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Candidate biomarkers for mosquito age-grading identified by label-free quantitative analysis of protein expression in *Aedes albopictus* females

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ABSTRACT

We applied a "shotgun" approach based on nanoliquid chromatography–high resolution mass spectrometry associated to label free quantification (LFQ) to identify proteins varying with age, independently from the physiological state, in *Aedes albopictus*, a mosquito species which in the last decades invaded temperate regions in North America and Europe, creating concerns for associated high nuisance and risk of arbovirus transmission. The combined "shotgun" and LFQ approach was shown to be highly suitable to simultaneously compare several biological samples, as needed in a study aimed to analyze different age-groups and physiological states of adult mosquito females. The results obtained represent the first wide-scale analysis of protein expression in *Ae. albopictus* females: >1000 and 665 proteins were identified from few micrograms of crude protein extracts of mosquito heads and thoraxes, respectively. Six of these proteins were shown to significantly vary from 2- to 16-day-old females, independently from their physiological state (*i.e.* virgin, mated, host-seeking, blood-fed, and gravid).

Biological significance: Mosquito-borne diseases, such as malaria, dengue and other arboviroses, are a persistent cause of global mortality and morbidity, affecting hundreds of thousands of people. Billions of people living in tropical areas are at risk of being bitten every day by an infective mosquito female and the spread of tropical species such as *Aedes albopictus* to temperate areas is creating alarm in the northern hemisphere. Mosquito longevity is a critical factor affecting mosquito-borne pathogen transmission cycles and the mosquito capacity to transmit pathogens. However, large scale analyses of the age structure of mosquito field populations is hampered by the lack of optimal age-grading approaches. Our findings open new perspectives for the development of reliable, simple and cheap protein-based assays to age-grade *Ae. albopictus* females and, most likely, other mosquito species of higher medical relevance, such as the main dengue vector, *Aedes aegypti*, and the major Afrotropical malaria vectors. These assays would greatly contribute to epidemiological studies aimed at defining the actual vectorial capacity of a given mosquito species. Moreover, they would be very valuable in assessing the effectiveness of mosquito control interventions based on the relative ratio between young and old individuals before and after the intervention.

1. Introduction

Mosquito population age structure is a key element for assessing the actual vectorial capacity of species capable of hosting human and/or zoonotic pathogens, as well as for evaluating the effect of mosquito control interventions. Large-scale analysis of field population age structure is however hampered by the lack of optimal mosquito age-grading approaches.

Traditional morphological approaches are very labor-intensive, as they require dissections and microscopic examination of ovaries to reveal minute anatomical changes occurring during female gonothrophic cycles. Moreover, while methods that offer simple two-category predictions (*e.g.* observation of ovarian tracheation after first oviposition and of midgut meconium) provide high-accuracy classifications, methods that offer the separation of multiple age categories (*e.g.* counting of ovariolar dilatation after each oviposition) have low accuracy [1].

To overcome these limits, different kinds of approaches have been proposed in the last years for the most medically relevant species, *i.e.* the major Afrotropical malaria vectors, *Anopheles gambiae*, and the main dengue vector, *Aedes aegypti*. These approaches include gas chromatographic analysis of cuticular hydrocarbons [2–5], near infrared (NIR) spectra instantly collected from cuticle scans [6,7], and gene transcriptional profiles measured by quantitative RT-PCR [8–12]. The latter two approaches in particular have been proven accurate both on laboratory reared and wild mosquitoes, but both require very careful

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manipulation and preparation of samples and relatively high-tech and expensive equipment, which limits their exploitation to well-equipped laboratories.

The use of proteins as "aging biomarkers" could represent a good alternative to the limitations of current mosquito age-grading approaches, if such biomarkers can be detected using widely adopted methods such as ELISA. The proofs of concept for the use of protein "aging biomarkers" comes from a seminal study by Fleming and co-authors [13], who revealed age-related alterations in protein expression in *Drosophila melanogaster*, and by more recent studies based on 2-D fluorescence difference gel electrophoresis (followed by spot identification using mass spectrometry) which identified a few proteins varying with age in *Ae. aegypti* [14] and in two *Anopheles* species [15]. In particular, in *Ae. aegypti* one protein (*i.e.* Anterior Fat Body protein) was found to be present in significantly lower concentration in 34-day-old females than in ≤18-day-old ones, suggesting that it may represent a good candidate biomarker to identify females who lived long enough to transmit the dengue virus. It is relevant that results were confirmed by semi-quantitative Western-blot. However, the analyses did not take into account possible differences related to physiological variations.

The aim of the present work was to apply a shotgun approach for the identification and quantification of mosquito proteins varying with age independently from the physiological status in order to provide grounds for more valuable protein-based age-grading approaches. The approach consists in protein identification by "shotgun" proteomics (LC–HR ESI–MS and MS/MS) — which allows identification of a large number of proteins from a crude extract of very small biological samples in a single experiment [16] — followed by relative quantification through label free quantitation (LFQ). The latter method allows the measuring of significant protein variations within a complex mixture or across an entire proteome in a single experiment, which makes it cheaper than quantification methods based on labeling strategies [17,18] and optimal for comparing multiple samples [19,20]. We focused the analyses on *Aedes albopictus* females, an important vector of arbovirus [21], which in the last decades invaded temperate regions in North America and Europe [22,23], creating concerns for the first cases of autochthonous arbovirus transmission [24–26] and for the high nuisance determined by its diurnal and aggressive biting behavior [27].

2. Materials and methods

2.1. Mosquito rearing

Analyzed samples originated from *Ae. albopictus* eggs collected in the campus of Sapienza University by 24 ovitraps in September–October 2013. Larvae and adults were reared in the insectary (T = 25 ± 1 °C, RH = 60 ± 5% and 14:10 hour photoperiod). Adults were kept in 25 cm cubic cages and fed with 10% sugar solution *ad libitum*. Female samples were collected at 10 different ages and/or physiological states, placed alive at −80 °C and kept at the same temperature until protein extraction (Table 1). The virgin female samples were obtained by separating females from males immediately after adult emergence. The 2–3 day old mated samples were selected by isolating mating couples and freezing the females immediately after mating. The 4-day old females were selected among those landing on the hand of a human volunteer (host-seeking samples) and those allowed to bite, which were frozen immediately after the blood-meal (blood-fed samples). The 8-day-old females were selected among those which laid (I oviposition samples) or did not lay eggs (no oviposition samples) when placed in single plastic containers lined with adsorbent paper. The 12-day old females were selected among those allowed to take a first blood-meal and to oviposit, provided with a second blood-meal opportunity and frozen before (no oviposition samples) or after oviposition (II oviposition samples) in single plastic containers lined with adsorbent paper. The 16-day-old females were selected among those allowed to take two blood-meals and to oviposit after these, provided with a third blood-meal opportunity and frozen before (no oviposition samples) or after oviposition (III oviposition samples) in single plastic containers lined with adsorbent paper.

Table 1
Lists of samples used for each age group and physiological state.

Age (days)	Physiological state	Blood-meal
2	Virgin	0
2–3	Mated	0
4	Host seeking	0
4	Blood-fed	1
8	I Oviposition	1
8	No oviposition	1
12	II Oviposition	2
12	No oviposition	2
16	III oviposition	3
16	No oviposition	3

2.2. Reagents

Ammonium bicarbonate, DTT, iodoacetamide, sodium chloride, formic acid, acetonitrile, trifluoroacetic acid, acetic acid, and thiourea were from Sigma-Aldrich (Milano, Italy), while Tris and urea were from Euroclone. Trypsin was purchased from Promega (Sequencing Grade Modified Trypsin) and Lys-C from Thermo Scientific (MS grade). The hand-made desalting/purification STAGE column were prepared using three C18 Empore Extraction Disks (3M).

2.3. Protein sample preparation and digestion

For each age and/or physiological state, heads and thoraxes with wings but without legs were dissected and pooled immediately before protein extractions. Eventually, 2 sample series from head samples (A_1 = 10 pools of 8 individuals and A_2 = 10 pools of 6 individuals) and one series from thorax samples (B = 10 pools of 8 individuals) were prepared. The pools were crushed in a mortar under liquid nitrogen and the proteins extracted with 6 M urea/2 M thiourea in Tris–Cl 50 mM pH 7.4. The protein extracts were centrifuged at 14,000 rpm for 40 min at 4 °C and the supernatants were collected for the analysis. The total amount of protein in each sample was assessed by the Bradford colorimetric assay [28] with the "Bio-Rad Protein Assay" kit using serial dilutions of bovine serum albumin to generate a standard curve. Protein sample concentration was measured by an Infinite PRO 200 reader (TECAN).

Protein digestion was carried out on 15 μg protein extracts. Reduction of disulfide bridges was performed by treating samples with DTT (1 μg of DTT/50 μg of proteins for 30 min at RT), followed by alkylation (5 μg of iodoacetamide/50 μg of proteins for 20 min at RT in the dark), as described by Foster and co-workers [29]. Protein samples were diluted 3 times with 500 mM ammonium bicarbonate, to increase pH and reduce the concentration of urea/thiourea. A first enzymatic digestion was performed by incubating the samples with LysC in a 1:50 ratio (w/w) for 3 h at 37 °C. The digestion products were then incubated with trypsin in a 1:50 ratio (w/w) overnight at 37 °C. The digested samples were then acidified by adding trifluoracetic acid and desalted on STop And Go Extraction (STAGE) tips [30]. The eluates were concentrated and reconstituted to 20 μL in 0.5% acetic acid, prior to HPLC–MS analyses.

2.4. Mass spectrometric analysis

The peptide mixture of each sample series was submitted to a nanoLC–nanoESI–MS/MS analysis on an Ultimate 3000 HPLC (Dionex, San Donato Milanese, Milano, Italy) coupled to a LTQ-Orbitrap mass spectrometer (Thermo Fisher, Bremen, Germany). Fractions containing 2.25 μg protein (corresponding to ~0.15 heads and ~0.05 thoraxes) were injected directly on a self-made nanocolumn packed with an