Sexual selection in *Drosophila silvestris* of Hawaii

(behavior/evolution/courtship/reproduction)

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ABSTRACT   Previous discovery that *Drosophila melanogaster* females tend to discriminate in mating against phenotypes of earliest courting males prompted a study of the Hawaiian species *D. silvestris*. Tibial bristle variation in males from opposite coasts of the island of Hawaii functions in courtship, and the possibility that females can distinguish males differing in the tibial trait is explored. Mating tests, designed to give each female and male an alternative choice between two individuals of opposite sex every 30 min, consisted of intrapopulation tests with a strain derived from an eastern (Kilauea) population and interpopulation tests between that strain and one derived from a western (Kahuku) population. Males were given initial combat tests, with "winners" then used in mating (except one test with "loser" males). Matings (52–55%) were classified into categories according to the readiness of the female to mate and sequence of courtship. Low-threshold females (accepting the first male after less than four courtship bouts) occurred at 30–35%. Among intrapopulational tests, females (with higher threshold) accepted first- and second-courting males about equally (25:36, respectively), but for male success in mating, the winning of initial intermale combats and the uniformity of courtship effort tended to be important criteria. Among interpopulation tests, homogamic matings were nearly equal (25% each), but heterogamic matings contrasted in that Kilauea females were reluctant to mate with Kahuku males (14%), while reciprocal matings occurred most frequently (34%). Females favored males second to court, particularly when a Kilauea male (with extra tibial bristles) was the second male. Thus a morphological feature likely to be influential in mating is demonstrated to be so; and sexual selection is operating via male–male combat plus discrimination in favor of particular opposite-sex individuals in this species.

The rare-male mating advantage has been observed in several species of *Drosophila* (1), and its behavioral basis can be accounted for by female avoidance of the phenotype characteristic of the earliest courting male (2, 3). Thus females may tend to act as selective agents against majority phenotypes among males if those phenotypes are distinguishable to the females and if majority males tend to be first to court. *Drosophila melanogaster* females demonstrated this selective behavior more when heterozygous than when homozygous (4), an indication that a diversity of genotypes in natural populations of *Drosophila* is probably necessary for expression of such discrimination ability. A few questions come to mind, once this avoidance tendency has been demonstrated for one species:

(*i*) How general is this tendency among *Drosophila* females? Hawaiian picture-wing species are known to have elaborate courtship and mating behavior (5–8) and are likely candidates for expressing sexual selection. Can discriminating tendecies in either sex be demonstrated in these recently evolved species?

(*ii*) Carson and Bryant's (9) discovery of tibial bristle variation in males from populations of *D. silvestris* inhabiting east or west

coast areas of the big island (Hawaii) provides us with a natural feature that clearly functions in courtship behavior, namely in the stage of courtship called "head under wing" (see below). By using multiple choice mating with laboratory stocks derived from these wild populations, we explore the possibility that females can distinguish males that differ in the tibial bristle trait by testing for evidence of discrimination by the females.

(*iii*) If we extrapolate from using genetic or artificial markers with known behavioral effects, we can expect a vast number of behavioral differences to be expressed in genetically polymorphic populations. Assuming that males differ in their courtship signals, do females detect individual differences and discriminate between any male first to court and a male that courts subsequently? Does the female need the experience from more than one male before her threshold is lowered sufficiently for acceptance?

Beyond these questions, evidence of ethological differentiation could indicate incipient isolation within *D. silvestris* between the east coast populations, which have evolved an increased number of foreleg tibial bristles in males, and the west coast populations, which resemble ancestral species (9). Just how sexual selection might have functioned as a pivotal force in cases of incipient speciation of Hawaiian *Drosophila* needs elucidation.

MATERIALS AND METHODS

A method used for estimating the performance of males in courtship and female acceptance for species like *D. melanogaster* (2–4) is to observe a single female courted by several males and to record sequences of courtship along with quantification of courtship bouts. However, in Hawaiian species such as *D. silvestris*, males display lek behavior (5), and if two or more males are confined in the same space, they quickly become combative towards each other. Our objective being to ascertain female acceptance conditions, it was necessary to design the experiments so that each female would have the opportunity to experience courtship from two males without the complication of fighting between them. Thus the procedure was as follows: In each mating chamber a male was introduced, followed 15 min later by a virgin female. The pair was given 30 min of observation. If no mating occurred, the female was aspirated out to an adjoining chamber (male no. 2) for the next 30 min. At the next time period the female was transferred back to the original chamber (male no. 1). Thus females were alternated every 30 min within a pair of chambers, with males remaining resident in each chamber, and each female saw two males in succession. We wanted to find out whether acceptance of males is related to the order of courtship (sequence of males experienced by the female). If the female tends to *avoid* the male first to court, then presenting her with just two males in succession will allow a null test for ascertaining whether random selection (giving ½:½) is true.

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Ten observation chambers constructed of Plexiglas (20 × 20 × 10 cm), with a green backing and plastic sponge floor moistened with about 10 ml of water, open on top with a muslin screen and removable plastic lid (10), were placed in two rows of five each for viewing. A small petri dish containing Wheeler–Clayton food medium was placed in each chamber. Lighting was by eight overhead 40-W broad spectrum daylight fluorescent lamps (Duro-test, Vita-lite), and room temperature was kept at 22°C.

D. silvestris samples had been collected in 1977 (9); two isofemale lines derived (i) from a collection (U28T2) at Kilauea Forest Reserve (elevation 1615 m) and (ii) from a collection (U26B9) at Kahuku Ranch (elevation 1220 m), representing the east coast and west coast populations, respectively, were maintained in the University of Hawaii laboratory. A sample of 10 wild males collected at the Olaa Tract of Hawaii Volcanoes National Park (about 8 km east of Kilauea at 1250 m) in July 1980 were also tested. The U28 and U26 strains were maintained by mass mating. Between 1 and 7 days after emergence from the pupa, sexes were separated, stored in food vials, six to eight flies per vial, until they were 3–4 weeks of age (18°C). Two days before testing, males were isolated, one male per vial, to prevent fighting.

At the start of testing, 20 males were set out, 2 males per chamber, to allow them an initial period for combat, head-to-head approach, supining of wings, jousting, fighting, and one male's decamping. The apparent "winner" after two encounters was allowed to remain in the chamber, while the "loser" was removed to a storage vial at 18°C. This selection of winners led to more mating success and thus more uniform results than simply testing males selected entirely at random (see Results). After 15 min, a virgin female was introduced into each chamber, and a 30-min observation period began. Courtship activity (frontal display, head-under-wing, and copulation attempts) and female responses (wing rowing, slashing with forelegs, decamping, or acceptance) were recorded (see refs. 5–8). If no copulation occurred during the period, the female was aspirated out and placed in the adjoining chamber. Each chamber was surveyed regularly during the 30 min to record the start (frontal display) and end (decamping) of each courtship bout. If the female mated during the 30 min, she was removed to a food vial for storage and subsequent dissection for evidence of sperm transfer. At the end of the time period, the female from the adjoining chamber (if unmated) was placed in with the male that had just mated. A fresh virgin female was added to the chamber from which the unmated female was taken. Time spent in copulation was recorded. A copulation was considered an acceptance by the female if the male either transferred sperm or (if no sperm) the male had stayed coupled to the female for at least 2 min with no sign of a struggle by either fly.

Usually four 30-min periods (occasionally three or five periods) constituted sufficient observation for a day. At the end of the last period for the day, females were either alternated to adjoining chambers (first 4 weeks of testing) or left with the same male overnight (all interpopulational tests and last 2 weeks of intrapopulational U28). All flies in chambers were then "put to sleep" by covering all chambers with black cloth. Because mating activity depends considerably on vision in this species, no mating occurs in the dark. All observations were made between 0800 and 1100.

Intrapopulation tests (U28, Kilauea) first employed 20 winner males in separate tests of 10 each followed by 10 loser males. Ten wild Olaa males were tested with U28 females for comparison with laboratory-raised males. After the interpopulational tests, U28 tests were repeated with four sets of 10 males each.

Interpopulational tests (U28 × U26) were done as follows: males from U26 placed in odd-numbered chambers and males from U28 in even-numbered were allowed to joust initially within strains; a winner male was left in each chamber. During the first set of tests, each female introduced for the initial 30-min period was from the same population as the male; if unmated she was aspirated into the adjoining chamber to be courted by a male from the alternate population. In the subsequent set of tests, each female was introduced initially to a male from the other strain. Thus by the end of 5 weeks, an equal number of independent sets of tests had been initiated with intrapopulational and interpopulational pairs. Whenever a female mated, she was replaced at the end of the 30-min period with an unmated female from the adjoining chamber destined to be introduced at that time. A new female of the appropriate population was placed in the chamber from which the unmated female had been moved.

RESULTS

Intrapopulational Tests. Total mating observed for U28 (Kilauea) females (× U28 males and × wild Olaa males) are given in Table 1, listed according to six criteria, which are explained as follows: If a female accepts the first male to court during the initial period, she may either have a low threshold (ready to mate with a minimum of courtship) or she may have a moderate threshold but may discriminate in favor of the first male when courted further by him. Examination of initial period mating with respect to the number of courtship bouts before acceptance revealed a bimodal distribution: 31 females mated after one to three bouts of courtship (15 after a single bout, 11 after two bouts, 5 after three bouts), none mated with four bouts, while 13 mated after five to seven bouts. For this reason of bimodality along with other reasons (see Discussion), we have separated these two groups of females into (a) low-threshold and (b) mating to the first male after further courtship in the first time period. In the next 30-min period (c) a female may accept the second male to court (either because she favors him for some courtship element that he supplies or because her threshold is brought down by more courtship than the first male provided). After three or more periods, the female may mate with any of the following: (d) a male who was the only one to court, (e) the male who had been the first to court, or (f) the male second to court.

Out of 185 females tested, 102 mated (55.1%), of which 71 had moderate to high thresholds and thus needed at least five bouts of courtship. Of these, 25 accepted the male first to court (b + e in Table 1), and 36 accepted the second male (c + f). We cannot conclude that there is significantly greater accep-

Table 1. Number of matings by U28 (Kilauea) females courted by U28 or by wild Olaa males grouped according to criteria* of courtship sequence in time periods

Type males	One or two periods			Three or more periods			Total
	a	b	c	d	e	f	
U28	9	4	8	4	4	3	32
U28 (repeat)	17	7	7	2	7	9	49
Wild Olaa	5	2	3	4	1	6	21⁻
Total	31	13	18	10	12	18	102

* a = low threshold; b = × first male after five or more courtship bouts; c = × second male during period 2; d = × only male that courted; e = × first-to-court male in period 3 or later; f = × second-to-court male in period 3 or later.

tance of the second over the first male. The numbers in the two categories are too small for statistical significance, and we cannot then conclude that the females favored either courting male.

In spite of this equality of males overall, when observing matings one gets the impression of individual differences among males both in their intensity of courtship and their mating success. Whether there is any general relationship between mating success and courtship intensity can be ascertained from examining the courtship bout records, which are averaged in Table 2. In order to quantify courtship intensity, an index was assigned with a value of 1 for any courtship bout consisting only of frontal display and with a value of 2 for any bout with a display of head-under-wing. The latter behavior element involves an escalation of courtship from circling (frontal) display to wing vibration and stimulating the female's abdomen by the tibiae. A mean bout index of 3 could refer either to three bouts of frontal display or to one bout of frontal display followed by a head-under-wing. Table 2 indicates a consistent average bout index (3.6–3.7) for both winner and loser U28 males with nearly the same range. Nevertheless, the loser males achieved only a single mating over 3 days and 8 30-min periods compared with 31 copulations by the winners in two sets of tests over 8 days. Thus dominance in the initial fighting between mates apparently leads to some success at mating. On the other hand, nine wild Olaa males achieved more total matings with less average courtship than the U28 strain males. Therefore no claim should be made that mating success results simply from greater courtship intensity; in fact the opposite is more likely, from the fact that males that were consistently refused tended to compensate by courting more intensely, and also certain highly successful males were inactive until in one or two short bouts they were accepted by females (see footnotes to Table 3).

In the third column of Table 2, the range in variance of bouts between time periods within males indicates a wide spread between uniformity of bouts and diversity (fluctuations) in every set of 10 males. In order to examine the data for any association between number of copulations and mean bout index or with the variance between 30-min periods, these variables are arranged in Table 3 accordingly. There is no apparent trend in mean courtship effort (bouts); males that copulated most had

Table 2. U28 and wild Olaa males' courtship mean bout index for 30 min, variance between 30-min periods, and frequency of copulations for sets of 10 males

Set (10 males)	Mean bout index (range)	Bout variance range (within males)	No. periods	No. males	No. copula-tions
Winners (first sample)	3.61 (0.8–6.2)	0.96–21.62	11	2 3 5	3 2 1
Losers	3.65 (0.5–7.4)	1.14–24.70	8	1 9	1 0
Winners (second sample)	3.71 (0.4–7.8)	1.12–14.69	8–18	1 3 1 5	4 3 1 0
Wild Olaa	1.30	0.00–11.24	10–26	2 1 1 5 1	6 3 2 1 0

Table 3. Courtship bout index and variance in bout index between 30-min periods arranged according to number of copulations (U28 and wild Olaa males)

Copulations	No. males	Mean bout index	Variance range within males
6	2*	2.25	4.34–11.24
4	1	3.40	8.73
3	6†	3.83	3.45–14.69
2	4‡	2.78	0.96–14.05
1	12§	2.76	0.90–21.62
0	15¶	2.98	1.12–24.70

* Wild Olaa males: one mated every day except one, the other did not court until the last period of the second day and from then on every female he courted accepted him.
† Including 1 wild Olaa male.
‡ Including 1 wild Olaa; a U28 male courted only twice and mated each time.
§ Including 5 wild Olaa males.
¶ Including 1 wild Olaa male that failed to court (omitted from the bout index).

no significant difference in average courtship bouts from males that failed to mate. Male mating success is not correlated with average number of courtship bouts performed. As for the variance in bouts between periods, males that achieved two or more matings were somewhat more uniform in performance (with variances for 11/13 males ranging from 0.96 to 11.2) than males that achieved either just one mating or none (with variances for 21/27 males ranging between 2.1 and 24.7). Thus it appears that uniformity of courtship may influence a male's success in mating but his courtship intensity is of little consequence as long as it exceeds a minimum. In fact the small amount of effort expended by some successful males was a surprise to us.

Individual differences in mating activity were not exclusively attributable to males' properties. Females differed in being courted (relative "desirability"). While there was no significant difference in the average number of courtship bouts (±SEM) received by mated (3.96 ± 0.52 bout index, n = 14) vs. unmated (3.65 ± 0.44 bout index, n = 27) females during the initial weeks of testing, some females were consistently courted (receiving an average bout index per 30 min of 6–7) by both males while others were avoided by both males (receiving an index of 0.5–1.5) even after 10 periods.

Interpopulational Tests. Matings that occurred between or within strains U26 and U28 are given in Table 4, listed according to the same courtship sequence criteria used in Table 1. Out of 186 females tested 97 mated (52.1%), of which 34 were low threshold and the remainder (63) needed at least five bouts of courtship. Turning our attention first to the total matings for each combination of mates, we notice the homogamic matings are nearly equal (27 U26 selfed: 23 U28 selfed), but the heterogamic matings are unequal ($\chi^2 = 6.89$, 1 degree of freedom,

Table 4. Number of matings by U26 (Kahuku) and U28 (Kilauea) females courted by males from these strains grouped by the courtship criteria of Table 1 and by the four combinations of mates

	U26 females						U28 females					
	a	b	c	d	e	f	a	b	c	d	e	f
U26 males	9	2	3	4	3	6	8	1	1	1	1	2
Subtotal			27						14			
U28 males	12	4	10	0	2	5	5	0	5	2	2	9
Subtotal			33						23			

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$P < 0.01$). Kilauea females are reluctant to mate with Kahuku males, but Kahuku females mate readily with Kilauea males. Thus there is some evidence for an asymmetrical partial isolation between these strains.

When the criteria of courtship sequence are applied to the females needing more than minimal courtship, those accepting the first-to-court male ($b + e$) are consistently less than those accepting the second-to-court male ($c + f$) for all combinations but particularly when U28 males court (lower row, Table 4). Whether this difference might result from females tending to avoid the first male to court or from their tendency to favor a simple difference in courtship from the first male is discussed in the next section. In any case the total matings in these two categories are significantly unequal $b + e = 15$, $c + f = 41$.

There is some contrast between the homogamic mating categories: for U26 matings, only 14/27 (52%) could be regarded as cases in which females may have had a "choice," while for U28 16/23 (70%) could be so regarded. In the latter, most of the matings were by the second male to court; in other words, if a U26 male courted first, the U28 female was more likely to mate with a male of her own strain. From the total matings of the two strains of females, it is apparent that under these conditions U28 females simply tended to mate less (37) than U26 females (60). Thus the change in male apparently promoted more mating for Kilauea females than there might have been with no change. As for U26 (Kahuku) females with more low-threshold (a) and no-choice (d) homogamic matings, the change of male was apparently stimulatory also but with a heterogamic result in contrast to U28 females.

In average bouts of courtship, U26 males courted with an average index of 3.1 irrespective of female strain. However, U28 males courted less on the average (bout index 2.5 in 6/8 independent sets of tests) with U26 females than with their own strain females (bout index 3.2). It should be noted from Table 4 that matings were most where courtship was least. Here again courtship intensity is either uncorrelated or negatively correlated with mating success.

DISCUSSION

Throughout these mating tests it is evident that opportunities for sexual selection abound. In *D. silvestris*, males that win initial combat contests tend to be more successful in mating than "losers"; this tendency probably follows from lek behavior in which success at holding a lek may well be a condition for acceptance by a female. In addition, from the courtship bout data, we note that males performing courtship with some consistency mate more frequently than those with variable or erratic courtship. Females tend to accept particular males, and in turn females appear to vary in their desirability for being courted. Thus sexual selection in both aspects (male–male rivalry and discrimination in favor of particular opposite sex individuals) is demonstrated.

A behavioral basis for the rare-male mating advantage in *D. melanogaster* (2, 3) was found attributable to a tendency for females towards avoidance of the type of male first to court. Owing to the technical problem with *D. silvestris* of not being able to use more than one male per chamber, we could not test multiple males of two types simultaneously; and the modification we have employed by sequentially allowing each female an experience from separate males carries advantages (focus on individual differences) as well as disadvantages (problems in interpretation of sequence) compared with the multiple-male technique. Criteria for classifying mated females to one of six categories according to courtship sequence are critical to further arguments. When a female mated immediately after the first bout of courtship, there could be no question that she had a low threshold.

The question of whether she has exercised any discrimination in favor of that male is unanswerable because there has been no alternative for her to choose from. Because the distribution of matings for the first 30-min period is bimodal with a cluster of females accepting after from one to three courtship bouts, we interpret this cluster as a homogeneous low threshold group. After further courtship (more than four bouts) by the same male and/or then courtship by the second male, the female may be said to have needed extra courtship to bring down her threshold. We cannot be justified in making assumptions about her discrimination in favor of first or second male until she has at least experienced both males' courtship. Thus three periods of alternation would appear to be a minimum for considering the null hypothesis (equal matings by first vs. second courting male) as a test for avoidance. Unfortunately with *D. silvestris*, our sample number of matings may be too restrictive for satisfactory answers (as with categories e and f in Table 1). However, in Table 4 the ratio of e:f is 8:22, which is significant ($\chi^2_{(Yates)} = 5.6$, $P = 0.03$), indicating the second courting male to be favored. Rather than simply discard the data of categories b and c in Tables 1 and 4, we consider that those matings can be characterized in either of two ways: (*i*) the female's threshold was brought down by courtship beyond the initial three bouts irrespective of which male courted (no "choice"), or (*ii*) the female was discriminating in favor of one of the males. Matings in the second 30 min cannot be considered "avoidance" of the first male because the female would need to be challenged again by the first male and then be rejected again. Nevertheless, we may consider it plausible to assume that a female tends to accept a male *different* from the first to court. A mere change of experience could bring down her threshold. Unfortunately we have no control experiment for testing numbers of matings from change vs. no change in males. (Category d, in which only one male courted, was not designed as a control and cannot be used efficiently as such.) However, we can logically assume the following: if the proportion of discriminating females remains about constant irrespective of which male is accepted, then an excess in category c (second male) would imply that male to be favored simply because he is different from the first to court. In Table 1 (intrapopulational tests) c is greater than b and also f is greater than e, but neither is significantly so. In contrast, Table 4 (interpopulational) indicates an excess of c over b as well as f over e, so that in total (41:15 for second vs. first male to court, respectively) there is an impressive indication that females favor males different from the first to court.

In the case of matings within the Kilauea strain, neither first- nor second-courting males are significantly favored, but we have no objective criterion that would tell us in what respects males might differ in their behavioral elements; consequently we could expect female discrimination only if individual males were indeed differentiated by females in some unknown way or ways. In contrast, the interpopulational tests presented males alternatively that differed in their tibial bristle rows and geographic origin. Expression of the second-to-court preference ($c + f$ categories) is slight but suggestive with U26 males (upper row, Table 4) but significant with U28 males (lower row). Thus it appears that a morphological feature likely to influence courtship and mating acceptance has actually done so.

For interpopulational matings there is no significant positive assortative mating ($\chi^2 = 0.48$); thus strains derived from allopatric populations on opposite coasts of the island of Hawaii cannot be said to display ethological isolation in the usual sense. However, the number of heterogamic matings contrasts conspicuously with the number of homogamic: U26 females accept a greater number of U28 males than the reciprocal (U28 females × U26 males). This fact indicates that males with the extra row

of tibial bristles (and likely difference in courtship elements) are as acceptable to U26 females as their own strain males, if not more so. To U28 females there is a tendency to accept their own males when those are second to court but they respond poorly or not at all (except for low-threshold females) to males from the other strain. Thus there is an asymmetrical case of partial isolation here (see also refs. 11–15).

Kaneshiro (14, 15) has proposed a hypothesis of phylogeny for cases of asymmetrical mating preferences between allopatric populations of *Drosophila* species. However, both U26 and U28 isofemale strains have been cultured too long in the laboratory for us to have confidence in whatever behavioral details they may still retain from their natural populational origin. Significant shifts in mating behavior have occurred in similar strains previously (12, 13). Probably some "domestication effect" has compounded their natural behavioral elements. Thus we shall not attempt to draw analogies with Kaneshiro's examples at this point but simply offer our present technique as useful for future application to flies derived more closely (F_1 progeny) from the natural populations.

In conclusion, the ingredients for operation of sexual selection (discrimination in favor of particular mates) are in evidence particularly when males differ in known features that have a likely function in mating behavior. We suggest that mating discrimination and the system of selection associated with it are operating on a genetically complex balanced polymorphism, which we conceive as a polygenic system under stabilizing selection. A major reorganizational shift in this balance may occur if the population, or a spatially isolated portion of it, undergoes selective change and random genetic drift after reduction in effective population size (16). A new polygenic balance may arise as the basis of an altered sexual recognition system and then be maintained by sexual selection. The evolutionary steps by which this process can occur might be few and rapid. After the change, if contact with the older, larger population that has remained allopatric is reestablished, a genetic basis for ethological isolation would exist. Accordingly, an event of incipient speciation may have occurred already.

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