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ULTRASTRUCTURE OF THE EGGS OF *CULICOIDES MOLESTUS* (DIPTERA: CERATOPOGONIDAE)

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ABSTRACT. The eggs of *Culicoides molestus* (Skuse) are described and illustrated by scanning and transmission electron microscopy. Eggs are elongate with a slight dorsoventral curvature. No outer chorionic tubercles are present. Aeropyles are present in large numbers at the anterior end and in lower numbers at the posterior end and lateral regions. The chorion has 5 layers. An outer, rough, proteinaceous layer covers a smoother inner surface, which in turn encloses a layer of columns and meshwork that appears capable of containing air. These columns are underlain by an additional 2 layers. The aeropylar region, in combination with the chorionic meshwork, appears to provide a plastron that may aid in the survival and development of inundated eggs.

KEY WORDS *Culicoides molestus*, egg morphology, chorion, aeropyle, biting midge, electron microscopy

INTRODUCTION

Culicoides molestus (Skuse) is a major pest in coastal southeastern Queensland and northern New South Wales, Australia (Kettle et al. 1979, Kay and Lennon 1982). Larvae have been recovered from sandy beaches and require fairly clean sand, such as is found in canal estates (a constructed labyrinth of coastal canals, usually lined with beach). These larvae do not have the association with surface-tunneling crabs (*Mictyris livingstonei* McNeill) that is present for larvae *Culicoides subimmaculatus* Lee and Reye and the 2 species are rarely sympatric (Kettle et al. 1979, Marks and Reye 1982). Oviposition has been assumed to occur where larvae have been found and this assumption is strengthened by the poor dispersal of the species as adults (Kettle et al. 1979); however, no published data exist for egg recovery and the description of the eggs is lacking. We investigated 3 canals on the Gold Coast, a major residential, tourist, and recreation area of southeastern Queensland, for presence of *C. molestus* eggs.

A number of studies address the ultrastructure of eggs of *Culicoides* species but none have looked at the structure of the chorion using transmission electron microscopy (TEM). Using scanning electron microscopy (SEM), eggs of *Culicoides circumscriptus* Kieffer, *Culicoides imicola* Kieffer, and *Culicoides gejgelensis* Dzhafarov have been described by Day et al. (1997); *Culicoides brevitarsis* Kieffer by Campbell and Kettle (1975) and Kariya et al. (1989); *Culicoides arakawae* (Arakawa), *Culicoides oxystoma* Kieffer, *Culicoides punctatus* (Meigen), *Culicoides sumatrae* Macfie, *Culicoides actoni* Smith, and *Culicoides maculatus* (Shiraki) by Kariya et al. (1989): and *Culicoides variipennis* (Coquillett) by Nunamaker et al. (1987). The surface structure of the eggs of all 11 species display tubercles. In our study we report a different surface structure for the eggs of *C. molestus* and investigate the ultrastructure of the chorion using both SEM and TEM.

MATERIALS AND METHODS

Eggs of *C. molestus* were collected from sand banks along a canal estate on the Gold Coast (Southport, Queensland, Australia: 27°59′S, 153°25′E). Sand banks were sampled for *C. molestus* in April and May of 1997, 5 days after a full or a new moon phase. Layers of sand were removed from various locations across the beach, near midtide level, packed into separate containers, and transported into the laboratory for egg extraction. Particles lighter than sand were flooded out of the samples using a water jet that suspended them within a volumetric cylinder. Then, the water was run into a tube fitted with fine stainless steel mesh (100-μm apertures) at the base. The contents were transferred into a petri dish to be sifted for eggs under a dissecting microscope. Larvae and pupae were extracted using the same protocol. Eggs were allowed to eclose in saline agar medium (7–8% agar in autoclaved canal water from the same site as the sample) and reared to adult for identification. Using light microscopy, eclosed larvae were compared to extracted larvae. Pupae were allowed to emerge and adults were identified, also under the light microscope. Adult midges were collected on the beach, above the sites of egg collection, using humans as bait. All samples were identified as *Culicoides molestus*.

Two techniques were used to prepare extracted eggs and eggshells for SEM. Eggs were fixed in modified Karnovsky's fixative (2.5% glutaraldehyde and 2.0% formaldehyde in 0.1 M sodium cacodylate buffer), washed in buffer, and postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer before being dehydrated in an acetone series and critical point dried. Alternatively, eggs were dehydrated in acetone to 100% and air dried. Forty-nine eggs were prepared according to this 2nd protocol

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and the best-preserved ones were selected for measurement. Egg size was unaffected by technique. Determination of the chemistry of the outer layers of the chorion was achieved using 2 further techniques: eggs were placed in 5% aqueous proteinase K at 37°C for 45 min (technique modified from Bodley and Wood 1996), washed in about 10% Decon 90 for 30 min, then sonicated for 30 sec while in the detergent, or eggs were placed directly into about 10% Decon 90 (Decon Laboratories Limited, Hove, East Sussex, United Kingdom) detergent for 1 h and sonicated for 30 sec. Eggs from both treatments were then fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, washed in buffer, and postfixed in 1% osmium tetroxide prior to dehydration in ethanol and critical point drying. All samples were mounted on double-sided adhesive tape and coated with gold to a thickness of 7–10 nm before viewing using a JEOL 6300 or 6400F SEM (Japan Electron Optics Laboratory, Tokyo, Japan) at 5 kV.

Eggs were prepared for TEM by fixing in modified Karnovsky's fixative in 0.1 M cacodylate buffer, washing in buffer, postfixing in 1% osmium tetroxide in 0.1 M cacodylate buffer, dehydrating in a series of acetone, and embedding in Spurr resin. Sections were cut with glass and diamond knives, stained with uranyl acetate and lead citrate, and viewed using a JEOL 1010 TEM (JEOL USA, Peabody, MA) at 80 kV.

Terminology used follows Day et al. (1997), Hinton (1981), Linley et al. (1991), and Harbach and Knight (1980).

RESULTS

The eggs of *C. molestus* are dark brown to black, elongate structures with a slight dorsoventral curvature (Figs. 1a, 1b). Egg dimensions are given in Table 1. Freshly extracted eggs were occasionally embedded in a clear, jellylike substance that, when fixed in place, obliterated surface detail. Bacteria were seen in this layer (Fig. 1c). Eggs without this layer showed a rough surface structure (layer 1) that appeared to peel back in some regions to expose a smooth, continuous layer, marked by a pattern of low-relief, domelike papillae (Fig. 1d). In places this smooth layer was seen to be broken away from the papillae (Fig. 1d). A thin layer (layer 2) joins the tops of the papillae, which are the rounded tops of columns.

Eggs treated with detergent (Decon 90) alone showed no change to the outer layers of the chorion but when eggs were pretreated with proteinase K and then detergent, the outer, rough layer was removed (Fig. 1e). This indicates that the outer layer is proteinaceous in nature. The inner, smoother layer that was revealed was unaffected by the treatments and therefore has a different chemical construction from the outer layer.

A poorly developed anterior micropylar dome is present and the surface at this end contains many aeropyles and is often fenestrated (Figs. 2a, 2b). A smaller number of aeropyles occurs along the egg and at the posterior end (Fig. 2c). Eclosion occurs at the anterior end, below the micropylar cap, which remains unbroken, and proceeds through a slit along about one third of the length of the egg (Fig. 2d). Eclosion of eggs was observed under the light microscope so this split is not a preparation artifact. Using SEM, no obvious hatching line is seen in intact eggs.

The cross section of the egg, viewed with TEM (Fig. 2e), shows the rough, scalelike outer layer, which may be absent but when present can be up to 2.4 μm thick (layer 1). A smooth, thin (20- to 140-nm) layer (layer 2) lies beneath this outer layer and a row of columnlike struts (layer 3) supports layer 2. The columns sometimes appear overlain by the thin layer 2 (Fig. 2e) and in other regions the layer is not distinct from the columns. The columns are about 380 nm high (Figs. 2e, 2f). They sit upon a finely lamellate layer (layer 4) that is usually about 40 nm thick but is sometimes seen to be constructed of 2 such layers and is about 80 nm thick (Fig. 2f). This lamellate layer covers an inner, homogeneous, layer (layer 5) that is about 405 nm thick (data obtained from 10 measurements). The larva lies adjacent to this inner layer. In all the chorion has 5 layers.

DISCUSSION

The egg surface structure of *C. molestus* is distinctly different from the 11 other species that have been studied by SEM (Table 1). All previously studied species have a covering of tubercles (also referred to as ansulae, hairlike papillae, granular papillae, or outer chorionic tubercles). Outer chorionic tubercles are prominences of the outer chorion (Harbach and Knight 1980). Sometimes these are arranged in rows, as in *C. circumscriptus*, *C. gejgelensis*, and *C. imicola* (Day et al. 1997); and *C. arakawae*, *C. oxystoma*, *C. punctatus*, *C. sumatrae*, and *C. actoni* (Kariya et al. 1989). In other cases the tubercles are scattered over the entire surface, as in *C. brevitarsis* (Campbell and Kettle 1975), *C. maculatus* (Kariya et al. 1989), and *C. variipennis* (Nunamaker et al. 1987). No outer chorionic tubercles are present in *C. molestus*. The surface of the chorion, beneath the rough outer layer, is relatively smooth, depending on the degree of collapse of the layer overlying the chorionic meshwork. Where papillae are seen, they are evident as the tops of the inner chorionic meshwork/columns and are not a separate structure on the outer chorion. Tubercular projections have generally been regarded as a plastron, to aid in air exchange and their absence in *C. molestus* raises the question as to how these eggs survive inundation.

No TEM studies of the chorion of a *Culicoides* species have been reported, although hints as to the structure for *C. variipennis* are available from SEM

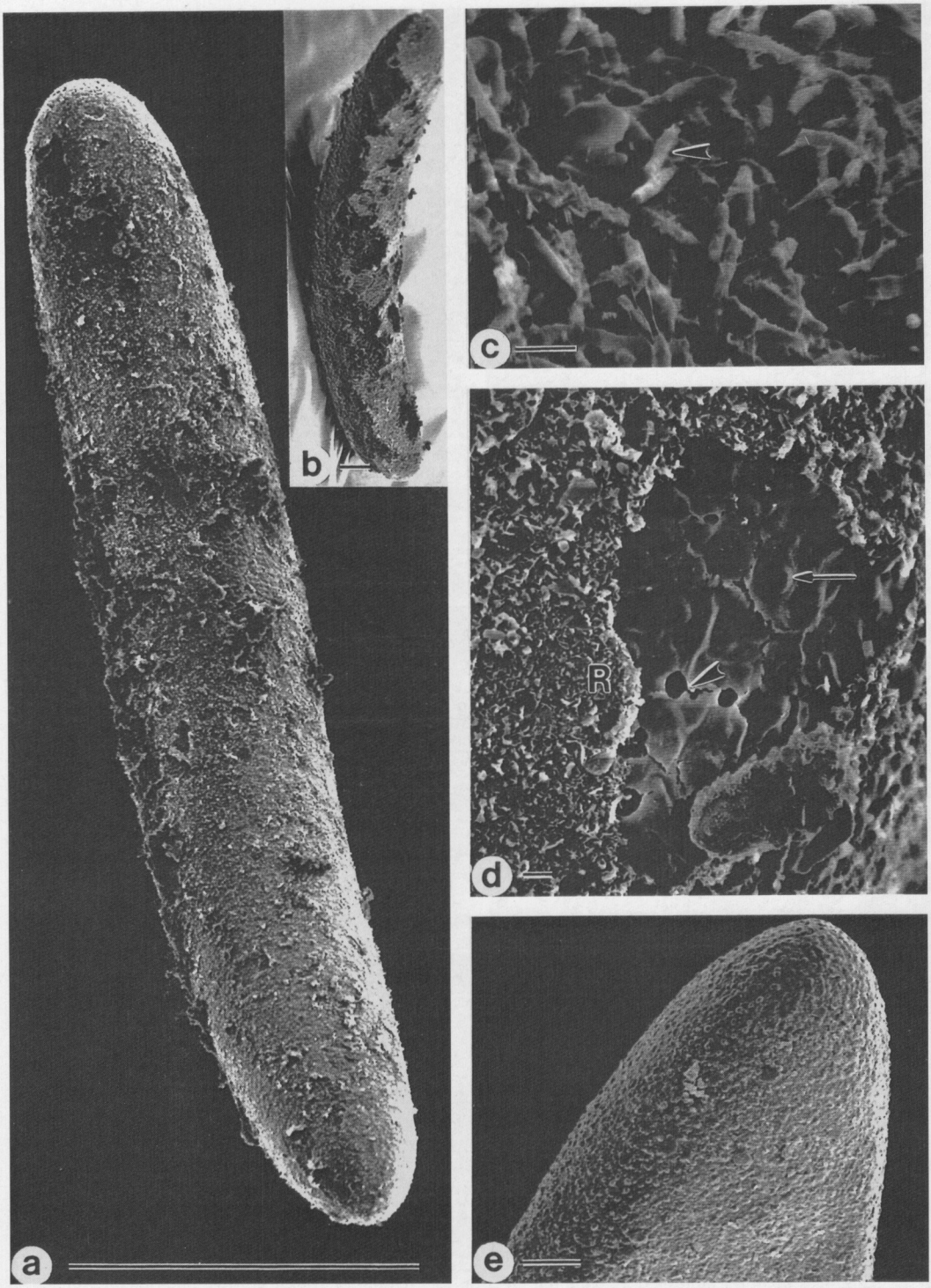

Fig. 1. Egg of *Culicoides molestus*. a. Scanning electron micrograph of ventral surface showing absence of tubercles. b. Lateral view showing dorso ventral curvature of egg. c. Bacteria found on egg surface (arrowhead). d. Detail of chorion showing rough outer surface (layer 1) (R), smooth inner surface (layer 2) marked with domelike papillae (arrow), and breaks (arrowhead) that reveal the thin nature of layer 2. e. Chorion after treatment with proteinase K, and Decon 90, showing layer 2. Scale, 100 μm (a), 20 μm (b), 10 μm (e), 1 μm (c, d).

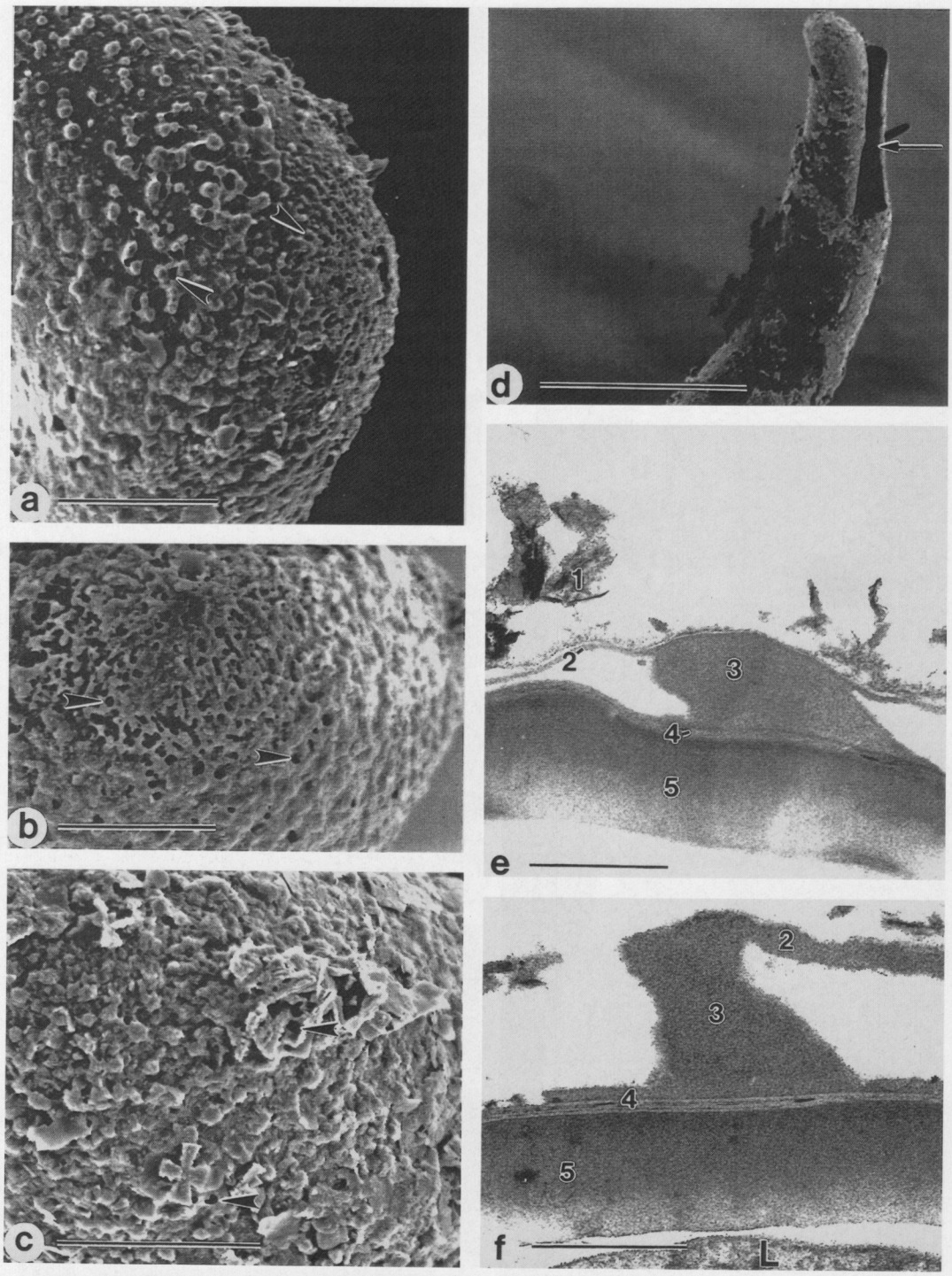

Fig. 2. Egg of *Culicoides molestus*. a. Scanning electron micrograph of lateral view of anterior micropylar dome and aeropyles (arrowheads). b. Dorsal view of anterior micropylar dome and aeropyles (arrowheads). c. Posterior end showing aeropyles (arrowheads). d. Eclosion slit (arrow). e. Transmission electron micrograph transverse section of chorion showing 5 layers (layers 1–5). f. Transmission electron micrograph transverse section of chorion showing no outer layer 1, thick layer 2, lamination and doubling of layer 4, and larva (L). Scale, 100 μm (d), 10 μm (a, b, c), 500 nm (e, f).

Table 1. Egg dimensions (μm) for *Culicoides* species for which scanning electron microscopy data are available.

Species[1] (number)	Length		Width		Length to width ratio		Reference
	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE	Range	
Culicoides brevitarsis	290 ± 2	?	73 ± 2	?	~4	?	Campbell and Kettle (1975)
Culicoides circumscriptus (10)	403 ± 3	384–413	54 ± 1	47–60	7.4 ± 0.2	6.8–8.6	Day et al. (1997)
Culicoides gejgelensis (11)	321 ± 5	285–341	47 ± 1	41–52	6.9 ± 0.1	6.1–7.5	Day et al. (1997)
Culicoides imicola (10)	363 ± 5	334–391	65 ± 1	63–69	5.6 ± 0.1	5.3–6.0	Day et al. (1997)
Culicoides molestus (13)	333 ± 2	322–356	62 ± 2	47–75	5.5 ± 0.2	4.5–7.0	This paper
Culicoides variipennis	400	?	68	?	~5	?	Nunamaker et al. (1987)

[1] *Culicoides arakawae, C. oxystoma, C. punctatus, C. sumatrae, C. actoni,* and *C. maculatus* are reported as having eggs 300–500 μm long (Kariya et al. 1989).

study (see Nunamaker et al. 1987). We have shown that in *C. molestus* a chorionic network and its associated spaces exist below the outer chorion. Evidence exists of a layer of tubercles seen to underlie the base upon which the outer layer of tubercles sit in *C. variipennis*, but a separation of the chorionic layers was not unequivocally demonstrated in this case (Nunamaker et al. 1987). Demonstration of such a space in *C. molestus* adds support to the theory that this separation also exists in *C. variipennis* and it may exist in eggs of other *Culicoides* species as well. Hinton (1981) describes such columns within the chorion as serving as an air-containing meshwork. The lack of an obvious external plastron in *C. molestus*, coupled with our observation that the eggs can survive, grow, and eclose when constantly inundated, leads us to the conclusion that the inner network of columns and their connection to aeropyles, particularly the extensive anterior aeropylar region, enables efficient respiration. Aeropyles are usually found to be hydrofuge and resist water entry and so can act as a plastron when covered with water (Hinton 1981). However, aeroplyes have to be sufficiently numerous to provide a large area for effective gas exchange. We believe that the anterior aeroplyar region in eggs of *C. molestus* may well be large enough to function, in combination with the chorionic air spaces, as a plastron.

Eggs of *C. molestus* are similar in size to those of other species (see Table 1). Because egg sizes of species overlap, size is not particularly useful for distinguishing eggs of different species. Day et al. (1997) were hopeful that surface morphology of eggs could be used to separate *Culicoides* species but concluded, as a result of their study, that this would not be possible for most *Culicoides* species. In contrast, Kariya et al. (1989) found that the surface of the eggs from each of the 7 species they studied had a specific pattern that could be useful in species identification. Evidence from our study supports the view that surface morphology of eggs may be extremely useful in determination of particular species. *Culicoides* eggs are now known to fall into 3 broad types (and many more refined categories) on the basis of external structure: tubercles in rows along the egg (*C. circumscriptus*, *C. imicola*, and *C. gejgelensis* [Day et al. 1997]; *C. arakawae*, *C. oxystoma*, *C. punctatus*, *C. sumatrae*, and *C. actoni* [Kariya et al. 1989]); scattered tubercles with no pattern (*C. brevitarsis* [Campbell and Kettle 1975, Kariya et al. 1989] and *C. variipennis* [Nunamaker et al. 1987]) or with variable patterns (*C. maculatus* [Kariya et al. 1989]); and eggs with no tubercles (*C. molestus*). Morphology may be used to discriminate species within these types; however, more quantification is needed.

Culicoides molestus occurs in a different habitat from the other species discussed here. It is marine and eggs are found in sand that is often inundated, whereas eggs of the other 11 species occur either in open muddy areas, dung, or damp organic soil, depending on the species (Day et al. 1997, Kariya et al. 1989, Kettle 1995). Kariya et al. (1989) proposed that habitat may be linked to egg morphology, specifically that eggs covered with hairlike papillae (scattered tubercles) may be laid in drier areas, such as cow dung, than those with tubercles in rows. These authors related surface structure to adherence of the eggs rather than to respiration. This trend has one contradiction: *C. imicola* has rows rather than scattered tubercles (Day et al. 1997) and is found in dung (Kettle 1995). However, on the basis of structure of eggs of *C. molestus*, morphologic differences may reflect terrestrial versus marine habitats and further studies of eggs of marine or esturine *Culicoides* species are warranted. *Culicoides molestus* often occurs alone but one species with which it can overlap is *C. subimmaculatus* and we intend to investigate the surface morphology of the eggs of this species for comparison with those of *C. molestus*.

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REFERENCES CITED

Bodley, H. D. and R. L. Wood. 1996. Ultrastructural studies on elastic fibres using enzymatic digestion of thin sections. Anat. Rec. 172:71–87.

Campbell, M. M. and D. S. Kettle. 1975. Oogenesis in *Culicoides brevitarsis* Kieffer (Diptera: Ceratopogonidae) and the development of a plastron-like layer on the egg. Aust. J. Zool. 23:203–218.

Day, J. F., D. Duzak, Y. Braverman, A. Chizov-Ginzburg and J. R. Linley. 1997. Ultrastructure of the eggs of *Culicoides circumscriptus*, *Culicoides gejgelensis*, and *Culicoides imicola*. (Diptera: Ceratopogonidae). J. Am. Mosq. Control Assoc. 13:76–83.

Harbach, R. E. and K. L. Knight. 1980. Taxonomists' glossary of mosquito anatomy. Plexus Publishing Inc., Marlton, NJ.

Hinton, H. E. 1981. Biology of insect eggs. Pergamon, New York.

Kariya, Y., S. Imai, T. Ishii and T. Morii. 1989. Ultrastructural comparison of surface structure of seven *Culicoides* species (Diptera: Ceratopogonidae) from Japan. Jpn. J. Sanit. Zool. 40:55–59.

Kay, B. H. and T. Lennon. 1982. Seasonal prevalence and bionomics of biting midges (Ceratopogonidae) at ocean shores, New South Wales. J. Aust. Entomol. Soc. 21: 207–216.

Kettle, D. S. 1995. Medical and veterinary entomology, 2nd ed. CAB International, Wallingford, Oxon, United Kingdom.

Kettle, D. S., E. J. Reye and P. B. Edwards. 1979. Distribution of *Culicoides molestus* (Skuse) (Diptera: Ceratopogonidae) in man-made canals in south-eastern Queensland. Aust. J. Mar. Freshwater Res. 30:653–660.

Linley, J. R., M. J. Geary and R. C. Russell. 1991. The eggs of *Aedes funereus*, *Aedes notoscriptus* and *Aedes alternans* (Diptera: Culicidae). Proc. Entomol. Soc. Wash. 93:592–612.

Marks, E. N. and E. J. Reye. 1982. An atlas of common Queensland mosquitoes with a guide to common Queensland biting midges. Queensland Institute of Medical Research, Brisbane, Australia.

Nunamaker, R. A., C. E. Nunamaker and B. C. Wick. 1987. A scanning electron microscopic study of the eggs of *Culicoides variipennis* (Diptera: Ceratopogonidae), pp. 884–885. *In:* G. W. Bailey (ed.). Proc. 45th Annu. Meet. Electron Microsc. Soc. Am., San Francisco, CA.