Age-dependent susceptiblility to ivermectin and gene expression following an ivermectin blood meal in the malaria vector Anopheles gambiae

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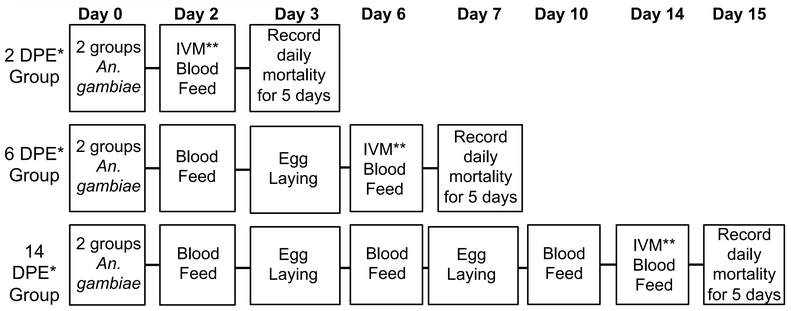
Introduction

Plasmodium species responsible for malaria disease are transmitted between humans by the bites of infected *Anopheles spp.* mosquitoes. Vector control is the most effective tool for reducing malaria transmission, but current methods have various shortcomings including insecticide resistance and inability to target exophagic vectors, creating the need for novel ways to target disease vectors. Ivermectin has been proposed as a novel malaria transmission control tool based on its insecticidal properties and unique route of acquisition through human blood. It has been previously shown that this drug, when administered to people by mass drug administration (MDA) for the treatment against onchocerciasis and lymphatic filariasis in Africa, is lethal to wild *Anopheles gambiae* that bite villagers (Sylla et al. 2010) and transiently interrupts the transmission of the parasite (Alout et al. 2014). Due to its short pharmacokinetic persistence, many vectors will ingest a sub-lethal dose if they bite people more than 2 days post treatment (Kobylinski et al 2010). It is important to understand the gene expression and other molecular events in the vector following IVM ingestion to further characterize the drug's effect on mosquito physiology, its efficacy against differently-aged mosquitoes and thus its effect on population structure, and the potential for mosquito resistance.

Methods

Mosquito Blood Feeding Timeline



*DPE: Days Post Emergence

**10 mg/mL Ivermectin in DMSO stock solution diluted in PBS and added to defibrinated calf blood for a final concentration of 11.75 ng/mL. Control blood contained same dilution starting with 100% DMSO.

Mortality Statistics

- Survival analysis of bioassays was performed on pooled replicates for each age (Log-rank test) to test for significant mortality from IVM
- Comparison of mortality between age groups was analyzed using the Mantel-Haenszel Hazard Ratio (HR) with 95% confidence intervals to account for control mortality

Whole Transcriptome Analysis using RNA-seq

- Total RNA extracted from 10 individual females from the 2DPE and 6DPE age groups and each blood feed state
- 12 libraries prepared using Illumina's TruSeq Stranded mRNA Sample Prep Kit
- Sequenced on a HiSeq 2500 platform with 2x100bp paired end reads
- GMAP and GSNAP were used for indexing and alignment to the AgamP3.22 genome build and Trimmomatic for quality control and removal of singletons
- Statistical analysis was performed using the edgeR package for R software. Differential expression (DE) analysis was then performed using the glmLRT procedure

Real-time Quantitative PCR validation

- External validation of RNA-seq results was performed on selected transcripts that showed significant differential expression following an IVM blood-meal in the 2DPE age group
- Same total RNA samples used for RNA-seq library preparation as well as samples from a recently-colonized strain (IRSS) from Burkina Faso and wild *Anopheles gambiae s.s.* (Kodeni) captured in the Kodeni district of Bobo Dioulasso, Burkina Faso
- 2DPE mosquitoes were blood fed on the arm of a human who had taken a standard oral dose of IVM (150 µg/kg) 36-hours prior, while the control group fed on a different, untreated human.

Results

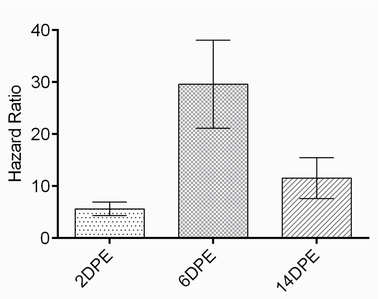


Figure 1. Mantel-Haenzel hazard ratio associated with IVM ingestion in three age groups. Mantel-Haenzel Hazard ratio computed by comparing each IVM treated group to its corresponding control. Bars represent the 95% confidence interval of the hazard ratio.

Mortality induced by the discriminating IVM concentration was **lowest in 2DPE** (HR=5.47; 95% CI: 4.20 – 7.14, N=258), **highest in 6DPE** mosquitoes (HR=27.89; 95% CI: 19.84 – 39.02, N=200), while the mortality was intermediate in 14DPE mosquitoes (HR=11.49; 95% CI: 7.54 – 17.52, N=112).

IVM-containing blood meals resulted in **asymmetrical transcriptional changes** with the **majority** of significantly DE transcripts showing **up-regulation** at both ages. More genes were differentially-regulated in response to IVM in the 6DPE group relative to the 2DPE group, and the fold-changes were overall higher.

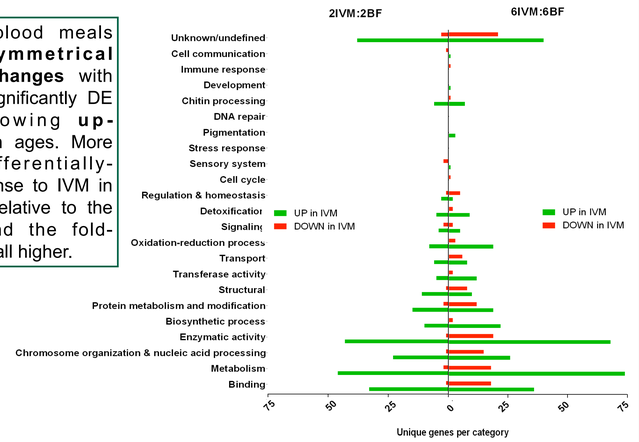


Figure 2. Gene Ontology terms associated with DE genes following IVM ingestion. GO terms were gathered with g:Profiler and manually sorted into the 23 functional categories. Only biological process and molecular function terms were used, cellular component terms were removed. Individual transcripts were allowed to fall into multiple categories, based on the GO terms returned, to more accurately reflect the multi-functional aspect of many enzymes.

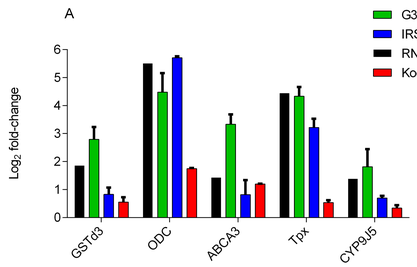


Figure 3. RT-qPCR validation of RNA-seq results. Five genes were selected for DE confirmation in the same RNA samples used for RNA-seq (G3), a laboratory raised colony from west-Africa (IRSS), and wild-caught mosquitoes from the Kodeni region of Burkina Faso (Kodeni). Log_2 fold-change comparison with standard deviation (does not apply to RNA-seq results).

Fold-change values in the **naturally-exposed mosquitoes** from Burkina Faso (IRSS and Kodeni) appear to **generally agree** with RNA-seq, however the **degree of up-regulation was generally less** and lowest in the wild-type (Kodeni) mosquitoes.

Results Cont.

Genes of interest

Niemann-Pick Type C genes

- The Niemann-Pick type C-2 (NPC2) family of genes were among the most highly up-regulated transcripts in our experiments in response to blood feeding only and to IVM ingestion
- AGAP002847 is among the most highly up-regulated genes in response to IVM ingestion at both ages, but is doubled in 6DPE (160-fold) vs. 2DPE (80-fold)".
- AGAP002848 is modestly up-regulated in response to IVM ingestion at both ages (17-fold and 11-fold, respectively)
- The IVM-responsive NPC2 genes (AGAP002848 and AGAP002847) appear to be evolutionarily distinct from the blood meal-responsive NPC2

Peritrophic matrix associated genes

- DE of 45 putative peritrophic matrix-associated genes upon IVM ingestion in both age contrasts suggests that PM gene transcription is responding directly to IVM treatment.
- In general the 2DPE contrast showed both an increased number of significantly DE transcripts as well as higher fold-changes than the 6DPE contrast, but interestingly, both age contrasts had DE genes unique to their own age group.
- Gene ontology analysis revealed down-regulation of chitin processing transcripts upon blood-feeding but up-regulation of this category after an IVM ingestion
- The less IVM-susceptible age group (2DPE) shows overall higher DE transcription among PM-associated genes in response to IVM than the more IVM-susceptible age group (6DPE), perhaps contributing to their increased tolerance to IVM toxicity.

Immune response genes

- The older age contrast showed significant DE among 19 immune-related transcripts and most were modestly up-regulated. Only 4 immune function genes were significantly DE in the 2DPE
- In general the 2DPE contrast showed both an increased number of significantly DE transcripts as well as higher fold-changes than the 6DPE contrast, but interestingly, both age contrasts had DE genes unique to their own age group.

Conclusions

- Our data shown that **older *An. gambiae* mosquitoes that have ingested previous blood meals are more susceptible to IVM** compared to young mosquitoes which have not ingested a prior blood meal.
- Following from this difference, **DE analysis showed that IVM in a blood meal induces significant transcription of more than 100 genes**, most of which are up-regulated. There was no single gene or group of genes that clearly explained the age-related susceptibility differences.
- It is clear that **IVM in a blood meal mostly induced transcription** of non-canonical genes, including **PM-associated genes, immune response genes and NPC genes**.
- The data suggest **complex midgut interactions resulting from IVM ingestion** that likely involves blood meal **digestion** physiological responses, **midgut microflora**, and **innate immune** responses.
- **More complex interactions** between IVM and the significant DE genes mentioned above **are likely** as **some genes have clear linkages across the groups** we have highlighted

Acknowledgements

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References:

Sylla et al. 2010. Mass drug administration of ivermectin in south-eastern Senegal reduces the survivorship of wild-caught, blood fed malaria vectors. *Malaria J.* 9:365. **Alout et al. 2014.** Evaluation of ivermectin mass drug administration for malaria transmission control across different West African environments. *Malaria J.* 13:417. **Kobylinski et al. 2010**. The effect of oral anthelmintics on the survivorship and re-feeding frequency of anthropophilic mosquito disease vectors. *Acta Trop.* 116:119–126