Unidirectional reproductive incompatibility between \textit{Aedes} (\textit{Protomacleaya}) \textit{brelandi} and \textit{Aedes} (\textit{P.}) \textit{hendersoni} (Diptera: Culicidae).

David B. Taylor
George B. Craig, \textit{University of Notre Dame}
Unidirectional Reproductive Incompatibility Between Aedes (Protomacleaya) brelandi and A. (P.) hendersoni (Diptera: Culicidae)

DAVID B. TAYLOR1 AND GEORGE B. CRAIG

Department of Biology, University of Notre Dame, Notre Dame, Indiana 46556

ABSTRACT

Forced mating was used to determine reproductive compatibility between two species in the Triseriatus Group of Aedes (Protomacleaya). In the cross between A. hendersoni Cockerell females and A. brelandi Zavortink males, only 7% of the females laid eggs that developed embryos. Furthermore, <5% of the eggs from single-female clutches in which one or more eggs did embryonate were fertile. In the reciprocal cross, A. brelandi females crossed to A. hendersoni males, 70% of the females laid fertile eggs and >90% of the eggs from those females became embryonated. Approximately 80% of the females were inseminated in both of the reciprocal crosses. No chromosomal abnormalities were observed in the hybrids. Incompatibility between the A. brelandi male genome and the A. hendersoni female cytoplasm appeared to be the cause of the reduced fertility.

The Triseriatus Group of the aedine subgenus Protomacleaya is composed of three sibling species of mosquitoes. This group is distributed throughout the United States east of the Rocky Mountains and extends into the Great Basin region. Aedes (P.) hendersoni Cockerell and A. (P.) triseriatus (Say) are sympatric throughout the eastern half of this range; Aedes hendersoni alone is found in the western portion. The third species in the group, A. (P.) brelandi Zavortink, is known only from the Chisos Mountains in western Texas (the type-locality of A. brelandi in 1972), but it has been reported from several other areas as well (Zavortink 1972). The genetics and population biology of A. hendersoni and A. triseriatus are well studied (Breed 1968, Truman and Craig 1968, Saul et al. 1978, Matthews and Craig 1980, Munstermann et al. 1982, Matthews and Munstermann 1983). However, almost nothing is known about the biology of A. brelandi. When Zavortink described A. brelandi in 1972, he stated that "Aedes brelandi is most closely related to A. hendersoni." Aedes brelandi and A. hendersoni are morphologically similar. Females differ primarily in the color of the setae on the scutum. Male genitalia are indistinguishable. Larvae can usually be separated by the number of branches on hairs 4b- and 4c-X (Zavortink 1972).

Electrophoretic analyses indicate that A. brelandi is very closely related to A. hendersoni. Munstermann et al. (1982) found genetic distances (Nei 1972) of <0.05 between A. brelandi and two strains of A. hendersoni, one from Colorado and the other from Michigan. These genetic distances are below those usually encountered between sibling species of Drosophila (Ayala et al. 1974a,b) and mosquitoes (Bullini 1982, Bullini and Coluzzi 1982). Preliminary interspecific crossing studies between A. brelandi and A. hendersoni indicated that the two species are compatible genetically with no sterility appearing in the hybrids (Munstermann 1980). However, subsequent crosses showed fertility in only one direction.

Due to the morphological, biochemical, and genetic similarities between A. brelandi and A. hendersoni, the validity of A. brelandi as a species distinct from A. hendersoni has been questioned. Though no biological criteria unambiguously define the specific status of allopatric populations, postcopulatory reproductive isolation indicates that the populations have diverged to the extent that reversion back to a single panmictic population is unlikely. The following experiments demonstrate unidirectional, postcopulatory reproductive isolation between A. brelandi and A. hendersoni, thus verifying that A. brelandi is a species distinct from A. hendersoni.

Materials and Methods

Strains of Mosquitoes. The CHISOS strain of A. brelandi was used for all of the crossing experiments. This strain was collected in June of 1978 from live-oak tree holes in the Chisos Basin of Big Bend National Park in western Texas (the type-locality of A. brelandi). Specimens of CHISOS were compared with the type-material of A. brelandi at the U.S. National Museum. The morphology of CHISOS agreed with that of the A. brelandi type-specimens for each of the diagnostic characters listed by Zavortink (1972). Two strains of A. hendersoni were used. AUSTIN was collected from

1Current address: American Embassy (Tuxtla), P.O. Box 3087, Laredo, TX 78041.
Scrapings collected in 1976 from Pueblo County, Colo. To date, no one has succeeded in establishing a free-mating laboratory colony of A. brelandi, and free-mating colonies of A. hendersoni, and A. brevipes must be maintained in large cages (60 by 60 by 180 cm) with several thousand individuals. All of the strains used in this study were maintained by forced copulation at the Vector Biology Laboratory, University of Notre Dame.

**Forced Copulation.** The forced copulation technique used in this study was a modification of that developed by McDaniel and Horsfall (1957). Mosquitoes were mated 1–2 weeks after emergence. Females were allowed to take a blood meal from anesthetized white mice 1–3 days before copulation. Male mosquitoes were anesthetized by chilling in an ice bath. Males were then impaled through the side of the thorax with a minute pin mounted on a wooden applicator stick, decapitated, and held over a damp paper towel. A blood-fed female was anesthetized with nitrogen gas and placed ventral side up on a depression slide with her genitalia hanging over the edge. A male was manipulated to bring his genitalia into contact with those of the female at about a 135° angle. When the proper orientation was achieved, the male clasped the female and sperm transfer ensued. Males released the females 10–20 s after clasping. Each male was used to inseminate two females. Approximately 80% of the females were inseminated successfully. The forced copulation procedure was done with the aid of a dissecting microscope at a magnification of ca. 7×.

**Mosquito Rearing.** Single female crosses were used for all of the hybridization experiments. The progeny of an individual female were referred to as a family. Each family within a cross was scored individually and treated as a replicate. For most of the crosses, 100 females were copulated. After copulation, females were held in 4-liter plastic cages and given a mixture of honey and celulose as a carbohydrate source. A piece of damp paper towel was placed on top of each cage to maintain the humidity and give the mosquitoes a source of water. The cages were held in an insectary at 21°C, 80% RH, and a photoperiod of 18:6 (L:D). Ten days after blood-feeding (3 days past the time of normal oviposition), females were transferred individually to 30-ml glass shell vials and given a mixture of honey and cellulose as a carbohydrate source. No carbohydrate sources were provided in the oviposition vials. Females oviposited within 1 to 3 days. After eggs were allowed to embryonate for 2 weeks, balsa wood strips were examined grossly and those 50 with the most eggs were chosen for the fertility experiments. Eggs were hatched by removing the cotton plug from the bottom of the vial, returning the balsa egg strip to the vial and adding 20 ml of water and 1 ml of liver powder solution (60 g liver powder in 1,000 ml water) to stimulate hatching. First instars were removed every 24 h, counted and placed into 450-ml larval rearing cups with 300 ml of water and 1 ml of liver powder solution. Larvae were removed from the hatching vials and added to the original rearing cups until no further eggs hatched during a 24-h period. Egg strips were then immersed for 5 min in modified Trpis bleach (Trpis 1970) (NaClO2 [90 g]/glacial acetic acid [20 ml]/distilled water [1,000 ml]). The bleach cleared the egg chorion, allowing eggs with unhatched embryos to be separated from those containing only yolk. Larvae were held in an insectary at 21°C and fed 1 ml of liver powder solution as required. Pupae were removed every 4 days from the larval rearing containers and placed in emergence containers with net chimneys. Adults were counted and separated by sex after all had emerged.

**Cytological Techniques.** Polytenie chromosomes in aedine mosquitoes cannot be used for cytological analyses because preparations are usually of poor quality. Therefore, meiotic chromosomes were used to detect chromosomal differences or incompatibilities that may exist between A. brelandi and A. hendersoni. Three-day-old male pupae were dissected in a drop of deionized water on a siliconized slide. The testes were transferred to a drop of 2% lactic-acetic orcein stain for 10 min; then they were squashed under an 18-mm cover slip. The cover slip was sealed with clear nail polish. Slides were stored at 100°C for up to 6 months. Slides were examined on a phase contrast microscope (Zeiss) utilizing a 100× objective (Neofluor).

**Aedine mosquitoes have three pairs of similar, metacentric chromosomes. Mosquitoes in the Tri-**

**seriatus Group have one small pair of chromosomes, I, and two large pairs, II and III. The two large chromosome pairs cannot be differentiated routinely. Therefore, they were grouped together for scoring the chiasmata frequencies.**

**Insemination Experiments.** To correlate the status of the sperm in the female reproductive tract with the egg embryonation rate, approximately 100 females were used for each of the reciprocal interspecific crosses. The females were force-mated and maintained in the same manner as in the fertility studies. Only the SOCOL strain of A. hendersoni was used in these experiments. Twenty-five females were dissected on days 1, 3, and 7 after copulation to determine the insemination rate and the condition of the sperm in the female reproductiv tract. The remaining females were placed individually in oviposition vials on day 10. After oviposition, on day 12, these females were dissected individually to associate the condition of the sperm in the reproductive tract with the embryonation rate of the eggs. After embryonating for 2 weeks, the eggs were bleached in modified Trpis bleach and the number of eggs containing embryos was recorded.
Table 1. Fertility of intra- and interspecific crosses between A. brelandi and A. hendersoni

<table>
<thead>
<tr>
<th>Cross</th>
<th>Eggs/family</th>
<th>Families with embryonated eggs</th>
<th>Eggs embryonated</th>
<th>First instar to adult</th>
<th>Sex ratio (♂:♀)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>z ± SE</td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>z ± SE</td>
</tr>
<tr>
<td>B·B</td>
<td>106.0 ± 4.6</td>
<td>50</td>
<td>74</td>
<td>50</td>
<td>62.4 ± 2.47</td>
</tr>
<tr>
<td>H·H</td>
<td>114.3 ± 2.7</td>
<td>50</td>
<td>60</td>
<td>50</td>
<td>60.2 ± 1.44</td>
</tr>
<tr>
<td>H·H²</td>
<td>108.8 ± 2.8</td>
<td>50</td>
<td>68</td>
<td>50</td>
<td>72.4 ± 2.30</td>
</tr>
<tr>
<td>B·H</td>
<td>108.2 ± 4.1</td>
<td>50</td>
<td>70</td>
<td>50</td>
<td>80.7 ± 2.28</td>
</tr>
<tr>
<td>B·H²</td>
<td>96.2 ± 4.3</td>
<td>50</td>
<td>70</td>
<td>50</td>
<td>3.3 ± 0.31</td>
</tr>
<tr>
<td>H·B</td>
<td>105.3 ± 6.5</td>
<td>50</td>
<td>7</td>
<td>98</td>
<td>4.8 ± 1.07</td>
</tr>
<tr>
<td>B·(B·H)</td>
<td>94.7 ± 3.7</td>
<td>50</td>
<td>78</td>
<td>50</td>
<td>78.3 ± 1.44</td>
</tr>
<tr>
<td>H·(B·H)</td>
<td>106.1 ± 3.2</td>
<td>50</td>
<td>82</td>
<td>50</td>
<td>51.4 ± 4.14</td>
</tr>
<tr>
<td>(B·H)·B</td>
<td>108.8 ± 3.2</td>
<td>50</td>
<td>68</td>
<td>50</td>
<td>90.2 ± 0.64</td>
</tr>
<tr>
<td>(B·H)·H</td>
<td>104.0 ± 2.6</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>90.9 ± 0.70</td>
</tr>
<tr>
<td>(B·H)·F₂</td>
<td>105.1 ± 3.3</td>
<td>50</td>
<td>72</td>
<td>50</td>
<td>87.9 ± 0.41</td>
</tr>
</tbody>
</table>

○ B, A. brelandi; H, A. hendersoni.

Includes only those families with one or more fertile eggs.

AUSTIN strain of A. hendersoni.

Significantly different from 1:1 by χ² test.

Results

The results of the intraspecific control crosses and the interspecific experimental crosses are presented in Table 1. The mean number of eggs per female was not significantly reduced in the interspecific crosses when compared with intraspecific crosses (F = 3.1, df = 1, n = 351, P > 0.05). In the intraspecific crosses, 60 to 74% of the females laid one or more fertile eggs, and between 60 and 72% of the eggs from families with at least one fertile egg were embryonated. In the cross between A. brelandi females and A. hendersoni males, 70% of the females laid at least one fertile egg and 80% of the eggs of those families developed embryos. These results were within the range of those observed in the intraspecific control crosses. However, in the reciprocal cross where A. hendersoni females were mated to A. brelandi males, only 7% of the females laid fertile eggs and only 3–5% of the eggs from those females eventually developed embryos. The total egg embryonation rate for this cross was less than 0.5%. These results were consistent in crosses with both the AUSTIN and SOCOL strains of A. hendersoni.

First instar to adult survivorships for the interspecific hybrids were equal to, or greater than, those for the intraspecific control crosses. The sex ratios did not differ significantly from the expected 1:1 for the parental and A. brelandi female × A. hendersoni male F₁ crosses (χ², df = 1 for each cross). Too few progeny were recovered from the A. hendersoni female × A. brelandi male F₁ crosses to calculate a χ² statistic. The sex ratio was distorted towards females in all of the backcrosses and the F₂ cross with the exception of the cross between A. brelandi females and F₁ males.

Back- and F₂ crosses using hybrid progeny of A. hendersoni females and A. brelandi males were not possible due to the difficulty in obtaining sufficient numbers of those F₁ progeny. The other possible F₂ and backcrosses, only the cross between A. hendersoni females and the F₁ male progeny of the A. brelandi female × A. hendersoni male cross showed reduced fertility. In this cross, 42% of the females produced at least one embryonated egg, with 51% of those eggs becoming embryonated. These results were intermediate between those of the control crosses and the incompatible interspecific cross. The maternal and paternal cytoplasms in this cross were of the same origins as in the incompatible F₁ cross. The genomes of the sperm varied from 100% A. hendersoni to 100% A. brelandi, with 50% of the sperm being predominantly one species and 50% predominantly the other.

The F₁ progeny from both reciprocal crosses were morphologically intermediate to the parental species. The scutal setae were primarily dark. However, some light and intermediate setae were observed also. The genital morphology of the hybrids was normal.

The insemination study was conducted to determine the cause of the infertility between A. brelandi and A. hendersoni.

Table 2. Insemination and egg embryonation rates for crosses between A. brelandi and A. hendersoni

<table>
<thead>
<tr>
<th>Cross</th>
<th>A. brelandi × A. hendersoni</th>
<th>A. brelandi</th>
<th>A. hendersoni</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Females with active sperm in spermathecae on day</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>80</td>
<td>25</td>
<td>80</td>
</tr>
<tr>
<td>3</td>
<td>72</td>
<td>25</td>
<td>84</td>
</tr>
<tr>
<td>7</td>
<td>80</td>
<td>25</td>
<td>88</td>
</tr>
<tr>
<td>12</td>
<td>75</td>
<td>32</td>
<td>76</td>
</tr>
<tr>
<td>Females with active sperm laying fertile eggs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>93</td>
<td>25</td>
<td>90</td>
<td>29</td>
</tr>
<tr>
<td>Eggs embryonated from females that laid fertile eggs</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
hendersoni females and A. brelandi males. Between 72 and 88% of the females for both reciprocal interspecific crosses had active sperm in their spermathecae 1, 3, and 7 days after mating (Table 2). No differences were observed in either insemination rate or sperm activity between the two crosses. Of the A. brelandi females mated to A. hendersoni males with active sperm in their spermathecae, 96% laid one or more fertile eggs and 90% of the eggs from those females developed embryos. In the reciprocal cross, where A. hendersoni females were mated with A. brelandi males, only 3 of the 29 females with active sperm in their spermathecae produced 1 or more fertile eggs and 40% of the eggs from those 3 females developed embryos.

Cytological examination of more than 1,000 meiotic nuclei in A. brelandi–A. hendersoni F₁ hybrids did not reveal any abnormal configurations indicative of chromosomal differences between these 2 species. An analysis of chiasmata frequencies in A. brelandi, A. hendersoni and their F₁ hybrid revealed no significant differences in the total number of chiasmata per nucleus (F₁ = 1.33, df = 2, n = 517, P > 0.05) (Table 3). However, differences were observed in the distribution of chiasmata. A. brelandi had significantly fewer chiasmata in the first pair of chromosomes than either A. hendersoni or the F₁ hybrid (F₁ = 28.83, df = 2, n = 517, P < 0.05) and more in pairs II and III (F₁ = 12.45, df = 2, n = 517, P < 0.05).

### Discussion

Results of hybridization studies indicate unidirectional postcopulatory reproductive incompatibility between A. brelandi and A. hendersoni. Fewer than 0.5% of the eggs laid by A. hendersoni females mated to A. brelandi males are fertile. Examination of female spermathecae indicates that A. brelandi males are capable of copulating with A. hendersoni females and that normal sperm transfer ensues. Aedes brelandi sperm in the female spermathecae of A. hendersoni appear normal with no reduction in activity apparent. Thus the incompatibility expresses itself between the movement of the sperm out of the spermathecae and early embryogenesis. The directionality of the incompatibility indicates a mechanism of cytoplasmic origin. Such mechanisms have been demonstrated to be due to rickettsia-like organisms in the Culex pipiens L. and Aedes scutellaris (Walker) species groups (Yen and Barr 1973, Trpis et al. 1981). However, the fertility pattern of the A. brelandi–A. hendersoni backcrosses differs from the pattern of maternal inheritance normally observed with cytoplasmic factors (Laven 1959). Based upon cytoplasmic inheritance, the backcross between A. hendersoni females and F₁, (A. brelandi × A. hendersoni) males should be completely incompatible. However, ca. 25% of the eggs from this cross were fertile. This is much higher than the 0.3% observed in the incompatible F₁ cross, but 50–60% lower than the fully compatible crosses.

The fertility pattern observed in the backcrosses between A. brelandi and A. hendersoni does not eliminate the cytoplasm as a factor in the incompatibility observed. However, the partial fertility of the A. hendersoni × F₁, (A. brelandi × A. hendersoni) cross does necessitate the involvement of nuclear factors. The mechanism that best explains these results invokes an interaction between the nuclear genome of the A. brelandi sperm and the cytoplasm of the A. hendersoni egg. This mechanism explains the intermediate fertility of the A. hendersoni × F₁, (A. brelandi × A. hendersoni) cross as well as the unidirectional nature of the incompatibility in the F₁ crosses. A similar mechanism was proposed by Motara and Rai (1977) to explain the incompatibilities observed between Aedes aegypti (L.) and Aedes mascarenensis MacGregor.

The demonstration of unidirectional postcopulatory reproductive incompatibility between A. hendersoni and A. brelandi confirms the status of A. brelandi as a species distinct from A. hendersoni. The incompatibility of the A. brelandi × A. hendersoni crosses, in which 50% of the progeny do not reach reproductive status, is actually greater than that observed in A. triseriatus × A. hendersoni crosses, in which 25% of the progeny do not reach reproductive status (Truman and Craig 1968, Taylor 1982).

Examination of chiasmata frequencies and meiotic configuration reveals no evidence of chromosomal repatterning during the divergence of A. brelandi and A. hendersoni. The chiasmata frequency in A. brelandi–A. hendersoni F₁ hybrids was actually higher than that observed in the parental species. In addition, despite the examination

### Table 3. Chiasmata frequencies and percentage bivalents achiasmate for A. brelandi, A. hendersoni and their F₁ hybrids

<table>
<thead>
<tr>
<th>Cross</th>
<th>Nuclei scored (n)</th>
<th>% No. of chiasmata/bivalent</th>
<th>Chiasmata/nucleus (total)</th>
<th>% Bivalents achiasmate</th>
<th>% Nuclei achiasmate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chromosome I</td>
<td>Chromosomes II &amp; III</td>
<td>Chromosome I</td>
<td>Chromosomes II or III</td>
<td></td>
</tr>
<tr>
<td>B-B</td>
<td>176</td>
<td>0.90 ± 0.06</td>
<td>1.63 ± 0.03</td>
<td>4.16 ± 0.09</td>
<td>37.7</td>
</tr>
<tr>
<td>H-H</td>
<td>144</td>
<td>1.32 ± 0.05</td>
<td>1.41 ± 0.04</td>
<td>4.13 ± 0.10</td>
<td>5.4</td>
</tr>
<tr>
<td>B-H</td>
<td>200</td>
<td>1.44 ± 0.05</td>
<td>1.44 ± 0.05</td>
<td>4.32 ± 0.09</td>
<td>10.6</td>
</tr>
</tbody>
</table>
of more than 1,000 hybrid nuclei, no meiotic config-
urations indicative of chromosomal rearrange-
ments were observed. Previous studies have shown that chiasma frequencies can be used as an index of chromosomal divergence in mosquitoes (Dev and Rai 1985) and in salamanders (Spurway and Callan 1960); the more reduced the hybrid chias-
mata frequency, the greater the divergence of the chromosomes. Little or no chromosomal repatterning has occurred since the divergence of these two species.

The divergence of A. brelandi from A. hender-
soni was a recent event in geological terms. The range of A. brelandi is limited to the Chisos Mountains of western Texas. The Chisos Mountains are currently surrounded by the Chihuahuan Desert, which represents a formidable geographical barrier between A. hendersoni and A. brelandi. Despite intensive efforts (J. Long and D. B. Taylor), neither A. brelandi nor A. hendersoni was found in the nearby Davis Mountains, approximately 150 km north of the Chisos Moun-
tains, although Aedes muelleri Dyar and Ortho-
podomyia alba Baker, both tree-hole mosquitoes sympatric with A. brelandi in the Chisos Moun-
tains, were found in abundance. The climatic his-
tory of West Texas is complex. During the late Pliocene, the climate was hot and dry, similar to the present conditions. During the kansasian glacial period, about 1 million years before the present, West Texas was forested and permanent lakes were present. Since that time, the climate has deteriorated, with brief pluvial periods during each of the glacial advances. The last pluvial period was the Wisconsin, which ended about 10,000–12,000 years ago. Whether there was sufficient rainfall at that time for deciduous forests to form is uncertain. However, the water runoff is known to have increased, allowing growth of trees along presently dry waterways and permitting the movement of tree-hole-breeding mosquitoes. The present arid conditions have been continuous for about 10,000–12,000 years (Frye and Leonard 1957, 1965, MacGinitie 1958, Wollin et al. 1971, Wright 1971). These data indicate that A. brelandi and A. hender-
soni have been isolated for 10,000–12,000 years.

The divergence of A. brelandi and A. hender-
soni was a recent event geologically and probably the result of habitat discontinuity due to climatic deterioration. Subsequent to geographical isolation, the species have changed little morphologi-
cally, genetically, or chromosomally. However, during that time, incompatibility between the ge-
nome of A. brelandi and the cytoplasm of A. hender-
soni has rendered the two species partially re-
productively incompatible.

Acknowledgment

We thank J. Long for his assistance in collecting Aedes brelandi and L. Munstermann for his review and helpful comments on this manuscript. This research was sup-
ported by National Institutes of Health Research Grant No. A-02753.

References Cited


MacGinitie, H. D. 1958. Climate since the late Cre-


Matthews, T. C., and L. E. Munstermann. 1983. Ge-
netic diversity and differentiation in northern pop-


Received for publication 4 January 1985; accepted 1 July 1985.