
<tr>
<th>RNA Sequence / Accession #</th>
<th>Input read</th>
<th>Reads removed</th>
<th>Overall mapping rate (%)</th>
<th>Alignment Pairs</th>
<th>% Concordant</th>
</tr>
</thead>
<tbody>
<tr>
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<td>28629865</td>
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<td>75.5</td>
<td>19784343</td>
</tr>
<tr>
<td>Control Rep. 2 / SRX1025916</td>
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<td>0.041</td>
<td>77.5</td>
<td>25992583</td>
</tr>
<tr>
<td>Control Rep. 3 / SRX1036840</td>
<td>35471224</td>
<td>13125</td>
<td>0.037</td>
<td>75.1</td>
<td>24490490</td>
</tr>
<tr>
<td>Ibuprofen Rep. 1 / SRX1029421</td>
<td>32575940</td>
<td>13833</td>
<td>0.039</td>
<td>77.1</td>
<td>22997040</td>
</tr>
<tr>
<td>Ibuprofen Rep. 2 / SRX1036845</td>
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<tr>
<td>Ibuprofen Rep. 3 / SRX1036846</td>
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<tr>
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<td>14330</td>
<td>0.041</td>
<td>79.5</td>
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<tr>
<td>Caffeine Rep. 2 / SRX1036843</td>
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<td>15643</td>
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<td>38445494</td>
<td>14610</td>
<td>0.038</td>
<td>78.1</td>
<td>27621725</td>
</tr>
</tbody>
</table>

**Table 2:** Summary of bed bug RNA sequences that mapped to its genome for Ibuprofen and control treatments.
and 170, respectively. There were 314 genes participated in binding 50% of the biological processes, with gene ontology scores of 284, 251 processes and single organism processes accounted for approximately

BLAST2GO are displayed in Figures 4, 5. Metabolic processes, cellular and organism functions. The pathway maps contained a network of molecular interactions and reactions that linked genes in the genome to gene products in the pathway [43,44]. KEGG pathway mapping for control vs Ibuprofen, identified 14 genes and 8 enzymes involved in the purine metabolism pathway, followed by pyrimidine metabolism pathway with 10 genes and 4 enzymes in the control vs Ibuprofen treatments. The enzymes identified in the pyrimidine metabolism pathway were in transferases and hydrolases class and the enzymes identified in the purine metabolism pathway were transferases and ligase. Of the 24 genes identified in those two pathways, 19 were significantly differentially down regulated that contained 10 in purine metabolism pathway and 9 in the pyrimidine metabolism pathway. The other five were significantly differentially up regulated, four in purine metabolism pathway and one in the pyrimidine metabolism pathway.

Top KEGG mapping output for control vs caffeine identified 45 genes and 19 enzymes differentially expressed involved in the purine metabolism pathway; 29 of these genes were differentially up regulated and 16 differentially down regulated. In the pyrimidine metabolism activity, followed by 183 genes in catalytic activities. These top two activities covered greater than 75% of the molecular activities of the genes that were significantly differentially expressed in the Ibuprofen treatment.

Data on caffeine vs control (untreated) indicated that of the 13,597 genes in the bed bug genome, there were 2,590 genes significantly differently expressed at the \( \text{Padj} <0.05 \) from DESeq and 2,670 genes from edgeR. When comparing the output from DESeq and edgeR, 2,161 significantly expressed genes were common, with 1,208 genes significantly up regulated and 953 genes significantly down regulated (Table 3). These genes were searched against the nr database of the NCBI using the BLAST algorithm to identify associated genes for the control vs Ibuprofen treatment. There was 67.42% positive hits of the genes submitted with similarity ranging between 37 - 99% to genes in the NCBI nr database.

Output MA-plots from DESeq for control vs caffeine (Figure 6) showed the log2 fold change of the distribution of the differentially expressed genes versus the means of normalized counts. Blue and green lines identify the boundaries of one and two log2 fold change of the genes up or down regulated and red points were of genes that were significantly (\( \text{Padj} <0.05 \)) expressed. The distribution of the significantly differentially expressed genes (Figure 7) indicated that 56% of the significantly differentially expressed genes were up regulated and 44% down regulated.

The top species among the five BLAST hit obtained from the NCBI nr database search for the control vs caffeine treatment were Bean Bug, \( R. \text{pedestris} \), Red flour beetle, \( T. \text{castaneum} \), Pea aphis, \( A. \text{pisum} \), Body louse, \( P. \text{humanus} \) and Alfalfa leafcutter bee, \( M. \text{rotundata} \). Biological and molecular functions of the BLAST genes are displayed in Figures 9 and 10, respectively. Metabolic processes, cellular processes and single organism processes accounts for top three biological processes with gene ontology scores of 945, 793 and 535, respectively. There were 997 genes participated in some binding activity, followed by 760 responsible for catalytic activities. These top two activities accounted for greater than 75% of the molecular activities of the genes that were significantly expressed in the caffeine treatment.

Blast2GO analysis

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway maps represented information on metabolism and various other cell and organism functions. The pathway maps contained a network of molecular interactions and reactions that linked genes in the genome to gene products in the pathway [43,44]. Top KEGG mapping output for control vs caffeine identified 45 genes and 19 enzymes differentially expressed involved in the purine metabolism pathway; 29 of these genes were differentially up regulated and 16 differentially down regulated. In the pyrimidine metabolism

among the BLAST hits obtained from the NCBI nr database search were Bean Bug, \( Riptortus \text{pedestris} \), Red flour beetle, \( Tribolium \text{castaneum} \) Herbst, Pea aphis, \( Acrystosiphon \text{pisum} \) Harris, Body louse, \( P. \text{humanus} \) L., and Jewel wasp, \( Nasonia \text{vitripennis} \) Walker (Figure 3) Biological and molecular functions of the BLAST genes identified by BLAST2GO are displayed in Figures 4, 5. Metabolic processes, cellular processes and single organism processes accounted for approximately 50% of the biological processes, with gene ontology scores of 284, 251 and 170, respectively. There were 314 genes participated in binding
Figure 3: Top BLAST hits of the species to which significantly expressed genes in bed bugs in control vs Ibuprofen treatment were mapped.

Figure 4: Biological functions to which significantly expressed genes in bed bugs in control vs Ibuprofen treatment were mapped.
pathway there were 22 genes and 9 enzymes, differentially expressed with all the significantly differentially down regulated.

**Gene expression validation**

Quantitative Real-time PCR resulted from the gene expression validation analysis of the genes tested agrees with data obtained from the RNA sequence analysis. The efficiency of these primers determined on the Applied Biosystems 7500 Fast Real-Time PCR System were 101%, with an $R^2$ value of 0.999 for both validation genes tests (Table 1). There were significant differences in the expression of genes between the control (untreated) and the Ibuprofen treatments. The cytochrome p450 9e2 was highly (19x relative quantization)
differentially expressed when compared to the cytochrome p450 9e2 in
the control and vitellogenin was expressed at 2x relative quantization
in the control, while for Ibuprofen it was at 0.45x relative quantization
(Figure 11A) There were also significant differences in the expression
of genes between the control and the caffeine treatment. In this case,
the cytochrome p450 9e2 was expressed at 0.6x relative quantization
in the control and 3x relative quantization in the caffeine. A similar
trend was observed for vitellogenin, which was expressed at 0.2x
relative quantization in the control and at 0.55x relative quantization
for caffeine (Figure 11B).

Discussion

This research is the second phase of a study by Narain and Kamble
[4] on the biological impacts of Ibuprofen and caffeine on bed bugs,
which focused on the differential gene expression in bed bugs 24 hr
after ingesting Ibuprofen and caffeine from reconstituted human blood.
For the Ibuprofen treatments, there was a 75% mapping rate of the
RNA-sequencing to the bed bug genome with 659 genes significantly
differentially expressed. Metabolic, cellular and single-organism
processes account for greater than 50% of biological functions, while

Figure 7: The distribution of the log2 fold change significantly expressed genes between control and caffeine treated RHB in bed bugs. Red bars represented down
regulated genes, blue bars up regulated genes.

Figure 8: Top BLAST hits of the species to which significantly expressed genes from bed bugs in control vs caffeine treatment were mapped.

Figure 9: Biological functions to which significantly expressed genes in bed bugs in control vs caffeine treatment were mapped.
binding and catalytic activities account for greater than 75% of the molecular functions. Most of the genes and enzymes regulated were involved in purine and pyrimidine metabolism. The top significantly down regulated genes were associated with heat stress, while top significantly differentially up regulated genes were stipulated with egg development.

Top KEGG mapping output identified 45 genes and 19 enzymes differentially expressed involved in the purine metabolism pathway, 29 of these genes were differentially up regulated and 16 differentially down regulated. The 19 enzymes were in the following classes: oxidoreductases (subclass acting on CH or CH₂ groups); transferases (subclass transferring phosphorus-containing groups); hydrolases (subclass acting on ester bonds, acting on carbon-nitrogen bonds and acting on acid anhydride); and lyases (subclass carbon-nitrogen lyases and phosphorus-oxygen lyases). In the pyrimidine metabolism pathway, there were 22 genes and 9 enzymes differentially expressed with all the significantly differentially down regulated. The nine enzymes were in the following classes: transferases (subclass transferring one-carbon groups and transferring phosphorus-containing groups); hydrolases

Figure 10: Molecular function of the significantly expressed genes in bed bugs in control vs caffeine treatment.

Figure 11: Quantitative RT-PCR validation of bed bugs genes for (A) Ibuprofen and (B) caffeine (error bars=std. err. Red=significantly differentially down regulated, Blue=significantly differentially up regulated gene) Different letters signify significant differences.
Among the top 10 significantly differentially up regulated genes were serine protease, homeobox protein, protein 5nuc-like, lipophorin precursor, and three predicted hypothetical proteins. Serine proteases are enzymes that cleave peptide bonds in proteins, with serine at the active site. They are involved in various physiological processes, such as digestion, development, immunity and defense [45,46]. Jayachandran et al. [46] fed a trypsin-like serine protease to cotton bollworm, Helicoverpa armigera Hubner and recorded a 70% reduction in fecundity and 40% larval mortality. In this study, the increase expression of serine protease may have had an impact on the bed bug fecundity because there was a 47% decrease in the number of eggs laid. Homeobox proteins are regulatory genes that act as transcription factors which recognize and bind to specific DNA sequences [47]. They were shown to play key roles in embryogenesis, such as setting up an anterior-posterior gradient in the egg of the fruit fly (D. melanogaster), and in cell differentiation [48]. Not much is known about the protein 5nuc-like, but the nuc-1 protein was shown to facilitate the apoptotic DNA degradation in many cells [49]. Lipophorin functions as the major lipid transport protein between different organs in insects. In the tsetse fly, Glossina morsitans Westwood, the primary nutrients within tsetse milk are lipids and proteins with amino acids and sugars as minor components. Knockdown of lipophorin (gmlp) by RNA interference resulted in reduced hemolymph lipid levels and delayed oocyte development [50]. In Drosophila spp, lipophorin has lipid-linked morphogens on its surface that are required for long-range signaling activity, control growth and pattern during development [51]. Insects produce and store lipophorin exclusively in the fat body which functions analogously to mammalian liver and participates in detoxification [52]. The insect fat body is also essential in energy storage and utilization. In addition, it is an organ of great biosynthetic and metabolic activity. The fat bodies synthesize most of the hemolymph proteins and circulating metabolites, such as storage proteins used as amino acids reservoir for morphogenesis, or vitellogenins for egg maturation [53]. Up regulation of the lipophorin precursor may have been required for the number of eggs produced, since Ibuprofen was shown to influence egg and ovary development.

For the significantly differentially down regulation, genes associated with heat stress, hsp20, hsp70, and alpha-crystallin b chain, were among the top ten. These genes code for stress protein, which may have responded to the exposure of the bed bugs to Ibuprofen in their diet.

The overall mapping rate of the RNA-sequencing to the bed bug genome for the caffeine treatment was 78%, with a 71% concordant. Comparison of the differentially expressed genes for control vs caffeine revealed 2161 genes significantly differentially expressed (1,208 up and 953 down) between the control and caffeine treatments. Proteins associated with heat stress (hsp20, hsp70, and alpha-crystallin b chain) were among the top 10 down regulated genes, while among the up regulated genes, were proteins associated with apoptosis immunity and metabolism, such as lipase, lysosomal aspartic protease, and zinc finger protein 512b-like and Kruppel-like. Using RNAi to identify the exact function of a few of the highly differentially expressed genes and control of the regulation of these genes, could aid in bed bug population management.

The ten top genes down regulated in the control vs caffeine treatment were mainly heat shock protein or related proteins (alpha-crystallin b chain). Among the top ten genes up regulated was lipase member m. Lipases are water-soluble enzymes that catalyze the hydrolysis of ester chemical bonds in water-insoluble lipid substrates. Lipase member m is an extracellular lipase, necessary for keratinocyte differentiation. They also function in the metabolism of circulating lipoproteins [54,55]. C-type lectin, which is a superfamily of extracellular proteins with diverse functions, whose most important role is the binding of calcium ion [45,56]. C-type lectin was also involved in cell death (apoptosis) and immune response in the organism [57,58]. Also among the up regulated genes were hydroxysteroid dehydrogenase, a ‘short-chain’ dehydrogenases/reductase active on OH-groups of steroids [59]. Additional functions assigned to ‘short-chain’ dehydrogenases/reductase is in intermediary metabolism, enzymes participating in lipid hormone and mediator metabolism [60]. In addition, there were homeobox proteins in the top 10 up differentially regulated genes. These regulatory genes act as transcription factors that recognize and bind to specific DNA sequences [48]. Another of the top 10 up regulated gene was the lysosomal aspartic protease, whose functions comprise the bulk protein degradation, antigen processing, proprotein processing, prohormone processing, degradation of matrix constituents in the extracellular space, and initiation of apoptotic processes [61]. Completing the top ten up regulated genes for the control vs caffeine treatment were zinc finger protein 512b-like and Kruppel-like factors which are highly related to zinc-finger proteins that are important components of the transcriptional machinery and take part in numerous cellular functions, including cell proliferation, apoptosis, differentiation, and neoplastic transformation. Additionally, they function as activators or repressors depending on the promoter or co-regulators with which they interact [62,63]. The caffeine the bed bug consumed may have increased apoptosis, increase cell death may stimulate an immune response, and therefore, this would explain the increase in expression of most of these top 10 up regulated genes.

In conclusion, differential gene expression analysis using Next Generation Sequencing of the bed bug RNA was performed using the first available assembly of the bed bug genome and gene annotation (version 0.5.2). The RNA extraction for the gene expression analysis was done at a single point (24 hr) after the bed bugs were allowed to feed for 45 minutes on 200 ppm Ibuprofen and 40 ppm caffeine in reconstituted human blood. Between 74.5 and 79.6% of all left and right reads were mapped to the bed bug genome. The number of genes differentially expressed varied from 2,161 for control vs caffeine of which 56% were up regulated, to 659 for control vs Ibuprofen treatment (52% up regulated). When the differentially expressed genes were searched against the NCBI nr database, there were a 67.4 and 95.5% hits for control vs caffeine and Ibuprofen, respectively. Gene ontology from BLAST2GO returned R. pedestris (Bean bug) for control vs Ibuprofen and for control vs caffeine.

The RNA sequence data produced from these analyses was submitted to the NCBI and could be used for additional analysis such as identifying splice junctions or isoforms of genes. Also if mapped against the human genome may produce some information, such as blood type. Additionally, it may be able to identify sibmions of the bed bugs. Genes that have been shown to influence egg production in previous studies were also found to be significantly differentially regulated in this study with RNAi technology these genes could be used to help in bed bug management.

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