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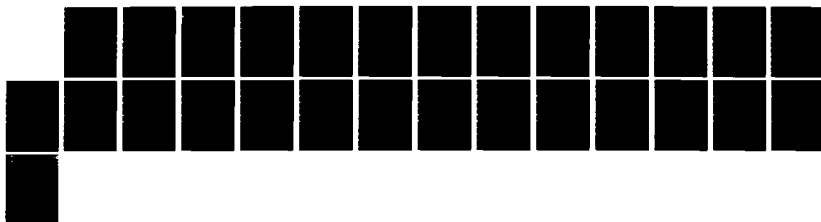
PHEROMONAL CONTROL OF BITING MIDGES (CULICOIDES SPP)
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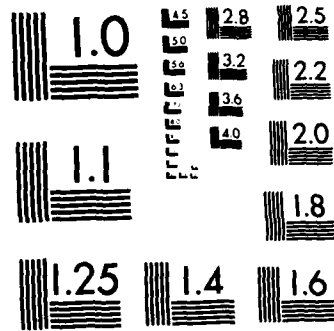
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FINAL TECHNICAL REPORT

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PHEROMONAL CONTROL OF BITING MIDGES
(CULICOIDES SPP.)

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ABSTRACT

✓ The male Culicoides melleus (Coquillett) (Diptera: Ceratopogonidae) is able to orientate extremely rapidly to the copulatory position on a C. melleus female and, remarkably, assumes an equivalent position on a mosquito female (Aedes taeniorhynchus). To test for possible similarity between the midge and mosquito females, the ability of their different bodily parts to stimulate sexual response from C. melleus females was assayed. Males were known to respond sexually to a contact pheromone. Males showed increasing response posteriorly on both female C. melleus and A. taeniorhynchus, and greater response to ventral positions than dorsal. Experiments involving transpositions of bodily parts of C. melleus females prove that orientation, at least in the later stages of precopulatory behavior, is to cues on the female abdomen. It is suggested that the orientation behavior of the C. melleus male is achieved in response to contact pheromone gradients on the female body.

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I. INTRODUCTION

Biting midges, principally of the genus Culicoides, are important as vectors of human and animal diseases. Particularly, in the last decade, they have gained significance in relation to human health owing to the occurrence of numerous epidemics of Oropouche virus infection in human populations in the Amazon basin. The virus is transmitted in the urban cycle by Culicoides paraensis. As pests, also, biting ceratopogonids are a great hindrance to coastal recreation, development and industry, and cause annoyance and reduce efficiency at coastal military installations.

A deficiency in research with these insects over the past 20 years has been the failure to concentrate effort in fundamental investigations of biology and behavior. When an analysis of the sexual behavior of Culicoides melleus was undertaken, the males were found to respond to the females on contact, and subsequent investigation revealed the presence of a relatively non-volatile contact pheromone, which could be extracted in hexane. The purpose of the research program here reported was to search for the existence of both attractant and contact pheromones that mediate sexual behavior in Culicoides, with a view to possible modification of behavior for control effect. Emphasis was placed on C. melleus and also, initially, C. variipennis since this species could be held relatively easily in laboratory culture.

II. OBJECTIVES

The principal objectives of the study were as follows.

- (1) Confirmation of bio-assay as valid method of investigation of stimulants for C. melleus.
- (2) Specific chemical identification of the C. melleus contact pheromone(s).
- (3) Synthesis of the C. melleus pheromone(s).

- (4) Bio-assay of synthesized materials and investigations of possible synergistic effects if more than one stimulant is involved.
- (5) Structure-activity relationships using synthesized stimulant compounds.
- (6) Detailed investigation of the exact role of the contact pheromone(s) in the sequence of sexual behavior, including examination of whether pheromone gradients on the female body provide cues for male orientation.
- (7) Investigation of effects of melleus pheromone on the behavior of other species, especially C. furens, a major pest in coastal areas.
- (8) Examination of cuticular hydrocarbons of other species, initially - C. furens, C. variipennis; assay and identification of sex pheromones if present.
- (9) Use of already constructed olfactometers in the search for possible attractant substances, initially in C. melleus and C. variipennis.

The objectives divide, essentially, into 2 groups which may be considered under the headings, respectively, of chemistry (objectives 2, 3, 4, 5) and behavior (objectives 1, 6, 7, 8, 9). Not all have been addressed as the amount of work is prohibitive.

Chemistry - objectives 2, 3, 4 and 5

Progress made on these objectives, with a full description of methods and experimental results; has been given in Annual Technical Report No. 2 (April, 1982).

Behavior - Objectives 1, 6, 7, 8 and 9

An investigation has been made of the relationship of the age of C. melleus females to their capacity to stimulate males sexually. These studies also examined whether killing the females or removing their wings affected the stimulus perceived by males. The methods, results and interpretation of these

experiments have been reported and fully discussed (Annual Technical Report No. 2, April, 1982) and now published (Section VII). Subsequently, attention was turned specifically to Objective 6 and the question of the possible role of contact pheromones in cuing precopulatory orientation in C. melleus. The introduction, rationale, methods and results of this work are given in detail as follows.

Males of the biting midge C. melleus (Coquillett) are stimulated to initiate copulation when they encounter and touch females (Linley and Adams, 1972, 1974). The sexual response is elicited by a hexane-soluble contact pheromone (Linley and Carlson, 1978) but is also highly unspecific. Preliminary observations showed that conspecific males were highly stimulating, and so also were males and females of Culicoides furens (Poey), male and female mosquitoes (Aedes taeniorhynchus (Wied.) and Culex nigripalpus (Theobald)), a bibionid (Dilophus orbatus Say) and female stable flies (Stomoxys calcitrans (L.)).

The sexual behavior of the male C. melleus immediately prior to copulation has been described previously (Linley and Adams, 1972). On touching a female, the male becomes highly excited and completes a very brief "pursuit," which leads to several of the male's feet being placed on the female's body. As the male clammers entirely onto the body of the female he immediately begins orientation to the copulatory position and simultaneously completes ventral bending of the abdomen, hypopygial rotation and opening of the claspers (Linley and Adams, 1972). The behavior suggested that orientation was cued by properties of the female body perceived through the male's feet, as was the contact pheromone (Linley and Carlson, 1978). In recent work (Linley and Carlson, 1983), male response was found to diminish when females were killed by freezing, or had their wings removed, but a high proportion of

males still achieved copulation and orientation was not impaired. Thus, when dead, legless and wingless females were mounted on a minuten pin inserted dorsally just behind the head, males (wingless, to prevent escape) allowed to clamber onto them immediately achieved orientation and copulation. Unexpectedly, however, orientation to the equivalent position could be achieved by males released onto tethered female mosquitoes, A. taeniorhynchus. The female midge and mosquito apparently shared a property necessary for male orientation. Since a contact pheromone was known to stimulate male response, its involvement in orientation seemed possible. A suitable assay technique for the pheromone was already available (Linley and Carlson, 1978), thus tests were designed to detect differences in stimulus perceived by male C. melleus allowed to contact different parts of the bodies of female C. melleus and A. taeniorhynchus. Supporting experiments examined the position of the male's feet on the female abdomen and the effect of reversing the normal relationships of bodily parts.

III. MATERIALS AND METHODS

Culicoides melleus pupae were collected from the larval habitats, separated by sex and induced to emerge when required as previously described (Linley and Adams, 1972). Prior to use, males were kept individually in vials to prevent exhaustion from their repeated attempts to copulate with one another. Males used in the experiments were aged 1-6 h old and females were 2-26 h old. Adult A. taeniorhynchus were reared from eggs laid by wild-caught adults and were used in the experiments when 1-7 days old.

The assay method was as described previously (Linley and Carlson, 1978). Male C. melleus were lightly anaesthetised with ethyl acetate and tethered to the blunt end of a minuten by a minute drop of shellac attached to the dorsum. The insects were oriented at right angles to the pin, which was

inserted into a 1 cm cube of balsa wood for easy manipulation. Each male's feet could be allowed, selectively, to touch and manipulate any part of the female insect under test. A scoring protocol assigned one point for each of the 3 progressive stages of male sexual response, which almost invariably occur in the following order - 1) rotation of terminalia, 2) acute ventral curvature of the abdomen and, 3) opening of claspers (Linley and Carlson, 1978).

Each position tested on a female consisted of an area that could be straddled by the legs of the assay male. The positions assayed did not overlap, or only slightly, and because of the greater size of the mosquito females, more positions were available on them than on the midges. The limits of each position are shown more clearly later. In each test, the number of males (a set) mounted varied from 2-5, corresponding to the number of positions to be tested. Also, the number of sets was a multiple of the number of positions because the responsiveness of mounted males had been found to diminish with time (Linley and Carlson, 1978). Thus, to avoid bias, the order in which males were exposed had to be distributed equally between test positions. In practice, males were allowed 5 min to recover from anaesthesia, then each was exposed on his first position with the other positions following in turn (rotation). Superimposed on this, the starting position of the first male to be tested was rotated so that all positions were exposed to an equal distribution of males in terms of how long they had been mounted. There were 3 successive exposures of 5 sec, per male, at each position, each exposure separated by an interval of 1 min. All 3 exposures were completed, then the observer progressed immediately to the next male on the next position. Males were held during exposure with the head directed anteriorly with respect to the female being assayed.

Both midge and mosquito females used for assay were killed by freezing at -20°C for 30 min. The wings and legs were removed with a needle-knife and the insects mounted on pins inserted from the anterior part of the thorax, just behind or below the head, through most of the length of the abdomen. When areas involving the head were to be tested, the pin was placed in the lower thorax to prevent interference with the male's feet during test. Pins bearing mounted females were pushed into a small mass of modelling clay attached to a tapered, 1.5 in. length of balsa wood (Fig. 1A), which could be turned to a convenient position for assay under a stereomicroscope. When it was desired to assay differences between dorsal and ventral positions, males had to be prevented from reaching around the curvature of the female body to touch more lateral areas. Accordingly, 2 test females (midge or mosquito) were mounted in contact, with the sagittal axis of each tilted somewhat inwardly or outwardly (Fig. 1A), so that almost all contact by the male would be with the desired surface.

In a series of 3 experiments, the normal orientations of certain parts of the female abdomen were altered. Parts of freeze-killed females were cut cleanly with a fragment of razor blade and reassembled, with very sharp forceps, in the required positions on a supporting minuten. The delicacy of this procedure sometimes required more than a single attempt to reconstruct a 'female' with only minor distortion. Details of these rearrangements are made clear later.

To examine the positioning of the male's feet in the final precopulatory attitude (when orientation manoeuvres had just ceased), the following method was used. A wingless and legless C. melleus female was mounted on a minuten and wingless males were released in turn, onto the female's body. As each male assumed his final copulatory position, at or momentarily after union of

the genitalia, the insects were photographed from the left side with strobe illumination and a Zeiss 40 mm Luminar lens adapted to a Hasselblad camera. The positions of the male's feet were subsequently mapped from projected negatives.

IV. RESULTS

Non-transfer of pheromone between positions

There was initially some concern as to whether rotation of males might transfer pheromone from position to position. This possibility was considered unlikely and, in 1 group of data obtained with A. taeniorhynchus females, it was possible to check for evidence of incrementing 'contamination'. The experiment (see below), rotated males between 5 positions along the length of the mosquito. The terminal portion of the abdomen proved to be 5.8 times more stimulating than the head and anterior thorax. At the third, fourth and fifth rotations, the head position was contacted by a male following 1, 2 or 3 previous contacts of males rotated from the highly stimulating posterior abdomen. If pheromone had been transferred anteriorly from this position, a progressively greater response would have been expected at the head at rotations 3, 4 and 5. This effect did not occur. For the 100 males tested, there were 300 contacts and possible responses (of any degree) at each position. The actual numbers of responses observed were 1, 4 and 0 for rotations 3, 4 and 5, respectively. Thus, pheromone was not transferred between positions to any degree detectable by the assay.

Response to Aedes taeniorhynchus females

The results of these assays are depicted in Fig. 1B and C. In the first group, C. melleus males contacted 5 positions, corresponding closely to the bracketed limits, along the length of the A. taeniorhynchus female. A single mosquito was mounted and males were brought up to contact from the dorsal

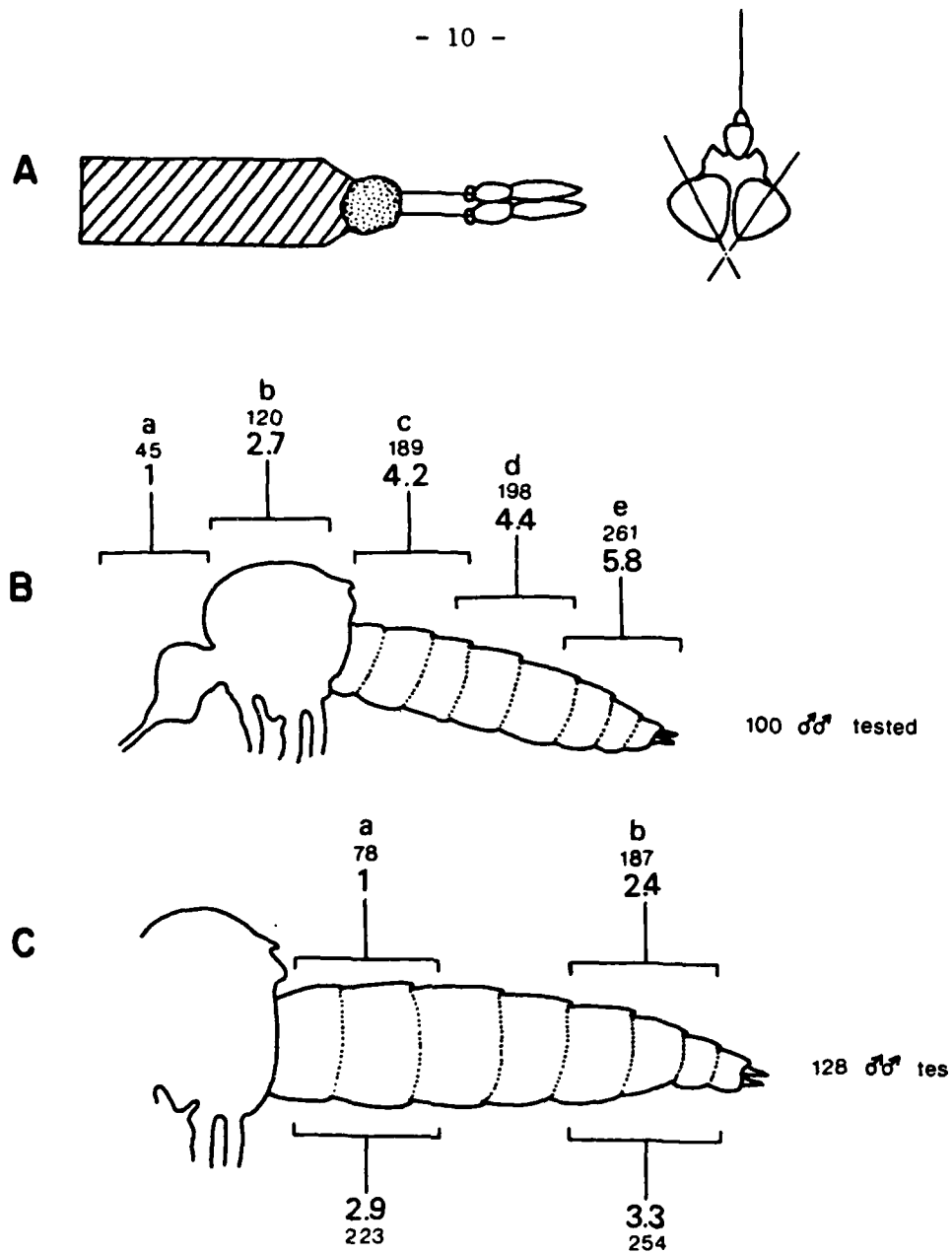


Fig. 1. A (left) method of mounting two pinned females; (right) transverse view of mounted females and staddling assay male, to show inclination of sagittal axes of females and male, accordingly, able to touch only dorsal (or ventral) surfaces. B, C, results of assays of sexual responses of *C. melleus* males on *Aedes taeniorhynchus* females; brackets show positions assayed, small numbers are response scores, large numbers the ratio of scores.

TABLE 1. Numbers of sexual responses (of any degree) of *C. melleus* males on different bodily positions of *Aedes taeniorhynchus* and *C. melleus* females.

Data	No.	No. responses at position (Figs. 2, 3)					χ^2
	responses possible	a	b	c	d	e	
Fig. 2B	300	16	38	65	66	90	72.5*
Fig. 2C	384	48	95	105	113		35.5
Fig. 3A	264	29	62				14.
Fig. 3B	276	79	123				15.7

*P < 0.001

side. One hundred males made contact at each position. The response score (points) increased posteriorly (Fig. 1B) and was 5.8 times as great at the posterior end of the abdomen as at the head/anterior thorax. The proportions of responses (of any degree, observed/possible), in accordance with the point scores were not homogeneous between positions (Table 1). It should be noted that Table 1 does not consider point scores; each exposure of a male was tallied simply for whether any sexual response at all (of any degree) occurred, allowing the proportions of observed/possible responses to be expressed.

The second group of tests (Fig. 1C) assayed primarily for differences between dorsal and ventral positions on the anterior and posterior parts of the abdomen. Two mosquitoes were mounted and 128 male C. melleus were used. There were highly significant differences between positions as regards proportions of possible responses (Table 1). Posterior sites, as previously, were the most stimulating (Fig. 1C). In addition, ventral positions on both the anterior and posterior parts of the abdomen elicited more reaction than dorsal positions. Paired statistical tests, using the proportions of male responses (Table 1) showed that the anterior dorsal abdomen produced a much lower response from males than either the anterior ventral position (a x c, $\chi^2 = 26.5$, $P < 0.001$), or the posterior ventral position (a x d, $\chi^2 = 19.0$, $P < 0.001$). The posterior ventral position was the most stimulating (Fig. 2C), but statistically the proportion of responses of any degree was not significantly higher than either the anterior (c x d, $\chi^2 = 0.4$, n.s.) or posterior dorsal area (b x d, $\chi^2 = 2.1$, n.s.).

Response to C. melleus females

When 88 males were assayed on single pinned C. melleus females to test the head-thorax position versus the abdomen, the latter proved 2.1 times more

stimulating in terms of response score (Fig. 2A) and, accordingly, the proportion of males showing any response was significantly greater (Table 1). Two pinned females assayed side by side to isolate dorsal and ventral positions showed the ventral position to be 1.7 times more stimulating (Fig. 2B) and, a significantly higher proportion of males showed some reaction on the ventral part of the abdomen (Table 1).

Non-involvement of eyes or antennae in orientation

The pheromone eliciting male response had been shown to be a cuticular hydrocarbon perceived through sensilla on the male's legs (Linley and Carlson, 1978). Extensive observations of the behavior (Linley and Adams, 1972, 1974) had suggested that the eyes and antennae of the male played no role, either in recognition of a female in close proximity or at contact, or during male orientation. The possible involvement of these sensory systems was investigated as follows.

Fifteen males were lightly anaesthetised and the antennae removed with a needle-knife between the pedicel and scape (this removes the 13 flagellomeres and Johnston's organ). Each male was placed in a Plexiglas observation cell described by Linley and Adams (1972). When 1 female was added to each cell, 13 of 15 males had achieved copulation within 5 min and response or orientation by males did not seem impaired. When 15 intact males, each enclosed with a single female, were placed in total darkness before any contact had been made, 11 of 15 had achieved copulation within 5 min. The antennae, certainly, and eyes very probably, were not needed for reception of sexual stimulus or for successful completion of orientation. Perception and use, for orientation, of some form of radiation is not unequivocally eliminated by these experiments, but seems unlikely.

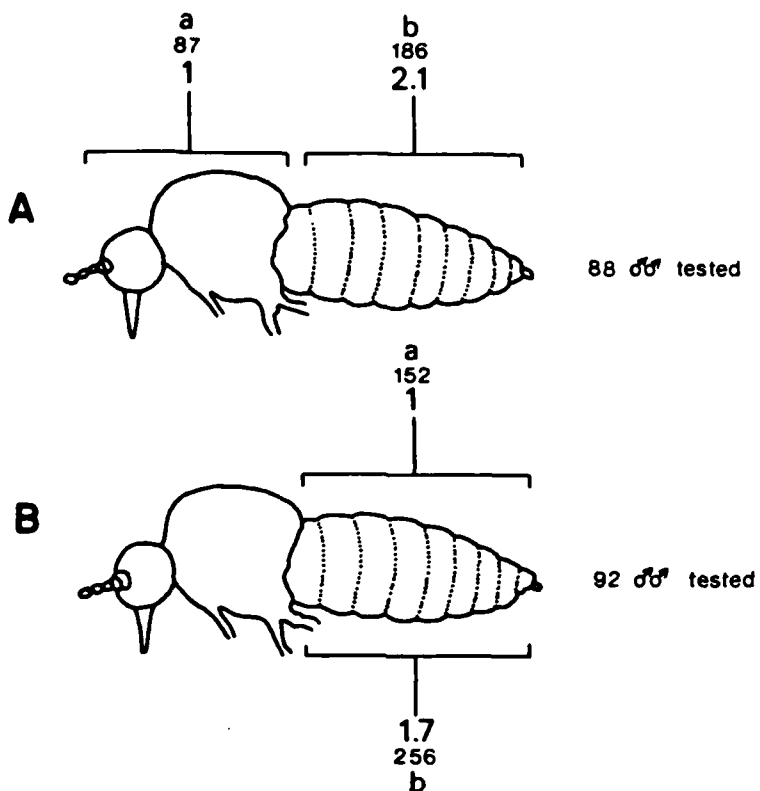


Fig. 2. A, B, results of assays of sexual responses of C. melleus males on C. melleus females. Conventions as in Fig. 1.

Transposition of bodily parts

To determine whether cues on the female body were used in orientation, 3 transpositions involving the abdomen were made. The response of 15 males in each case, was observed. The first transposition tested the effect of inverting the terminal portion of the female abdomen bearing the genital through 180° (abdomen cut through segment 7, see Fig. 4). All the males positioned themselves correctly with respect to the normally oriented anterior portion of the abdomen and apparently were not influenced by the inverted position of the female genital opening. In the reverse arrangement, with the anterior portion inverted and the abdominal tip in the normal orientation, all males again assumed correct orientation relative to the position of the anterior part of the abdomen. The final transposition tested the effect of leaving the terminal part of the female abdomen in the normal orientation, but reversing the longitudinal orientation of the anterior part, while maintaining its *normal dorso-ventral alignment*. Males in this test found it difficult to manoeuvre to the correct final position, adjusted to the 'expected' location of the female genitalia, because of the presence of the adjoining thorax. The significant result was that all the males moved to the correct, but reverse posterior end. The experiments showed, in summary, that in the final stages of positioning, when all the male's feet were in contact with the abdomen only, orientation in both directional axes was to cues on the female abdomen, excluding the small terminal portion bearing the genital opening.

Placement of the male feet

From the photographs, the positions of the feet of 29 males, at or momentarily after the moment of genital union, were mapped and are shown in Fig. 3 A (any overlapping symbol was omitted). The symbols show, for the fore, middle and hind legs, the position of the terminal tarsal segment on the

left side of the female. The right side is equivalent. Most tarsal segments of each leg were appressed to the abdomen and generally aligned parallel to the longitudinal abdominal axis. The position of the terminal tarsal segment is used simply as a measure of the consistency in positioning of the male's feet. For determining the average tarsal position, the abdomen was divided longitudinally into 3 sections, numbered from the ventral side. This allowed each segment, also numbered, to be divided into 3 approximately rectangular pieces in each of which the x and y coordinates of each mapped symbol could be estimated.

The average positions of all 3 terminal tarsi were in abdominal segment 4 (Fig. 3B), with the front leg somewhat more anteriorly placed. Placement of the fore, middle and hind feet was progressively in the dorsal direction, such that in relation to strengths of stimulus, the male would perceive the strongest signals from the fore feet and the weakest from the hind feet. This would be true even if stimulus was received through sensilla on other or all tarsal segments, since the legs generally lay parallel to the longitudinal abdominal axis. An important element of information not evident in Fig. 3 is that among the 29 individual map records, all males had their 3 feet, respectively, in this basic sequence relative to the dorso-ventral stimulus differentials.

Of the 3 feet, the position of the fore foot was somewhat more consistent (Fig. 3A), with many of the contact points clustered in the ventral third of abdominal segment 4, and only 1 point in a slightly more dorsal position. Middle and hind leg points were scattered somewhat more widely (Fig. 3A).

V. DISCUSSION

The male C. melleus is capable of achieving precopulatory orientation on both C. melleus and A. taeniorhynchus females. Both of these, when assayed

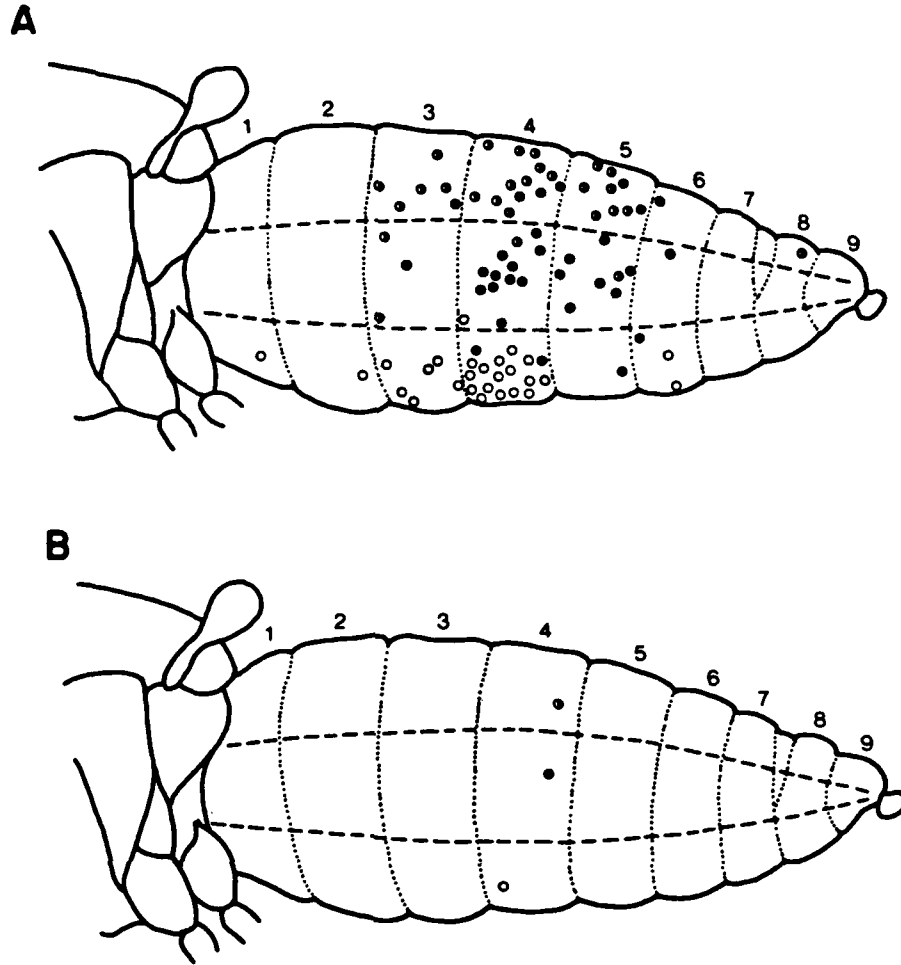


Fig. 3. Positions, on the left side of the female abdomen (segments numbered), of the terminal tarsal segment of the fore (open circles), middle (filled circles), and hind (half-filled circles) legs of *C. melleus* males in the final precopulatory position. A, the individual positions; B, the average positions.

by C. melleus male sexual response, have comparable bodily patterns of contact stimulus intensity. The eyes (almost certainly) and antennae of the C. melleus male are not required for response or orientation, and transpositions of abdominal parts prove that orientation, at least in the later stages, is to cues on the female abdomen. These observations strongly suggest that pre-copulatory orientation is achieved in response to bodily gradients of contact pheromone. There are, however, possible alternatives that remain untested. One involves the perception of bodily shape in permitting males to achieve orientation. The female abdomen in both midge and mosquito tapers posteriorly and could conceivably present a common feature for orientation, but this mechanism would not explain correct dorso-ventral alignment, as observed on both insects, especially as the female genital opening is not involved. The transverse sectional shape of the abdomen could be important, but this idea invokes an extraordinarily subtle male ability to differentiate form. Also, the female mosquito abdomen seems distinctly more tapered ventrally than is the case with the midge. Shape does not seem useful, moreover, in explaining how males achieve movement to the posterior end of both midge and mosquito females when these are substantially different in size and yet would have to provide the same 'shape cues' to the C. melleus male. Another possibility is that the direction of the abdominal setae provides the cue for male orientation, as shown in the staphylinid, Aleochara curtula Goeze (Peschke, 1979). The abdomen of the C. melleus female bears very many small microtrichia and relatively few rather large and unevenly distributed setae, all rearwardly directed. For such structures to be involved in orientation would, however, require the male C. melleus to derive equivalent tactile cues from much larger and differently distributed setae (and perhaps scales) on the

mosquito abdomen. It is unlikely that this takes place, or that it could form a basis for correct dorso-ventral positioning.

For the present, a hypothesis of movement along a stimulus gradient provides a simpler basis for male orientation and one which can explain the entire orientational sequence. The final position of the male with feet positioned in a consistent alignment on the female abdomen, implies strongest stimulus to the front feet, least to the hind feet. Thus, with the apparent anterior-posterior gradients on both midge and mosquito the male who continuously adjusts his position and moves in the direction that increases stimulus at the fore feet relative to the other two, will from any starting point achieve (by tropotaxis, Fraenkel and Gunn, 1961) the precopulatory position. A more complete analysis of this idea might be attempted, in which the positions of the feet at every change in an orientational sequence are recorded, and the observed subsequent turning motions of the male compared with the pattern of stimulus being received at all 6 feet.

In its broader implications, the behavior of C. melleus males may be representative of at least some species. There are others, for example Culicoides nubeculosus (Kremer et al., 1979), Culicoides variipennis (Coq.), Culicoides riethi Kieff. (Boorman, 1974), and Culicoides wisconsinensis Jones (Mullens and Schmidtman, 1981), in which the adults also copulate on contact without need for flight. Contact pheromones and similar mechanisms of orientation are probably involved. Culicoides nubeculosus females also produce a volatile, air-borne attractant pheromone (Kremer et al., 1979; Ismail and Kremer, 1980). In many and almost certainly most Ceratopogonidae, however, formation of male swarms is the usual behavior, and meeting of the sexes normally takes place in the air. How males manoeuvre to achieve copulation under these circumstances has not been described.

In mosquitoes, there is some evidence that pheromones are important in the sexual behavior. Kliever et al. (1966) reported the existence of a volatile pheromone produced by female Culiseta inornata (Williston). It was claimed that this compound attracted males, but Lang and Foster (1976) were unable to confirm this effect and Lang (1977) concluded, instead, that a contact pheromone was present, which permitted recognition of conspecific females and stimulated attempts at copulation. The possible role of the pheromone in assisting male orientation was not evaluated. Again with regard to conspecific recognition, Nijhout and Craig (1971) concluded that contact pheromones may have a role in ensuring reproductive isolation between species of Aedes (subgenus Stegomyia). Males were attracted to females by wing beat sound (Roth, 1948) or an artificial source of equivalent frequency, but would depart after brief contact if the female was not of the same species, rather than continue to copulation. Nijhout and Craig (1971) suggested that pheromone "...keeps the male avid while in contact with the female..." and also that it stimulates males to increased flight activity after contact. In these mosquitoes, pheromone is apparently not essential to continued sexual activity once contact is made, because males could be induced to proceed with normal copulatory movements on being attracted to abnormally loud flight sounds generated by a tuning fork (Nijhout and Craig, 1971).

There are several extensive studies of sexual behavior in mosquitoes, but important details remain unclear. Little has been done to examine the sequence of male manoeuvres on females once contact has been made in the air. The positions of the female legs in the flying attitude, since mosquito legs are rather long, are probably important. Blockage of probable chemoreceptors on the male tarsal segments significantly reduced recognition of conspecific females (Nijhout and Craig, 1971) and Charlwood and Jones (1979)

have shown the mechanical importance, in male Anopheles gambiae Giles, of the presence and positioning of the male front tarsal claws, which are used to grapple the legs (usually fore legs) of the female in flight. It is likely that the legs are an important site of contact pheromones in mosquitoes, as suggested by the work of Lang (1977), who found leg sets of female Culiseta inornata to be highly stimulating to males. An evaluation of leg interactions might add considerably to understanding both the mechanical and chemical factors involved in orientation. It remains for the present an open question as to whether abdominal cues are involved. The cuticular compounds to which male C. melleus respond on mosquitoes may well be unimportant in the sexual behavior of the mosquitoes themselves. Lang's (1977) failure to extract the Culiseta inornata pheromone with hexane, which did remove an active compound from C. melleus, suggests that mosquito and midge sex pheromones are structurally different.

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