Published in final edited form as:

Arch Insect Biochem Physiol. 2009 August ; 71(4): 236–253. doi:10.1002/arch.20318.

MICROARRAY ANALYSIS OF GENE EXPRESSION CHANGES IN FEEDING FEMALE AND MALE LONE STAR TICKS, *AMBLYOMMA AMERICANUM* (L)

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Abstract

A collection of EST clones from female tick *Amblyomma americanum* salivary glands was hybridized to RNA from different feeding stages of female tick salivary glands and from unfed or feeding adult male ticks. In the female ticks, the expression patterns changed dramatically on starting feeding, then changed again towards the end of feeding. On beginning feeding, genes possibly involved in survival on the host increased in expression as did many housekeeping genes. As feeding progressed, some of the survival genes were downregulated, while others were upregulated. When the tick went into the rapid feeding phase, many of the survival genes were downregulated, while a number of transport-associated genes and genes possibly involved in organ degeneration increased. In the males, the presence of females during feeding made a small difference, but feeding made a larger difference. Males showed clear differences from females in expression, as well. Protein synthesis genes were expressed more in all male groups than in the partially fed females, while the putative secreted genes involved in avoiding host defenses were expressed less.

Keywords

tick; salivary gland; gene expression; feeding; mating

Introduction

Ticks are obligate ectoparasites that feed on blood from mammals, birds and sometimes reptiles (Sonenshine 1991). Female ixodid ticks are long-term feeders that remain attached to a host for as long as two weeks in the adult phase. Because of this long term attachment, they need to manipulate the host in various ways to keep blood flowing and to prevent the host from harming or removing them. They have evolved an extensive series of secreted salivary gland proteins, peptides and small molecules to achieve these goals, by preventing blood coagulation, keeping capillaries open, reducing inflammation and pain and suppressing the immune response (Bowman and Sauer 2004; Wikel and Alarcon-Chaidez 2001). In addition, they need to dispose of the excess water taken in with the blood meal,

which they do by secreting it through the salivary glands back into the host (Sauer et al. 1995).

The adult female tick *Amblyomma americanum* goes through a remarkable developmental program after starting to feed and mating. From a weight of 4–5 mg, after 10–14 days in the slow feeding phase she attains a weight of 200–250 mg, then undergoes a fast phase of feeding in which her weight doubles or triples in 12 to 24 hrs. She then drops off the host to lay her eggs and dies (Sonenshine 1991). Since the tick may have to wait a considerable time after molting before a host comes along, the unfed tick may not express many of the genes necessary for feeding and making eggs. These are expressed in response to attachment, feeding and mating. Various activities, such as salivary gland fluid secretory ability, are greatly stimulated after attachment, and reach a peak at the end of the slow phase of feeding (Sauer et al. 1989). Enzymes, such as dopamine-sensitive adenylyl cyclase (Schramke et al. 1984) and cAMP phosphodiesterase (McMullen et al. 1983) show a similar or inverse behavior. Both the overall protein composition and the mRNA content of the salivary gland change dramatically (McSwain et al. 1982; Shelby et al. 1987). Mating is required for the female to progress through these changes. We hypothesize that the unfed tick, in order to conserve resources, holds metabolic and secretory processes “in reserve” until it finds a host and starts feeding. She would require a certain number of gene products to be available to maintain life, and others to accomplish activities, like collecting water from the air, that she would not need after finding a host. She may also need some of the feeding success-oriented genes available from the start in order to avoid immediate host defenses, but probably not all. After finding a host, she would be able to shut down expression of a few genes needed for life off the host, and would need to start up expression of many of the other genes she would need to feed successfully, in addition to those she would need from the very start. Since mating is required for producing and laying eggs, much of this program will not proceed in its absence. In addition, general metabolic activity will increase to handle the large amount of blood ingested, leading to increased expression of many housekeeping genes. As feeding progresses, there may be changes to adjust to the host defense mechanisms initiated in response to the parasite and possibly to allow for replacement of proteins that have been countered by antibodies. To allow for the rapid phase of feeding, ticks require greater salivary secretory ability, excretion, and digestion, which may be reflected in further increases in expression of proteins already expressed. At about the time of the fast phase of feeding, a program for degrading the internal organs sets in to provide nutrients for making eggs (Sonenshine 1991). Many of the factors necessary on the host may become redundant. Salivary gland degeneration should require genes involved in apoptosis and protein degradation to be expressed.

In the case of the males, because of their different lifestyle, the expression program is likely to be different. While the unfeds have the same problem of living for long periods off the host, the feeding males are intermittent feeders compared to the long-term more voracious feeding females, so may not require the same array of defenses as the females. Likewise, they do not grow so large, so do not need the large metabolic capacity the females do. Also, since they don't lay eggs, there is no obvious necessity of breaking down their organs. Males also show some changes on feeding and possibly on mating (Anyomi et al. 2006; Bior et al. 2002; Weiss and Kaufman 2004). These changes may be involved in the phenomenon of mate protection proposed by Wang et al. (1998).

Many methods exist for determining the relative expression of various genes in an organism under different conditions. One that has achieved a lot of use recently is the microarray, in which oligonucleotides or cDNA fragments are attached to glass or other substrates and hybridized to a mixture of cDNAs, made from the organism of interest under two different conditions (Shalon et al. 1996). These cDNAs are labeled so they can be distinguished,

typically by fluorescence. The degree of hybridization of a particular spot on the array is a measure of the abundance of that RNA in the organism under a particular condition (Freeman et al. 2000) and is typically standardized by determining the ratio of intensities under the two different conditions. This method has been extensively validated and gives results similar to older methods, such as northern blotting, but can survey a large number of genes at once. The method has been used to determine the effects of acaricides on gene expression in *Rhipicephalus microplus* (Saldivar et al. 2008) and the role of a protective antigen in *Ixodes scapularis* (de la Fuente et al. 2008).

In this study, we have constructed a microarray from a set of cDNAs found in our EST (expressed sequence tag) study (Aljamali et al. 2009). This array has been used to survey the expression of these ESTs through the adult female feeding cycle, and also to investigate the effect of feeding on adult male ticks.

Materials and Methods

Growth of ticks

A. americanum ticks were grown by the method of Patrick and Hair (1975). Adult male and female ticks were grown on sheep. For the female study, approximately 40 female unfed ticks were placed on sheep in a sock along with 20 males. Females were removed at various stages of growth, or allowed to grow to repletion, as indicated below. Separate groups of ticks were grown for the biological replicates, using different host animals. For the male tick study, males were allowed to feed in a sock with females, as above, for 10 days, to give group F. Another group was grown on sheep in a sock without females, and again allowed to feed for 10 days to give group W. The U group was not fed as adults. Ticks were provided by the OSU Tick Rearing Facility.

cDNA glass chip fabrication

Plasmid DNA templates were chosen from three cDNA libraries (Aljamali et al. 2009). These libraries include one made from poly(A) RNA isolated from female ticks at around 200 mg, another made from this library by normalizing, and one made from total RNA isolated from female ticks in a variety of growth stages, including unfed and replete. About 10 ng each of 1145 purified plasmid DNAs chosen from the three libraries were amplified by PCR in 100 µl total volume using Hot Start Platinum® Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) and the appropriate 5' and 3' sequencing primers. The amplification was done according to the program 94°C for 2 min, 26 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min, followed by a final extension step at 72°C for 20 min. The PCR samples were purified using Multiscreen™ filter plates (Millipore, Bedford, MA, USA) according to the manufacturer's instructions and random purified PCR products were electrophoresed on a 1% agarose gel to estimate the yield. The purified PCR products were transferred to 384 well microarray printing plates (Genetix, New Milton, UK), dried at 42°C for 6 hours and reconstituted with printing solution (0.45 M NaCl, 0.045 M sodium citrate, pH 7) to a final concentration of ~150 ng/µl. Eight ArrayControl™ control standards (Ambion, Austin, TX, USA) were reconstituted with printing solution to a final concentration of 160 ng/µl. The cDNAs were printed on ArrayIt™ SuperAmine substrates (TeleChem, Sunnyvale, CA, USA) using a Cartesian pix 5500 (Genomic Solutions, Ann Arbor, MI, USA) microarrayer in 56% relative humidity. Each PCR product and standard was printed four times on each slide. After drying, the slides were baked at 85°C for two hours and stored at room temperature in a desiccator. Prior to hybridizations, slides were washed with 1% SDS then boiled for two minutes in sterile water, snap-cooled in ice-cold 95% ethanol and then dried by 10 sec centrifugation in an ArrayIt™ slide centrifuge.

(TeleChem, Sunnyvale, CA, USA). The quality of the printed slides was investigated by Syto61 staining according to a standard protocol.

RNA Extraction/Labeling/Hybridization

For the female ticks, salivary glands were dissected from five different adult stages of tick feeding; unfed ticks (UF), early fed ticks with weight <50 mg (EF), partially fed ticks with weight 50–200 mg (PF), fast feeding ticks with weight 300–550 mg (FF) and replete detached ticks (Rep). The unfed ticks were taken from the rearing facility's stock, in different batches. For the other stages, at least four independently grown sets of ticks were used. The reference RNA for females was a pool isolated from 400 partially fed female ticks in the weight range 50 – 200 mg, as for the PF group. For the male ticks, salivary glands were dissected from the U, F and W groups. The reference for the males was a sample of RNA from partially fed females, as above, grown at the same time. The salivary glands were stored in RNAlater™ (Ambion, Austin, TX, USA) at –20°C till the RNA extraction. The total RNA was extracted using an RNAqueous™ -Mini kit (Ambion, Austin, TX, USA) for most samples and a Midi kit for the pooled reference RNA. RNA concentrations were measured using a RiboGreen® RNA quantitation kit (Molecular Probes, Eugene, OR, USA) or by absorbance at 260 nm. ArrayControl™ control RNA spikes (Ambion, Austin, TX, USA) were added to the RNA samples before labeling to hybridize with the control standards on the slides. Indirect labeling of RNA from females was performed with a 3DNA® Array 50™ DNA Dendrimer Cy3™/Alexa Fluor 647™ kit (Genisphere, Hatfield, PA, USA), while males were labeled with the Alexa Fluor 546/Alexa Fluor 647 kit. The labeling specificity is achieved through the binding of each of the two dyes to the specific cDNAs as the primer oligo dT used in the reverse transcription reaction for each RNA sample contains a capture sequence specific for either the Cy3, Alexa546 or Alexa647 dye (Stears et al. 2000). Ten µg of total RNA samples were precipitated with ethanol overnight at –20°C. RNA pellets were reconstituted with water and the cDNA synthesis was done according to the manufacturer's protocol using SuperScript™ II RNase[–] reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with a dye-specific oligo dT primer. cDNA resulting from one of the groups using a particular dye-specific capture sequence was mixed with that from the reference group with a different capture sequence and hybridized in a formamide-based hybridization solution in a total volume of 25µl. Hybridization was performed overnight at 42°C in CMT™ hybridization chambers (Corning, Lowell, MA, USA). After hybridization, slides were washed, then fixed in 95% ethanol. The second hybridization with the 3DNA™ reagents containing the dyes was performed in a formamide solution containing anti-fade reagent in a total volume of 25µl for 4 hours at 42°C after which the slides were washed and dried. For the female ticks, all hybridizations used independent RNA isolations from independently-grown ticks. For the males, there were two independent RNA samples from independently-grown ticks for the F and W groups and one for the U. These were all hybridized once, except for one of the F samples, which was hybridized twice.

Data analysis

Slides were scanned after hybridization in a ScanArray Express confocal laser scanner (Perkin-Elmer, Waltham, MA, USA) using the software that came with the instrument. The resulting tiff files were analyzed using GenePix Pro, v. 4.0 (Molecular Devices, Sunnyvale, CA, USA) software to find spots, determine local backgrounds and integrate spot intensities. Statistical package R (<http://www.r-project.org>), and library LIMMA (Smyth et al. 2003) (Linear Models for Microarray Data) were used for data filtering, data transformation, background correction, normalization, calculation of consensus correlation for technical replicates within a slide, linear model fitting, and statistics calculation. Genepix-flagged spots and spots whose intensities after background subtraction were lower than 200 in both

channels were filtered out prior to normalization and linear model fitting. Background was corrected using RMA, a non-linear method used in the Affymetrix package and included in the LIMMA library as a method for background correction. The log₂ of the resulting intensity data was used to calculate the gene expression value, log₂ (treatment/control), which was used for further linear model fitting and statistical calculations. Local print-tip loess normalization was used for within-array normalization and quantile normalization was used for across-array normalization. Comparison of quantitative real-time reverse transcriptase polymerase chain reaction (qPCR) and microarray results suggested that this normalization significantly overestimated the expression in the unfed females. The Array Control internal standards were used for normalization of this stage because the results using them agreed much better with the qPCR results.

The consensus correlation for technical replicates within a slide was a robust average from the correlation of each individual EST clone, which was estimated by fitting a mixed linear model for each clone's technical replicates within one slide. Each clone was fitted by a linear model, and the coefficients describe the difference between cDNA sources hybridized to the array. The P-value from moderated t-statistics after Benjamini and Hochberg correction for false discovery rate was used to identify statistically significant differentially expressed ESTs. The false discovery rate was 10%. The Tigr MultiExperiment viewer (TMEV) (Saeed et al. 2003) was used to conduct hierarchical clustering and K-Means clustering to identify patterns of gene expression. Twelve clusters were used for the females because of the number of possibilities with 5 groups. For the males with 3 groups, 6 clusters provided enough differentiation. Only clones with adjusted p-value <0.01 for the log ratio in at least one condition were included in clustering analysis.

The Gene Ontology (GO) categories (<http://www.geneontology.org/>) found in each cluster or expression class were compared for over- or under-representation to those of the whole array using the program Gostat (Beißbarth and Speed 2004) with a P value < .05 considered significant.

These results have been deposited in GEO with accession number GSE5010. The files under this accession include the difference results as well as the identities of the genes in the clusters for the female and male experiments.

qPCR

qPCR was performed using SYBR-GREEN chemistry in an AB7500 Real- Time PCR machine (Applied Biosystems, Foster City, CA, USA). Eight contigs were selected, representing seven different clusters from the microarray analysis and one with no significant changes in any stage. Primers (Table 1) were designed using software provided with the instrument. Each was tested on RNA extracted from each of the female feeding stages. Equal amounts of RNA by absorbance were used in the amplifications. The loading standard was DR766684, ribosomal protein S13. Not all determinations gave successful amplifications, as judged by melting behavior of the product or lack of a linear portion of the amplification curve, though all primers gave successful amplifications enough times for a statistically valid average. For each contig, the difference between a feeding stage's ΔC_t value and that of the partially fed stage was calculated for comparison with the microarray data. This is equivalent to a log₂ ratio value. The values for each contig were analyzed by a one-factor analysis of variance, and the 95% confidence interval for each stage calculated from the mean square of the error variance. Since members of some of the contigs were present on the microarray more than once, all the representatives were averaged to obtain the comparison values, and the confidence interval was calculated from the one factor analysis of variance as for the qPCR results. For the contig containing DR766616, two of the microarray expression patterns were similar and the third was quite different. Only the two

similar ones were used for the comparison. For the contigs only represented on the microarray once, the confidence limit was taken from the significance analysis described above.

Results and Discussion

Female feeding stages

Progression—To study the expression profile of the genes identified through the female salivary gland EST project, we arrayed probes representing 1145 EST's on glass slides. We compared hybridizations of each of five female feeding stages (unfed ticks: UF; early fed ticks with weight <50 mg: EF; partially fed ticks with weight 50–200 mg: PF; fast feeding ticks with weight 300–550 mg: FF; and replete detached ticks: Rep) with that of the reference RNA using RNA samples extracted from four independently grown batches of ticks for each stage including partially fed ticks similar to those used for the reference RNA. The latter hybridizations were done to validate the reference RNA sample extracted from a large pool of partially fed ticks in representing ticks from this feeding stage. When these results were compared with those obtained for RNA samples from the same growth stages either by Northern blotting or qPCR, it was found that the lowess and quantile normalization used gave results that did not agree for the unfed female ticks, though those for other stages showed better agreement (see below). In addition, the log ratios for the internal control RNA's using this normalization were not close to zero for this stage, though they were for the others. Because of this discrepancy, the internal control RNA's were used to normalize the unfed females, while the statistical method was used for the other stages and the males.

Valid results were obtained for 1062 ESTs on the array. Only 20 (1.9%) were identified as significantly different in the four independent biological samples of partially fed relative to the reference sample. The dye effect probably does not account for these differences as the dye was interchanged in two out of the four experiments and is less of a problem in the two-hybridization system we used than in techniques where the dye is attached directly to the cDNA (Stears et al. 2000). 199 ESTs were not significantly changed in any stage and included some involved in oxidative phosphorylation, a few housekeeping genes (adenosine kinase, aconitase), some ribosomal proteins and the 16s mitochondrial ribosomal RNA. However, mitochondria increase greatly during feeding (Binnington 1978; Krolak et al. 1982). The failure of the statistical normalization for the UF group suggests that a substantial percentage of the ESTs change expression as feeding starts, which may mask these changes.

The biggest change in gene expression occurs between the unfed and early fed female ticks (Table 2), and this is almost entirely an upregulation, with very few genes decreasing significantly. Genes increasing in expression include a number of putatively secreted proteins, including several of the p36 immunosuppressants, and a number of protease inhibitors (possible anticoagulants). Also increasing are a number of housekeeping genes, such as ATP synthase, cytochrome oxidase, glyceraldehyde phosphate dehydrogenase, phosphoglucose isomerase, cyclophilin G and actin. In progressing from the early fed stage to the partially fed stage about an equal number of genes increase and decrease. Genes increasing expression include the histamine binding proteins (HBPs) and several more protease inhibitors (e.g., KUN-2). Genes decreasing include several p36 immunosuppressants (most of the ones increasing between UF and EF), several different protease inhibitors (e.g., KUN-5), several mitochondrial proteins and several ribosomal proteins. On going from the partially fed stage to the fast feeding stage, there are many more genes increasing in expression than decreasing. Upregulated genes include several mitochondrial genes and 12s rRNA, several transport-associated proteins such as a chloride channel and Na/K ATPase, calpain, ubiquitin, actin, some protease inhibitors, and some

proteins involved in protection from reactive oxygen. Genes downregulated include a number of putative secreted proteins, including some p36 immunosuppressants and HBPs, as well as some protease inhibitors, including KUN-2, and cyclophilin G. Between the fast feeding and replete females, almost nothing changes.

Cluster Analysis—Clustering is a statistical procedure for grouping targets with similar patterns of expression. Hierarchical clustering showed groups of ESTs having similar expression patterns. It confirmed that the FF and Rep groups are most similar, while the EF group was most different from the others. The data were analyzed using K-means clustering with 12 groups (Fig. 1). No cluster shows a significant difference in expression between FF and Rep, though clusters 2, 11 and 12 suggest a trend to lower expression in Rep, and clusters 3, 8 and 10 suggest a trend to more expression in Rep. UF is expressed less than control in all but clusters 1 (more) and 8 (same). EF does not include zero within its error bar only in clusters 12 (higher) and 2 and 9 (lower).

Cluster 1 is the only one that shows greater expression in UF than PF, but has too few identified ESTs to show a clear pattern as to what these may be. Clusters 2 and 11 have a similar expression pattern with UF, FF and Rep under-expressed. Cluster 2 shows a significant over-representation of the GO (Gene Ontology) terms avoidance of host defense and amine binding activity, and includes several histamine-binding proteins, while 11 has a number of other putative secreted tick proteins and protease inhibitors, including KUN-2. Clusters 3, 6 and 10 show a similar expression pattern with UF under-expressed and FF and Rep over-expressed. These clusters show a number of electron transport proteins, ion channels, ribosomal proteins, actin and protease inhibitors, including KUN-5. Clusters 4 and 7 show low expression in UF with little difference elsewhere. Cluster 7 shows significant overrepresentation of the GO terms avoidance of host defenses, amine binding and serine endopeptidase inhibitor activity, and contains histamine-binding proteins and p36 immunosuppressants. Both clusters show some housekeeping ESTs, such as hexokinase. Clusters 5 and 12 show UF, FF and Rep less expressed and EF more expressed, but show no obvious pattern as to what kinds of ESTs are expressed. They contain cyclophilin G and ixosin. Cluster 8 shows equal UF expression and higher FF and Rep expression. It has 12s mitochondrial ribosomal RNA. Cluster 9 has ESTs with low expression in all stages except PF and contains several putative secreted tick proteins.

These patterns of expression largely agree with the expectations outlined. The biggest change happens at the initiation of feeding, where a large number of genes increase their expression. A small number decrease expression, and these may be ones that are needed for life off the host. As the female tick feeds, more genes are turned on and some are turned off. In the early stages, the upregulated genes include those likely to be involved in countering host defenses, but as the tick goes into the rapid feeding stage, many of these genes are turned down or off, and another set of genes, those required for producing eggs, are turned up. General metabolic genes remain at a high rate of expression for almost the entire feeding cycle.

Salivary glands from female ticks contain both histamine agonistic and antagonistic activities (Chinery 1981; Chinery and Ayitey-Smith 1977). While it is crucial for ticks to sequester histamine at the feeding site and prevent its strong proinflammatory effects, ticks may also induce histamine release to increase the permeability of blood vessels and increase blood uptake. Mulenga et al. (2003) have previously reported a functional histamine release factor (HRF) expressed in the salivary glands of *Dermacentor variabilis* and later Mulenga and Azad (2005) identified a highly similar homologue for HRF in *A. americanum*. The increase in an HBP homologue was also shown in the salivary glands from *A. americanum* on day one after feeding, which substantially dropped on day 3 (Mulenga et al. 2007).

Homologues for both HRF and HBP were up-regulated in EF relative to UF (clusters 11 and 12). Our data agree with previous reports and support a fine control for histamine activity in the early stage of feeding. It is noteworthy that several homologues for *I. scapularis* secreted proteins with possible HBP function, found in clusters 2, 7 and 9, have slightly different expression profiles. Using RNA interference against a single gene, we were able to suppress only 30% of the histamine binding activity of salivary glands *in vitro* (Aljamali et al. 2003). The above findings support the existence of several tick HBPs in salivary glands with differential expression during early and slow feeding (EF and PF).

One of the most common classes of activities found in the EST project is protease inhibitors, including homologues for tissue factor pathway inhibitors, putative disintegrins and thrombin inhibitors (Aljamali et al. 2009). Several of these are upregulated in the EF and PF stages, with the levels going down on going into the FF stage. The expression pattern is complex, with some of these increasing between EF and PF, and others decreasing. Possibly, there are different functions needed at different times, or new proteins are turned on to replace ones that have been neutralized by the host immune system.

Two peptide homologues with putative antibacterial function were upregulated in EF and PF female ticks relative to other stages. Ixosin (cluster 5) (Yu et al. 2006) and ixodidin (cluster 11) (Fogaca et al. 2006) have higher expression levels in EF and PF, respectively. Due to the prolonged feeding time of the female tick, the tick-host interface constitutes an advantageous milieu for bacterial contamination within the disrupted layers of the skin epithelium. These two tick peptides may play a key role in inhibiting bacterial growth especially at the tick-host interface, where host immune response is already weakened due to several factors secreted in tick saliva (Wikel and Bergman 1997). These tick factors might include homologues for immunosuppressant from different tick species, which were mainly expressed in the EF and PF ticks (clusters 7 and 11).

Many protein homologues are upregulated in FF and Rep ticks relative to all other feeding stages. A major group is proteins possibly involved in the degeneration of salivary gland occurring in the late feeding stage and in replete female (Kaufman 1991). Among these ESTs, distributed mainly in clusters 3 and 6, are homologues for calpain 3, proteasome subunit and ubiquitin-like proteins. Interestingly, the expression of these ESTs changed in the same direction, with higher expression in Rep compared to FF. This suggests that the expression of factors mediating protein degradation and possibly gland degeneration increases in the FF stage, when ticks are still attached to the host, then continues to increase in Rep. Moreover, two homologues for thymosin and P8 stress protein were up-regulated in Rep. Both of these proteins have been linked to regulation of apoptosis (Fan et al. 2006; Malicet et al. 2006). Finally, homologs to glutathione S-transferase and peroxiredoxin with putative antioxidant function were highly expressed in FF and Rep ticks, which may alleviate high toxicity of reactive oxygen species that are known to mediate cell death (Ryter et al. 2007). A second group of proteins that are over-expressed in FF and Rep are the ion transport proteins. These include Na/K-ATPase beta subunit, chloride channel 2 and vacuolar H⁺-exporting ATPase, which may be involved in osmoregulation during the feeding period, during which ticks increase at least 100-fold in size due to fluid intake. We also found that the expression of all four homologues for cytoskeleton actin followed the same trend of up-regulation in FF and Rep ticks. It was recently argued that reduction in the dynamics of actin cytoskeleton might have a role in releasing reactive oxygen species from the mitochondria that ultimately results in cell death (Gourlay and Ayscough 2005). Nevertheless, the rise in actin expression may also contribute to a higher level of osmoregulation.

Males

The three groups of male ticks (unfed: U, fed in the absence of females: W, fed in the presence of females: F) were compared to the female PF stage, as for the female groups. Seven hundred five ESTs of the 881 with good data from the unfed group were significantly different in at least one of the groups. Approximately 55% were changed relative to the female PF control in any one group, with roughly equal numbers greater or less in expression (Table 3). The U group showed a slightly higher percentage of significant differences and a slight bias towards down-regulation, while the W group showed a slightly lower percentage of differences and the F group showed a bias towards up-regulation. In contrast to the female groups, these groups tended to have expression different in the same direction relative to the control, with a very small percentage (zero for F compared to W) changing directions between groups (Table 4).

In calculating the differences and their significance for the male groups to each other, we found a rather small number of ESTs to be significantly different in one group relative to another. For W compared to F, 30 ESTs were significantly different, for F compared to U, 11 were and for W compared to U only one EST was significantly different. This one EST was also different between F and U. Of the ESTs different between F and U, 9 were also different between F and W. These results suggest the W group is more similar to the U than to the F. The one EST differing between W and U is a WD40 domain-containing protein. Proteins with this domain have functions such as signal transduction, pre-mRNA processing and cytoskeleton assembly (Smith et al. 1999). The ESTs differing between W and F are mostly unknown, but include a histamine release factor, negative elongation factor and deoxycytidine deaminase.

ESTs differing from the PF reference in the U group but not the W or F include an osteonectin homolog which is more expressed and several mitochondrial proteins which are less expressed. Proteins significantly over-expressed in all male groups compared to the reference female PF include a large group of ribosomal proteins and 12s mitochondrial ribosomal RNA. Proteins significantly under-expressed in all male groups compared to the female PF reference include several potential protease inhibitors, a 9.4 kd secreted protein, the mechanosensory abnormality protein, and the α -microglobulin/bikunin precursor.

Hierarchical clustering suggests the W and F groups are more similar than the U is to either. This is confirmed by the K-means clustering (Fig. 2), where none of the six clusters shows a significant difference between W and F, but clusters 2 and 4 show a tendency to higher expression in U, and clusters 3, 5 and 6 show a tendency to lower expression in U.

Clusters 1 and 2 show down-regulation of all male groups in comparison with the PF female. Cluster 2 is significantly overrepresented for the GO term serine endopeptidase inhibitor. In addition these clusters contain homologs to a number of secreted proteins identified in other tick species, including HBPs and protease inhibitors. Possibly, the intermittent feeding of the males does not require the array of counters to the host defenses that the female feeding does.

Cluster 3 shows only minor differences in expression of any group compared to the female PF control and contains some cation transporters and electron carriers. Clusters 4, 5 and 6 all show higher expression in all male groups compared to the female PF control, with cluster 6 showing very large changes. This group shows the majority of the ribosomal proteins, calpain, ixosin and actin. Cluster 5, with the smallest difference shows several electron transport proteins. The group of ESTs over-expressed in all male groups is overrepresented for the GO term structural constituent of ribosome. Thus the males appear to have a greater protein synthesizing capacity than the females. It should be noted that the EST project from

which the ESTs on the array were selected was done with female feeding ticks, so there may be male specific ESTs not present and not detected.

Array validation

To validate the microarray results, we chose 8 ESTs from various female clusters to verify their expression patterns with qPCR (Fig. 3, Table 5). One problem with an experiment of this sort is what to use as a standard. In the growing tick, essentially everything actually changes during the feeding cycle, so things like actin or glyceraldehyde phosphate dehydrogenase would not be good choices. The microarray experiment implicitly uses total RNA as the standard, though the statistical normalization assumes a majority of the samples do not change relative expression, and so normalizes against a number of presumably constant mRNA's. For the qPCR, we used rRNA as a standard by loading equal amounts of total RNA. In order to check this, we also used clone DR766684, ribosomal protein S13, which in the microarray study showed very little change in expression in any stage, as a standard. These two agreed well. Major changes in expression were seen in the same direction in both methods, though the magnitude of the change in the microarray results was generally smaller than that in qPCR. The confidence limits for the methods were fairly large, so small changes were mostly not significant. However, there was one case in which the methods clearly disagreed: Rep for DR766649. In other possible cases of disagreement, one of the values was not significant because of the wide confidence limit: EF for DR766684 and DR766596. Our results agree with the findings of other studies where the gene expression measured by microarray and other methods were incompletely correlated (Taniguchi et al. 2001).

Within the estimated errors of the data, the qPCR and microarray results mostly agree, at least in direction, if not in magnitude. In general, the qPCR results are more extreme than the microarray ones. This may result from the tendency of microarray values to be limited because too small or too large signals are discarded in the analysis. Because the samples for the microarray were selected fairly early in the analysis of the EST library, in some cases, several samples belonging to the same contig were chosen to put on the array. Some of these were used in the qPCR test, as indicated in Table 5. This fact provides another check on the consistency of the data, and, for the most part, ESTs that should be similar gave similar results on the microarray. However, there were some examples where this was not true. One is DR766616, with three representatives on the microarray, where two gave similar results, and the third was quite different from these. The values in Figure 3 only use the two similar results. These three ESTs have slightly different sequences, so it is possible that they are actually different genes, with different expression patterns. The qPCR primers are identical to one of the more similar pair.

In order to explore the consistency further, several other contigs, with representation from 3 to 38 cDNA's on the array were analyzed by analysis of variance (anova) to determine their 95 % confidence limits. Considering all the data, the distance of the limits from the average varied from 0.26 log₂ ratio for 16s mitochondrial ribosomal RNA with 38 cDNA's to 1.72 for a homolog to a *Haemaphysalis longicornis* protein with 3 cDNA's. There was a general, but imperfect inverse correlation between the magnitude of the limit and the number of cDNA's. In many of these, there was a single discordant sample that was responsible for much of the size of the confidence interval. If this sample was removed, the size between the limits became much smaller, and the values for expression of this sample in the different stages were mostly out of the 99% confidence limits calculated for the remaining values. The *H. longicornis* EST, for example, had a confidence interval of 0.71 for the two most similar samples (although with this few samples, the discarded one can not be beyond the 99% confidence limits of the remaining two). A striking example was cytochrome b, where the interval was 0.72 for 8 samples, and dropped to 0.15 with most discordant one removed.

In the case of the 16s rRNA, there was no markedly discordant sample: taking out the most discordant one only lowered the confidence limit to 0.23 from 0.26. In the case of the mechanosensory abnormality homologs, the most discordant sample had a sequence considerably longer than the others, and was only 88-89% identical in the region of overlap, so may be a different gene. However, in the case of cytochrome b, the discordant sample was a duplicate of one of the other samples, spotted twice on the array, so the difference must result from differences in the hybridization conditions at different points in the array, though these must have happened similarly in the replicate arrays. Another possibility is that these are not, in fact, duplicates, but were recorded incorrectly.

Conclusions

The female tick shows major changes in gene expression on beginning feeding. Genes involved in defenses against the host defenses, such as anticoagulants, immunosuppressants and histamine binding proteins increase soon after feeding commences, as do some housekeeping genes. As feeding progresses, some of these genes start decreasing in expression, and by the end of the feeding cycle, in the fast feeding tick, most have decreased expression to a low level. A new set of genes starts to be expressed in this late phase, some of them clearly involved in the transformation to egg production, such as ones involved in protein degradation and apoptosis, while others have a less obvious need at this stage, such as mitochondrial genes and transporters. These changes mostly finish before the tick finishes feeding and detaches. The male tick shows only a small number of changes in gene expression during the course of feeding and mating, but the overall pattern of expression is markedly different from that of the female, with less expression of counters to host defenses, and more capacity for protein synthesis. However, this array probably does not have male-specific genes, which may have distinct expression patterns. The microarray technique agrees reasonably well with other methods of assessing gene expression, but not perfectly. It shows less overall change than qPCR. Internal agreement of duplicate genes on the array is good.

Acknowledgments

We thank the OSU Protein/DNA Resource Facility and the OSU Tick Rearing Facility for assistance in this project. We thank Margaret K. Essenberg and Rolf Prade for helpful comments on the manuscript. Support was provided by USDA-NRI grant 990-2129. This publication was made possible by Grant Number P20RR016478-06 from the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH). This paper was approved by the Director of the Oklahoma Agricultural Experiment Station.

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Figure 1.

Expression patterns in the 12 clusters from K-means clustering for the female groups. Bars show the mean and standard deviation of the mean $\log_2$ ratio value for the group. Groups are shown in the order UF, EF, PF (reference), FF and Rep. Each panel indicates the number of ESTs in the cluster.

Figure 2.

Expression patterns in the 6 clusters from K-means clustering for the male groups. Bars show the mean and standard deviation of the mean $\log_2$ ratio value for the group. Groups are shown in the order U, W and F. Each panel indicates the number of ESTs in the cluster.

Figure 3.

Comparison of results by qPCR and microarray.

Panels show the results from the qPCR (darker shading on left) and microarray (lighter shading on right) for the clones identified in Table 5. The units are the difference in ΔC_t value for indicated class less PF control for the qPCR and $\log_2$ (average class intensity/average pooled PF reference intensity) for the microarray. The error bars show the 95% confidence limits calculated from the appropriate analysis of variance.

Table 1 Primers for qPCR^a

EST	Primer sequences
DR766616	AGGTACGAGAAGTGCGTGCA GCAGTTGTTTTCTCCGCAGG
DR766641	GACACAAGCATGGCTTTTCGA GGCGATGAAATCCTTAGCGA
DR766665	TGGTGCTTGCAAATAGTGGC ACATCGGCGTCGAGTAGTT
DR766684	TCATCTTGGTGGAGAGCCGT TGGCCTTGTAATAGCGCACA
DR766649	GCCCCGTGCAGAGTAGCA GCGCATTTCTGTCAGAAAGAG
CX764416	ATTCGGAGGCCTGAGAAAGC CCGCCATTTACAACACTTCCA
DR766596	TGCACCCCATACCTCTGTCA GAAACACCACCAGAAGGCACTC
CX764160	CCACGGTGCGGTATCTGTT TCTTGTGACAAGCTCGGTGC

^a Sequences for forward and reverse primers shown 5' to 3'

Table 2

Number of ESTs showing significant expression changes in going from one female feeding class to another^a

	UF	EF	PF	FF	Rep
UF		428	456	370	379
EF	8		229	116	181
PF	9	191		384	397
FF	9	143	269		3
Rep	8	162	273	2	

^aClasses: UF = unfed; EF = early fed, < 50 mg; PF = partially fed, 50 – 200 mg; FF = fast feeding, 300 – 550 mg; Rep = replete. The numbers give cDNAs with significantly increased expression in the class on the left compared to that on top.

Table 3

Fraction of ESTs showing significant expression changes in male ticks relative to a reference RNA from a pool of partially fed female ticks^a

	U	W	F
Signif. ^b	58.3	51.0	55.1
Up ^c	28.4	24.9	29.1
Down ^d	29.9	26.1	26.0
Not Sig. ^e	41.7	49.0	44.9

^a Classes: U = unfed; W = fed in the absence of females; F = fed in the presence of females.

^b Percent of the total valid hybridized ESTs showing a P value < 0.01 for average log2 ratio

^c Expression in the indicated group significantly higher than in the PF reference RNA

^d Expression in the indicated group significantly lower than in the PF reference RNA

^e Percent of the total valid hybridized spots showing a P value > 0.01 for average log2 ratio

Table 4

ESTs from male ticks whose expression differed in the same or opposite directions relative to the partially fed female reference in comparisons of two treatments^a

		Same ^b		
		U	W	F
Opposite ^c	U		34.4	34.7
	W	0.9		43.0
	F	2.1	0.0	

^aPercent of total valid hybridized ESTs. Classes as in Table 2

^bChanges in row and column class both increase or both decrease (upper right triangle)

^cOne class increases and other decreases (lower left triangle)

Table 5

Clones used in comparison of qPCR and microarray

EST ^a	Identity	Number on array ^b	Degrees of Freedom in qPCR	Cluster ^c
DR766616	Unknown	3(2)	10	8
DR766641	ATP/ADP Translocator	2	13	6
DR766665	Unknown	2	9	10
DR766684	Ribosomal protein S13	1	13	-
DR766649	KUN-5	8	9	4
CX764416	Histamine binding protein	1	11	11
DR766596	Unknown	1	10	5
CX764160	Syntaxin 6	1	12	4

^a GenBank accession number^b Number of ESTs on the array contained in the contig containing this EST^c K means cluster number (Figure 1). EST DR766684 showed no significant differences in any class, so was not included in the clustering