Laboratory colonisation of *Aedes geniculatus*

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Abstract: *Aedes geniculatus* (Diptera: Culicidae) is a container-breeding mosquito species widespread throughout Europe and adjacent regions. Similar to other container-breeding mosquitoes, this primarily mammophilic species could be a vector for several pathogens of medical or veterinary importance. We here report for the first time a laboratory colonisation of *Ae. geniculatus*. Eggs and larvae were collected from forest edges in the area around Zürich, Switzerland. The colony could be established by maintaining adults at room temperature in a large cage where mating occurred. Females took blood meals through a pig intestine membrane in a restricted space. After embryo development for 8 – 14 weeks at 10 °C, 'hatching in installments' was induced. Our established colony of *Ae. geniculatus*, now in its sixth generation, facilitates future studies on basic ecology and physiology of this species and its vector capacity traits. ***Journal of the European Mosquito Control Association* 34: 1-4, 2016**

Keywords: *Aedes geniculatus*, colony, container-breeder, blood-feeding, pig intestine membrane

Introduction

Aedes (Dahliana) geniculatus (Olivier) is a Palaearctic mosquito species which is widespread throughout Europe and is also reported from North Africa and countries in the Middle East (Dahl & Blackmore, 2001; Schaffner *et al.*, 2001; Knio *et al.*, 2005; Azari-Hamidian *et al.*, 2009; Becker *et al.*, 2010; Muller *et al.*, 2012). This poorly studied species mainly occurs in deciduous forests (Dahl & Blackmore, 2001; Schaffner *et al.*, 2001) where older trees provide tree-holes as larval habitats which in Europe may also contain larvae of *Ae. (Hulecoeteomyia) japonicus (Theobald)*, *Anopheles plumbeus* Stephens and *Culex torrentium* Martini (Yates, 197; Bradshaw & Holzapfel, 1992; Schmidl *et al.*, 2008; Damiens *et al.*, 2014). These species may also breed in artificial containers, like *Cx. pipiens* Linnaeus, *Ae. (Stegomyia) albopictus* (Skuse) and *Ae. koreicus* sensu Reinert (2000) (Schaffner & Mathis, 2011; Versteirt *et al.*, 2012; Montarsi *et al.*, 2013). Larvae of *Ae. geniculatus* seem to tolerate a broad spectrum of water quality, e.g. with regard to pH and contents of organic matter (Dahl & Blackmore, 2001; Schaffner *et al.*, 2001; Schmidl *et al.*, 2008; Becker *et al.*, 2010).

In its northern range, *Ae. geniculatus* can overwinter in the larval stage (Yates, 1979). However, primarily eggs undergo a diapause and can hatch 1.5-2 years after they have been laid (Yates, 1979). Presence of first instar larvae during all seasons (Yates, 1979) and repeated stimulation of hatching in the laboratory (Armbruster *et al.*, 2000) confirms a 'hatching in installments' behaviour which is typical for certain aedine-type mosquitoes (Becker *et al.*, 2010). Female densities are highest in July/August when most eggs are laid (Yates, 1979); thus *Ae. geniculatus* is thought to be univoltine in its northern range (Yates, 1979; Dahl & Blackmore, 2001). Virtually nothing is known about the biology of more southern populations. Larval and imaginal development may continue during mild winters (Schaffner *et al.*, 2001; Becker *et al.*, 2010) and up to two generations may occur per year (Schaffner *et al.*, 2001).

Though mainly thought to be mammophilic (Yates, 1979; Armbruster *et al.*, 2000; Schaffner *et al.*, 2001; Becker *et al.*, 2010), *Ae. geniculatus* was also reported to feed on birds and reptiles (Schaffner *et al.*, 2001). *Aedes geniculatus* is usually a rare species (Yates, 1979; Azari-Hamidian *et al.*, 2009; Schaffner & Mathis, 2011; Lühken *et al.*, 2014) but can locally be highly abundant (Becker *et al.*, 2010; Damiens *et al.*, 2014) and become a nuisance to humans (Schaffner *et al.*, 2001). In a study in southern Germany, 94% of 50 tree-holes (mainly beech trees) investigated contained *Ae. geniculatus* larvae, with a maximum of 303 larvae in a tree-hole accommodating 2.5 litres of water (Schmidl *et al.*, 2008).

Aedes geniculatus is a laboratory vector of yellow fever virus, Eastern equine encephalitis virus (Yates, 1979) and West Nile virus (WNV) (Vermeil *et al.*, 1960). However, the species was not considered a relevant vector during WNV outbreaks due to low population density (Romi *et al.*, 2004) or based on modelling which revealed that habitat suitability of *Ae. geniculatus* was negatively associated with equine disease (Mughini-Gras *et al.*, 2014). The species might act as a vector for canine *Dirofilaria* spp. as it produced infective stages of *D. immitis* following experimental feeding on an infected dog (Petruschke *et al.*, 2001), and it was attracted by dog-baited traps (Pollono *et al.*, 1998).

Here we report the first establishment of a laboratory colony of *Ae. geniculatus*, completing six full generations. This native species is ecologically similar to the container-breeders *Ae. japonicus* and *Ae. koreicus* which are invasive (Schaffner *et al.*, 2001; Versteirt *et al.*, 2012; Montarsi *et al.*, 2013; Kampen & Werner, 2014) and which may transmit pathogens such as *Dirofilaria* spp. or WNV (Turell *et al.*, 2001; Montarsi *et al.*, 2015).

Materials and Methods

Mosquito eggs and larvae were repeatedly collected with 14 ovitraps to which a polystyrene block (5 x 5 x 2.5 cm^3) was added as oviposition support and from 5 tree-holes at each of two forest edges in the area around Zürich, Switzerland (47°23'49"N, 8°33'14"E; 47°30'31"N, 8°29'22"E), an area with

high population densities of *Ae. japonicus* (Wagner, 2011; Balestrino *et al.*, 2015), from 2012 to 2013.

Table 1: Breeding conditions of the *Aedes geniculatus* colony.

Mosquito stage	Factor	Breeding conditions
Larvae	Rearing	• In pans[1] containing 500 ml dH_2O • At RT[2], with daylight and an additional light source[3] • Addition of Tetramin[4] every second day (30, 60 and 120 mg for L1, L2, and L3-L4 larvae) at maximum densities of 200 larvae per pan • Transfer of pupae to adult cage
Adults	Maintenance	• In large cage[5] at RT[2] with daylight and an additional light source[3] • Addition of a 5% glucose solution offered on cotton sticks protruding from a cup
	Blood meal	• Transfer of females from the large cage[5] to a small cage[6] for feeding • Heparinised sheep blood using a Hemotek feeding system[7] with a pig intestine membrane and a piece of the synthetic lure 'iGu'[8]
	Oviposition	• Addition of a black cup[9] in a shaded corner of the cage, coverage of inner cup with seed germination paper, addition of tap water to ¾ of total volume and 5-10 g Tetramin[4] • Exchange and storage of seed germination paper with eggs every two weeks
Eggs	Storage	• Moist storage protected from light on seed germination paper for 8 to 14 weeks at 10 °C in a plastic cup (300-400 ml) sealed with Nescofilm®
	Hatching	• Submersion of stored eggs on papers in 500 ml dH_2O at RT[2] in pans[1] supplemented with 30 mg Tetramin[4] • Incubation for 1 week • Removal of papers from water, keeping papers attached to bottom side of plexiglass plate covering the pan[1] and incubation for 1 week • Repetition of paper submersion in water and incubation (2x) • Transfer of 1st instar larvae into a new pan[1]

[1]Pans (25 x 18 x 5 cm^3) covered with plexiglass plates
[2]RT, room temperature (20 – 22 °C)
[3]Artificial long day regime (16h, without dawn and dusk phases)
[4]Ground 'Tetramin BioActive® formula flakes' (Tetra GmbH, Melle, Germany)
[5]Large cage (61 x 61 x 61 cm^3; BioQuip, Rancho Dominguez, USA)
[6]Small cage (17.5 x 17.5 x 17.5 cm^3; BugDorm-41515; MegaView Science Co. Ltd., Taichung, Taiwan)
[7]Hemotek™ (Hemotek Ltd, Lancashire, UK) heating up to 37 °C
[8]A quarter piece of iGU® Combi FRC 3003 (Silva GmbH & Co. KG, Lübeck, Germany)
[9]'Ramona', 850 ml (Luwasa®, Interhydro AG, Allmendingen, Switzerland)

The polystyrene oviposition supports with the field-collected aedine eggs were stored at 10-15 °C in a zip-bag containing a moist cotton tissue for 3-6 months. Then, they were repeatedly set to float on the water surface with eggs submerged to induce larval hatching.

Table 1 summarises the breeding conditions of the *Ae. geniculatus* colony. Larvae were raised in pans (25 x 18 x 5 cm^3), covered with plexiglass plates, in 500 ml dH_2O at room temperature (RT; 20–22 °C) with daylight and an additional light source (long day, 16h, without artificial dusk or dawn). Ground fish food was provided as nutrition (Tetramin BioActive® formula flakes, Tetra GmbH, Melle, Germany), i.e. 30, 60 and 120 mg for L1, L2 and L3-L4 larvae every second day at maximum densities of 200 larvae per pan. Food amounts were adjusted for smaller larval densities. Pupae were transferred to a cubic cage (61 x 61 x 61 cm^3; BioQuip, Rancho Dominguez, USA), and adults were kept at RT and long day conditions after eclosion with ad libitum access to 5% glucose solution offered on cotton sticks protruding from a cup. Field-collected larval stages and larvae hatched in the laboratory from field-collected eggs were identified with a morphological key for fourth instar larvae (Becker *et al.*, 2010) and using a programme for morphological mosquito identification (Schaffner *et al.*, 2001).

Results and Discussion

Aedes geniculatus readily mated at densities of approximately 50 adults in large cages (see below). At natural dusk, single males were repeatedly observed to perform flights in the lower third of the cage to which single females joined and mated. When establishing laboratory colonies of *Ae. japonicus* in the same cage type, artificial mating had to be done for the first generations (Williges *et al.*, 2008; Hoshino *et al.*, 2010). Free mating was not observed until adult densities increased up to 200–300 individuals (Williges *et al.*, 2008). Artificial mating was also done with *Ae. geniculatus* for three generations in a study addressing inbreeding using single pair matings (Armbruster *et al.*, 2000). In that study, blood feeding of the mosquitoes was done on human arms (Armbruster *et al.*, 2000). Yet, in another study, *Ae. geniculatus* readily fed on humans in the field but was reluctant to do so in the laboratory (Yates, 1979). In our study, females were never fed on a human arm. In the large maintenance cages (61 x 61 x 61 cm^3), the females of our colony did not accept blood meals using a Hemotek with different natural and artificial membranes (see below) or white mice. However, after transferring females to a smaller cage (17.5 x 17.5 x 17.5 cm^3; BugDorm-41515; MegaView Science Co. Ltd., Taichung, Taiwan), they readily fed on heparinised sheep blood using a membrane feeding system at 37 °C (Hemotek™, Hemotek Ltd, Lancashire, UK). The best feeding rates (40-60%) were observed using pig intestine as membrane and placing a quarter block of the synthetic lure iGu® Combi FRC 3003 (Silva GmbH & Co. KG, Lübeck, Germany), releasing R-1-octen-3-ol and ammonium bicarbonate, on top of the cage as a phagostimulant. This way, feeding rates were superior to other approaches (white mice or the use of chicken skin or Nescofilm® as membranes). Females were offered a blood meal every 1-2 weeks for one hour in a small cage, and they accepted it at the day of eclosion. Each gonotrophic cycle was estimated to be two weeks as inferred from females from a single cohort of eclosion kept in the maintenance cage. Females completing three gonotrophic cycles survived for approximately six weeks.

A black cup ('Ramona', 850 ml, Luwasa®, Interhydro AG, Allmendingen, Switzerland) was placed in a shaded corner of

the maintenance cage for oviposition. The inner side of the cup was covered with seed germination paper; tap water was added to ¾ of the total volume to the cup and supplemented with 5-10 mg Tetramin flakes as an oviposition stimulus. Bi-weekly, new paper was inserted and the removed paper with eggs was stored moist and protected from light in a plastic cup (300 or 400 ml) sealed with Nescofilm®. Hatching rates (see below) were best when eggs were stored at 10 °C for 8 to 14 weeks. Shorter and longer storage periods at this temperature or storage of eggs at RT for 8 to 14 weeks resulted in hatching rates <2%. Examination under a stereomicroscope revealed that <1% eggs were desiccated after 12 – 16 months storage at 10 °C. Thus, *Ae. geniculatus* eggs are very resistant to desiccation under our storage conditions.

Initial mating of field-collected mosquitoes (F0) yielded 25 eggs (F1) and the hatching rate was very low (i.e. 4%). Thus, the first two colony generations were established by mixing the colony with additional field-collected specimen to increase adult densities and to minimise inbreeding or founder effects (Armbruster *et al.*, 2000; Williges *et al.*, 2008). The current colony produces approximately 3,500 eggs per generation with hatching rates as specified below.

Hatching of larvae from the eggs on the germination papers was achieved by submerging the stored papers in pans containing water and 30 mg Tetramin (as described above) for one week at RT under a long day regime. The papers were then removed from the water and attached to the bottom side of the plexiglass plate covering the larval rearing pan. After another week, the papers were submerged again for one week. First instar larvae were transferred to a new pan and reared as described above. We allowed only two hatching intervals, as compared to seven in another study (Armbruster *et al.*, 2000), to select for an increase in hatching rates as discontinuous hatching is not favorable when large cohorts of mosquitoes are required for laboratory studies. Consequently, overall hatching rates improved during our study from 4% in the first generation (F1) to 41% (F5). A similar increase in hatching rate from 2% in the initial colonisation phase to above 50% was reported for *Ae. japonicus* (Hoshino *et al.*, 2010).

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