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Differentiation of *Aedes aegypti* and *Aedes notoscriptus* (Diptera: Culicidae) eggs using scanning electron microscopy

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ABSTRACT

Aedes notoscriptus and *Aedes aegypti* are both peri-domestic, invasive container-breeding mosquitoes. While the two potential arboviral vectors are bionomically similar, their sympatric distribution in Australia is limited. In this study, analyses of *Ae. aegypti* and *Ae. notoscriptus* eggs were enabled using scanning electron microscopy. Significant variations in egg length to width ratio and outer chorionic cell field morphology between *Ae. aegypti* and *Ae. notoscriptus* enabled distinction of the two species. Intraspecific variations in cell field morphology also enabled differentiation of the separate populations of both species, highlighting regional and global variation. Our study provides a comprehensive comparative analysis of inter- and intraspecific egg morphological and morphometric variation between two invasive container-breeding mosquitoes. The results indicate a high degree of intraspecific variation in *Ae. notoscriptus* egg morphology when compared to the eggs of *Ae. aegypti*. Comparative morphological analyses of *Ae. aegypti* and *Ae. notoscriptus* egg attributes using SEM allows differentiation of the species and may be helpful in understanding egg biology in relation to biotope of origin.

1. Introduction

Aedes notoscriptus mosquitoes are capable of transmitting Ross River virus (RRV), Barmah Forest virus (BFV), and Rift Valley fever (RVF) (Sota and Mogi, 1992; Turell and Kay, 1998; Watson and Kay, 1998, 1999; Watson et al., 2000; Harley et al., 2001). *Ae. aegypti* is a known vector of dengue and is also a potential vector of RRV (Watson and Kay, 1999; Harley et al., 2000). *Ae. aegypti* distribution is limited to tropical and sub-tropical environments while *Ae. notoscriptus* is found throughout the south-west Pacific islands and in all states of Australia, including many Australian off-shore islands (Lee et al., 1982).

While the two species are bionomically similar, the only region of *Ae. notoscriptus* and *Ae. aegypti* sympatry in Australia is in tropical north-east Queensland, where the two species appear to coexist in equilibrium (Tun-Lin et al., 1999). *Ae. notoscriptus* and *Ae. aegypti* are both peri-domestic, container-breeding mosquitoes, laying their eggs at or above the water line on the edge of natural and artificial receptacles, where eggs may remain dry between inundations. This ability to resist desiccation enables persistence of the species within their current ranges (Kearney et al., 2009; Williams et al., 2010). Mosquito egg desiccation resistance has also been strongly correlated with egg size, suggesting morphological bases for the trait (Sota and Mogi, 1992).

Comparative intraspecific egg studies of several anopheline mosquitoes have revealed variations in egg surface morphology which have been correlated with geographical differentiation of the populations (Linley et al., 1993a, 1996; Lounibos et al., 1999). Furthermore, scanning electron microscopy (SEM) of eggs of four strains of the *Culex quinquefasciatus* species complex revealed variation in egg morphometrics, suggesting an influence of ecological variation (Suman et al., 2009). The chorion structure has been useful in distinguishing some *Stegomyia* mosquito species, through comparative SEM analyses (Matsuo et al., 1974; Linley, 1989).

Phylogenetic analyses of geographically distinct *Ae. notoscriptus* populations have recently suggested the species is rather a complex of genetic lineages (Endersby et al., 2013). Similarly, allelic variation has been noted in Australian populations of *Ae. aegypti* (Endersby et al., 2009). SEM analysis of *Ae. notoscriptus* eggs obtained from Sydney, Australia (Linley et al., 1991) and *Ae. aegypti* from Florida (Linley, 1989) have previously been conducted, however, inter- and intraspecific comparisons or statistical differentiations have not been made. In the present study, we utilised SEM to analyse the

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eggs of two Australian populations of both *Ae. aegypti* and *Ae. notoscriptus* from geographically and climatically distinct regions, to enable inter- and intraspecific comparisons of the egg morphology and ultra-structural morphology. We hypothesised that there would be significant differences in the eggshell morphology of these two mosquitoes, given they are distinct species, with variable geographic distribution. Eggshell analyses conducted using SEM will contribute to our understanding of invasive container-breeding mosquito ecology and eggshell morphology.

2. Materials and methods

2.1. Egg source

Ae. aegypti eggs were obtained from laboratory populations originating in Cairns and Charters Towers (Australia), maintained at 24 °C, 90% RH (Faull and Williams, 2015). At the time of initial egg collection, the colonies had experienced approximately 60 generations. As *Ae. notoscriptus* is a notoriously difficult species to establish in colony (Watson et al., 2000), wild-type eggs were obtained from two locations where established populations have been long resident, Sydney, NSW (Russell, 1986; Strid, 2008) and Adelaide, SA (Williams et al., 1999). *Ae. aegypti* and *Ae. notoscriptus* eggs were obtained following procedures outlined by Faull and Williams (2015) and Faull et al. (in press) respectively. Egg collections spanned 2010 and 2014 to ensure no cohort-specific effects.

2.2. Sample preparation and fixation

Eggs remained *in situ* on the oviposition substrate during preparation for scanning electron microscopy (SEM). Small swatches of the coffee filter paper substrate were cut to obtain small batches of eggs. Egg batches were obtained from the oviposition substrates at random, to ensure the samples included eggs of different female mosquitoes. Eggs were pre-fixed for at least one hour in 4% paraformaldehyde/1.25% glutaraldehyde in PBS +4% sucrose, pH 7.2, prior to dehydration in a graded ethanol series.

2.3. Sample drying methods

The eggs were dried by submission to the superdry CO_2 critical point drying (CPD) method (Nation, 1983) using a Balzers Critical Point Dryer (BAL-TEK model CPD 030). The flooding and venting process was performed for eight cycles to purge all ethanol. Finally, the eggs were immersed in CO_2 at 41 °C for 10 min for critical point drying, before slow venting.

Hexamethyldisilazane (HMDS) was used as an alternative drying method (Nation, 1983) for replicate batches due to egg surface damage during CPD. The HMDS method has previously been used for aedine mosquito eggs (Jarial, 2001). The eggs were immersed in equal parts HMDS and 100% ethanol for 10 min before immersion with 100% HMDS (10 min × 2) and air-drying at room temperature.

The Adelaide *Ae. notoscriptus* sample size was the largest (20 eggs) and also contained eggs prepared for SEM using both the CPD and HMDS drying techniques. T-tests were therefore performed on Adelaide *Ae. notoscriptus* morphological data (including ultra-structural morphology) to determine whether the drying methods resulted in any variation in attribute structure and consequent measurement (Table 1).

The lack of any significant variations in morphometrics in Adelaide *Ae. notoscriptus* eggs between the critical point and hexamethyldisilazane techniques is consistent with previous comparative method studies (Bray et al., 1993; Araujo et al., 2003). We are therefore confident there was no significant effect of drying technique choice on SEM results and measurements obtained from all specimens, and recommend the use of HMDS in the SEM preparation of mosquito egg samples to reduce surface damage.

Table 1
Egg attribute comparison between SEM drying methods: critical point drying (CPD) and hexamethyldisilazane (HMDS) in Adelaide *Ae. notoscriptus* eggs.

Attribute	CPD	HMDS	*P*-value
Length:Width	3.33	3.46	0.29
Micropylar diameter	37.2	38.34	0.5
Micropylar collar width	7.98	6.9	0.18
Anterior EN width	2.1	1.8	0.28
Post EN width	1.8	1.7	0.37
Anterior OCC-L	12.5	12.1	0.31
Posterior OCC-L	12.99	13.3	0.63
Anterior OCC-W	18.1	17.1	0.14
Posterior OCC-W	18.8	18.9	0.88
Anterior CT diameter	6.9	6.5	0.29
Posterior CT diameter	6.7	6.6	0.75

All values are in mean and are given in μm.
Abbreviations: EN, exochorionic network; OCC-L, outer chorionic cell length; OCC-W, outer chorionic cell width; CT, central tubercle.

2.4. Scanning electron microscopy (SEM)

Eggs were mounted for microscopy by attachment of the oviposition substrate to SEM stubs, using double-sided tape. Stub surfaces were then sputter coated with carbon/gold. Images of the eggs were obtained using Philips XL30 scanning electron microscope. Sample sizes varied between species and populations with 15 and 20 *Ae. notoscriptus* eggs from Sydney and Adelaide respectively, and 15 and 19 *Ae. aegypti* eggs from Cairns and Charters Towers respectively.

2.5. Egg morphological analyses

ImageJ Software version 1.48 (ImageJ, Wayne Rasband, National Institutes of Health, USA) was used to analyse the eggs and obtain morphological and ultra-structural morphometrics. Terminology for the analysis of egg morphology was adopted from Linley (1989), Linley et al. (1991) and Farnesi et al. (2009), whereby the orientation of eggs is as follows: the surface of the egg facing the substrate is dorsal, the opposite side is ventral, the pole possessing the micropylar apparatus is anterior and the slightly narrower, tapered pole is posterior. Terminology regarding the layers and morphological attributes of mosquito eggshells is a matter of literary controversy. Therefore we conform to revised nomenclature (Harbach and Knight, 1978; Monnerat et al., 1999; Rezende et al., 2008).

2.5.1. Egg morphometrics

Seven attributes of each egg were analysed. The length and width of the eggs was measured, from which the length to width ratio was calculated. Further morphological parameters measured included egg width at 1/3 anterior end, width at 1/3 posterior end, micropylar apparatus diameter and micropylar collar width.

2.5.2. Egg ultra-structural morphometrics

To analyse egg ultra-structural morphology, ten topographic attributes of each egg were measured, including outer chorionic cell width and length (10 replicates of each), from which the cell length to width ratio was also calculated. Replicate measurements (n) of each ultra-structural attribute were obtained from both the anterior and posterior poles of each egg. The exochorionic network width was measured (5 replicates per egg pole), as was the central tubercle diameter (10 replicates per pole). Central tubercle density was calculated as number of tubercles within a randomly selected 900 μm^2 area at both the anterior and posterior pole of each egg.

Table 2
Comparative egg morphology and ANOVA results.

Attributes	*Aedes aegypti*		*Aedes notoscriptus*		*P*-value
	Cairns ($n = 15$)	Charters Towers ($n = 19$)	Sydney ($n = 15$)	Adelaide ($n = 20$)	
Whole egg					
Egg length	554.41 ± 36.56	562.62 ± 30.85	565.40 ± 15.07	570.50 ± 20.61	0.376
Egg width	167.65 ± 7.05ab	160.15 ± 9.73b	148.04 ± 7.39c	170.37 ± 11.03a	<0.001
Egg width at 1/3 anterior end	161.82 ± 6.08ab	157.54 ± 9.13b	146.31 ± 7.31c	166.01 ± 11.93a	<0.001
Egg width at 1/3 posterior end	152.03 ± 5.8a	146.95 ± 5.5a	130.54 ± 6.84c	152.02 ± 12.49a	<0.001
L:W[a]	3.31 ± 0.18a	3.52 ± 0.27b	3.83 ± 0.16	3.36 ± 0.23ab	<0.001
Micropylar region					
Apparatus diameter[a]	33.49 ± 3.9a	34.19 ± 5.4a	37.00 ± 1.39ab	37.48 ± 3.30b	0.006
Collar width[a]	6.37 ± 0.93a	6.13 ± 1.42a	7.17 ± 0.87ab	7.70 ± 1.60b	0.0012

All values are in mean ± standard deviation and are given in μm (excluding L:W ratio). No significant differences denoted by letters, as determined by Tukey's post-hoc analysis.
[a] Attributes used within PCA analysis.

2.6. Statistical analyses

A one-way analysis of variance (ANOVA) was performed to determine attribute variations between populations of the two species and Tukey's post-hoc analyses were performed to determine variations ($P < 0.05$) between each of the four sample groups, for each morphological and ultra-structural morphological parameter, using GraphPad Prism version 6.01.

A principal component analysis (PCA) was performed incorporating morphological and ultra-structural morphological data using Stata version 11.0 (StataCorp, College Station, TX). Egg length and width values were not included in the PCA as these attributes may be influenced by other life history stages, particularly larval food supply, density and temperature, in turn determining adult size and consequent egg size (Steinwascher, 1984). Derived characters including egg length to width ratio however, can be useful in differentiating mosquito populations (Linley et al., 1993b). Where applicable, ratio values as opposed to individual attribute measurements were therefore included in the PCA to control for any potential influence of life history. Attributes used within the PCA are denoted in Tables 2 and 3.

3. Results

3.1. SEM drying method comparison

The ultra-structural morphology of Adelaide *Ae. notoscriptus* egg attributes did not differ between SEM drying methods (Table 1).

3.2. Egg morphology

The eggs of both species are broadly cigar shaped, prolate spheroids (Fig. 1). Egg length did not differ between the four populations (Table 2) while egg width of Sydney *Ae. notoscriptus* differed from Adelaide ($P < 0.0001$), and both populations of *Ae. aegypti* (Cairns $P < 0.0001$; Charters Towers $P = 0.002$). Adelaide *Ae. notoscriptus* egg width also differed significantly from Charters Towers *Ae. aegypti* ($P = 0.005$) but not from Cairns. Cairns and Charters Towers egg width did not vary ($P = 0.094$). Width at the anterior third varied between all groups ($P < 0.027$) except Adelaide *Ae. notoscriptus* and Cairns *Ae. aegypti* ($P = 0.544$) and Cairns and Charters Towers *Ae. aegypti* ($P = 0.535$). Width at the posterior third for Sydney *Ae. notoscriptus* eggs was significantly different when compared to Adelaide, Cairns and Charters Towers ($P < 0.0001$).

Table 3
Comparative analysis of egg ultra-structural morphology and ANOVA results.

Attributes	*Aedes aegypti*		*Aedes notoscriptus*		*P*-value
	Cairns ($n = 15$)	Charters Towers ($n = 19$)	Sydney ($n = 15$)	Adelaide ($n = 20$)	
Anterior surface					
EN-W ($n = 5$)[a]	1.59 ± 0.54a	1.87 ± 0.58b	2.58 ± 0.49c	2.05 ± 0.51b	<0.0001
OCC-W ($n = 10$)	23.62 ± 3.58a	23.43 ± 2.77a	18.73 ± 3.05b	17.95 ± 2.76b	<0.0001
OCC-L ($n = 10$)	13.58 ± 2.31a	12.96 ± 2.65abc	13.96 ± 2.26ab	12.44 ± 2.04c	0.0022
OCC-R ($n = 10$)[a]	0.58 ± 0.11a	0.56 ± 0.13a	0.76 ± 0.23b	0.70 ± 0.12c	<0.0001
CT					
Diameter ($n = 10$)[a]	8.44 ± 1.29a	9.00 ± 3.53a	8.12 ± 1.28ab	6.77 ± 0.95b	0.002
Density[a,b]	3.73 ± 0.46a	3.95 ± 0.52ab	4.33 ± 0.49b	4.25 ± 0.44b	0.002
Posterior surface					
EN-W ($n = 5$)[a]	1.44 ± 0.40a	1.91 ± 0.67b	2.35 ± 0.46c	1.75 ± 0.44ab	<0.0001
OCC-W ($n = 10$)	22.82 ± 3.2a	21.05 ± 2.86b	18.89 ± 2.80c	18.79 ± 2.69c	<0.0001
OCC-L ($n = 10$)	13.34 ± 2.59	12.86 ± 2.51	13.30 ± 2.25	13.06 ± 1.92	0.414
OCC-R ($n = 10$)[a]	0.59 ± 0.12a	0.62 ± 0.12a	0.72 ± 0.14b	0.70 ± 0.12b	<0.0001
CT					
Diameter ($n = 10$)[a]	8.12 ± 1.33a	7.54 ± 1.04ab	7.84 ± 1.13ab	6.67 ± 0.98c	<0.0001
Density[a,b]	3.60 ± 0.51	3.58 ± 0.61	3.67 ± 0.62	3.95 ± 0.39	0.125

All values are in mean ± standard deviation, and are given in μm excluding Density.
Abbreviations: EN-W -– exochorionic network width; OCC-L – outer chorionic cell length; OCC-W – outer chorionic cell width; OCC-R – outer chorionic cell length:width; CT – central tubercles.
[a] Attributes used within PCA analysis.
[b] Density of tubercles in 900 μm^2 area. No significant difference denoted by letters, as determined by Tukey's post-hoc analysis.

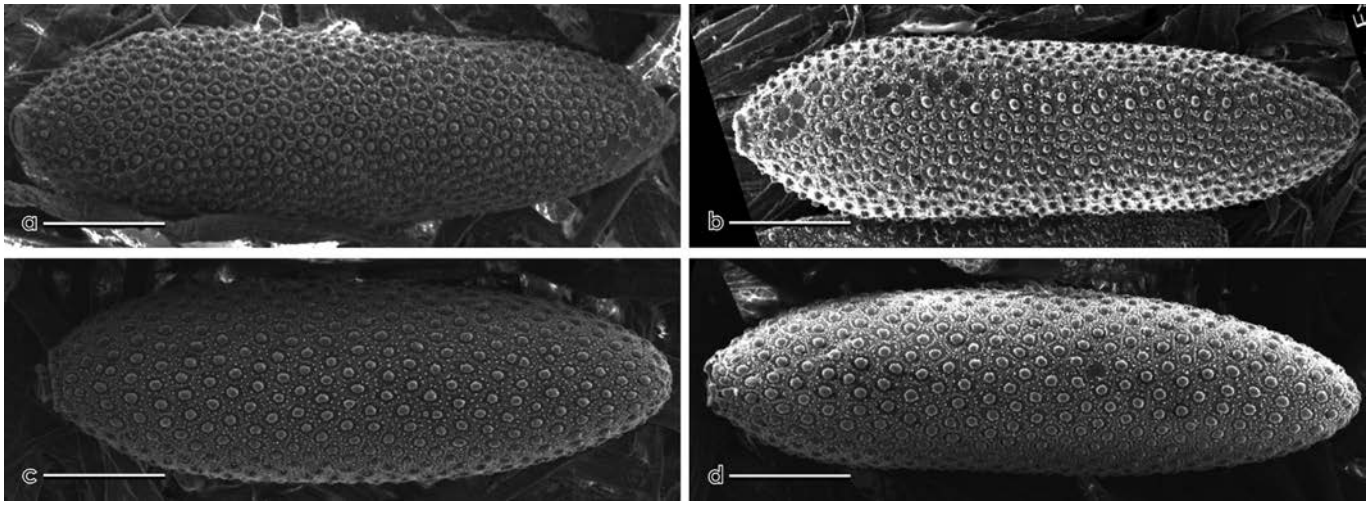

Fig. 1. SEM of entire egg. (a) Adelaide *Ae. notoscriptus*; (b) Sydney *Ae. notoscriptus*; (c) Cairns *Ae. aegypti*; (d) Charters Towers *Ae. aegypti*. Scale 100 μm.

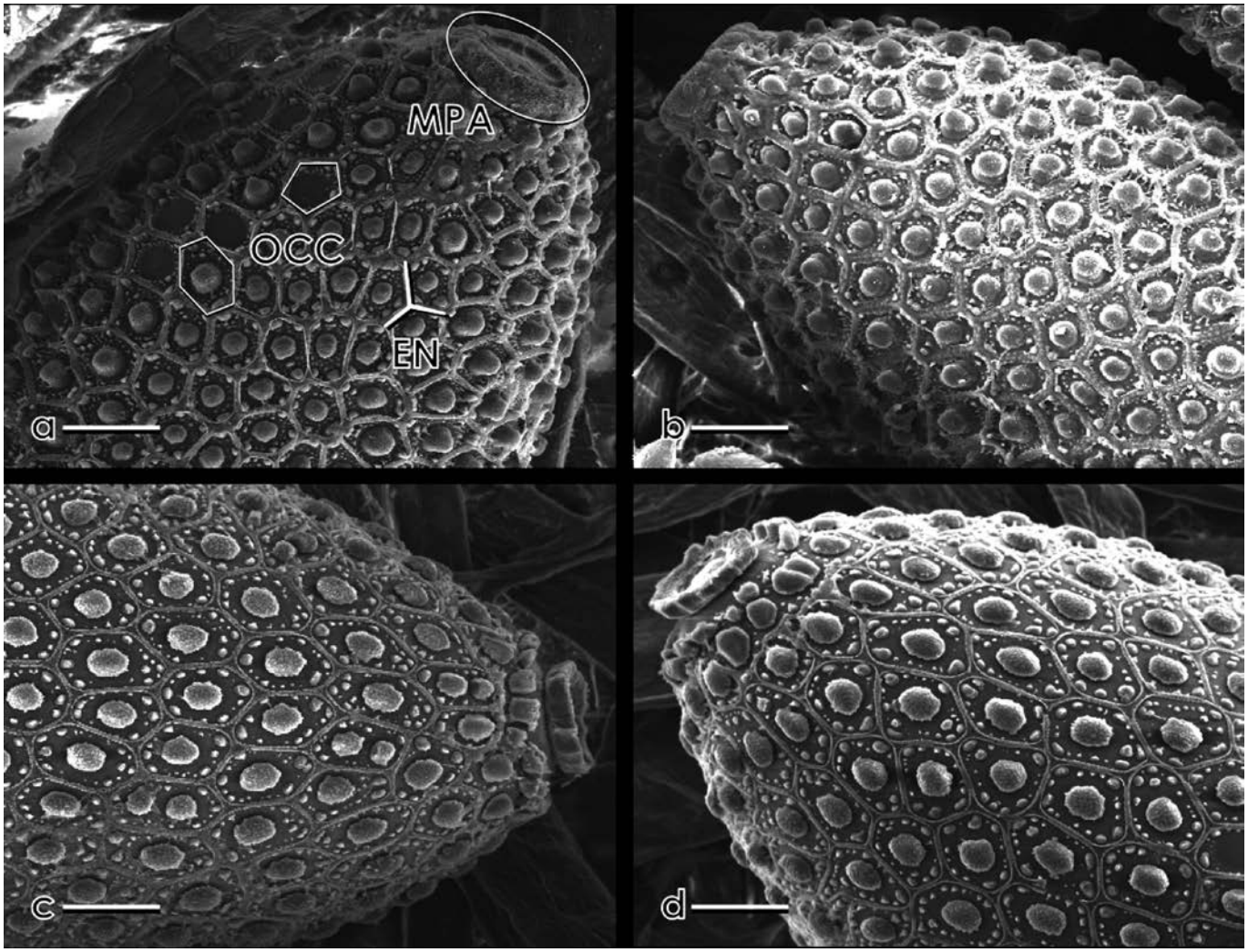

Fig. 2. SEM of anterior egg surface depicting the exochorionic network (EN), micropylar apparatus (MPA) and outer chorionic cells (OCC) of (a) Adelaide *Ae. notoscriptus*; (b) Sydney *Ae. notoscriptus*; (c) Cairns *Ae. aegypti*; (d) Charters Towers *Ae. aegypti*. Scale 25 μm.

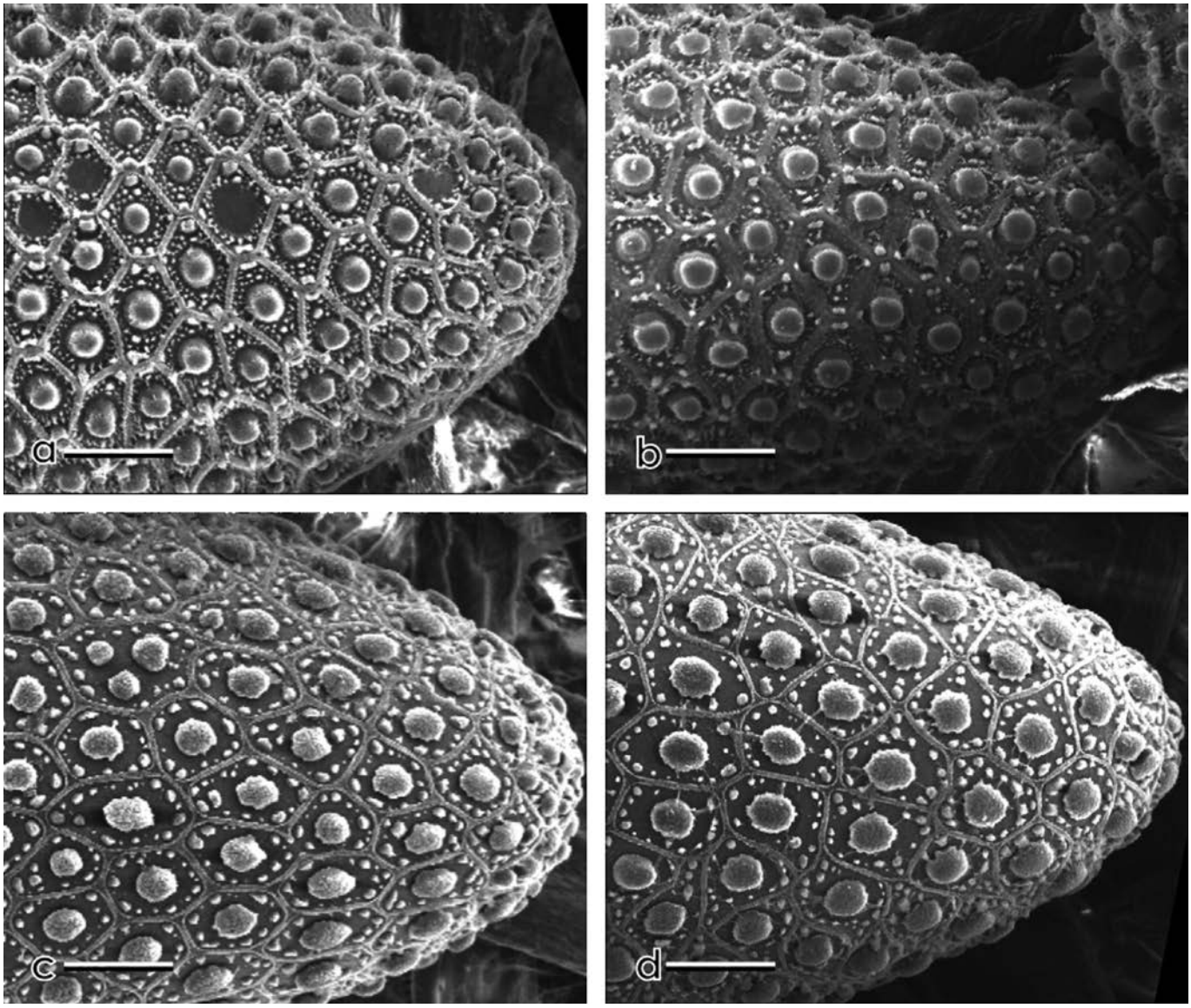

Fig. 3. SEM of posterior egg surface depicting the exochorionic network (EN) and outer chorionic cells (OCC) of (a) Adelaide *Ae. notoscriptus*; (b) Sydney *Ae. notoscriptus*; (c) Cairns *Ae. aegypti*; (d) Charters Towers *Ae. aegypti*. Scale 25 μm.

Length to width ratio varied between all populations ($P < 0.029$) excluding Adelaide when compared to both Cairns and Charters Towers *Ae. aegypti* ($P > 0.1$). Micropylar apparatus diameter (Fig. 2) varied between Adelaide and Cairns ($P = 0.018$) and Charters Towers ($P = 0.048$). Similarly, Adelaide *Ae. notoscriptus* micropylar collar width varied from Cairns ($P = 0.018$) and Charters Towers ($P = 0.002$). The eggs of both Cairns and Charters Towers *Ae. aegypti* possessed an anterior ring, devoid of tubercles and exochorionic network (EN), immediately surrounding the micropylar apparatus. In contrast, the tubercle and EN topography of both Sydney and Adelaide *Ae. notoscriptus* eggs was contiguous with the micropylar apparatus (Fig. 2).

3.3. Egg ultra-structural morphology

The general ultra-structural morphologies of the eggs of *Ae. notoscriptus* and *Ae. aegypti* are largely consistent with the descriptions by Linley et al. (1991) and Linley (1989) respectively. General outer chorionic cell morphology differed between the eggs of *Ae. aegypti* and *Ae. notoscriptus* with the edges and corners of *Ae. notoscriptus* outer chorionic cell fields more defined than *Ae. aegypti* cells (Figs. 2–4). Outer chorionic cells of *Ae. aegypti* and *Ae. notoscriptus* were mostly hexagonal, occasionally pentagonal and rarely heptagonal, and quadrilateral cells were also observed in *Ae. aegypti* eggs (Figs. 2 and 3). *Ae. aegypti* cell fields occasionally contained two or more central tubercles (Figs. 2c, 3c and d) while *Ae. notoscriptus* cell fields contained only one central tubercle (Figs. 2a, b, 3a and b).

Minute tubercles were observed immediately adjacent to the exochorionic network (EN) structure in the eggs of both *Ae. aegypti* and *Ae. notoscriptus* (Figs. 2 and 3). Some spoke-like connections between central and minute tubercles were observed in *Ae. notoscriptus* cell fields but such structures were largely absent in *Ae. aegypti* cell fields (Figs. 4 and 5).

Anterior EN width varied between all eggs ($P \leq 0.04$) excluding Adelaide and Charters Towers ($P = 0.99$). Posterior EN width varied between all groups ($P \leq 0.009$) excluding Adelaide *Ae. notoscriptus* and *Ae. aegypti* eggs from both Cairns ($P = 0.09$) and Charters Towers ($P = 0.58$).

Anterior outer chorionic cell width varied between all eggs ($P < 0.0001$) excluding Sydney and Adelaide *Ae. notoscriptus*

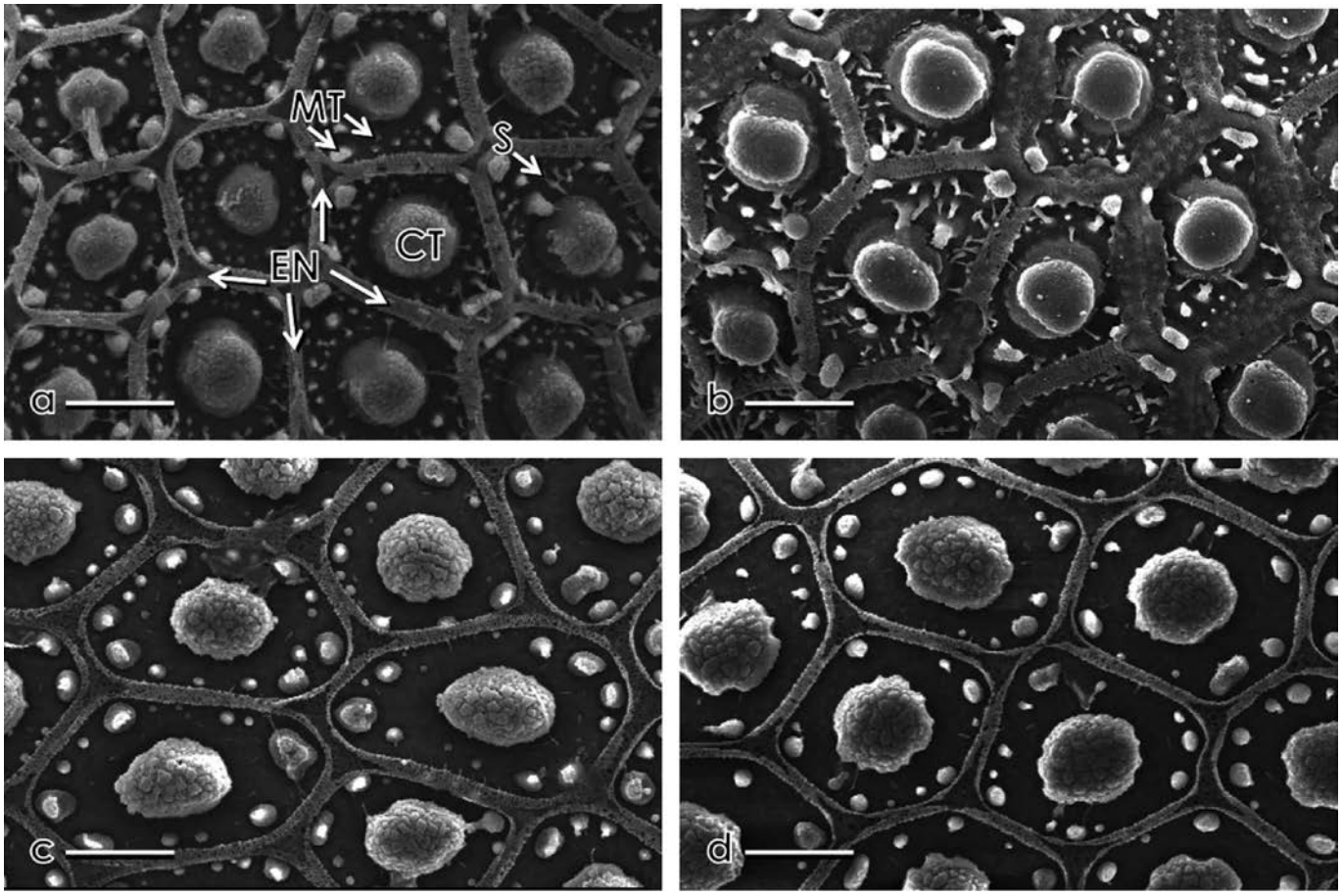

Fig. 4. SEM of outer chorionic cells (OCC) and associated large central tubercles (CT), minute tubercles (MT), spoke-like connections (S) and exochorionic network (EN) for (a) Adelaide *Ae. notoscriptus*; (b) Sydney *Ae. notoscriptus*; (c) Cairns *Ae. aegypti*; (d) Charters Towers *Ae. aegypti*. Scale 10 μm.

($P = 0.46$) and Cairns and Charters Towers *Ae. aegypti* ($P = 0.89$). Posterior outer chorionic cell width varied between all groups ($P \leq 0.01$) excluding Sydney and Adelaide *Ae. notoscriptus* ($P = 0.998$). Variations in anterior outer chorionic cell length only existed between Adelaide *Ae. notoscriptus* and the Sydney population, and Cairns *Ae. aegypti* ($P = 0.003$ and 0.038 respectively). Posterior outer chorionic cell length did not vary between any group ($P > 0.7$).

Anterior central tubercle diameter varied only between Adelaide *Ae. notoscriptus* and Cairns and Charters Towers *Ae. aegypti* ($P = 0.039$ and 0.0012 respectively). Posterior central tubercle diameter of Adelaide *Ae. notoscriptus* varied from Sydney *Ae. notoscriptus* ($P = 0.0005$), and both populations of *Ae. aegypti* (Cairns $P < 0.0001$; Charters Towers $P = 0.008$). Anterior central tubercle density for Cairns *Ae. aegypti* eggs varied from both populations of *Ae. notoscriptus* (Adelaide $P = 0.013$; Sydney $P = 0.006$) while posterior tubercle density did not vary between any of the populations (Table 3).

3.4. Principal component analysis

Principal components one and two accounted for 28 and 17% of total variation respectively. The principal component analysis (Fig. 6) highlighted distinctions within both principal components one and two, between the eggs of *Ae. notoscriptus* Sydney and Adelaide populations, and the eggs of *Ae. aegypti* Cairns and Charters Towers populations, with the four egg groups occupying separate quadrants.

4. Discussion

Our results demonstrated significant variation in length to width ratio between *Ae. aegypti* and *Ae. notoscriptus* eggs, which could be useful for differentiation, as suggested by Linley (1989). Furthermore, intraspecific variations in egg length to width ratio have previously enabled differentiation between multiple *Anopheles aquasalis* populations (Linley et al., 1993b) as well as several *C. quinquefasciatus* populations (Suman et al., 2009). Our results also demonstrated between-population variations in egg length to width ratio within both species studied here.

Outer chorionic cell morphology has previously been useful in morphometrically distinguishing some *Aedes* species (Linley et al., 1992). Anterior outer chorionic cell width varied between all eggs excluding Sydney and Adelaide *Ae. notoscriptus*, and Cairns and Charters Towers *Ae. aegypti*. Additionally, posterior outer chorionic cell width varied between all groups excluding Sydney and Adelaide and therefore outer chorionic cell width may be, in this case, species specific. The presence of an anterior ring surrounding the micropylar apparatus is also useful in differentiating *Ae. aegypti* eggs from *Ae. notoscriptus* eggs.

Variations in outer chorionic tubercle diameter have previously enabled differentiation of some *Culex* (Suman et al., 2008) and *Aedes* species (Hinton and Service, 1969; Matsuo et al., 1972). Furthermore, intraspecific variations in tubercle size have enabled distinctions between populations of *Anopheles* species (Linley et al., 1993a, 1996). In the present study, inter- and intraspecific variations in central tubercle diameter support the use of this attribute in the distinction of mosquito eggs.

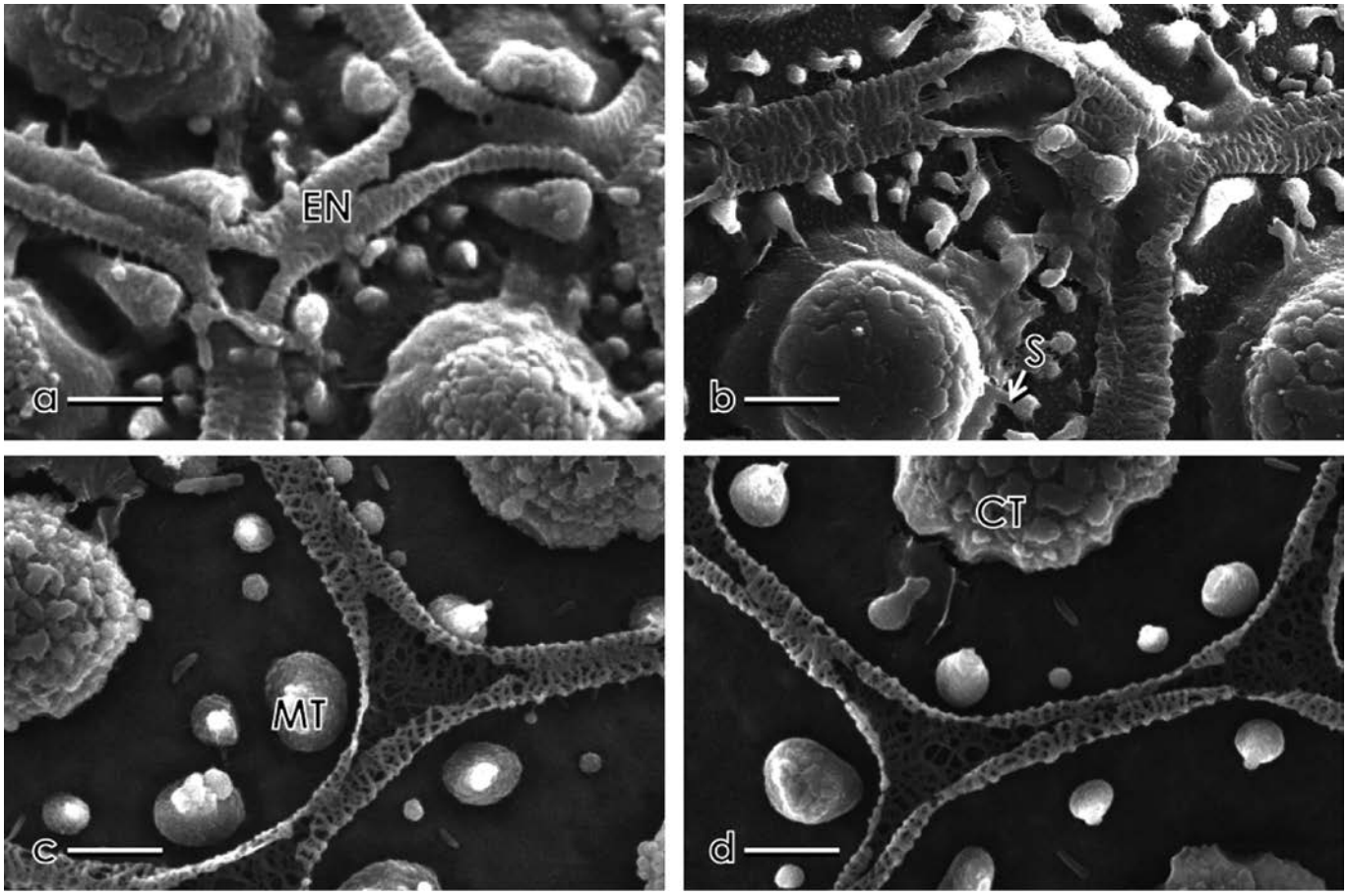

Fig. 5. SEM of exochorionic network (EN), minute tubercles (MT), large central tubercles (CT) and spoke-like connections (S) in (a) Adelaide *Ae. notoscriptus*; (b) Sydney *Ae. notoscriptus*; (c) Cairns *Ae. aegypti*; (d) Charters Towers *Ae. aegypti*. Scale 2.5 μm.

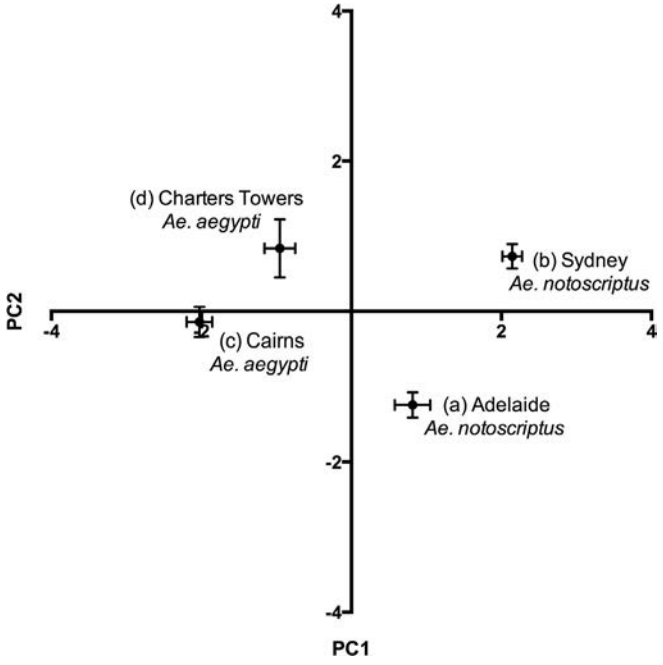

Fig. 6. Principal component analysis of egg morphology for *Ae. notoscriptus* from Sydney and Adelaide, and *Ae. aegypti* from Charters Towers and Cairns.

The general shape and morphology of the eggs of *Ae. aegypti* and *Ae. notoscriptus* were largely consistent with the descriptions by Linley (1989) and Linley et al. (1991) respectively. The ventral cell fields of Sydney *Ae. notoscriptus* contained only one large central tubercle, consistent with SEM analyses of this population by Linley et al. (1991). Conversely, the ventral cell fields of *Ae. aegypti* eggs of Cairns and Charters Towers origin occasionally contained two or more large central tubercles whereas *Ae. aegypti* of Florida origin were only reported to contain a single central tubercle (Linley, 1989), suggestive of regional variations in intraspecific egg surface morphology.

Ae. notoscriptus eggs from Sydney and Adelaide both possessed spoke-like connections between minute and central tubercles, consistent with previous SEM analyses of the species' eggs (Linley et al., 1991). The minute tubercles of Cairns and Charters Towers *Ae. aegypti* did not possess such connections, contrary to SEM analyses of Florida strains (Linley, 1989). In addition, the minute tubercles and EN structures (<2 μm width) remained independent in Australian populations of *Ae. aegypti* while the comparatively wider EN (>4 μm) reportedly overlay many of the minute tubercles in observations of Florida strains (Linley, 1989), further representing regional intraspecific variations in egg ultra-structural morphology. Regional variations in egg morphology and ultra-structural eggshell morphology may reflect adaptation to local biotope. Further studies involving samples obtained throughout the ranges of both *Ae. notoscriptus* and *Ae. aegypti* mosquitoes may further confirm the existence of morphological differences that

would allow the characterisation of different populations and the potential for local adaptation of the eggshell.

The principal component analysis highlighted the considerable variation in egg morphology between *Ae. aegypti* and *Ae. notoscriptus* mosquitoes. Adelaide and Sydney *Ae. notoscriptus* eggs were not associated with *Ae. aegypti* egg morphology and were distinguishable from each other suggesting strong diversity of *Ae. notoscriptus* intraspecific egg morphology. Egg morphology between Charters Towers and Cairns *Ae. aegypti* varied to a lesser extent and the increased continuity of structural variants in *Ae. aegypti* eggs when compared to *Ae. notoscriptus* may be reflective of reduced variation in biotopic origin. Intraspecific egg morphology in *Ae. notoscriptus* length to width ratio, EN width, outer chorionic cell width and posterior central tubercle diameter may distinguish the Sydney and Adelaide populations. Australian populations of *Ae. notoscriptus* are known to be genetically distinct (Endersby et al., 2013) and the egg morphology variances may suggest that Sydney and Adelaide populations are from separate genetic lineages, however future phylogenetic analyses of the two populations would be required to confirm this.

This investigation supports the hypothesis of intraspecific variation in egg morphology in both *Ae. aegypti* and *Ae. notoscriptus* mosquitoes. Furthermore, our results demonstrate the usefulness of studying the morphology of *Aedes* eggs with SEM. This study provides a comprehensive inter- and intraspecific comparative analysis of the eggs of Australia *Ae. aegypti* and *Ae. notoscriptus*, providing attributes by which to statistically differentiate them, and increases our understanding of the egg morphology of these invasive mosquito species.

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