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THE CHECK IS IN THE MALE: MALE MOSQUITOES AFFECT FEMALE PHYSIOLOGY AND BEHAVIOR

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ABSTRACT. The accessory glands of male mosquitoes may produce substances that are transferred to females during mating and alter female physiology and behavior. The effects of male substances include the inhibition of subsequent female mating behavior, stimulation of oviposition and preoviposition behaviors, and the inhibition of host-seeking behavior. The circadian rhythmicity of females can also be altered and their metabolic priorities restructured, making them more likely to reproduce. The specific components that affect the female have yet to be completely identified, but the published reports are summarized.

KEY WORDS Culicidae, mating, male accessory gland substances, behavior, host-seeking

INTRODUCTION

Most studies of insects focus on the female of the species, perhaps because that sex produces a reproductive product, the egg, that can potentially be manipulated with the goal of population control. The focus on females has been even more exaggerated in mosquito biology because in addition to producing eggs, they feed on blood and have the potential to transmit agents of disease. Compared to the voluminous research done on female mosquitoes, males are poorly studied and often ignored for lack of any practical reasons to know them better. Yet, the males contribute substantially to many aspects of female behavior and physiology and indirectly influence parasite transmission through their effects on the female.

From a teleologic viewpoint, the primary reason that male mosquitoes exist is to mate with females. Parthenogenesis is rare in mosquitoes (Craig 1957, Kitzmiller 1959) despite its many advantages, but it is not always beneficial to literally put all your eggs in one basket when living in a changing environment, and the recombination that occurs during sexual reproduction provides the long-term advantages of genetic variability. Along with the internal fertilization that accompanies sexual reproduction comes an opportunity for males to transfer other products to females in addition to sperm. In many insects, sperm are transferred within a gelatinous spermatophore produced by the male accessory glands, but spermatophores are absent in male mosquitoes. The accessory glands instead manufacture substances that are relocated to the female during mating and can serve several purposes. Their primary function is as a transport medium for sperm, but these substances also may nourish the sperm while they reside in the female, can form mating plugs in the female that may interfere with subsequent mating, supplement the female's nutritional state, and provide chemical signals to the female that mating has occurred. This last function may be accompanied by physiologic changes in the female that are induced by male substances. This review will discuss the many ways that these male accessory gland substances that are transferred during mating can alter the behavior and physiologic state of female mosquitoes.

POSSIBLE REASONS FOR THE EVOLUTION AND PRODUCTION OF MALE ACCESSORY GLAND SUBSTANCES

Vigorous competition occurs among the males of a species for the insemination of females and the subsequent transmission of their genes to ensuing generations, resulting in a strong selective pressure for males to produce substances that may give them a reproductive advantage. Preventing a female from mating with other males and expediting her reproductive processes insures that the progeny produced by that female will carry some of his genes. Competition also occurs between conspecific males and females, who may be at odds because they do not always share the same ultimate goals. For example, although males benefit by frequent copulation with a variety of females, repeated mating by a female may exhaust her nutritional reserves and make her more susceptible to predation. Mating may even reduce the immune response to parasites (Siva-Jothy et al. 1998). Additionally, the male components may prevent the female from remating and thereby assure the paternity of the 1st male, despite it being in the best interests of female reproduction to sample the products of other males that might possibly be genetically superior. It is clearly to the benefit of the males when they can affect female metabolism to cause the females to use their metabolic reserves for reproduction instead of their own survival to increase the probability that eggs will develop. However, females can also gain from the interaction, using the male products as signals that mating has occurred and thus preventing them from expending energy on laying eggs that could not be fertilized and therefore would not be viable.

Eberhard (1996) presented evidence for the role of "cryptic female choice" in the evolution of male products intended to manipulate females and the discrimination among the various males whose reproductive substances might be utilized. With cryptic female choice, the female selectively chooses paternity by males that display a particular trait over others that lack it, and does so after physically copulating with them. This female choice is an important selective pressure for the evolution of new and improved substances that males produce to insure that their genes do survive.

These complex relationships that are associated with sexual reproduction may have resulted in the rapid evolution of structures and substances used by both males and females to increase their reproductive success (Eberhard 1985, Arnqvist 1998). According to the sexual selection hypothesis, the conflict between males and females can lead to the evolution of new substances by males that better assure their paternity. Females can coevolve the means to select which males will fertilize their eggs. The evolutionary divergence of genes for the production of male substances that has come under selective pressure from sexual selection has been well documented in *Drosophila* (Whalen and Wilson 1986, Aguadè et al. 1994, Rice 1996, Tsaur et al. 1998). In mosquitoes, the behavior of females and their status as vectors can be affected by this objective of male paternity assurance.

FUNCTIONS OF MALE ACCESSORY GLAND SUBSTANCES IN FEMALE MOSQUITOES

Inhibition of mating behavior

Patterns of receptivity in female insects vary from single mating to several degrees of multiple mating (Thornhill and Alcock 1983). In species that engage in multiple mating, a cyclical receptivity may occur in which males are rejected for a period after each successful encounter, a rejection that is generally based on some consequence of the male's behavior. In mosquitoes, single mating appears to be most common; mating once dramatically reduces the tendency of most female mosquitoes to become subsequently inseminated by another male. This contention is supported by an abundance of field and laboratory evidence demonstrating that single mating is the rule (Gillies 1956, Goma 1963, Bryan 1968, Jones 1973, Bullini et al. 1976, Mahmood and Reisen 1980, Reisen et al. 1984, Baimai and Green 1987, Gomulski 1990, Villarreal et al. 1994, Yuval and Fritz 1994).

Craig (1967) first reported the permanent inhibition of female receptivity in *Aedes aegypti* (L.) by male accessory gland substances. He implanted unmated *Ae. aegypti* females with testes, segments of the male digestive tract, or male accessory glands, and observed an almost total absence of insemination in those containing accessory glands after the females were caged with normal males. Spielman et al. (1967) demonstrated that the bursa copulatrix, normally the 1st destination of sperm before they enter the spermatheca where they permanently reside, did not fill if the female was already mated or implanted with a male accessory gland. Craig (1967) also showed the effect was nonspecific, with the male accessory glands of several other species inducing refractory mating behavior in female *Ae. aegypti.* Subsequent studies suggested some degree of specificity; although the male accessory glands of *Aedes triseriatus* (Say) were effective in inhibiting mating in *Aedes atropalpus* (Coquillett), they were not effective in *Ae. aegypti,* and *Ae. aegypti* glands were ineffective in *Ae. triseriatus* (Ramalingam and Craig 1976). The mechanism that prevents reinsemination in *Ae. aegypti* may be related to the difficulty males have in grasping the cerci of mated females (Gwadz et al. 1971a). The target of male accessory gland substances appears to be the terminal abdominal ganglion, which innervates the genitalia (Gwadz 1972). The involvement of the brain in this regulation was suggested by the localization of radioactively labeled male products within both the heads and abdomens of female *Culex tarsalis* Coquillett (Young and Downe 1987). However, the transection of the ventral nerve cord in females and the disruption of nervous transmission between the terminal ganglion and the brain does not prevent insemination (Gwadz 1972), making it unlikely that the brain is implicated in female mating.

In spite of the overwhelming field data suggesting monandry, several reports have been made of polyandry in female mosquitoes under certain circumstances. These include the several hours immediately after the 1st mating before the male accessory gland substances have had time to have an effect (Craig 1967). When copulation is interrupted or when mating occurs with a previously depleted male so that the transfer of male accessory gland substances is not complete, the inhibition of mating may not be established (Gwadz and Craig 1970). Reports also have been made of the renewal of sexual receptivity in *Ae. aegypti* females after they underwent several gonotrophic cycles (Williams and Berger 1980). Young and Downe (1982) also demonstrated the increased likelihood of remating after a previous gonotrophic cycle, but they employed the transfer of a radioactive label from labeled males to unlabeled females. However, using a similar protocol with an additional set of controls consisting of *Ae. aegypti* females exposed to radioactively labeled *Anopheles* males (Dickinson and Klowden 1997), some transfer of label was also observed even though the mosquitoes could not have mated, suggesting that previous reports may not have considered the nonspecific movement of label resulting from nonsexual contact. In the previous studies, this transfer might have been interpreted as occurring through mating. Dickinson and Klowden (1997) concluded that monogamy in female *Ae. aegypti* was probably the rule even after several gonotrophic cycles, a conclusion also reached by Reisen et al. (1984) for *Cx. tarsalis.* However, in experiments in which the bursae were directly examined for sperm as a criterion for multiple mating, a small percentage of remating by female *Ae. aegypti* was indeed observed (Spielman et al. 1967, Williams and Berger 1980). It is arguable whether any of these laboratory experiments are relevant to the issue of whether remating naturally occurs in field populations, given that crowding in small cages may lead to multiple mating in *Anopheles culicifacies* Giles, whereas multiple mating does not occur in larger cages (Mahmood and Reisen 1980).

Male accessory gland substances interfere with reinsemination in many anophelines by the production of a so-called mating plug (Gillies 1956, Giglioli 1963, Giglioli and Mason 1966). The mating plug occupies the common oviduct for 1–2 days and has been reported to prevent subsequent male products from entering, although a small proportion of field-collected mosquitoes do retain double plugs as evidence of multiple mating attempts (Giglioli and Mason 1966). It is not known how reinsemination in some *Anopheles* continues to be inhibited once this plug is dissolved, because unlike in aedines, male accessory gland substances themselves fail to induce mating refractoriness in many species in this genus (Klowden, unpublished data). However, multiple mating in natural populations of anophelines seems to be a rare event in spite of the rapidity and apparent ease at which mating can occur soon after adult emergence in some species (Lounibos et al. 1998). Although female *Ae. aegypti* may copulate soon after emergence, they are unable to develop mating competence and do not retain semen during copulation until after juvenile hormone is released at 1–2 days postemergence (Lea 1968, Gwadz et al. 1971b). In the field, many aedines generally fail to mate until this competence develops (Edman et al. 1972, Lea and Edman 1972). In contrast is the unusually precocious behavior of female *Opifex fuscus* Hutton. Adult males search for female pupae and copulate with females as they emerge (Bates 1949).

Stimulation of oviposition

Once eggs mature, they are much more likely to be deposited if the female has mated. Gillett (1955) speculated that seminal fluid entering the spermathecae stimulated ovulation and oviposition in *Ae. aegypti,* but Leahy and Craig (1965) first demonstrated the actual involvement of the male accessory glands in this mating effect. The stimulation of oviposition was not very specific, with glands from *Aedes albopictus* (Skuse), *Culex pipiens* Linnaeus, and *Drosophila melanogaster* Meigen showing activity in unmated gravid *Ae. aegypti* females; however, but glands from male coleopterans in the genus *Tenebrio* had no effect when implanted into *Ae. aegypti* hosts (Leahy and Craig 1965, Leahy 1967). The active principle from the glands, called matrone (Fuchs et al. 1968), consisted of 2 fractions designated α and β (Fuchs et al. 1969, Fuchs and Hiss 1970), with the β fraction having the greatest effect on the stimulation of oviposition (Hiss and Fuchs 1972).

Switchover to preoviposition behavior

Once eggs develop, the behavior of the female mosquito again changes. Rather than readily responding to stimuli from hosts for a blood meal, the gravid female becomes more sensitive to chemical and physical stimuli emanating from the oviposition site, in effect allowing her to alter her priorities so she caters to the needs of her offspring rather than herself. In *Ae. aegypti,* this change in behavior is mediated by a humoral factor produced during oogenesis (Klowden and Blackmer 1987), but it only occurs in mated females. Unmated females that have fed on blood and matured their eggs do not respond to methyl propionate, one component of the bouquet of oviposition site stimuli (Yeh and Klowden 1990). Thus, in order for the female to engage in behavior that will ultimately deposit eggs in a location where they will survive, she must have at least 3 inputs, including one from male accessory gland substances. Required are stimuli from the oviposition site, a humoral factor associated with egg development, and a signal from male accessory gland substances verifying that mating has occurred and that the eggs that are to be laid will be fertilized. The chemical nature of this preoviposition trigger is not known.

Circadian rhythmicity

Some female mosquitoes display a behavioral rhythmicity that is altered after mating. Female *Anopheles gambiae* Giles display a bimodal pattern of flight activity in the laboratory, with one peak shortly after the onset of the scotophase and a 2nd at the end of the scotophase. The 1st activity peak, which correlates with the time of mating, is reduced in females after mating occurs (Jones and Gubbins 1977). Jones and Gubbins (1978) speculated, without evidence, that this alteration in circadian behavior in *An. gambiae* was caused by the transfer of male accessory gland substances. Similarly, changes in circadian activity patterns of *Ae. aegypti* (Jones 1981) and *Anopheles stephensi* Liston (Rowland 1989) came about as a result of mating. However, male accessory gland substances again were only speculated to be responsible for the behavioral changes and were not causally linked. Given the absence of effects of male accessory gland substances on mating behavior in *An. gambiae* (Klowden, unpublished data), it is important to repeat these circadian experiments in this species with male accessory gland substances to establish whether they are indeed involved. The data are more definitive for several members of the *Cx. pipiens* complex, where extracts of male accessory glands were found to be specifically responsible for modifying the circadian flight activity of *Culex quinquefasciatus* Say (Jones and Gubbins 1979) and the circadian activity patterns of both *Cx. p. pallens* Coquillett and *Cx. p. molestus* Forskal (Chiba et al. 1990, 1992).

Regulation of reproduction

Egg maturation in mosquitoes is a complicated process that requires the coordination of a number of environmental and physiologic events (Klowden 1997). Some species that diapause as adults do not produce eggs during certain times of the year even if they happen to ingest blood (Mitchell and Briegel 1989). The production of vitellogenin is metabolically expensive, and it is in the best interests of the female to begin doing so only when she has acquired sufficient reserves, much of which come from the blood that is ingested. Male accessory gland products seem to modulate the physiologic response of the female, making her more likely to produce eggs. This phenomenon has also been described in the blood-sucking hemipteran *Rhodnius prolixus,* which develops fewer eggs when unmated (Davey 1967). Presumably, the mated female invests more of the blood meal in oogenesis, whereas the unmated female uses some for reproduction but reserves the remainder of the energy to power the locomotion necessary to find a mate (Davey 1989). Lounibos (1994) noted a correlation between the ability of several species of *Anopheles* to develop eggs when they were unmated and the corresponding volume of the male's accessory glands. He suggested a possible functional relationship between the size of the gland and the physiologic involvement of the gland products in reproduction, with species that had small glands being less dependent on the male contributions than species with larger glands. *Anopheles albimanus* Wiedemann, having the smallest volume of male accessory glands, showed no effects on male-stimulated oogenesis.

When adult female *Ae. aegypti* were maintained under conditions of suboptimal nutrition, they were more likely to mature a batch of eggs when they were mated or injected with a homogenate of male accessory glands than when they were unmated (Klowden and Chambers 1991). This effect was only observed after the unmated females that were maintained on water after emergence ingested small blood meals; with larger blood meals egg development was no different than for females maintained on sucrose. The male substances did not seem to modulate reproduction by bolstering the nutritional reserves of mated females. Levels of glycogen, protein, and lipid did not differ between mated and unmated females, and heating the glands inactivated their effects, although heating presumably did not alter the nutritional content of the glands. The effect also was not dependent on the concentration of male accessory glands injected. Although the glands from *Ae. albopictus* were active in stimulating egg development, those from *Aedes taeniorhynchus* (Wiedemann) and *Aedes bahamensis* Berlin were not. This specificity, along with the absence of concentration effects, also indicates a signal role rather than a nutritional role for these male substances because the raw nutritional content of the glands from different species is unlikely to differ substantially. A similar effect of male accessory glands was observed in nutritionally deprived *Ae. albopictus* (Klowden 1993). The nutritional state of the male also affects the tendency of females to develop eggs, perhaps by his failure to produce certain components in his ejaculate. When starved males were mated to starved females that had ingested 3 μl of blood, the females were less likely to develop eggs than were starved females mated to well-fed males (Klowden and Chambers 1991). Thus, male accessory gland substances apparently can direct the metabolic priorities of the female, causing her to use her limited metabolic reserves for egg development only after she has mated and the eggs she develops can be fertilized. This mechanism prevents her from wasting her metabolic resources on eggs that would not be viable.

In autogenous species, which are capable of maturing a small initial batch of eggs without requiring the exogenous protein from a blood meal, the female's decision to partition existing resources into either maintenance or reproduction can similarly be modulated by mating or more specifically by the action of male accessory gland substances (O'Meara and Evans 1977, O'Meara 1979, O'Meara and Petersen 1985, O'Meara and Mook 1990). The response of *Ae. taeniorhynchus* females to this male signal was nonspecific, because the glands from *Aedes sollicitans* (Walker) males were also effective (O'Meara and Evans 1977).

Modulation of host-seeking behavior

Before a blood meal can be ingested, the female mosquito must activate behaviors that will place her in the vicinity of a host. The sequence of behaviors that comprise this movement in response to host stimuli are collectively referred to as host-seeking behavior. Host-seeking behavior seems to be the default behavior in most anautogenous mosquitoes in that it occurs during a circadian window of activity whenever the female receives stimuli from potential hosts. However, at times the behavior is regulated endogenously, dependent on the physiologic state of the female. The terminal step in host-seeking behavior is biting, and Lavoipierre (1958a, 1958b) observed that mating suppressed the biting behavior of gravid *Ae. aegypti.* Judson (1967) correlated this suppression of biting specifically with male accessory gland components. Host-seeking behavior in *Ae. aegypti* is similarly inhibited by the hormone *Ae. aegypti* Head Peptide I, released during oogenesis (Klowden and Lea 1979, Brown et al. 1994), but the presence of male accessory gland substances is required for the behavioral inhibition to be fully expressed (Fernandez and Klowden 1995). Because male *Ae. aegypti* tend to congregate in the vicinity of a host, this mechanism of reduced host-seeking inhibition in gravid unmated females seems to provide them with a means of returning to the host to increase their chances of encountering males for mating (Hartberg 1971).

Anopheline physiology is quite different from the established model for mosquitoes, which has been based on *Ae. aegypti* (Briegel 1990). Multiple egg batches have been reported (Briegel and Hörler 1993), and no mechanism for inhibiting host-seeking behavior during oogenesis was apparent in the somewhat limited number of anophelines that have been studied (Klowden and Briegel 1994). Therefore, in the absence of several male-induced effects, the role of male accessory glands in modulating the behavior in mosquitoes of the genus *Anopheles* is somewhat questionable, and indeed we have been unable to identify any behavioral function for male accessory glands in female anophelines (Klowden, unpublished data). In *Ae. aegypti,* which generally feed during the day when their hosts are active, the behavioral system that has evolved minimizes risky interactions with the host except if the gravid female is not inseminated. Unmated females that would be unable to lay fertile eggs take more chances and reapproach the host in an attempt to find a male. The anophelines, on the other hand, tend to feed during evening hours when hosts are less defensive and apparently not as much selective pressure has occurred to evolve a system of behavioral suppression. Field evidence indicates that a large proportion of *An. gambiae* and *Anopheles funestus* Giles populations feed multiply (Beier 1996).

Male nutrition does not affect the control of female host-seeking behavior, as it does reproduction. Females that were mated to poorly nourished *Ae. aegypti* males, which produced a reduced accessory gland contribution, showed no increase in host-seeking behavior as a result of the reduction of these male products (Fernandez and Klowden 1995).

Identity of male accessory gland substances

Compared to the wealth of information available on the identity of male accessory gland components and the genes that code for them in *Drosophila* (Wolfner 1997, Wolfner et al. 1997), relatively little is known about the specific fractions in mosquitoes. One reason for the dearth of information might be the original terminology applied to the substances. The name matrone (Fuchs et al. 1968) has been retained as a generic term that refers to the active fraction of male accessory glands present in all mosquito species, suggesting that only one active substance occurs in all of them. In *Drosophila,* several peptides and genes have been isolated and identified, many of which have very different functions in the female (Wolfner et al. 1997). A similar situation is likely to exist in mosquitoes, given the activity of heterologous male gland products for the activation of different female functions. For example, male accessory glands from *Ae. albopictus* stimulate reproduction and the inhibition of host-seeking in *Ae. aegypti,* but not preoviposition behavior (Yeh and Klowden 1990, Klowden and Chambers 1991, Fernandez and Klowden 1995). This activation of some behaviors and not others, depending on the donor gland, suggests that several components may be present in the males of a few species, some of which are absent in males and inactive in females of other species. The term matrone conveys the notion that a single active component mediates all these diverse processes in many species, and the term has outlived its usefulness.

The morphologic and ultrastructural characteristics of the glands from several mosquito species have been described (Lum 1961, Dapples et al. 1974, Ramalingam and Craig 1978, Ramalingam 1983). Because the glands are capable of replenishment after sexual activity, male *Ae. aegypti* are able to transfer accessory gland secretions to an average of 16 or 17 females (Foster and Lea 1975). Fuchs et al. (1968, 1969) partially isolated and characterized the active components of male accessory glands of *Ae. aegypti* from whole-body extracts and demonstrated that they consisted of proteins separable into 2 components with estimated molecular weights of 30,000 and 60,000. Williams et al. (1978) used the same protocol and arrived at estimated molecular weights between 50,000 and 100,000. Beginning with isolated male accessory glands instead of whole male bodies, Young and Downe (1987) determined that the active component regulating female receptivity in *Cx. tarsalis* had a molecular weight of 2,000. Recently, a peptide with a molecular weight of 7,600 was found to be responsible for both inhibiting mating and modulating host-seeking behavior in *Ae. aegypti* (Lee and Klowden 1999). The same peptide weakly stimulated oviposition. Although no function or transfer to females was demonstrated, both juvenile hormones I and III were identified as being synthesized by the male accessory glands of *Ae. aegypti,* and male *Culex nigripalpus* Theobald, *Anopheles rangeli* Gabaldon, Cove García and Lopez, and *Anopheles trinkae* Faran were shown to synthesize juvenile hormone III (Borovsky et al. 1994). Another way exists in which male accessory gland substances might be involved in the regulation of juvenile hormone titers. In *Drosophila,* the so-called "sex peptide" from males not only stimulates production of juvenile hormone in the female

(Moshitzky et al. 1996), but surprisingly also does so in a lepidopteran (Fan et al. 1999).

Reasons for studying males in a female-dominated field

Male mosquitoes are generally disregarded because they play no direct role in parasite transmission, except perhaps in the venereal infection of females (Thompson and Beaty 1977, Ovenden and Mahon 1984), but the substances the males produce are instrumental in modulating the behavior of females and thus their ability to transmit agents of disease. In the process of manipulating the female mosquito to assure that the male's genetic contribution survives, male accessory gland substances affect several aspects of female behavior that can influence her potential as a vector. Understanding the physiologic mechanisms that regulate mating and remating is also important if genetically altered males are to be used for the introduction of new genes into a population or if the sterile male method of control is to be implemented. Finally, because of the rapid rate of evolution of male substances, these may be partially responsible for the reproductive isolation that ultimately leads to speciation. Thus, it may be possible to use the pattern of production of male accessory gland substances of a group of species to determine their relatedness and ancestry.

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