Use of Transcriptional Age Grading Technique to Determine the Chronological Age of Sri Lankan Female Dengue Mosquitoes; *Aedes Aegypti* and *Aedes Albopictus*

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Abstract

Background: *Aedes aegypti* and *Ae. albopictus* are important vectors of human diseases such as dengue, chikungunya, and zika. In Sri Lanka, they have been responsible for transmitting the dengue virus. One of the most important parameters that affect the likelihood of arbovirus transmission is the age structure of the mosquito population. However, mosquito age is difficult to measure with accuracy. This study aims to construct Multivariate calibration models using the transcriptional abundance of three age responsive genes: *Ae15848* (calcium-binding protein), *Ae8505* (structural component of cuticle), and *Ae4274* (fizzy cell cycle/cell division cycle 20).

Methods: Transcriptional age grading technique was applied to determine the chronological age of *Ae. aegypti* and *Ae. albopictus* female mosquito strains from Sri Lanka using the age responsive genes; *Ae15848*, *Ae8505*, and *Ae4274*. Further to investigate the influence of temperature on this age grading technique. Expression levels of these three genes were quantified using reverse transcription qualitative PCR (qRT-PCR) and results were normalized against the housekeeping gene *ribosomal gene S17* (*RsP17*).

Results: The expression of *Ae15848* and *Ae8505* decreased with the age of mosquitoes and, showed the most significant and consistent change while expression of *Ae4274* increased with age. The multivariate calibration models showed more than 80% correlation between expression of these age responsive genes and the age of female mosquitoes at both temperatures. At 27^0C the accuracy of age predictions using the models was 2.19 (±1.66) and 2.58 (±2.06) days for *Ae. aegypti* and *Ae. albopictus* females, respectively. The accuracy of the model for *Ae. aegypti* at 23^0C was 3.42 (±2.74).

Conclusions: An adult rearing temperature difference of 4^0C (23^0C to 27^0C) did not significantly affect the age predictions. The calibration models created during this study could be successfully used to estimate the age of wild *Ae. aegypti* and *Ae. albopictus* mosquitoes from Sri Lanka.

Background

Aedes aegypti and *Ae. albopictus* are important vectors of human diseases such as dengue, chikungunya, and zika. Among these diseases, dengue is the most rapidly spreading mosquito-borne viral disease that is endemic in more than a hundred and twenty countries in the world including Sri Lanka. Among several factors, the age structure of the dengue vector population has been identified as one of the most sensitive parameters that influence the epidemiology of the disease.

Based on experimental observations the average extrinsic incubation period (EIP), the incubation period of the dengue virus within the mosquito's body before it is transmitted to the human body is 8–12 days [1, 2, 3, 4}. Joy et al. [2] have stated that wild populations with 10% − 27% of females that can survive more than the EIP are more likely to establish dengue virus transmission within that area. Hence, knowing the age structure of the wild populations in an area is vital in decision making especially in vector control programmes.

As there is no specific antiviral vaccine against dengue, efficient and environmentally friendly vector control measures are needed to prevent the disease from spread and outbreaks throughout the world. Introduction of artificially infected *Ae. aegypti* females with life-shortening *Wolbachia pipienis* (*w*MelPop) into the field has been introduced as one of the most promising dengue mosquito eradication technique in the world due to failures in traditional vector control measures. *Wolbachia* has the ability either to block the dengue virus inside the mosquito body and/or decrease the female mosquito life span than the EIP of the dengue virus. This environmentally friendly method of vector control has been successfully practiced in several areas in the world especially in Australia [5]. Knowledge of the age of the vector population is important before designing and releasing *w*MelPop infected individuals into natural populations to determine the effectiveness of this method.

Morphological, biochemical, and molecular-based age grading techniques have been developed in determining the age of female mosquitoes. However, the use of both morphological and biochemical methods have become questionable due to their inability and inaccuracy in measuring the age of mosquitoes older than the EIP. The transcriptional age-grading technique has now been identified as the most accurate and precise approach in determining the chronological age of mosquitoes [3, 6, 7].

Quantification of expression levels of genes that show a variation in its expression with the age of the female mosquito is the basis of this technique. The transcription scores of these age-responsive genes of laboratory-reared mosquitoes of known ages are then feed into a multivariate calibration model which could be later used in age predictions of field/wild individuals. The mosquitoes used in the construction of the calibration model must be from the same mosquito strain of the field population where the researcher is planning to apply the technique [6]. The analysis of age responsive genes during these studies have shown that this technique accurately detects the age of *An. gambiae* [8, 9, 10] and *Ae. aegypti* [2, 3, 4, 11, 12] older than 15 days, that is more than the EIP period. Trials, using mosquitoes reared in field cages, have concluded that the gene expression profiles of *Ae. aegypti* female mosquitoes could determine the age with an accuracy of ± 5 days of the actual age [11, 13, 2].

The orthologues of the eight age responsive genes *i.e. Ae- 4274, Ae-4679, Ae 4916, Ae-6639, Ae-7471, Ae-8505, Ae-12750, Ae-15848* selected from *Drosophila melanogaster* have initially been used to predict the age of female *Ae. aegypti* mosquitoes under both laboratory and field conditions [6, 14, 15, 16]. According to mosquito transcriptional age grading studies *CG-8505/ Ae- 8505/AAEL003259* (*Pupal cuticle protein 78E putative*) and *SCP-1/ Ae.-15848/AAEL008844* (*calcium binding protein, putative*) displayed the largest and significant decrease in expression levels with the age of female mosquitoes while expression levels of *fizzy/ Ae-4274/AAEL014025* (fizzy *cell cycle/ cell physiology, putative*) significantly increased with the age. Hence these three genes; *Ae- 8505* and *Ae.-15848* and *Ae-4274* have been identified as the most informative age-responsive genes that could be used in the transcriptional profiling of mosquitoes [2, 11, 13]. Cook et al. [6] have identified *Ae- 8505 Ae-15848* and *Ae-4274* genes as the most reliable age responsive genes and recommended to use these three genes for future age determination studies. The gene *RsP17* (*40S ribosomal protein s17*) that showed an insignificant variation with the age has been exclusively used as the reference gene in normalizing the samples in these studies. Further, the

expression of these genes has are independent of blood feeding, egg laying, digestion, and reproductive status of the mosquitoes [6, 8, 17].

However, this approach needs further validation and optimization based on the geographical region, as the mosquito populations may have sequence polymorphism which only affects the reliability of gene expression analysis. Therefore, Cook et al. [6] suggested creating separate models for mosquitoes in different geographical regions. Further, fluctuation in the environmental parameters such as temperature, humidity, photoperiod, *etc.* was also to be considered as these might affect the transcriptional abundance of age responsive genes [6]. Hence, models constructed considering all or majority of these limiting factors will increase the accuracy and precision of the transcriptional age grading method.

At present dengue has become one of the major causes of hospitalization and deaths in Sri Lanka. The year 2017 reported the largest dengue outbreak (≈ 186,000 cases) in the country which is around 4.3 times an increase in the average number of cases that have been reported from 2010 to the 2016 period [18]. Control of dengue vector populations in Sri Lanka is primarily based on the application of adulticides and larvicides. However, most of these programmes have been challenged due to the development of insecticide resistance by both the dengue vectors [19, 20]. Hence, the Sri Lankan government has planned to release *w*MelPop infected dengue vectors into the field [21]. However, so far no attempts have been made in Sri Lanka to determine the chronological age and age structure of any of the dengue vectors which is essential before implementing this programme. Both primary and secondary dengue vectors, *Ae. aegypti* and *Ae. albopictus* are found in Sri Lanka. Although *Ae. albopictus* was considered as a rural secondary dengue vector in the past has now invaded urban areas as well and has become the most dominant *Aedes* species in most of the areas in the country [20]. However, so far none of the research in the world has focused on determining the age structure of *Ae. albopictus* populations.

Hence, the proposed work aimed to construct multivariate calibration models using the transcriptional abundance of three age responsive genes; *Ae15848*, *Ae8505*, and *Ae4274* to determine the age structure of *Ae. aegypti* and *Ae. albopictus* female mosquito strains from Sri Lanka and to investigate the influence of temperature on the expression levels of these age responsive genes of *Ae. aegypti* females.

Methods

Establishment of mosquito colonies and sample collection

Blood-fed mosquitoes collected from Sri Lanka were used to obtain eggs to establish initial colonies of *Ae. aegypti* and *Ae. albopictus*. Colonies were maintained at an insectary with ambient conditions of 27 ± 2°C temperature, 70 ± 10% relative humidity (RH), and a photoperiod of 12:12 (L:D) which is similar to the field sites.

Another *Ae. aegypti* colony [using the eggs from the above *Ae. aegypti* colony at 27°C)] was established at 23 ± 2°C (70 ± 10% RH and of 12:12 photoperiod) to determine the effect of temperature on the

transcriptional age grading technique. For all the colonies larvae were given similar conditions (similar larval densities and larval food to each tray).

Unfed adult females of both species reared at 27□C were collected at 8-time points *i.e.*1, 5, 9, 13, 17, 21, 25, 29 days. Unfed *Ae. aegypti* females reared at 23□C were collected from 5-time points *i.e.* 1, 5, 9, 13, and 17 days. Thirty individuals were collected from each time point. All samples were snap-frozen at -80 ^{0}C for molecular analysis.

RNA extraction and cDNA synthesis

RNA was extracted from pools of 10 individual mosquitoes of a given age group (3 replicates per each age class) using the Arcturus® PicoPure RNA Isolation kits (Thermo Fisher Scientific, Waltham, MA, US) following the manufacturers' protocol. All the steps were done while keeping the samples on ice and centrifugation steps were carried out at 4^{0}C. These samples were treated with RNase-free DNase (Qiagen Hilden Germany) to remove the DNA contaminations from the RNA samples. Samples were stored at -80 ^{0}C for molecular analysis.

cDNA was synthesized by reverse transcription using extracted RNA as the template. SuperScript III first-Strand Synthesis System (Invitrogen) was used for cDNA synthesis following the manufacturers' protocol.

Validation and quantification of age responsive genes using qRT-PCR assay

Three age responsive genes *i.e. Ae. 15848* (a gene involved in calcium-binding), *Ae. 8505* (a gene involved in producing a structural component of cuticle), *Ae. 4274* (a gene involved in fizzy cell cycle/cell physiology) and, a housekeeping gene *Rsp17* were selected for qRT-PCR analysis according to previous studies [6]. Primer sequences given in Cook et al. [6] were used to amplify these three genes in *Ae. aegypti*. Primers for *Ae. albopictus* were designed using Primer 3 software (version 0.4.0) [22, 23] (Table 1).

Table 1
Primer sequences and the product length of the candidate genes and housekeeping genes

Gene name	*Aedes aegypti* (Primer 5'-3')	Product length (bp)	*Aedes albopictus* (Primer 5'-3')	Product length (bp)
Ae 15848	F-CGAAGAGTTCAAGGATGCCG R-TCTATGCTGACCAGACCGTC	135	F-GATGAGATCTCTGCCCTTGC R -TCGGAGTAGGACTTGCCAAC	103
Ae 8505	F-ATCATCTGCCAACTCCACCA R-ATCCGGCAGTCAGAGTGAAA	115	F-CGCTCAAGATAGCAACATCG R-TATGATGACGTCGCTGGTGT	130
Ae 4274	F-GGACGCTTAGCGGGAAGAC R-TTGGCGTTTGGGATTTACCT	81	F-GCCCGATATCATCAACGACT R-CCCTCCTTCGTTCTCGTACA	142
Rsp17	F-GCAGCTGGACTTCAACAACA R-AACAACATCCCAACTGCACC	139	F-CAGGTCCGTGGTATCTCCAT R-TCCACTTCGATGATGTCCTG	104
F- Forward, R- Reverse				

After validating the three age responsive genes and the housekeeping gene of both species, quantitative real-time polymerase chain reaction (qRT-PCR) assays were continued to quantify the expression levels of all these genes using the MX3005 qPCR system (Agilent Technologies). Each qRT-PCR reaction mixture (20 µl) was prepared using 1 µl of cDNA, 10 µl Brilliant green Ultra-fast SyBr Green qPCR master mix (Agilent), 0.6µl of primer (10 mM), 7.8 µl nuclease-free water. The thermal cycling conditions were conducted with denaturation at 95 ^{0}C for 3 minutes followed by 40 cycles of 10 seconds at 95 ^{0}C, 10 seconds at 60 ^{0}C, and a last step 95 ^{0}C for one minute, 55 ^{0}C for 30 seconds, and, 95 for 30 seconds. Three biological and three technical replicates were performed for each age class and each gene.

Data analysis

Data were analyzed using MxPro qPCR software (Agilent Technologies) to obtain the Cycle Threshold (Ct) value which is the measure of the expression of genes. Fold Change (FC) of each age responsive gene at each time point, relative to the one-day old mosquitoes were calculated using the $2^{-\Delta\Delta CT}$ method [24] incorporating the PCR efficiency.

Gene expression value/Ct value of each candidate gene at each time point was normalized to the reference gene (*Rsp17*) by calculating the log contrast values using the equation given below [6].

Log contrast Xi = log 10 $[(X_i / X_{total})/(X_{ref}/ X_{total})]$

Where, X_i = Mean Ct value of gene X

X_{ref}= Mean Ct of the reference gene *Rsp17*

X_{total} = Sum of the Ct values for all genes from an individual

One-way ANOVA was conducted (using the transcriptional abundance data/Log contrast values of each experiment) to determine any significant variation in the expression of the gene with the age of mosquitoes using the MiniTab 15 version.

A multivariate calibration method described by Cook et al. [6, 3] was used to predict mosquito age. Canonical redundancy analysis was conducted, for each experimental design, using the normalized gene expression values (Log contrast) of all three candidate genes using the SAS statistical software (SAS University edition). Previously written syntax by Cook et al. [6], was modified and used in all the analyses. A calibration model, which explains the strength of the linear relationship between the expression of all the three candidate genes and mosquito age was constructed using the linear regression of the first redundancy variate generated during the redundancy analysis. A nonparametric bootstrapping (1000 bootstraps) method was used to assess the sampling error [95% confidence intervals (CI)], to validate the constructed calibration models and to predict mosquito ages. The median point of the CI intervals was considered as the likely predicted ages for mosquitoes [6]. The residual value (the difference between the predicted age and the actual age) was used to assess the accuracy of each model. The expression data of one experimental design was cross-checked with other models *eg*. *Ae. albopictus* age was predicted using *Ae. aegypti* model and *vice versa*, to determine the possible use of one model for age prediction.

Results

Expression levels of age responsive genes of *Ae. aegypti* and *Ae. albopictus* at 27 ^{0}C

All three genes, *Ae15848*, *Ae8505* and *Ae4274* were differentially expressed in both species and the fold change (relative to the expression of one day old mosquitoes) obtained are presented in Fig. 1. Gene *Ae15848* and *Ae8505* of *Ae. aegypti* respectively showed the highest FC of 0.173 and 0.01 at day 5 and lowest FC of 0.020 and 0.002 at day 29 respectively. For the same genes *Ae. albopictus* had the highest fold change at day 5 (FC for *Ae15848* = 0.10 and *Ae8505* = 0.028) and lowest at day 29 (FC for *Ae15848* = 0.030 and *Ae8505* = 0.007). For *Ae. aegypti*, the FC of gene *Ae4274* was highest at day 21 (FC = 8.802) and the lowest FC of 2.938 at day 5. *Aedes albopictus* showed the lowest FC at day 13 (7.973) while largest FC of 14.876 at day 21.

The transcriptional profiles and abundance values (logcontrast of Ct) of each candidate gene at each experimental design are shown in Figs. 2 and Table 2. The expression levels are inversely proportional to the logcontrast of normalized Ct values. The lowest logcontrast value/transcriptional abundance of *Ae15848* gene for both *Ae. aegypti* [(-0.090) ± 0.016] and *Ae. albopictus* [(-0.074) ± 0.012] was at day 1 and highest was at day 29 [*Ae. aegypti* = 0.032 ± 0.033 and *Ae. albopictus* = 0.30 ± 0.004]. The transcriptional abundance of genes *Ae15848* and *Ae8505* increased with the age of both species, that is the expression of both the genes decrease with the mosquito age. Expression levels of *Ae15848* was always greater than that of *Ae8505* at each time point.

The expression of *Ae4274* increased with the age of mosquitoes unlike the other two genes. For both the mosquito species the lowest expression of *Ae4274* was at day 1 (0.138 ± 0.025 and 0.143 ± 0.013 respectively for *Ae. aegypti* and *Ae. albopictus*). The highest expression was at 21 day old age class, and the logcontrast values were 0.087 ± 0.012 for *Ae. aegypti* and 0.89 ± 0.007 for *Ae. albopictus*. The expression levels of this gene were lower compared to *Ae15848* and *Ae8505* in almost all the time points (Table 2).

According to one-way ANOVA test results all the three genes showed a significant change in the expression with the age of female mosquitoes (Table 3). The expression of *Ae15848* (*Ae. aegypti* $F = 12.34$, $p < 0.05$, *Ae. albopictus* $F = 43.94$, $p < 0.05$) and *Ae8505* (*Ae. aegypti* $F = 19.62$, $p < 0.05$, *Ae. albopictus* $F = 52.04$, $p < 0.05$) showed the most significant and more consistent change in its expression with the age of mosquitoes compared to the gene *Ae4274* (*Ae. aegypti* $F = 8.50$, $p < 0.05$, *Ae. albopictus* $F = 9.12$, $p < 0.05$). Analysis using two-way ANOVA showed that, the expression of *Ae15848* ($F = 34.99$, $p < 0.05$) and *Ae8505* ($F = 36.69$, $p < 0.05$) is significantly greater in *Ae. aegypti* than that for *Ae. albopictus* while *Ae4274* did not show any significant difference in its expression between these two species ($F = 0.37$, $p = 0.547$).

Table 2
Logcontrast of Ct/Transcriptional abundance recorded for *Ae. aegypti* and *Ae. albopictus* females at 27^0C

Age (days)	Logcontrast of Ct of *Ae15848*		Logcontrast of Ct of *Ae8505*		Logcontrast of Ct of *Ae4274*	
	Ae. aegy.	*Ae. albo.*	*Ae. aegy.*	*Ae. albo.*	*Ae. aegy.*	*Ae. albo.*
1	-0.090 ± 0.016	-0.074 ± 0.012	-0.040 ± 0.004	-0.006 ± 0.007	0.138 ± 0.025	0.143 ± 0.013
5	-0.048 ± 0.008	-0.025 ± 0.005	0.080 ± 0.009	0.086 ± 0.002	0.112 ± 0.003	0.100 ± 0.006
9	-0.037 ± 0.011	-0.013 ± 0.008	0.074 ± 0.024	0.096 ± 0.003	0.101 ± 0.012	0.095 ± 0.008
13	-0.026 ± 0.022	0.012 ± 0.014	0.080 ± 0.022	0.116 ± 0.012	0.101 ± 0.022	0.108 ± 0.014
17	-0.008 ± 0.009	0.018 ± 0.012	0.087 ± 0.017	0.108 ± 0.006	0.104 ± 0.007	0.104 ± 0.010
21	-0.015 ± 0.017	0.015 ± 0.002	0.076 ± 0.023	0.110 ± 0.006	0.087 ± 0.012	0.089 ± 0.007
25	-0.010 ± 0.009	0.025 ± 0.009	0.077 ± 0.004	0.117 ± 0.009	0.092 ± 0.015	0.093 ± 0.009
29	0.032 ± 0.033	0.030 ± 0.004	0.117 ± 0.026	0.118 ± 0.005	0.095 ± 0.017	0.100 ± 0.008

Table 3
Results of one-way ANOVA of *Ae. albopictus* and *Ae. aegypti* from different experimental designs

Gene	F value			
	Ae. aegypti Eight age classes	*Ae. albopictus* Eight age classes	*Ae. aegypti* in 23^0C, five age classes	*Ae. aegypti* in 27^0C five age classes
Ae15848	12.34*	43.94*	42.44*	14.41*
Ae8505	19.62*	52.04*	11.55*	30.74*
Ae4274	8.50*	9.12*	10.46*	5.73*
*Significant ($p < 0.05$)				

Expression levels of age responsive genes of *Ae. aegypti* at 23 ^{0}C

Expression levels of the three age responsive genes; *Ae15848*, *Ae8505* and, *Ae4274* were determined at 23^0C for five age classes (1, 5, 9, 13 and, 17 days) of female *Ae. aegypti* and the transcriptional profiles obtained are shown in Fig. 3. The expression patterns of all three genes at 23^0C were similar to those of *Ae. aegypti* mosquitoes at 27^0C, that is the expression of *Ae15848* and *Ae8505* decreased with the age of mosquito while *Ae4274* showed an increase in its expression with the mosquito age. The expression of the *Ae15848* gene was highest at all the five-time points (log contrast value ranging from 0.073 ± 0.005 at day 29 to -0.071 ± 0.017 at day 1) compared to the other two genes. The transcriptional abundance of *Ae8505* was highest in 29 day old mosquitoes (0.151 ± 0.009) and lowest in 1 day old mosquitoes (0.014 ± 0.038). The expression of *Ae4274* increased with the age of mosquitoes at both temperatures (transcriptional abundance ranging from 0.138 ± 0.013 to 0.201 ± 0.008). Similar to 27^0C results, the change in expression levels of the three genes with female mosquito age was significant at 23^0C (Table 3). *Ae15848* (23^0C $F = 42.44$, $p < 0.05$) showed a more consistent change in its expression with age of mosquitoes compared to *Ae8505* (23^0C $F = 11.55$, $p < 0.05$) and *Ae4274* (23^0C $F = 10.46$, $p < 0.05$). Further, the transcriptional abundance was greater for *Ae. aegypti* at 23^0C compared to 27^0C indicating a lower expression at 23^0C (Fig. 3).

Multivariate calibration models and age predictions

Aedes aegypti and *Ae. albopictus* from 27^0C

Calibration models were generated separately for each species using multivariate canonical redundancy analysis of the transcriptional abundance of the three genes; *Ae15848*, *Ae8505*, and *Ae4274*. Figure 4a and 4b show the graphs drawn between the first redundancy variate and the actual age of *Ae. aegypti* and *Ae. albopictus* females.

A nonparametric bootstrapping (1000 bootstraps) procedure was used to validate the model generated for each species and, to obtain the 95% confidence intervals (CI) and age predictions (Cook *et al*., 2007). According to the calibration model results, the actual age of both *Ae. aegypti* ($R^2 = 0.8108$, $p < .0001$) and *Ae. albopictus* ($R^2 = 0.8990$, $p < .0001$) females showed a strong positive correlation with the first redundancy variate. The graphs between the predicted age derived from the model and the actual age of *Ae. aegypti* and *Ae. albopictus* are shown in Figs. 4c and 4d. The mean residual value (the difference between the actual age and the predicted age) was 2.19 (± 1.66) for *Ae. aegypti* and 2.58 (± 2.06) days for *Ae. albopictus*.

Further to check the species specificity of models created, data of *Ae. albopictus* was used as the test data on the model generated for *Ae. aegypti* to predict the ages of *Ae. albopictus* at each time point *vice versa*. The age of *Ae. albopictus* predicted using the *Ae. aegypti* model overestimated (mean residual value of 6.77 ± 1.41 days) (Fig. 4e) the age of *Ae. albopictus* while *Ae. albopictus* model estimated the age of *Ae. aegypti* 7.4 ± 1.41 days (residual value) lower than the actual age of the mosquito (Fig. 4f).

Aedes aegypti from 27^0C and 23^0C

Linear age prediction models were generated separately for female *Ae. aegypti* at 23^0C and 27^0C using the normalized expressions of the age responsive genes for 5 age classes, 1, 5, 9, 13 and 17 days. Similar to the calibration model for mosquitoes at 27^0C the model developed at 23^0C also showed a strong linear positive correlation with the age of female mosquitoes (23 ^{0}C; $R^2 = 0.9222$, $p < 0.0001$) (Fig. 5a and 5b). According to the nonparametric bootstrap method, the residual value for *Ae. aegypti* at 27^0C was 2.19 (± 1.66) days and at 23^0C was 3.42 (± 2.74) (Fig. 5c and 5d).

To test the effect of temperature on the age prediction accuracy, cross predictions were conducted. When the age of *Ae. aegypti* females reared in 27^0C were cross-checked with the model generated for *Ae. aegypti* females in 23^0C, the age of *Ae. aegypti* was overestimated [residual value 3.93 (± 3.00) days] (Fig. 5e). The 27^0C model under-estimated (mainly the younger age classes showed the highest deviation) the age of *Ae. aegypti* females from 23^0C [residual value of 5.82 (± 3.57)] (Fig. 5f).

Discussion

Transcriptional age grading is a molecular-based technique with high precision and accuracy that could be used to determine the chronological age of mosquitoes. However, the models developed using this approach should be validated and optimized for mosquito strains from different geographical areas and adjusted for variations of environmental factors, especially temperature prevail in such areas [6]. Hence, the present study was conducted to develop a multivariate calibration model for *Ae. aegypti* and *Ae. albopictus* from Sri Lanka using transcriptional age grading technique that could be later used in estimating the age structure of wild mosquito populations. Further, the study attempted to understand the effect of temperature on this age grading technique.

In Sri Lanka, the highest number of dengue incidences and deaths every year is reported from the dry zone where the annual temperature is 27.5 ^{0}C covers a major part of the island and this is the area that reports. Further, the entire country experiences relatively high humidity averaging around 80% throughout the year [25]. Hence, the mosquitoes used in the present study were maintained in insectaries with 27^0C ± 2 and 80% ± 10 relative humidity simulating the field conditions. Transcriptional age grading studies on *Ae. aegypti* [2, 4] and *An. gambiae* [9] have clearly shown that the models constructed for mosquitoes reared in laboratory or semi-field conditions could be successfully used to assess the age of wild mosquito populations. Therefore, the multivariate calibration models that were constructed during this study will be a useful tool in predicting the age of wild populations of *Ae. aegypti* and *Ae. albopictus* in Sri Lanka.

For both Sri Lankan *Ae. aegypti* and *Ae. albopictus* the pattern of gene expression was similar to the previous reports *i.e.* reduction in the expression of *Ae15848* and *Ae8505* genes and an increase in *Ae4274* gene expression, with the age of the female mosquito [3, 4, 6, 8, 11]. In all the experimental designs, the change in expression of these three genes was significant with the age of female mosquito, further

proving the fact that these are among the most informative age responsive genes that could be successfully used in the transcriptional age grading approach.

The expression levels of all three genes were high in both *Ae. aegypti* and *Ae. albopictus* strains from Sri Lanka than the mosquito strains from other countries. Further, during the current study, the expression of *Ae15848* was significantly higher than the other two genes and showed a consistent change with the mosquito age. This gene has shown a similar strong negative correlation with the chronological age of female *Ae. aegypti* [2, 4] and *An. gambiae* [9] mosquitoes from other parts of the world. *Aedes aegypti* has shown a four-fold increase in the log contrast of the same gene from 1 to 29 day old mosquitoes in a study carried out in Northern Australia [4], and around 1.5 fold increase in mosquitoes from Central Vietnam [11] and Queensland, Australia [3]. Gene, *Ae8505* of both species showed a rapid decrease in gene expression from day 1 to day 5 and more consistent change thereafter, similar to previous reports. The expression change of gene *Ae4274* with the age of mosquitoes was significant although it vaires along with a small range as observed for *Ae. aegypti* females from Australia and Vietnam [3, 4, 11].

Compared to calibration models developed previously for *Ae. aegypti* by Cook et al. [3] ($R^2 = 0.73$) and Hugo et al. [4] ($R^2 = 0.72$), the two multivariate calibration models for both species used for the present study showed a strong correlation with mosquito age (R^2 for *Ae. aegypti* = 0.8108, $p < .0001$ and R^2 for *Ae. albopictus* = 0.8990, $p < .0001$). Although, Cook et al. [3], have stated that the accuracy of the age estimation using this technique is only ± 5 days for *Ae. aegypti* the accuracy was greater in this study for both species [2.19 (± 1.66) days for *Ae. aegypti* and 2.58 (± 2.06) days for *Ae. albopictus*]. Studies using the same approach have reported only a 3.17 (± 2.16) days and 4.3 (± 4.2) days accuracy in determining the age of *Ae. aegypti* [12] and *An. gambiae* respectively [8]. These facts indicate the high precision and accuracy of the age prediction models generated during the current study. Hence, this transcriptional age grading could be successfully used in the age structure determination of wild populations of both *Ae. aegypti* and *Ae. albopictus* strains of Sri Lanka. Further, these findings provide strong evidence that the transcriptional age grading must be validated and optimized for mosquito strains from different geographical locations as stated by Cook et al. [6].

The age of *Ae. albopictus* was overestimated when the data is cross-checked with the *Ae. aegypti* model and, the age prediction accuracy decreases significantly from 2.58 (± 2.06) days to 6.77 (± 1.41) days. *Ae. aegypti* age will be underestimated and age prediction accuracy decrease from 2.19 (± 1.66) to 7.4 (± 3.41) days when crosschecked with *Ae. albopictus* model. This analysis strongly supports the species-specific nature of the multivariate calibration models (although of the same genus) generated using the transcriptional age grading technique.

Previous research work has reported a 4^0C temperature difference as the minimum range that could have a significant effect on the survival of both mosquito larvae and adult [26, 27, 28, 29]. Hence, the model developed for *Ae. aegypti* colonies maintained at 27 ^{0}C was compared with mosquitoes reared at 23^0C (4^0C difference) to check the effect of temperature on this molecular based age grading approach. According to the results, the expression of the three age responsive genes for mosquitoes from 23^0C was

lower than mosquitoes from 27^0C. Around 92% of the gene expressions were age related at 23^0C while it is 81.01% at 27^0C. According to the analysis conducted using nonparametric bootstrap, the accuracy of age predictions did not show a considerable difference between the two temperatures (residual value 2.19 ± 1.66 days at 27 ^{0}C and 3.42 ± 2.74 days at 23 ^{0}C).

According to the results of the cross-validation, the age prediction accuracy of mosquitoes from 27 ^{0}C decreased slightly from 2.19 (± 1.66) days to 3.93 (± 3.00) days and from 3.42 (± 2.74) to 5.82 (± 3.57) days for mosquitoes reared in 23 ^{0}C. It could be stated that a temperature difference of 4 ^{0}C affects expression of age responsive genes to a lesser extent than the difference exists between two species under the same temperature. Similar observations have been reported by Hugo et al. [4] and have suggested generating separate calibration models for inter-seasonal variations for different regions that show a greater variation in climatic factors. Sri Lanka is a small island and does not show drastic changes in temperature between areas within the country. Hence, the models developed during this study could be used to determine the age structure of wild mosquito populations from the whole country. Depending on the average temperature at the time of investigation the most appropriate model to be used can be determined.

Studies will be conducted in the future to determine the age of wild mosquitoes using the multivariate calibration models generated during this study.

Conclusion

This is the first report from Sri Lanka on the use of the transcriptional age grading technique to determine the age of mosquitoes and the first study to develop a model for *Aedes albopictus*. The species-specific multivariate calibration models created using the age responsive genes, *Ae15858*, *Ae8505*, and *Ae4274* could successfully estimate the chronological age of wild *Ae. aegypti* and *Ae. albopictus* with higher accuracy than previously reported in other mosquito species. The age of mosquitoes could be predicted with an accuracy of nearly ± 3 days of their actual age, across the age spectrum. A drop in temperature from 27^0C to 23^0C did not have a very strong effect on the multivariate calibration models.

List Of Abbreviations

EIP- extrinsic incubation period

RH- relative humidity

qRT-PCR- quantitative real-time polymerase chain reaction

Ct- Cycle Threshold

FC- Fold Change

CI- confidence intervals

Declarations

Ethics approval and consent to participate

“Not Applicable”

Consent for publication

“Not Applicable”

Availability of data and materials

All data generated during this study are included in this published article

Competing interests

"The authors declare that they have no competing interests"

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Authors' contributions

CSW, SHPPK, LR and TCW conceptualized and designed the work. TCW, LSR and CSW conducted molecular laboratory work. TCW, WAPPdeS and CW analyzed the data. All contributed to the discussion and writing the manuscript. All authors read and approved the final manuscript.

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Figures

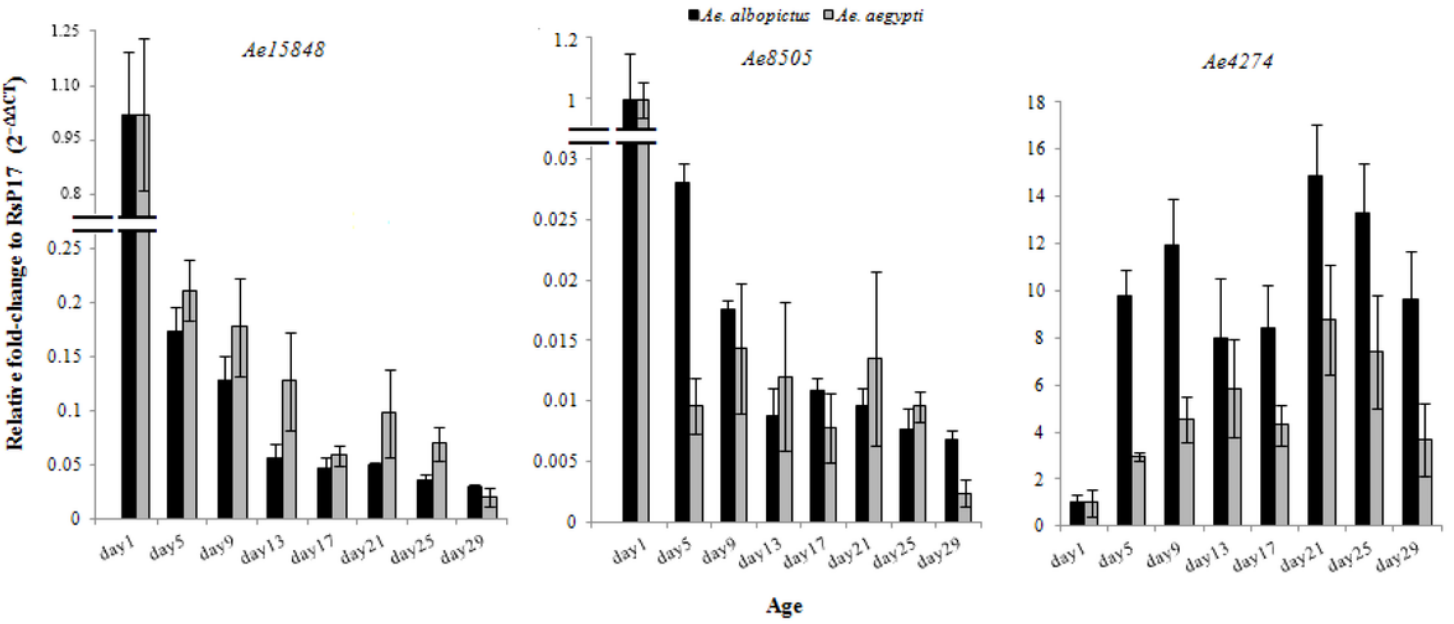

Figure 1

Fold change of age responsive genes Ae15848, Ae8505 and Ae4274 relative to the expression of one day old mosquitoes of Ae. aegypti and Ae. albopictus, as determined by Qrt-PCR analyses (Error bars represent SEM).

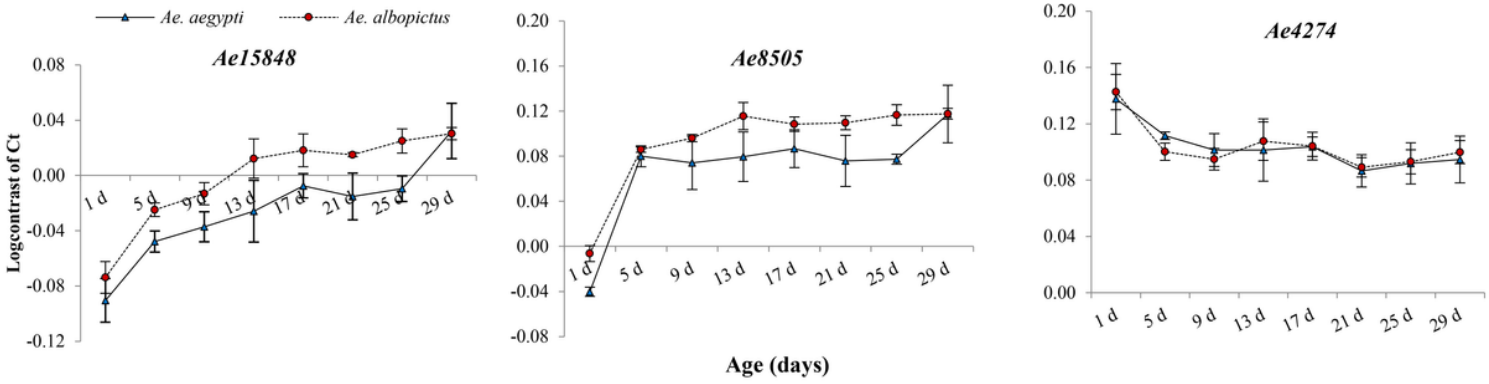

Figure 2

Logcontrast values obtained for the three age responsive genes Ae15848, Ae8505 and Ae4274 at each time point of Ae. aegypti and Ae. albopictus female mosquitoes reared in 27°C temperature.

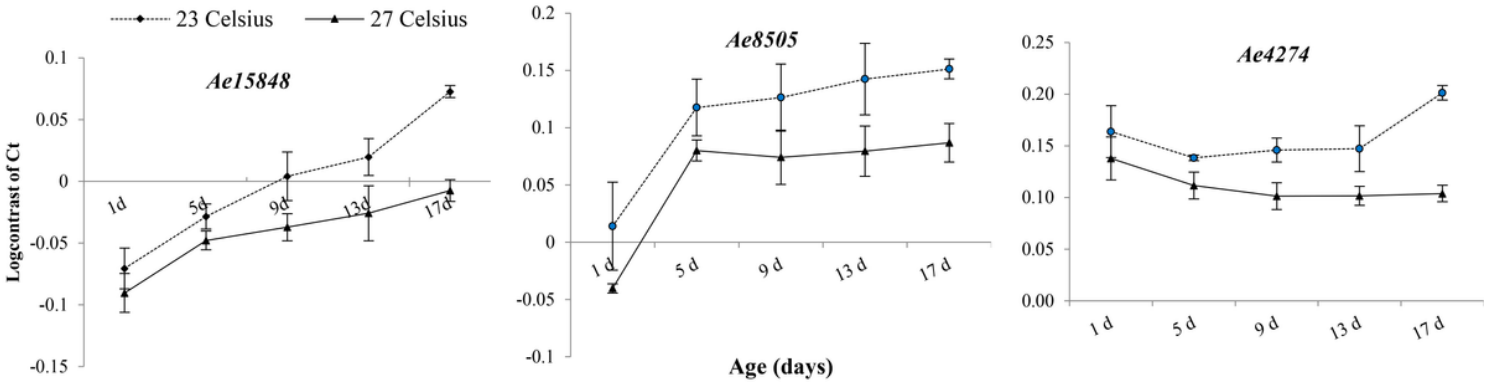

Figure 3

Logcontrast values obtained for the three age responsive genes, Ae15848, Ae8505 and Ae4274 at five age classes (1, 5, 9, 13 and 17 days) of Ae. aegypti females reared at 27°C and 23°C.

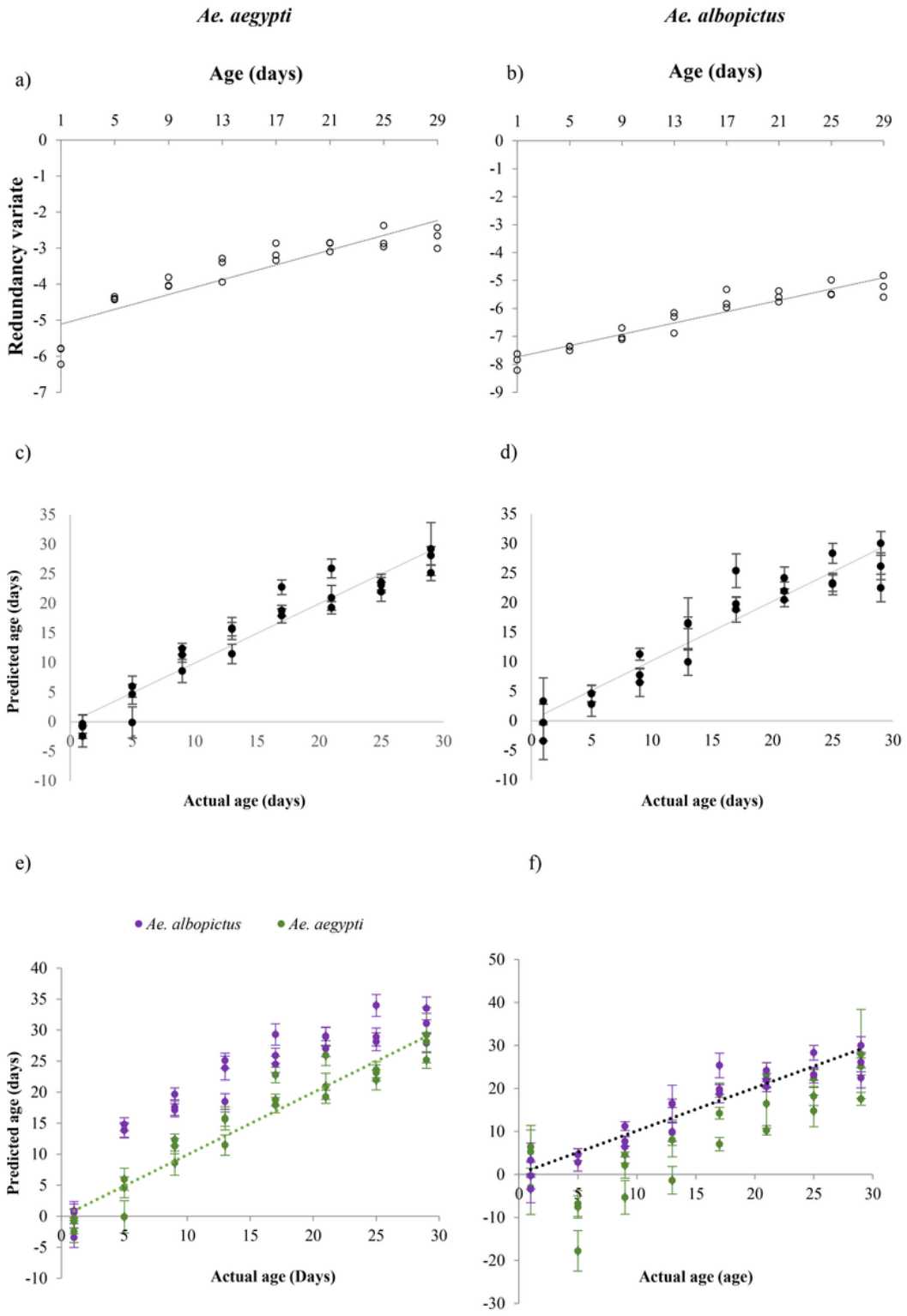

Figure 4

Calibration models generated for a) Ae. aegypti and b) Ae. albopictus female mosquitoes using the transcriptional profiles of the three age responsive genes; Ae15848, Ae8505 and Ae4274 c); Age predictions of c) Ae. aegypti d) Ae. albopictus female mosquitoes at eight age class; Cross validation, e) Ae. albopictus data checked on Ae. aegypti model f) Ae. aegypti data checked on Ae. albopictus model (The dash lines indicate where predicted age equals actual age).

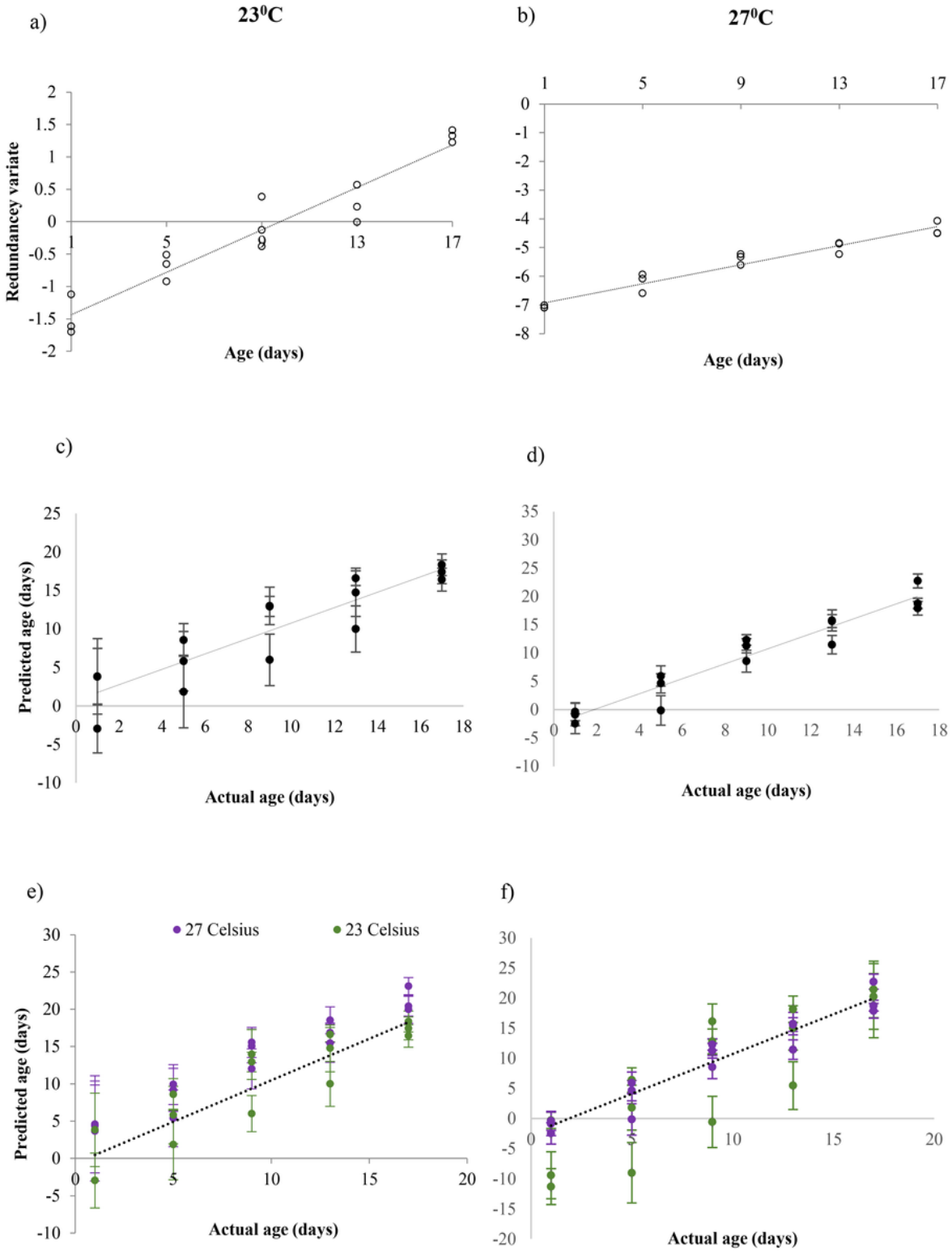

Figure 5

Calibration models generated for female Ae. aegypti mosquitoes reared at a) 230C and b) 270C using the transcriptional profiles of the three age responsive genes; Ae15848, Ae8505 and Ae4274 c); Age predictions of female mosquitoes Ae. aegypti reared at c) 23°C d) 27°C at five age class; Cross validation, e) 27°C data checked on 23°C model f) 23°C data checked on 27°C model (The dash lines indicate where predicted age equals actual age).

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