EXTRACHROMOSOMAL INHERITANCE OF CARBON DIOXIDE SENSITIVITY IN THE MOSQUITO CULEX QUINQUEFASCIATUS

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ABSTRACT

Mosquitoes from a laboratory colony of Culex quinquefasciatus from Matsu Island, China, develop irreversible paralytic symptoms after exposure to carbon dioxide at 1°. This CO₂ sensitivity is caused by an inherited infectious agent, probably a virus. Crossing studies between CO₂-sensitive and -resistant mosquitoes showed that the sensitivity trait is inherited extrachromosomally in a fashion strictly analogous to the hereditary transmission of sigma virus in Drosophila melanogaster. Sensitivity could be maintained through maternal transmission alone, despite nine generations of backcrossing of "stabilized" CO₂-sensitive females to males from a resistant strain. CO₂-sensitive males crossed to resistant females transmitted sensitivity to a portion of their F₁ progeny, and only the female F₁ sensitives were capable of further hereditary transmission.—Matsu, or a very similar hereditary infectious agent, is common in natural populations of Cx. quinquefasciatus on Oahu, Hawaii. Fifty-nine percent of the families reared from field-collected egg rafts contained CO₂-sensitive mosquitoes, and some families contained only sensitive mosquitoes.

INSECTS exposed to carbon dioxide gas normally experience only narcosis, from which they rapidly recover upon return to normal air. Carbon dioxide-sensitive insects react quite differently to even momentary exposure to pure CO₂ gas, becoming irreversibly paralyzed. The phenomenon of CO₂ sensitivity was first discovered in Drosophila melanogaster (L'HERITIER and TEISSIER 1937), where the trait was shown to be a symptom of infection by a hereditary virus named sigma, common in natural populations of flies. In nature, sigma virus is transmitted solely via male and female gametes, with virus and host genotypes strongly influencing the hereditary association (BRUN and PLUS 1980).

It has been shown that sigma virus and certain viruses pathogenic to vertebrates can induce CO₂ sensitivity when inoculated into mosquitoes (ROSEN 1980; TURELL and HARDY 1980; TURELL, HARDY and REEVES 1982a). Although some of the latter viruses can be efficiently maintained in mosquitoes by transovarial transmission (TESH and SHROYER 1980; TURELL, HARDY and REEVES 1982b), neither transmission through sperm nor CO₂ sensitivity of transovarially infected mosquitoes has been demonstrated (TURELL, HARDY and REEVES 1982a; D. A. SHROYER, unpublished data). ROSEN and SHROYER (1981) recently reported the discovery of natural CO₂ sensitivity in the cosmopolitan mosquito, Culex...
quinquefasciatus. In that preliminary communication we stated that the CO₂ sensitivity was maintained in laboratory populations of Cx. quinquefasciatus in a fashion analogous to the complex mode of inheritance of sigma virus in D. melanogaster and that the sensitivity was caused by an infectious agent which we called Matsu virus. The present paper details the mode of inheritance of CO₂ sensitivity in Cx. quinquefasciatus and provides evidence that Matsu or a very similar agent is common in field populations of this mosquito in Hawaii.

MATERIALS AND METHODS

Mosquito strains: Three laboratory strains of Cx. quinquefasciatus were employed in this study. MATSU: an unselected geographic strain originating from larvae collected on Matsu Island, China, in November 1975. Mosquitoes were CO₂ sensitive when first tested in July 1979. MATSU-CDS: derived from MATSU. Progeny of a single female were selected to initiate this subline. The selected family had displayed clear-cut expression of CO₂ sensitivity, with all tested mosquitoes showing complete paralysis. HOKAMA: a CO₂-resistant strain originating from a collection of egg rafts from Oahu, Hawaii, in July 1980. Progeny of the field-collected mosquitoes were tested for CO₂ sensitivity, and all sensitive families were discarded from this and the next laboratory generation. No additional selection was required to maintain CO₂ resistance.

Rearing methods: Larvae were reared on a suspension of beef liver powder at 28° and a 16 hr light: 8 hr dark photoperiod. Adults were maintained in 46 × 46 × 46 cm cages supplied with 10% sucrose as food. Blood meals for initiation of ovarian development were from restrained mice. For progeny rearings, newly laid egg rafts were transferred from the oviposition site to a separate container for hatching. Each egg raft was carefully examined after transfer to detect and remove any contaminating “loose” eggs from other disrupted egg rafts. This assured that the larvae reared were progeny of a single female.

In crosses between CO₂-sensitive and -nonsensitive (resistant) mosquitoes, parents were first sexed in the pupal stage and then examined daily until completion of emergence to reveal sexing errors and assure virginity. Large numbers of each sex were used for crosses, and when 2-3 weeks old the mated females were blood-fed to obtain progeny. Only first ovarian cycle progeny were employed.

Survey of field populations: Field collections of Cx. quinquefasciatus egg rafts were made from various parts of the island of Oahu, Hawaii, during 1980 and 1981. Each egg raft was hatched and reared separately and a sample of each set of progeny so obtained was tested for CO₂ sensitivity.

CO₂ sensitivity testing: Mosquitoes were placed in test tubes in groups of ten each and chilled on wet ice for 15 min (tube temperature approximately 1°). Each tube was then gassed 20 sec with pure CO₂ and left on ice for an additional 15 min. After the 15-min exposure to CO₂ mosquitoes were transferred to small well-ventilated cages and held at 23-25° for 2 hr before scoring for CO₂ sensitivity. CO₂-resistant mosquitoes recovered fully within 2 hr. Sensitive mosquitoes displayed a range of symptoms, including spasmodic twitching of legs, intermittent or sustained fluttering of wings, inability to maintain normal posture on horizontal or vertical surfaces or complete paralysis. Because symptoms occurred as a continuum, it is likely that individuals with subtle symptoms were sometimes improperly scored as “CO₂ resistant.” Conversely, exceptional individuals in otherwise CO₂-resistant groups showed symptoms mimicking CO₂ sensitivity but which were apparently due to nonspecific causes such as injury or senescence. Because of this inherent lack of precision in the CO₂ assay, a test sample of at least 20 mosquitoes was not considered sensitive unless more than 10% displayed characteristic symptoms. Except where noted, known sensitive and resistant Cx. quinquefasciatus were included in each test as controls. Mosquitoes were generally less than 2 weeks old when tested and in the case of females were nulliparous and not previously blood-fed. Equal numbers of males and females were tested, but since no consistent differences in sensitivity were noted sexes were pooled in the data reported.

RESULTS

Reciprocal crosses were made between the MATSU-CDS CO₂-sensitive strain and the HOKAMA resistant strain (abbreviated as M and H, respectively).
Before making the crosses we assayed a sample of 30 HOKAMA and ten MATSU-CDS families for incidence of CO₂ sensitivity. As was typical for these strains, most HOKAMA families were free of sensitive mosquitoes, whereas most MATSU-CDS families contained only CO₂ sensitives (Figure 1A and B). Virgin mosquitoes used for crosses came from nine completely sensitive MATSU-CDS families and from 18 HOKAMA families in which no CO₂ sensitives were observed. After the crosses were made and the female parents blood-fed, the resulting progeny were reared separately by family and tested for CO₂ sensitivity.

All 20 F₁(M × H) families were CO₂ sensitive, with 55–100% of the tested mosquitoes being sensitive (Figure 1C). When results from these families were pooled, 83% of all mosquitoes tested were sensitive. The CO₂ sensitivity trait displayed by these hybrids was inherited maternally. In contrast, only 45% of the F₁(H × M) families contained more than 10% sensitives, and the sensitive families contained a lower proportion of sensitives than the reciprocal hybrids (Figure 1D). The proportion of sensitives in offspring of transmitting MATSU-CDS males (i.e., the “valence” of sigma virus workers) ranged from 15–80%. Collectively, only 22% of the F₁ (H × M) mosquitoes tested were sensitive. Although transmission of CO₂ sensitivity was clearly less efficient through male than through female gametes, a significant amount of paternal transmission was nonetheless demonstrated.

Using the same parental strains, we made a second independent determination of the valence of MATSU-CDS males. Of 20 F₁(H × M) families tested for CO₂ sensitivity, 90% contained more than 10% sensitive mosquitoes (Figure 2). Valences of transmitting MATSU-CDS males ranged from 15–85% in this experiment.

F₁ hybrids between the HOKAMA and MATSU-CDS strains were backcrossed to CO₂-resistant HOKAMA mosquitoes in order to observe the extent of continued vertical transmission of the CO₂ sensitivity trait. The F₁(M × H) mosquitoes used in the backcrosses came from five families with 95–100% incidence of sensitivity, and the F₁(H × M) mosquitoes were from five families that were 40–80% CO₂ sensitive (Figure 1C and D). Backcross progenies were reared separately and tested for CO₂ sensitivity as before. F₁(M × H) females backcrossed to HOKAMA males produced offspring that were almost all CO₂ sensitive (90%), with individual families being 60–100% sensitive (Figure 3A). The reciprocal backcross gave markedly different results, with most H × F₁(M × H) families being CO₂ resistant and with no family containing more than 54% sensitives (Figure 3B).

When females from partially sensitive F₁(H × M) families were crossed to HOKAMA males only a portion of the backcross progeny were sensitive (Figure 3C). However, some of these backcross families contained mostly sensitive mosquitoes. In contrast, none of the H × F₁(H × M) families tested were CO₂ sensitive (Figure 3D). Unlike females that received their sensitivity from their fathers, paternally infected males were incapable of transmitting the trait further.

CO₂-sensitive F₁(M × H) hybrids were successively backcrossed to resistant HOKAMA males for a total of nine generations. At each generation, virgin
females were selected from five to ten families displaying the highest incidence of CO₂ sensitivity as well as the strongest symptoms (complete paralysis predominating). At least 365 females and 405 males were used in each cross. As shown in Figures 3A and 4, all 224 backcross families tested were CO₂ sensitive. In fact, over the nine generations of backcrossing 55% of the families contained only sensitives among the 40 mosquitoes sampled from each, and no decline in incidence of sensitivity was observed.

Mosquitoes from the ninth backcross were allowed to mate randomly among themselves to initiate a CO₂-sensitive subline in a genetic background predominantly derived from the HOKAMA strain. This subline retained its CO₂ sensitivity through 16 generations in the absence of any artificial selection (139 of 140 mosquitoes tested in the 16th generation were sensitive).
Soon after our initial discovery of Matsu-induced CO₂ sensitivity in Cx. quinquefasciatus we found that an identical sensitivity occurred in Hawaiian populations of the same mosquito. F₁ progeny of mosquitoes collected in 1980 from a site on the island of Oahu were tested for CO₂ sensitivity. Based on samples of 20 mosquitoes, each of seven F₁ families contained 25–80% sensitives. A laboratory colony originating from mosquitoes collected elsewhere on Oahu had been maintained for 26 generations in the laboratory before being selected for CO₂ sensitivity. A new subline of this strain based on a selection of three wholly CO₂ sensitive families has been maintained continuously for 24 generations without further selection and with no significant decline in sensitivity.

To more directly document the occurrence of CO₂ sensitivity in Hawaiian mosquitoes, from June to August 1981 we made collections of at least two intact Cx. quinquefasciatus egg rafts from nine locations on Oahu, most within or near the Honolulu metropolitan area. Except for unusually small families, 40 mosquitoes from each family were tested for CO₂ sensitivity.

Of 70 families reared from field-collected egg rafts, 41 were CO₂ sensitive. The proportion of sensitives varied with family and collection. Figure 5 shows the frequency distribution of sensitivity among families from the four largest field collections. Collections C and D (Figure 5) contained some families that were entirely CO₂ sensitive.

DISCUSSION

We have demonstrated that the carbon dioxide sensitivity observed in the MATSU strain of Cx. quinquefasciatus is caused by an infectious agent by
transmitting CO₂ sensitivity to CO₂-resistant *Cx. quinquefasciatus*, Toxorhynchites amboinensis, *Aedes albopictus* or *Ae. vexans* mosquitoes by inoculation of undiluted hemolymph or whole body triturates, or by ovary transplant (Rosen and Shroyer 1981). The sensitivity-inducing agent has also been adapted to replicate to high infectious titer in cloned cells of *Ae. albopictus*, and filtration studies are consistent with a viral agent (L. Rosen, unpublished data). Additionally, the remarkable similarities in modes of hereditary transmission displayed by sigma virus and this mosquito agent provide compelling circumstantial evidence that the latter is a viral agent similar to sigma virus. Yet, Matsu is clearly distinct from sigma virus, as we have been unable to induce CO₂ sensitivity in *D. melanogaster* by inoculation with the Matsu agent (L. Rosen, unpublished data).

The pattern governing hereditary transmission of the Matsu agent in *Cx. quinquefasciatus* is summarized in Figure 6. Crosses between CO₂-sensitive
FIGURE 4.—CO₂ sensitivity of individual families from repeated backcrosses of hybrid females to HOKAMA males. Superscripts in crossing formulas indicate the number of sequential crosses to HOKAMA males. Approximately 40 mosquitoes were tested from each family.

FIGURE 5.—CO₂ sensitivity of field-collected families from four sites on Oahu, Hawaii. Approximately 40 mosquitoes were tested from each family.
MATSU-CDS females and resistant HOKAMA males, and subsequent successive backcrosses of hybrid females to HOKAMA males established that Matsu can be efficiently maintained as a "stabilized" maternal infection as was first demonstrated in the sigma virus-Drosophila association (BRUN and PLUS 1980). Like most strains of sigma virus, Matsu also exhibits substantial transmission through male gametes. Female mosquitoes infected with Matsu by their fathers act as either stabilized or nonstabilized females, transmitting to all or only a portion of their offspring. But paternally infected males do not appear to further transmit the Matsu agent. Thus, the patterns of inheritance of Matsu and sigma virus (BRUN and PLUS 1980) are indistinguishable. Although stabilized maternal lines have been established in mosquitoes infected with bunyaviruses (TESH and SHROYER 1980; TURELL, HARDY and REEVES 1982b), vertical transmission through the male has not been found in a line of Ae. albopictus persistently infected with San Angelo virus (D. A. SHROYER, unpublished data).

Three experiments measuring extent of Matsu transmission from infected males to their progeny yielded somewhat different results. Individual male valences covered approximately the same range in the two comparisons of transmission by MATSU-CDS males, but in one case 90% of the $F_1(H \times M)$ progenies were CO$_2$ sensitive (Figure 2), whereas in the other experiment only 45% of the progenies were sensitive (Figure 1D). The $F_1(M \times H)$ males transmitted sensitivity to only 19% of the tested families (Figure 3B), and the highest filial infection rate observed was 54%. The valence of D. melanogaster males for sigma virus declines with male age (SIGOT 1953), and the minor differences in age at mating of the male mosquitoes in our experiments were consistent in

![Schematic summary of inheritance of CO$_2$ sensitivity in Cx. quinquefasciatus.](image-url)
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direction with this precedent (males were 4–8, 7–9 and 9–11 days old, respectively, in the experiments). It is also possible that the hybrid genome of the $F_1(M \times H)$ males contributed to their reduced valence, given the importance of the insect genotype in the Drosophila-sigma virus association (BRUN and PLUS 1980). Further study is required to determine the significance and origin of the observed variation.

A comparison of Figures 1B and C, 3A and 4 clearly shows the efficient transmission of the CO₂ sensitivity trait through the maternal line, but subtle differences are also apparent. The $F_1(M \times H)$ families and the families resulting from the initial backcross of that hybrid to HOKAMA males tended to contain more CO₂-resistant mosquitoes than either the MATSU-CDS parental families or the later backcross families. If real, this observation could be attributable to host genetic factors. However, we have found that the severity of symptoms among unambiguous CO₂ sensitives often differed from generation to generation and between unselected generations of the MATSU-CDS colony. Resistant individuals from those exceptional families might be infected but asymptomatic. They might also represent Matsu-free individuals resulting from the appearance of occasional noninfected germinal cells in the mother ("decrochage," ICONOMIDIS and L'HERITIER 1961).

Our discovery of CO₂ sensitives among colonized and field-collected mosquitoes from Hawaii indicates that Matsu or another very similar agent is common in natural populations of Cx. quinquefasciatus. Most of the egg rafts which we collected produced progenies that were at least partially sensitive. Wholly CO₂-sensitive field-collected progenies were also found, and these may have been the offspring of stabilized females. But despite the ready selection of pure-breeding sensitive strains, many of the field mosquitoes were CO₂ resistant. Sigma virus infection rates are also lower in natural populations than in caged populations, typically 10–30% (WILLIAMSON 1961; FÉLIX, GUZMÁN and DE GARAY ARELLANO 1971; FLEURIET 1976). FLEURIET (1980, 1981, 1982) has extensively investigated the roles of host refractory genes and virus modification of fitness parameters in the regulation of sigma virus incidence in nature.

We do not yet know whether CO₂ sensitivity is a common feature of global populations of Cx. quinquefasciatus. This species has one of the widest distributions of any mosquito, and it seems unlikely that the Matsu agent would occur only in the two insular locations sampled. In fact, Hawaiian Cx. quinquefasciatus populations are probably derived from an introduction from Mexico in 1826 (HARDY 1960). A worldwide survey for sensitivity in Cx. quinquefasciatus and other members of the Culex pipiens species complex would be of potential value in revealing the role of the Matsu agent in the natural history of the host mosquito.

Hereditary microorganisms like Matsu may function as efficiently transmitted cytoplasmic genes and confer important phenotypic effects on their hosts (L'HERITIER 1970; COSMIDES and TOOBY 1981). In insects such cytoplasmic traits include sex ratio distortion and mating compatibilities between populations (YEN and BARR 1971, EHRMAN and KERNAGHAN 1972). Aside from the production of CO₂ sensitivity under conditions not experienced in nature, the biological
consequences of Matsu infection for Cx. quinquefasciatus are unknown. Insect resistance to infection with microorganisms pathogenic to vertebrates is inherited maternally in two insects (BRAS-HERRENG 1981; TRPIK, DUHRKOPF and PARKER 1981). As Cx. quinquefasciatus is an important vector of several vertebrate pathogens, it is important to know how stabilized Matsu infection might influence vector competence of local populations for such pathogens. Particularly if Matsu is ultimately proved to be, like sigma, a virus of the family Rhabdoviridae, it is possible that it may confer some heterologous protection against other rhabdoviruses that are transmissible and pathogenic to vertebrates.

The discovery that natural populations of both D. melanogaster and Cx. quinquefasciatus commonly support hereditary microorganisms and that these microorganisms are maintained by identical rules of transmission suggests that other Diptera of medical or economic importance may harbor similar hereditary agents. Extensive studies of sigma-Drosophila relationships conducted over the past four decades (BRUN and PLUS 1980; TENINGES, CONTAMINE and BRUN 1980) have provided a model for understanding other insect-virus relationships. As illustrated by this and other studies (TESH and SHROYER 1980; TURELL, HARDY and REEVES 1982b), the sigma virus model of hereditary transmission appears to have wide applicability.

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