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Multivariate analysis of *Drosophila* courtship

(sequential structure/temporal stationarity/transmission/wild-type pairs)

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ABSTRACT   Courtship records of 15 pairs of *Drosophila me-lanogaster* were analyzed for temporal stationarity of courtship be-haviors, behavioral diversity, behavioral intercorrelations, se-quential properties, and information transmission for both sexes. Durations of one male behavior, "orient-back," and two female behaviors, "preen" and "stand still," were found to change from the first to the second half of courtship. Male diversity was greater than female diversity, and both were stationary over time. Corre-lation analyses failed to single out any particular male or female behaviors as being influential in controlling courtship duration. Male behavior sequences formed several multibehavior loops; fe-male behavior consisted of only a few terminal two-tuple transi-tions. Transmission analysis carried out on the joint male/female transition matrix showed a higher transmission rate from males to females (12%) than from females to males (7%). Potential ap-plications of this multivariate analysis to investigations of neuro-biological and evolutionary aspects of *Drosophila* courtship be-havior are proposed.

Within the genus *Drosophila*, courtship behaviors of both sexes are diverse and species specific (1). Species vary not only in the relative roles particular sensory modes play in mate selection but also with respect to the quantitative features of the presen-tation of sensory information during courtship interactions. For example, a number of species require light in order to mate, suggesting that visual stimuli are important in their courtships (2, 3). Furthermore, among closely related species, character-istic differences may exist in the amount of time spent perform-ing a particular behavior or the sequence in which behaviors occur (4).

Within a species, variation in the quantity or sequence of male behaviors has been shown to be associated with reduced male courtship success (5–8). Deviation from species-specific courtship structure somehow results in the inability of a male to stimulate female receptivity properly. Elucidation of the neu-rologic control and evolutionary history of interspecific and in-traspecific courtship interactions relies heavily upon the ability to characterize the structural components of courtship behavior.

The present study uses a series of multivariate analyses to provide a new picture of courtship of *D. melanogaster* which re-flects the sequential and temporal properties of the behaviors of both sexes as well as the transmission of information between them. The implications of these findings for future studies are discussed.

MATERIALS AND METHODS

Flies used in this study were descendents of a multi-female col-lection of wild *D. melanogaster* made at Tempe, AZ, in June 1979. Flies were reared under uncrowded conditions in half-pint milk bottles on standard cornmeal/molasses/agar medium at 24 ± 1°C and were used in mating experiments within three generations of their capture. Virgin males and females were separated under minimal ether anesthesia and stored in 8-dram vials with medium until used in experiments at 4 days of age. Flies were maintained on a cycle of 11 hr of light/13 hr of dark. In all experiments, a single 4-day-old virgin female was aspir-ated into a 1-inch-diameter (2.5 cm) round plexiglass and filter paper observation chamber containing one virgin male. The complete courtship was videotaped through a Wild dissecting microscope at ×120 by a JVC color video camera and JVC HR 3600 videorecorder using a light intensity of 210 footcandles (226 lux). Courtships not resulting in copulation within 10 min were not included.

A 19-inch Sanyo color television monitor was used for play-back of courtship records. A pair of observers logged in nine male and seven female behaviors simultaneously on an Ester-line Angus serial event recorder. Recorded male behaviors were: locomotion away from female, chase, chase and vibrate, orient-back, orient-front, orient-back and vibrate, orient-front and vibrate, lick, and copulation attempt. Most of these be-haviors have been defined (9). We have subdivided vibration into three categories depending upon the basic position of the male with respect to the female. We use the term "orient" in a modified sense from that originally used by Bastock (5). Dur-ing both orient-front and orient-back, the male's head is di-rected toward a stationary female, toward either her front or back half. We separated these because orient-front has the abil-ity to influence females in the visual mode. The female behav-iors were single wing flick, double wing flick, decamp, loco-motion, preen, stand still, and kick, most of which appear to be associated with rejecting the male.

Fifteen successful courtships ranging in length from 29.9 to 318 sec were analyzed for various multivariate relationships, behavioral stationarity over courtship, information transmis-sion, and sequential properties.

RESULTS

Temporal Stationarity. Each courtship record was divided into four equal segments. Two dependent measures were used to assess the stabilities of behavior probabilities over courtship length: (*i*) the relative frequency (total number of times a be-havior occurred divided by the total number of behaviors in a particular segment), and (*ii*) the relative duration (the total du-ration of a behavior divided by the total time of a particular courtship segment). The various male and female behaviors did not show any significant changes in relative frequency over the four quarters of the courtships. Relative duration was also gen-erally invariant with the exception of locomotion and preen in females and orient-back in males. When courtships were di-vided into their first and second halves (Table 1) rather than into quarters, female locomotion was found to decrease [$t(28) = -3.4, P < 0.01$] from the first to the second half of courtship,

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Table 1. Quantitative aspects of male and female behaviors in first and second halves of courtship

	% of courtship performing specific behavior			Mean duration (bout length), sec		
	1st half	2nd half	P	1st half	2nd half	P
Male behavior:						
Orient-front	1.4	3.2	>0.1	6.6	1.0	>0.1
Vibration	10.6	12.7	>0.1	3.0	1.6	>0.1
Chase	25.8	24.5	>0.2	3.0	2.4	>0.05
Orient-back	9.2	16.1	<0.01	1.2	1.8	<0.05
Female behavior:						
Locomotion	32.4	23.3	<0.01	9.0	4.8	<0.05
Preen	1.7	4.8	<0.05	1.2	2.4	<0.01

and both female preen [$t(28) = 2.5$, $P < 0.02$] and male orient-back [$t(28) = 3.7$, $P < 0.001$] increased in the second half.

The temporal stationarity of relative behavior probabilities also occurs for the behavioral diversity observed over courtship of each sex. Behavioral diversity is akin to variance but is measured on a nominal behavior scale like behavior categories or, more simply, the number of behavior changes per unit time. The Brillouin diversity index is a measure of behavioral diversity commonly used in the assessment of ecological diversity (10, 11). In the present case the Brillouin index was calculated over the courtship duration and normalized with the maximal diversity that could occur over the categories of behavior (10) so the index varied from 0 to 1 and was independent of the total number of behavior frequencies. Male and female indices over each one-eighth of courtship are shown in Table 2. In both sexes, the diversity did not change significantly over the courtship. Male diversity was consistently higher than female, and there was no correlation between male and female diversities during courtship ($r = 0.02$, $P > 0.1$). Male diversities of the longest and shortest courtships were found to not differ significantly (diversities 1.81, 1.83; $t(16) = 0.396$, $P > 0.1$).

Interrelations Between Courtship Variables. Individual correlations were calculated between time until mating and all nine male and seven female behavioral variables. Two general measures of each behavior were used for the correlations: (*i*) total duration and (*ii*) bout rate. Durations of all behaviors of males and females were highly correlated with courtship duration. The longer the time until mating, the longer the durations of

each behavior. No bout rates were correlated with mating time. Another variable strongly correlated with mating time was frequency of total courtship bouts, or the number of times the male "broke off" courtship behavior prior to mating. The more courtship bouts there were, the longer mating took to occur.

In addition to mating time, various male behaviors were found to be intercorrelated. Two measures of four behaviors (comprising 92% of the courtship time) resulting in eight variables were tested for intercorrelation. The four behaviors were total vibration (independent of male position), chase, orient-back, and orient-front (all independent of whether or not vibration was occurring simultaneously). The two measures of these four behaviors were total duration and bout rate.

The correlation matrix was submitted to maximum likelihood factor analysis (BMDP4M) to assess whether a simpler representation of either behaviors or measures could be obtained. Three factors were extracted and orthogonally rotated (varimax), and loadings less than 0.25 were replaced by 0 to obtain more interpretable factors (Table 3). A significant portion of the variance was accounted for by all three factors (62.6%). Loadings per variable were high in each factor but generally nonoverlapping with other factors. The factors tended to be dominated by either total duration or by bout rate rather than by the various behaviors. Factor 1 was composed of all durations with the highest loading in vibration and lowest in orient front. Rates of bouts of various behaviors also clustered and were found in factor 3, with the high loading in chase. Factor 2 was comprised of orient front rate (highest loading) and orient front duration which overlaps with factor 1.

Sequential Structure. Transition tables for all two-tuple transitions were prepared for males and females separately over all courtship sequences. These tables were treated as contingency tables (12) and a χ^2 test of whether transition frequencies dif-

Table 2. Brillouin diversity indexes

Courtship segment	N (changes)	Raw diversity	Normalized diversity
Males (9 behaviors)			
1	139	1.88	0.86
2	144	1.88	0.85
3	139	1.91	0.86
4	139	1.89	0.86
5	148	1.92	0.87
6	140	1.97	0.84
7	147	1.89	0.86
8	143	2.008	0.91
Females (7 behaviors)			
1	57	1.36	0.72
2	55	1.44	0.76
3	50	1.53	0.81
4	57	1.47	0.78
5	48	1.47	0.78
6	67	1.51	0.78
7	68	1.56	0.82
8	61	1.46	0.77

Table 3. Sorted rotated factor loadings (pattern) for male courtship behaviors

	Behavior	Factor 1	Factor 2	Factor 3
Vibration duration	1	0.954	0.000	0.000
Chase duration	2	0.822	0.000	0.000
Orient-back	3	0.755	0.000	0.000
Orient-front duration	4	0.607	0.591	0.000
Orient-front rate	8	0.000	0.913	0.000
Chase rate	6	0.000	0.000	0.717
Orient-back rate	7	0.000	0.000	0.484
Vibration rate	5	0.000	0.000	0.499
Eigenvalues		3.2	1.7	1.2
Variance accounted		2.6	1.337	1.074
Cumulative variance, %		36.5	50.4	62.6

fered from chance was performed. Only in the male transition matrix, using frequencies greater than five and frequencies greater than the predicted frequencies, was the overall χ^2 highly significant: males, $\chi^2(64) = 926$, $P < 0.001$; females, $\chi^2(36) = 29$, $P > 0.70$. The existence of significant individual cell transition frequencies was tested for by collapsing all cell frequencies around one particular cell and performing a χ^2 test (13) or by individual binomial tests with conservative significance levels. Both approaches agreed, showing several significant two-tuple transitions for males (Fig. 1A) and even a few for females (Fig. 1B), indicating that the probabilities of various behaviors cannot be independently calculated.

The validity of treating these transitions as first order Markov chains was tested by fitting a higher-order dependence model to all three-tuple transitions (14). The overall χ^2 square was non-significant, and even examination of relatively large individual cell frequencies in the three-tuple transition matrix showed no deviation from chance predictions, suggesting that a simple first-order Markov chain accounts for the sequential structure of mating success. Because the transition matrices fit the assumptions of a Markov chain process, the stationary probabilities of each behavior may be calculated. These probabilities represent the likelihood of returning to any behavior independently of which behavior started the sequence and independently of any moment in courtship. These probabilities of behaviors are the equilibrium probabilities of each behavior's depending on the probability of all other behaviors.

Computationally, the steady-state distribution can be found by finding the nth power of the transition matrix, given n is large. In the present case the transition matrix was multiplied against itself recursively ($p_{ij}^{n+1} = p_{ij}p_{ij}^{n}$). Although convergence was fast and both the male and female transition matrices converged in about 20 trials, both matrices were multiplied recursively 100 times. The steady state (assuming an ergodic Markov process) probabilities were used in the sequential diagram (Fig. 1) to represent the likelihood of occurrence of any particular behavior (diameter of circle). In order to represent the spatial position of each behavior in a sequential diagram less arbitrarily, the temporal contiguity of each behavior was assessed and scaled by using the Shepard–Kruskal multidimensional scaling algorithm (15). This method attempts to place a set of points (the behaviors in this case) in a space of low dimensionality while preserving at least the rank order of contiguities of all possible pairs of points. The contiguities of the behaviors were estimated by collapsing the transition frequencies into a lower-half triangular matrix. Thus, transitions A–B and B–A were treated equivalently.

For both male and female contiguity matrices, strong solutions were obtained (stress values, $1 - w^2$, were 0.01 for males and 0.025 for females) in two dimensions (Fig. 1). The female behavior in the contiguity space appears to be centered around "stand still." "Kick" is the greatest outlier in the space, and "preen" is almost directly opposite of "kick." "Chase" and "orient-back" are central to the male space. "Chase" also appears to be a pivotal point for "orient-back" and "vibrate and chase." There appear to be two loops, one beginning at "chase" and proceeding through "orient-back" to "vibrate and orient-back" to "lick" to "copulation attempt" and then returning to "chase." The alternate route is shorter and proceeds from "chase" to "vibrate and chase," to "lick," and to "copulation attempt" before returning again to "chase." Which high-probability route is taken depends upon whether the female is standing or moving. The "lick–copulation attempt–chase" sequence is common to both loops. Locomotion away from female and orient-front behaviors are less probable behaviors and tend to be located outside of the high-probability precopulation loops. Leaving the high-probability loops through locomotion leads to a loop between orient-front and orient-front and vibrate which can only return to the high-probability loops through vibrate and orient-back. This sequence of vibrate and orient-front to vibrate and orient-back might be best described as "circling" (9). Although orient-front behaviors occurred for more than half the male subjects, the sequential relationship between the orient-front behaviors and orient-back only appeared for the longest courtship records (3–5 min).

Transmission Rates. Because male and female courtships were recorded simultaneously, the behaviors of both sexes could be correlated with the previously described χ^2 technique. Transition tables for between male and female behaviors were constructed for the same instant in time, one behavior change before (relative to the females behavior) and one behavior change after (relative to the females behavior). χ^2 values calculated on all three matrices, using only cells greater than 5 and larger than chance frequencies, were all significant. The matrix associated with behaviors occurring in the same instant had the largest χ^2. There were no continuous loops (three-behavior triangle) as in the individual behavior structure, only terminal two-tuple transitions.

A transmission analysis was carried out on the joint frequency matrix of the male and the female. This procedure utilizes infor-
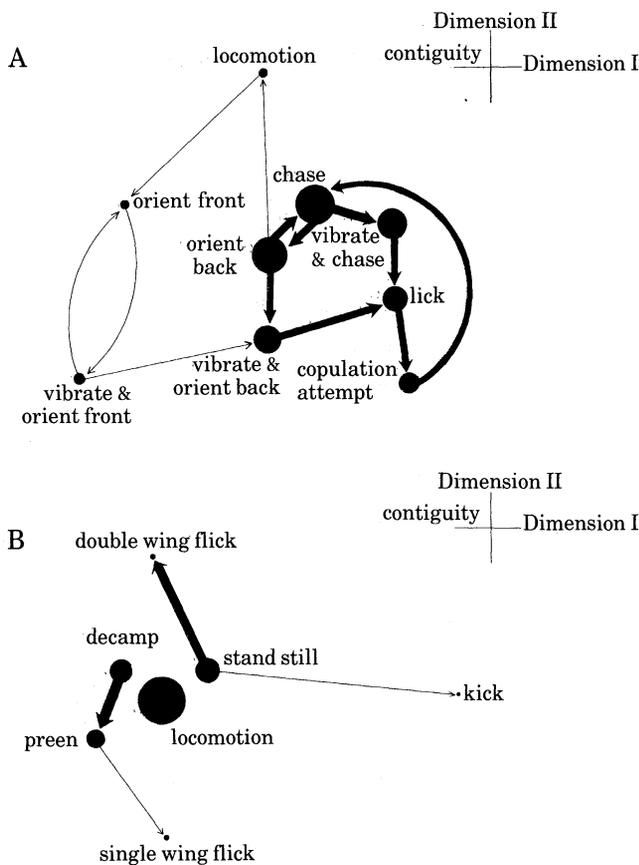
FIG. 1. (A) Male courtship. (B) Female courtship. Two-tuple sequential dependencies are represented by arrows between circles: the thickness of each arrow is proportional to the level of significance (from a binomial test) of the frequency of the particular transition (thick, $P < 0.0005$; thin, $P < 0.01$). The circles represent each behavior, and the diameter of each circle is proportional to the stationary probability of each behavior (A, chase, probability = 0.28; B, locomotion, probability = 0.46). Coordinate positions of each circle are derived from the multidimensional scaling solutions which place the behavior in the two dimensional contiguity space of all behaviors.

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mation indices to estimate the reduction in uncertainty of the behavior of one individual given the behavior of another individual during an interaction (16). In the present case, as expected, the information content of the males' behavior was almost twice that of the females. Regardless of the instant in time of the females' behavior, the transmission rate of the males was always greater. For example, knowing the males' behavior at the same moment reduced the uncertainty of the females' behavior by 12%, but the uncertainty of the males' behavior was only reduced by 7% with the knowledge of the females' behavior. The transmission rates and asymmetry were highest when calculated for female and male behavior occurring at the same time. When the females' behavior occurred either after or before the males' behavior, transmission rates dropped to half the value obtained when they were at the same instant (6% and 3%, respectively).

DISCUSSION

Once courtship began there was little change in the duration, the frequency, or the diversity of courtship behaviors examined. This observed temporal stationarity was violated by three behaviors. As courtship proceeded, the female spent less time in locomotion and more time preening. The male spent more time orienting back. It is unknown what causes the female to move less but her immobility and preening appear to be important for a copulation attempt to be successful. All successful copulation attempts (those terminating a record) were directed at immobile females. For *D. melanogaster*, this slowing down appears to be the only visible indication to a male of a female's willingness to mate, other than the final spreading of the vaginal plates. Unlike other species including the sibling species of *D. melanogaster*, *D. simulans*, in which a female will perform an obvious spreading of her wings in front of a male attempting to copulate, no overt female indications of receptivity are observed in *D. melanogaster* (1).

The existence of any specific behavior which might be important in controlling courtship duration is a major question in studies of courtship behavior. Bastock and Manning (17) proposed that female receptivity is enhanced by a gradual summation of certain courtship elements, especially vibration, and that, once a certain threshold is reached, a female will switch rather abruptly from an unreceptive to a receptive state. One way in which we sought to identify controlling behaviors was by looking for correlations between rates and durations of particular behaviors and length of courtship record. The high correlations for durations of all behaviors with record length failed to single out any particular behaviors. The absence of correlations between bout rates and record length suggests that the rate at which a behavior is performed is similarly unimportant. It is still possible, however, that some summation is occurring for several behaviors with respect to their quantity, frequency, or sequence.

Perhaps the most interesting finding from the factor analysis was that orient-front duration and orient-front rate were more closely correlated with each other than with any other behavior. Orient-front may serve quite a different function relative to other courtship behaviors. Orient-front tended to occur more frequently in longer courtships and toward the end of courtship records. Orient-front also appears more frequently and in longer bouts in courtships which, for various reasons, are unsuccessful (unpublished data). Spieth (1) suggested that this behavior may have evolved as a maneuver to prevent an unreceptive female from escaping. Orient-front was also singled out by the sequence analysis, being involved in a loop separate from the other behaviors. One obvious difference between orient-front and other behaviors is that, when the male is in

front of the female, visual information can be imparted to her. *D. simulans*, unlike its sibling species *D. melanogaster*, shows reduced mating in darkness (3), presumably because of the importance of visual cues in its courtship. *D. simulans* males do indeed spend a greater amount of time in orient-front and orient-front/vibrate type behaviors than do *D. melanogaster* males (unpublished data). The evolution of such vibration into more elaborate visual stimuli in this species has been suggested elsewhere (1).

Unlike other kinds of interactive encounters, such as aggression in which unstable motivational states produce an oscillation of behaviors (13, 18), *Drosophila* courtship is characteristically stable. This makes it particularly suited for stationary probability sequential analysis (Markov chain analysis). Low variances in behavioral duration and invariant diversity are characteristic of a fixed action pattern, a relatively fixed response in the presence of a female or female stimuli. Sequential structure is important because it provides predictability of the occurrence of units in the sequence. Such predictability may provide specific cues for individual interaction and be a determinant of courtship success. Absence of sequences and less stable diversity may be related to unsuccessful courtship.

The strong sequential patterns and relatively high information content of the male's behavior corresponds to the finding of a high transmission rate (26%) when transmission analysis is carried out on the males' transition matrix alone. Moreover, transmission analysis showed that reduction in uncertainty about both male and female courtship behavior at the same instant in time is more dependent on the male's behavior than on the female's. In fact, the male's own behavior influenced his subsequent behavior twice as much as it influenced the female's behavior. Although the particular transmission values for *Drosophila* courtship seem low, they are significantly different from 0 and they are not unlike those transmission rates found by Steinberg and Conant (19) in their analysis of male grasshopper interactions. Steinberg and Conant also found an asymmetry in the transmission rates between interactors; knowing the initiators' behavior reduced the uncertainty of the responders behavior by 22% whereas knowledge of the responders' behavior reduced the uncertainty of the initiators' behavior by 12%. Analogous to the present asymmetry of transmission rates, Steinberg and Conant concluded that initiators affected responders more than responders affected initiators. In the present case, transmission analysis in the instant before (relative to the female) and the instant after revealed that transmission rates dropped to half their values (6% and 3%) when compared to the same instant. This is even stronger support for the hypothesis that, during courtship, the *Drosophila* male proceeds through a relatively invariant action pattern which then cues female behavior.

The outcome of courtship depends upon the proper integration of numerous sensory and motor functions of both sexes and has definite consequences for the fitness of both individuals. The highly specific and complex nature of these courtship interactions poses some fascinating questions in the areas of neurobiology and evolutionary biology. What are the effects of genetic manipulation on various levels of the neural control of courtship? What are the roles of particular behaviors in sexual selection at the intraspecific level? How do behavioral isolating mechanisms arise during speciation? A prerequisite for obtaining meaningful answers to these questions is a method of describing *Drosophila* courtship in the most exhaustive terms possible. The above method provides a comprehensive way to measure the dynamics of courtship interactions and to investigate more fully the influence of genetic and nongenetic factors on the entire courtship process. A multivariate approach should reveal not only that males having a particular mutant vibrate

less but how that change affects other aspects of courtship structure and progress compared to an appropriate control.

Several testable predictions emerge from the picture of courtship painted here. One prediction is concerned with the remarkable stability of male diversity levels over courtship duration. If stable diversities are important for male success, males showing variable diversities should show reduced success or longer courtship records. Another prediction is that, if females reveal their receptivity by decreasing their locomotion and increasing preening, females that are known to be unreceptive should not show this reduction in locomotion and increase in preening. In addition, transmission rates might be expected to change. A multivariate approach should also be of value in examining the importance of particular sensory modes for courtship success. Although odors and sounds cannot be visually recorded in this system by the observer, it is possible to use genetic means of eliminating the ability to perceive olfactory, auditory, and visual cues of either sex and to examine the ensuing alterations in courtship structure and transmission rates.

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