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Graphical abstract

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Highlights

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Proteomic changes occurring in the malaria mosquitoes *Anopheles gambiae* and *Anopheles stephensi* during aging

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- The expression of some *A. gambiae* and *A. stephensi* proteins changes with age.
- Mass spectrometry was used to provide identity of age related proteins.
- Age related trends identified by 2-D-DIGE analysis were confirmed by Western blot.
- Antibodies developed against *Aedes* species cross-react with *Anopheles* proteins.

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ABSTRACT

The age of mosquitoes is a crucial determinant of their ability to transmit pathogens and resistance to insecticides. We investigated changes to the abundance of proteins found in heads and thoraces of the malaria mosquitoes *Anopheles gambiae* and *Anopheles stephensi* as they aged. Protein expression changes were assessed using two-dimensional difference gel electrophoresis and the identity of differentially expressed proteins was determined by using either matrix-assisted laser desorption ionization tandem time-of-flight mass spectrometry or capillary high-pressure liquid chromatography coupled with a linear ion-trap (LTQ)-Orbitrap XL hybrid mass spectrometer. Protein biomarkers were validated by semi quantitative Western blot analysis. Nineteen and nine age dependent protein spots were identified for *A. stephensi* and *A. gambiae*, respectively. Among the proteins down-regulated with age were homologs of ADF/Cofilin, cytochrome c1, heat shock protein-70 and eukaryotic translation initiation factor 5A (eIF5A). Proteins up-regulated with age included probable methylmalonate-semialdehyde dehydrogenase, voltage-dependent anion-selective channel and fructose biphosphate aldolase. Semi quantitative Western blot analysis confirmed expression patterns observed by 2-D DIGE for eIF5A and ADF/Cofilin. Further work is recommended to determine whether these biomarkers are robust to infection, blood feeding and insecticide resistance. Robust biomarkers could then be incorporated into rapid diagnostic assays for ecological and epidemiological studies. **Biological significance:** In this study, we have identified several proteins with characteristic changes in abundance in both *A. gambiae* and *A. stephensi* during their aging process. These changes may highlight underlying mechanisms beneath the relationship between mosquito age and factors affecting *Plasmodium* transmission and mosquito control. The similarity of changes in protein abundance between these species and the primary dengue vector *Aedes aegypti*, has revealed conserved patterns of aging-specific protein regulation.

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Q7 1. Introduction

Age affects several aspects of mosquito biology relevant to the transmission of mosquito borne disease. It has been shown that older mosquitoes are less susceptible to infection with filarial nematodes and infection increases the mortality rate of older mosquitoes to a greater extent than for younger mosquitoes [1]. *Plasmodium* malaria parasites can only be transmitted by a small fraction of *Anopheles* mosquito

population consisting of mosquitoes that survive long enough to support the development of the parasite to the stages infective to humans [2]. The period required for parasite development in the mosquito, known as the extrinsic incubation period, is approximately 9–14 d depending on *Plasmodium* species and ambient temperature [2]. During their development inside mosquitoes, pathogens such as *Plasmodium* [3] or dengue virus [4] have been reported to induce alterations in the mosquito proteome. It has also been reported that aging increases susceptibility to insecticides of otherwise resistant mosquito species [5–7]. This could be due to alterations in the expression of enzymes that regulate insecticide resistance such as Glutathione Transferase e2 (GSTe2) [5].

Aging in general is attributed to increase in oxidative and heat stress, protein misfolding and other events that can eventually lead to loss of cellular function or ability to deal with stress. Aging has therefore been defined as a deteriorative change over time that eventually causes

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mortality [8]. At older ages, deteriorative changes have been suggested to be due to a decreased force of natural selection [9]. According to the free radical aging theory, free radical by-products of normal cellular metabolism, such as reactive oxygen species (ROS), cause damaging oxidation of cellular molecules including DNA and proteins [10,11]. These oxidised molecules accumulate over time eventually leading to cellular and organ failure. Oxidoreductases can also be major contributors of ROS due to inefficient electron transfer. Superoxide dismutase is an antioxidant enzyme that catalyses the dismutation of the ROS superoxide into H_2O_2 and O_2 . H_2O_2 is also highly detrimental to the cell when left to accumulate; therefore it is converted into H_2O and O_2 by catalases. Recent studies in *Aedes aegypti* demonstrated changes in abundance of different isoforms of SOD2 during aging, suggesting that aging is associated with post-translational modification of this stress response enzyme [12].

Investigation of biochemical changes during aging in mosquitoes on a species by species basis provides the greatest opportunity for discovering age related biochemical changes and could elucidate the relationship between aging, infection, blood feeding and resistance to insecticides. Age associated protein changes can also provide useful biomarkers for the prediction of the age of mosquitoes and other insects, which facilitates ecological studies into insect populations and epidemiological studies into the pathogens they transmit. Changes to gene expression have formed the basis of a novel method of predicting mosquito age [13–15]. Recently, protein biomarkers of aging were identified in the dengue mosquito vector, *A. aegypti* [12]. Using a similar 2D Difference Gel Electrophoresis (DIGE) approach, we isolated age associated proteins in the major African malaria vector *Anopheles gambiae* s.s. (hereafter *A. gambiae*) and the major vector on the Indian subcontinent, *Anopheles stephensi*. The identity of the aging dependent proteins/peptide spots was then determined by either using matrix-assisted laser desorption ionization tandem time-of-flight (MALDI-TOF/TOF) mass spectrometry or capillary high-pressure liquid chromatography (CapHPLC) mass spectrometry. By comparing changes across two cohorts of mosquitoes for each species, we have identified proteins with robust age-dependent expression patterns including homologs of proteins with known association with aging in other species. Quantitative Western analysis was used to confirm the expression of selected candidate biomarkers.

2. Materials and methods

2.1. Ethics approval

Ethics approvals were obtained for routine blood feeding from the QIMR Berghofer Medical Research Institute (QIMR HEC P1162), Griffith University (ENV/29/09/HEC) and the Ifakara Health Institute (IHDC/EC4/CLN96/2004) human research ethics committees. Written consent was obtained from all volunteers who were involved in blood feeding, and volunteers were given the right to refuse to participate or withdraw from the experiment at any time.

2.2. Mosquito maintenance

Two biological replicates (two generations reared at different time points) of *A. gambiae* were reared at the insectary of Ifakara Health Institute, Tanzania and two biological replicates of *A. stephensi* were reared at the insectary of QIMR Berghofer Medical Research Institute, Australia at 27 °C, 70% humidity, 12:12 hr day:night lighting. Larvae were fed on Tetramin tropical flakes (Tetra Melle, Germany). Approximately 3000 pupae were required for each cohort. Pupae were transferred into cages measuring 40 × 40 × 30 cm for adult emergence. Adults were fed on 10% sugar solution daily and blood fed on a human volunteer for 15 min every 7 d. At least 40 female adults were collected 24 h post emergence and again at 9 and 17 d post-emergence for *A. gambiae* and at 9, 17 and 34 d post-emergence for *A. stephensi*.

Additional *A. stephensi* samples were collected at 5, 13 and 25 d post-emergence for validation of the age associated markers. .

2.3. Protein extraction and quantification

Only mosquito heads, thoraces and wings were included for protein extraction to exclude possible contamination from human blood proteins remaining in abdomens. Five females at 1, 9 and 17 d of age for *A. gambiae* and 1, 9, 17 and 34 d for *A. stephensi* were used. Extraction and quantification experiments were performed according to the procedure described in an accompanying manuscript [16] and the procedure described by Hugo and colleagues [12].

2.4. 2D-DIGE, Imaging and processing

The stock solutions of each of Cy2, Cy3 and Cy5 fluorescent cyanine dyes (GE Healthcare) was prepared by reconstituting the appropriate dye in 5 µl Dimethylformamide (Sigma Chemical Co. Ltd, St Louis, MO, USA) to a concentration of 1 nM. Four hundred picomoles of either Cy3 or Cy5 was added to each sample and 2400 pmol of Cy2 was added to the internal standard. Each sample pool was combined with 4.5 µl 100× ampholytes (Bio-Rad, Richmond, CA, USA), 5.6 µl Destreak reagent (GE Healthcare) and made up to 450 µl in 2D Buffer. Samples were pipetted onto 24 cm, pH 3–10 immobilised pH gradient strips (Bio-Rad) in rehydration trays and left at room temperature for 4 h for the strips to absorb the sample. Following absorption, the strips were transferred to a new focussing tray, overlaid with mineral oil and allowed to rehydrate overnight at room temperature. For first dimension separation, the strips were transferred to a dry protean iso-electric focusing tray. The strips were overlaid with fresh mineral oil and iso-electric focussing was performed using the following run conditions: current of 50 µA per strip, 250 v for 15 min, 1000 v for 5 h, 10,000 v for 4 h and 500 v to reach a total of 80,000 Vh⁻¹. Focused strips were equilibrated for 15 min in buffer I containing 6 M Urea, 0.375 M Tris-HCl pH 8.8, 2% [w/v] SDS, 20% glycerol and 2% [w/v] DTT and then for 15 min in buffer II containing 6 M Urea, 0.375 M Tris-HCl, 2% [w/v] SDS, 20% glycerol and 2.5% [w/v] iodoacetamide. The strips were placed on 12% acrylamide gels cast in 24 cm optically clear plates. Electrophoresis was performed in a Protean® Plus Dodeca cell (Bio-Rad) at 5 mA/gel for 15 min, 10 mA/gel for 15 min and 30 mA/gel until the dye front reached the bottom of the gel.

A. stephensi gels were scanned using a Typhoon FLA-9400 fluorescent imager (GE Healthcare) at 100 µm pixel resolution, using the 520 band pass (BP) 40 filter for Cy2, 580 BP 30 filter for Cy3 and the 670 BP 30 filter for Cy5 emission. *A. gambiae* gels were scanned using a FLA-9000 Starion fluorescent imager (GE Healthcare) at 100 µm pixel resolution using the BPB1 (530DF20) filter for Cy2, DGR1 (570DF20/665LP) filter for Cy3 and LPR (665LP) filter for Cy5. Fluorescent images were processed and analysed using Delta2D version 4.0 software (Decodon, Greifswald, Germany). Spots on gel images scanned from the same gel were then connected by direct warping and the spots on all gels aligned and normalised for intensity using the Cy2 channel (internal standard images) on each gel with the match vectors tool. All aligned images were fused and thereafter spot validations were transferred to all other images from this fused image as described by Hastie and colleagues [17].

2.5. In-gel digestion and identification

Protein spots for identification were manually excised from 2D gels using the method of Hugo and colleagues [12] as described in an accompanying manuscript [16]. In-gel tryptic digests were analysed by either MALDI-TOF/TOF mass spectrometry [18] or separated by CapHPLC and sprayed directly into the ion source of LTQ-Orbitrap XL hybrid MS [19].

2.6. Validation of selected age biomarkers using quantitative Western blot analysis

Age associated trends observed for selected proteins by 2-D DIGE analysis were validated by measuring protein abundance in independent mosquito lysates using semi-quantitative western analysis. Validations were performed for ASTEI06110-PA (ADF/Cofilin), methylmalonate-semialdehyde dehydrogenase mitochondrial acylating protein (MMSA), eukaryotic initiation factor 5A (eIF5A) and manganese superoxide dismutase (SOD2). Rabbit polyclonal antibodies were first raised by the Institute for Medical and Veterinary Science (Adelaide, Australia) against a recombinant GST fusion protein of *A. gambiae* MMSA and *A. stephensi* ADF/Cofilin prepared using the pGEX-6P-1 expression vector, expressed using competent BL21 *E. coli* cells and purified using Gathionlute Sepharose 4B (GE Healthcare) [20]. Affinity purified polyclonal antibodies against aging related *A. aegypti* protein SOD2 [12] and *Aedes albopictus* eIF5A [4] were also applied in semi-quantitative Western analyses to determine specificity and cross-reactivity.

Protein was extracted from aged mosquitoes as described above. Five microgrammes of protein was loaded for each sample. Six age points (1, 5, 13, 17, 25 d old) of individual *A. stephensi* were used. At least three biological replicates were tested for each protein. Each sample was represented in two lanes on each blot. Additionally, two pooled standard samples were included on each blot to enable standardised comparisons between gels. SDS PAGE was performed using 12% Mini protean TGX gel (Bio-Rad) at constant 200 V. Proteins were transferred onto Polyvinyl Difluoride membrane (Bio-Rad) using the Trans-blot® turbo™ transfer system (Bio-Rad) for 30 min at 25 V and 1A. Membranes were briefly rinsed in 100% methanol for 1 min and distilled water for 2 min before blocking with Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE, USA): PBS (1:1) for 1 h at room temperature while gently shaking. Primary antibodies were used at dilutions stated in Table 1. Membranes were incubated with the primary antibody for 1 h at room temperature followed by three washes of 5 min using PBS/T solution (0.1% Tween in PBS). Secondary antibodies were diluted in Odyssey blocking buffer: PBS (1:1) at the dilutions provided in Table 1 and added to the membranes. Membranes were incubated with the secondary antibody for 1 h at room temperature while shaking and were washed with PBS/T for five washes of 5 min each and then with 100% methanol before being dried. Membranes were scanned using an Odyssey infrared imager (Li-cor Lincoln, NE) at an optimised intensity without saturation of pixels on the 700 nm and 800 nm channels and 84 µm pixel resolution. Membranes were stained in Direct Blue 71 dye and scanned using an Epson expression 1640 XL scanner (Epson, Suwa, Nagano, Japan) at 600 dpi in greyscale.

2.7. Data analysis

Expression profiles from DIGE were analysed using the Delta 2D version 4.0 software package (Decodon, Greifswald, Germany). The included multivariate statistical analysis package was used to detect changes in protein abundances significantly associated with age. Normalised spot volumes were compared by analysis of variance (ANOVA) with 1000 permutations, applying an α value of 0.01 and standard Bonferroni correction for multiple testing chosen to minimise

false positives being selected for time consuming downstream processes [21].

Protein identification was performed by searching peptide peak lists detected by TOF/TOF mass spectrometry against *in-silico* databases of theoretical trypsin digests of published proteins using the MASCOT database search tool (Version 2.2, Matrix Sciences, London). The database was compiled from UniProtKB database on 22/05/2012 and from VectorBase database on 26/05/14. A mass tolerance of 100 ppm for peptide precursor ions and 0.8 Daltons for fragment ions was applied in all searches. Putative function of hypothetical proteins was inferred using VectorBase database. A detailed description of the procedure used to identify proteins by mass spectrometry is described in our accompanying manuscript [16].

Western blot bands were quantified using Odyssey version 3 software (Li-cor Biosciences). Total protein was determined based on the procedure described by Aldridge and colleagues [22]. Pixel volume was determined from a region from total-protein stained lanes using Multigauge version 3 software (Fujifilm, Midtown West, Tokyo, Japan).

To correct for gel loading error, protein quantities were standardised by dividing band pixel volume obtained for Western analysis bands by the pixel volume obtained for total protein. To correct for gel to gel variation, the mean standardised protein of the duplicates was divided by the mean of the gel standard duplicates. Data for mean standardised protein quantities were first normalised by a $\log_{10} [x + 1]$ transformation in statistical package for social sciences (SPSS) version 20 and then One Way ANOVA was used to test the difference of mean normalised protein quantity between ages by selecting age and normalised protein quantity as fixed and dependent variables in the model, respectively. The Tukey post-hoc test was applied to test for changes in band intensity among age groups.

3. Results

3.1. Age-related protein spots for *A. stephensi* and *A. gambiae*

A total of 856 and 693 spots were detected in cohort one and two of *A. stephensi* respectively (Fig. 1A). For *A. gambiae*, 773 and 587 protein spots were present on 2D fusion images from cohort one and cohort two, respectively (Fig. 1B). All the spots detected on 2D-DIGE images for both species are presented in our accompanying manuscript [16]. For both species, proteins were selected as candidate age markers only if a significant change was observed for that protein spot in both cohorts between at least two age groups. Out of all the spots detected in *A. stephensi*, 3.8% (N = 27) and 13% (N = 90) were differentially expressed with age in cohort one and cohort two, respectively, but only 19 proteins spots were shared between the two cohorts. Five of the *A. stephensi* age-related protein spots were up-regulated with age (protein spot identification numbers; 391,418, 532, 641 and 836) and the remainder were down-regulated with age (Fig. 2A and B). For *A. gambiae*, out of all the spots detected, 3.6% (N = 27) and 5% (N = 30) were found to significantly change in expression with age for cohort one and cohort two, respectively. However, only nine age associated spots were shared between the two cohorts. Three spots showed a steady increase with age (protein spot identification numbers; 539, 890 and 66,393) while the rest gradually decreased their expression with age (Fig. 2A and B).

Table 1

Primary and secondary antibodies and the dilutions used for semi quantitative Western blot analysis.

Primary antibody	Type	Dilution	Secondary antibody	Dilution
ADF	Rabbit pAs	1:1000	Goat α rabbit IR800CW (Li-cor)	1:20,000
MMSA	Rabbit pAs	1:1000	Goat α rabbit IR800CW (Li-cor)	1:20,000
SOD2	Rabbit pAb	1:500	Goat α rabbit IR800CW (Li-cor)	1:20,000
eIF5A	Rabbit pAs	1:5000	Goat α rabbit IR800CW (Li-cor)	1:20,000

pAs—polyclonal antisera.

pAb—polyclonal antibodies.

Fig. 1. 2-D DIGE protein map of the *A. stephensi* (A) and *A. gambiae* (B) head and thorax proteome showing candidate aging biomarker proteins identified by 2-D DIGE analysis from two cohorts for each species. Each panel shows a 2-D DIGE comparison of protein extracts from two age samples (1 and 17 d old). Spots appearing red represent proteins that are more highly expressed in the 17 d sample than the 1 d sample, whereas spots appearing green are more highly expressed in the 1 d sample. Yellow spots are proteins that are equally abundant in the two age samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Identification of age-related protein spots

Protein identification for more visible protein spots on 2D preparative Coomassie stained gels was achieved using MALDI-TOF/TOF MS whereas CapHPLC LTQ-Orbitrap XL hybrid MS was applied to identify less visible spots. Protein spots were then significantly matched to *A. stephensi* and *A. gambiae* proteins from UniProtKB and VectorBase databases. Protein identification with MALDI-TOF/TOF MS yielded clear top hits from the databases for those proteins that could be identified (Table 2). However, due to much higher sensitivity of the CapHPLC LTQ-Orbitrap MS there were hits to multiple proteins with MW and pI

values in close range to the estimated values from the excised protein/peptide spot. This was potentially due to the simultaneous detection of overlaying proteins within 2-D gels or due to incomplete focussing of non-target proteins along the horizontal (pI) or vertical (MW) axes. Due to these uncertainties, all proteins identified by CapHPLC LTQ-Orbitrap with MW values within ± 5 kDa and pI values within ± 1 of the actual protein are reported in an accompanying manuscript [16].

Ten *A. stephensi* and six *A. gambiae* in-gel tryptic digests were identified using MALDI-TOF/TOF MS and were significantly matched to *A. gambiae* and *A. stephensi* published sequences (Fig. 2 and Table 2). The remaining age associated spots were identified using CapHPLC

Fig. 2. Age associated changes in the expression of candidate aging biomarker proteins identified from the head and thorax proteome of *A. stephensi* and *A. gambiae* for cohort one (white columns) and cohort two (grey columns). Each age sample was comprised of a pool of five adult females. Columns show mean $\pm$ SD normalised spot volume from three replicate samples for *A. stephensi* and four replicate samples for *A. gambiae*. Analysis of Variance with Bonferroni correction was applied to identify significant changes in protein spot volume within each cohort. Protein spot volumes that differ significantly ($\alpha = 0.05$) between the different age groups within each cohort are marked with a different letter. Letters in bold blue and black relate to cohort one and two, respectively. **Panel A** shows proteins identified by MALDI-TOF and **panel B** shows proteins identified by CapHPLC LTQ-Orbitrap XL hybrid MS/MS. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2 (continued).

LTQ-Orbitrap XL hybrid MS (Fig. 2B) and Table 2 in [16]. Two spots (1452 and 66,139) were not visible on Coomassie stained preparatory gels and could therefore not be identified by either of the techniques. Nucleoside diphosphate kinase was found to be age responsive in both *A. gambiae* and *A. stephensi*. Four age responsive proteins identified in this study: ADF/Cofilin and eukaryotic translation initiation factor 5a identified in *A. stephensi* and heat shock and cuticular proteins identified in *A. gambiae* were also previously found to be age responsive in *A. aegypti* [12]. The mass spectrometry proteomics data have been deposited into the ProteomeXchange Consortium [23] database via the PRIDE partner repository with the dataset identifiers PXD002153 and 10.6019/PXD002153.

3.3. Validation of aging-biomarker candidates using quantitative Western blot analysis

3.3.1. *A. stephensi*

Aging trends in the abundance of four protein markers tested by semi quantitative western analysis matched abundance trends yielded

by 2-D DIGE analysis. The abundance of ADF decreased significantly with age ($F = 6.86$, d.f. = 5, $P = 0.001$; Fig. 3A). Comparison between age groups using Tukey post-hoc test indicated that the abundance of ADF in the 1 d old age group was significantly higher than all other age groups. Similarly, the abundance of eIF5A decreased significantly with age ($F = 3.79$, d.f. = 5, $P = 0.006$; Fig. 3B). The expression in 1 d old mosquitoes was significantly higher than all other age groups except the 5 d old age group. Although there was an observed increase in band pixel volume for both MMSA and SOD2 proteins, the change was not significant for either MMSA ($F = 2.20$, d.f. = 5, $P = 0.100$; Fig. 3C) or SOD2 ($F = 1.69$, d.f. = 5, $P = 0.188$; Fig. 3D).

4. Discussion

This study represents the first investigation to determine changes that occur within the proteomes of two major malaria vectors; the African and Asian malaria vectors, *A. gambiae* and *A. stephensi* respectively, during adult aging. Our examination of over 580 proteins for *A. gambiae* and 691 proteins for *A. stephensi* by 2-D DIGE analysis

Table 2

A. stephensi and *A. gambiae* age associated spots identified by MALDI-TOF/TOF-mass spectrometry and MASCOT database searching.

Spot ID	Vector base protein code (species name)	Putative identity/published Name	Score to the highest ranked hit (species name) ^a	Peptide sequence	Peptide score	% coverage	Calc. Mw [kDa]	Calc. pI
710	ASTEI06110-PA ^b (<i>A. stephensi</i>)	ADF/Cofilin	99% (<i>A. gambiae</i>)	R.YVIFYRDEK.Q K.QIDVEVIGDR.N R.NAEYDSFLEDIQK.G R.YGLDFEYMHQCQGTSESSK.K K.LFLMSWCPDTAK.V K.LFLMSWCPDTAK.V + Oxidation (M) K.MLYSSSFDAKK.S K.YIQATDLSEASR.E K.YIQATDLSEASREAVEEK.L K.YIQATDLSEASREAVEEKL.R.A R.EAVEEKL.R.A	30 20 105 57 84 41 56 53 20 14 26	64	17.6	6.74
708	ASTEI106894-PA ^b (<i>A. stephensi</i>)	Nucleoside diphosphate kinase	97.6% (<i>A. gambiae</i>)	R.GDLVCQVGR.N K.IIERFEAK.G R.TFIMIKPDGVQR.G R.TFIMIKPDGVQR.G + Oxidation (M) K.MLGATNPADSEPGTIR.G K.ERTFIMIKPDGVQR.G R.KMLGATNPADSEPGTIR.G	47 12 61 (27) 59 12 53	28	18.9	8.03
391	ASTEI10732-PA ^b (<i>A. stephensi</i>)	Fructose bisphosphate aldolase	97.8% (<i>A. gambiae</i>)	R.FADIGVENNEDNRR.Q K.KPTAQEIALATVTALR.R K.KPTAQEIALATVTALRR.T K.NTPSYQAIENANVLAR.Y R.IVPIVEPEILPDGDHDLER.C K.QIVAPGKILADESTATCGKR.F K.GVVDLMGSEGETTQGLDDLAAR.C	12 61 27 12 53 59 47	30	39.9	8.49
418	ASTEI10732-PA ^b (<i>A. stephensi</i>)	Fructose-bisphosphate aldolase	97.8% (<i>A. gambiae</i>)	R.FADIGVENNEDNRR.Q K.KPTAQEIALATVTALR.R K.NTPSYQAIENANVLAR.Y R.IVPIVEPEILPDGDHDLER.C K.IGKNTPSYQAIENANVLAR.Y K.GVVDLMGSEGETTQGLDDLAAR.C	63 27 76 74 15 57	25	39.9	8.49
565	ASTEI01617-PA ^b (<i>A. stephensi</i>)	14-3-3 Protein Zeta	97.5% (<i>A. aegypti</i>)	K.AKLAEQSERYDDMAQAMK.S K.LAEQSERYYDDMAQAMK.S K.LAEQSERYYDDMAQAMK.S + Oxidation (M) K.SVTETGVLSNEER.N K.SVTETGVLSNEERNLLSVAYK.N R.NLLSVAYKNVVGAR.R R.VISSIEQKTESSAR.K K.MKGDYYR.Y R.YLAEVATGETR.H R.HTVVDDSQAAQYDAFEISK.G K.MQPTPIR.L	26 78 20 76 37 25 31 15 54 168 21	33	36	5
607	ASTEI02598-PA ^b (<i>A. stephensi</i>)	Mitochondrial cytochrome c1 heme protein	98.6% (<i>A. gambiae</i>)	K.LSDYFSPYPNNEEAAR.A R.AANNNGAYPPDLSYIALAR.H + Deamidated (NQ) K.DVSTFLVWAAEPYHDER.K K.VHMGVIDIFTGK.K	37 49 49 98	16	34.1	8.83
695	ASTEI04191-PA ^b (<i>A. stephensi</i>)	Eukaryotic translation initiation factor 5a	99% (<i>A. gambiae</i>)	R.AEFDGSGKEIVCTVLK.S K.KYEDICPSTHNMDVPNVK.R K.VWNLANCK.L K.LWNTLAECK.Y K.YPDMILSSSR.D R.LWDLAAGQSTR.R R.DELSYGIPQKR.L R.LWDLAAGQSTR.R K.LTRDELSYGIPQKR.L R.FSPNHTNPIIVSAGWDR.V K.YTIQEDGHSWVSCVR.F R.FEDHTKDVLSVAFSVDNR.Q R.RFEDHTKDVLSVAFSVDNR.Q K.IDHLGHNGYLSVSPDGLCTSGGKDCRA + Deamidated (NQ) K.NGDPPQCLSLAWSTDGQTLYAGYSDNIIR.V + Deamidated (NQ)	65 47 47 57 50 49 57 11 53 75 100 40 62 65 67	28	17.9	5.21
515	ASTE000014-PA ^b (<i>A. stephensi</i>)	Guanine nucleotide-binding protein subunit beta-2-like 1 protein	99.7% (<i>A. gambiae</i>)	K.VWNLANCK.L K.LWNTLAECK.Y K.YPDMILSSSR.D R.LWDLAAGQSTR.R R.DELSYGIPQKR.L R.LWDLAAGQSTR.R K.LTRDELSYGIPQKR.L R.FSPNHTNPIIVSAGWDR.V K.YTIQEDGHSWVSCVR.F R.FEDHTKDVLSVAFSVDNR.Q R.RFEDHTKDVLSVAFSVDNR.Q K.IDHLGHNGYLSVSPDGLCTSGGKDCRA + Deamidated (NQ) K.NGDPPQCLSLAWSTDGQTLYAGYSDNIIR.V + Deamidated (NQ)	47 57 50 49 57 11 53 75 100 40 62 65 67	52	35.5	7.63
645	ASTEI04582-PA ^b (<i>A. stephensi</i>)	NADH:ubiquinone dehydrogenase	97.1% (<i>A. gambiae</i>)	K.YCEAQNNEFMICR.Q K.TCAQEFTQYANCLDK.S K.VVYPDATTPGLPDDYPRPESK.Y K.CVLDNMQLERPDYGYFCRA	40 84 51 33	37	20.6	6.9

(continued on next page)

Table 2 (continued)

	Spot ID	Vector base protein code (species name)	Putative identity/published Name	Score to the highest ranked hit (species name) ^a	Peptide sequence	Peptide score	% coverage	Calc. Mw [kDa]	Calc. pI
t2.72	532	AGAP009833-PA ^c	Voltage-dependent anion-selective channel	100%	K.GYHFGWLWK.L	41	23	30.6	8.6
t2.73		(<i>A. stephensi</i>)		(<i>A. gambiae</i>)	M.APPSYSDLGK.Q	22			
t2.74					R.EFGGLIYQR.C	35			
t2.75					K.YDLDDKDACV.R.A	37			
t2.76					K.VSFDGMFVPHTGSK.T + Oxidation (M)	79			
t2.77					K.VNNQSQIGLGYQQK.L + Deamidated (NQ)	37			
t2.78	539	AGAP002499-PA	Methylmalonate-semialdehyde dehydrogenase	NA	K.TVTQLWR.E	11	16	57.3	7.9
t2.79		(<i>A. gambiae</i>)			R.NWIPDLVER.A	25			
t2.80					K.TLIDAEGLVLR.G	19			
t2.81					R.GSFLGDCHFYGK.Q	36			
t2.82					R.CMALSTAVFVGEAR.N	20			
t2.83					R.AVSFVGSDQAGKYIYER.A	32			
t2.84					K.TNDWIDLHDPATNEVVTR.V	18			
t2.85	1073	AGAP007963-PA	Calcium binding protein	NA	K.QTCVGKPYSEFPK.A	47	23	22.3	4.7
t2.86		(<i>A. gambiae</i>)			R.YQELYAQFMGNENAK.C	58			
t2.87					K.MMDINNDGLVSIIEYR.Y	77			
t2.88	1130	AGAP006829-PA	Cuticular protein 59, RR-1 family	NA	K.GQYSLVEPDGSRV.T	44	16	21.6	6.5
t2.89		(<i>A. gambiae</i>)			K.YSYNYGVHDSLTGDKV.S	86			
t2.90					R.DGDVVKGQYSLVEPDGSRV.T	72			
t2.91	1324	AGAP007120-PA	Nucleoside diphosphate kinase	NA	R.GLVGQIMQR.F	41	25	18.7	6.8
t2.92		(<i>A. gambiae</i>)			R.GLVGQIMQR.F + 2 Deamidated (NQ)	(21)			
t2.93					R.GLVGQIMQR.F + Oxidation (M)	(10)			
t2.94					R.TFIMIKPDGVQR.G	62			
t2.95					R.TFIMIKPDGVQR.G + Oxidation (M)	(28)			
t2.96					R.GLVGQIMQRFEAK.G	12			
t2.97					K.MLGATNPADSEPGTIR.G	69			
t2.98					R.KMLGATNPADSEPGTIR.G	33			
t2.99	66393	AGAP002728-PA	Isocitrate dehydrogenase	NA	R.SLNLAIRK.E	11	28	45.8	8.4
t2.100		(<i>A. gambiae</i>)			R.MSDGLFLR.C + Oxidation (M)	30			
t2.101					K.LITEEASNR.V	25			
t2.102					K.YPEIKFEER.Y	57			
t2.103					K.FGIPQGAIDSVNR.N	(48)			
t2.104					K.FGIPQGAIDSVNR.N + Deamidated (NQ)	59			
t2.105					K.CSEYTNACDR.I	17			
t2.106					K.TLYDNVDVVTIR.E	57			
t2.107					K.EFNLYANVRPCR.S	25			
t2.108					R.YLDTVCLNMVQDPR.K + Oxidation (M)	19			
t2.109					R.DMAQKYPEIKFEER.Y + Oxidation (M)	39			
t2.110	66134	AGAP010876-PA	Heat shock protein hsp-70	NA	K.DAGQIAGLNVLR.V	85	13	69.5	5.5
t2.111		(<i>A. gambiae</i>)			K.VQSLVQEVFGR.Q	(81)			
t2.112					R.AKLETLVGDILKRT	61			
t2.113					K.SDIGEVLLVGMTR.M + Oxidation (M)	20			
t2.114					K.MLGSTFLVGIPAPR.G + Oxidation (M)	54			
t2.115					K.NAVITVPAYFNDSQR.Q + Deamidated (NQ)	(85)			
t2.116					K.VQSLVQEVFGRQPSR.A + 2 Deamidated (NQ)	74			

The location of *A. stephensi* spots is shown on Fig. 1A and the location of *A. gambiae* spots is shown on Fig. 1B. A mass tolerance of 100 ppm for peptides and 0.8 Daltons for fragment ions was applied in all searches. Peptides were accepted if the E-value was <0.05 and at least two accepted peptide identifications were required to assign protein identification.

^a All *A. stephensi* proteins are hypothetical proteins therefore inferred names correspond to the highest ranked hit to a homologous species indicated in brackets.

^b Hypothetical protein.

^c *A. stephensi* protein name was derived from the highest ranked hit to a homologous species.

demonstrated that only a small proportion of the proteins (between 3.6 and 13%) changed in abundance with age. Further, we confirmed age associated trends by identifying proteins that followed similar significant age-associated abundance changes between two independent cohorts and verified trends for two proteins by semi quantitative western analysis. Nineteen and nine proteins were shown to be significantly age associated in two cohorts of mosquitoes for *A. stephensi* and *A. gambiae*, respectively. In keeping with the dengue mosquito *A. aegypti* [12], at least twice as many proteins decreased in abundance during aging than proteins that increased in abundance. Sixteen candidate proteins were identified by matching peptide mass spectra to proteins within the VectorBase database. The remaining two candidates were not visible on Coomassie stained gels therefore could not be identified by either of the mass spectrometry approaches. Some of the age associated proteins identified in this study are associated with gene/protein families in which previous association with aging have been reported in other organisms.

Three of the up-regulated proteins identified in our study are metabolic enzymes. These include; a protein similar to fructose-1,6-bisphosphate aldolase (FBA), methylmalonate-semialdehyde dehydrogenase (MMSA) and the oxidoreductase isocitrate dehydrogenase (NAD⁺). Previously, Wang and colleagues found that genes encoding oxidoreductases are down-regulated with age in *A. gambiae* [14]. FBA is involved in the glycolysis pathway whereas MMSA and Isocitrate dehydrogenase (NAD⁺) play roles in pyrimidine metabolism [24] and the tricarboxylic acid cycle, respectively. The up regulation of metabolic proteins identified in this study could be a response to increased energy requirements of the cell to cope with increased age-related oxidative stress. A putative mitochondrial protein of *A. stephensi* with sequence similarity to cytochrome c1 was found to decrease with age. Cytochrome c oxidase activity also appeared to decrease with age in house flies [25]. We identified six candidate age biomarkers that had previously been implicated as age biomarkers in other mosquito species. 1) A heat shock protein-70, 2) a calcium binding protein, 3) ASTEI06110-P; a protein

Fig. 3. Change in aging biomarker protein abundances with the age of individual female *A. stephensi* determined by semi-quantitative Western blot analysis. Western blots, total protein controls and protein abundances for individual mosquitoes are provided for A. ADF, B. eIF5A C. MMSA and D. SOD2. Each Western blot is shown with corresponding Direct Blue 71 stained total protein (lower panels). Graphs show mean $\pm$ SD standardized protein abundances from four individual mosquitoes. Protein abundances that differ significantly between the different age groups ($P < 0.05$) are marked with a line.

with 99% sequence similarity to the Actin depolymerizing factor (ADF)/Cofilin superfamily, 4) a cuticle protein, 5) ASTE104191-PA; a hypothetical protein with 99% sequence similarity to eukaryotic translation initiation factor 5a (eIF5A) and 6) ASTE101617-PA; a protein with similarity to 14-3-3. Hsp-70 was also found to decrease with age in *A. aegypti* [12] whereas its expression increased with age in *D. melanogaster* [26]. Hsps are known to play various roles related to the maintenance of protein stability [8,27–31] hence age-specific induction of hsp-70 could be due to a number of factors including oxidative stress or due to other otherwise undetermined factors associated with aging [8,29–31]. The expression of the calcium binding protein decreased with age in *A. gambiae* consistent with previous findings at the transcript level in both *A. gambiae* [13,14] and *A. aegypti* [32]. Also with a decreasing age associated expression trend was *A. stephensi* ADF/Cofilin. ADF/Cofilin is involved in the regulation of microfilament turnover and depolymerisation of F-actin filaments as well as reorganization of cytoskeletal structure through Actin-interacting protein 1 [33–36]. An age-dependent down regulation of Actin Depolymerizing Factor protein was also observed in *A. aegypti* [12]. The age-associated decrease of ADF/Cofilin protein at early ages, now observed across two mosquito genera, illustrates a robust marker of early age and possibly a residual effect from the tissue remodelling that takes place during mosquito metamorphosis. The decreasing expression trend observed for a cuticle protein in *A. gambiae* follows a similar age-related expression trend to that previously observed for an insect cuticle protein in *A. aegypti* [12]. It has been reported that the abundance of some cuticular proteins are differentially regulated at different developmental stages [37]. Eukaryotic translation initiation factor 5A was strongly down-regulated with age in *A. stephensi* as it was in *A. aegypti* [12]. In eukaryotes, this protein is highly conserved and exists in different isoforms. In plants, initiation factor 5A isoforms are known to regulate senescence and cell division [38,39]. An isoform of 14-3-3 decreased with age in *A. stephensi* and was also found to decrease with age in *A. aegypti* [12]. 14-3-3 chaperones are known for their role in apoptosis

and cell survival [40,41]. 14-3-3 zeta is also a heat-shock related molecular chaperone that dissolves heat-aggregated proteins [42].

A number of candidate age-associated proteins have roles in detoxification and potentially insecticide resistance or immunity to infection. The most common pyrethroid resistance mechanism, known as “target-site” or “knockdown” resistance is characterised by mutation in the voltage gated sodium channel. It is defined by the ability of mosquito mutants to withstand pyrethroid insecticides for extended periods of time without being knocked down [43]. Alternatively, metabolic resistance involves the increased expression of enzymes involved in the metabolism, sequestration or excretion of insecticides. The expression of glutathione transferase e2 has been reported to decrease in older resistant mutants making them less tolerant to insecticide relative to the younger mosquitoes [5]. In this study, we have identified an up-regulated protein in *A. stephensi* which is most similar to a voltage-dependent anion-selective channel protein in *A. gambiae*. Other age associated biomarkers identified have been found at elevated abundances in infected mosquitoes hence possibly play roles in cellular immunity. These include eIF5A which was induced in dengue infected *A. albopictus* cells [4] and a 14-3-3 isoform which was upregulated following *Plasmodium* infection in *A. gambiae* [3]. Transcription of hsp-70 was also upregulated in blood fed *A. gambiae* mosquitoes but decreased in sugar-fed females [14]. Further work comparing expression levels at different ages is needed to investigate possible involvement in aging associated changes to insecticide resistance and infection susceptibility.

Aging specific trends identified by 2-D DIGE analysis were confirmed by Western analysis for two of the biomarkers tested. An age associated decrease between 1 and 5 d old was observed for ADF/Cofilin with no further changes at older ages. An age-dependent decrease in protein abundances of eIF5A also confirmed the trend observed from 2-D DIGE. However, given the previously discussed association of eIF5A with dengue infection [4], the response to *Plasmodium* infection should be established before this protein is applied as an age biomarker in

Anopheles. Furthermore, MMSA and SOD2 identified in *A. gambiae* and *A. aegypti*, respectively, were used to probe *Anopheles stephensi* to investigate the conservation of aging trends. Age dependent changes were not significant, validating the 2D-DIGE results for this species. The effect of temperature and blood feeding on the expression of aging biomarkers should also be investigated before these findings are generalized to field mosquitoes. If age dependent changes remain robust to these factors, the proteins could be targeted in antibody-based rapid diagnostic assays for the prediction of mosquito age. Proteins with abundance changes greater than 2-fold are likely to be most useful. A rapid diagnostic assay for the prediction of mosquito age in remote locations would be highly sought for the evaluation of vector control interventions in large field studies targeting these primary vectors of malaria. The observed cross-reactivity of *A. aegypti* and *A. albopictus* antibodies to *Anopheles* proteins was pleasing as it demonstrated the potential for a single assay to be applicable to diverse mosquito taxa.

In general, 2D DIGE together with mass spectrometry facilitated the identification of *Anopheles* proteins with characteristic expression patterns occurring during aging. The expression changes of candidate aging biomarkers were validated by semi quantitative western analysis on individual *A. stephensi* mosquitoes. The trends were consistent to those obtained by 2-D DIGE analysis of pooled samples and trends observed for *A. aegypti* [12]. This provides support for the use of the 2D-DIGE and mass spectrometry workflow for the identification of differentially expressed proteins in mosquito vector studies.

5. Conclusion

We have identified proteins undergoing aging specific expression changes in two major vectors of malaria. These may illuminate metabolic and other biochemical pathways underlying the relationships between mosquito age and several factors determining pathogen transmission and susceptibility to insecticides. Further, these changes may serve as useful biomarkers for predicting mosquito age, which would facilitate numerous investigations into the biology and epidemiological risk of wild mosquito populations. The conserved aging specific expression patterns of many of the proteins and the demonstrated cross-reactivity of antibodies between *Anopheles* and *Aedes* vector species suggest that this approach might be useful for the prediction of age-related protein changes in mosquitoes of other genera and insects more generally.

Author contributions

Conceived and designed the experiments: MTS, LEH, BHK, JIG, PED, RLK. Performed the experiments: MTS, LEH, JM, KAD. Analysed data: MTS, LEH, JM, KAD, MLH, GFK. Contributed reagents/materials/analysis tools: BHK, JIG, GFK, PED, RLK. Wrote the paper: MS, LEH. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no competing interests. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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