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Courtship in *Drosophila* mosaics: Sex-specific foci for sequential action patterns

(sexual behavior/fate mapping/gynandromorphs/behavioral genetics)

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ABSTRACT **Mosaic fate mapping is used to locate the foci determining sex-specific steps in the mating behavior of *Drosophila*. Male performance of following females and displaying wing vibration toward them requires that a focus inside the head be constituted of male tissue, regardless of the sex of the head sense organs, the legs, the wings, or the thoracic ganglion. For attempted copulation to occur, a second focus in the thoracic region must also be male. Courtship by males is induced by a posteriorly located focus in the female, but an anterior female focus determines receptivity to attempted copulation. The interplay of male and female foci in the complex behavioral sequence is delineated.**

In sexual courtship, a stimulus from one participant arouses the prospective mate, whose response in turn affects the first partner; mutual interaction progresses to a climax. The fruit fly *Drosophila melanogaster* displays an elaborate sequence (1), the main steps performed by the male being orienting toward and following the female, tapping the female's abdomen, extending and vibrating a wing to produce a species specific courtship song (2), licking the female genitalia, and attempting copulation. The female provides stimuli that provoke the male actions, and decides whether to be receptive to the male's efforts. These action patterns are performed expertly by flies without previous experience; they are largely programmed by the genes, which dictate the development of specific nervous networks. The problem addressed in this paper is to locate the sex-specific foci for various steps in intact animals, by the use of mosaics. The feasibility of such an approach was shown by Whiting and others (3, 4) in the wasp *Habrobracon*. Mosaic wasps with male head-thorax tended to court females, while ones with female head-thorax displayed the female-specific pattern of stinging a meal moth larva, the host for the wasp's eggs. Morgan and Bridges (17) recounted some early observations of courtship behavior in *Drosophila* mosaics.

In *Drosophila*, sex mosaics in which part of the body is male and part female can readily be produced by chromosome loss during early development of a XX female embryo, producing male XO tissue in part of the animal. The dividing line between male and female portions of the blastoderm may pass between any two sites fated to become specific body structures; the greater the distance between those two sites, the greater is the probability of that happening. Garcia-Bellido and Merriam (5) used this to construct a map of the blastoderm sites fated to become various structures. Such a map is shown in Fig. 1.

Hotta and Benzer (6) showed that the same principle can be used to map the focus of a behavioral mutant, i.e., the part that must be mutant for mutant behavior to be displayed. In this paper, the procedure is extended to a complex behavioral sequence to determine which parts of a fly must be male or female for various steps in courtship.

RESULTS

Male Following and Wing Vibration. Four hundred seventy-seven male-female mosaics were produced by the use of the w^{vC} unstable ring-X chromosome, which tends to be lost in some embryos at an early stage of development. Among female embryos initially having the w^{vC} chromosome and a second X chromosome carrying recessive marker genes, loss of the unstable chromosome generates autonomously male haplo-X parts in which the marker genes are expressed (10). The markers used were *yellow* (*y*) for the cuticle and bristles, *chocolate* (*cho*) for the eyes, and *Hyperkinetic* (Hk^1) for the thoracic ganglion. The Hk^1 gene causes the legs to shake under ether anesthesia; in mosaics, the independent shaking of each leg permits one to score the corresponding focus of the thoracic ganglion (6, 11). *y cho* Hk^1 males were crossed to $w^{vC}/y\ w\ spl$ females and gynanders originating from $w^{vC}/y\ cho\ Hk^1$ embryos were collected from the progeny. In these mosaics, the female parts had the genotype $+w^{vC}++/y+cho\ Hk^1$, producing normal cuticle color, normal eye color, and only mild leg shaking under ether. In the male parts, generated by loss of the w^{vC} chromosome, the markers *y* and *cho* were manifest, and strong shaking of a leg under ether revealed male tissue in the corresponding thoracic ganglion focus. For each mosaic, various body landmarks and the shaking behavior of each leg were scored. Since young males, or ones kept under crowded conditions, courted less proficiently (1), all flies were collected within 12 hr of emergence, kept in separate vials of cornmeal-yeast medium, and aged 4–7 days.

Each mosaic fly was tested by introducing it to a normal (Canton-Special wild type) virgin female beneath a watch glass (30 mm diameter, 2 mm inside height) on a white ceramic

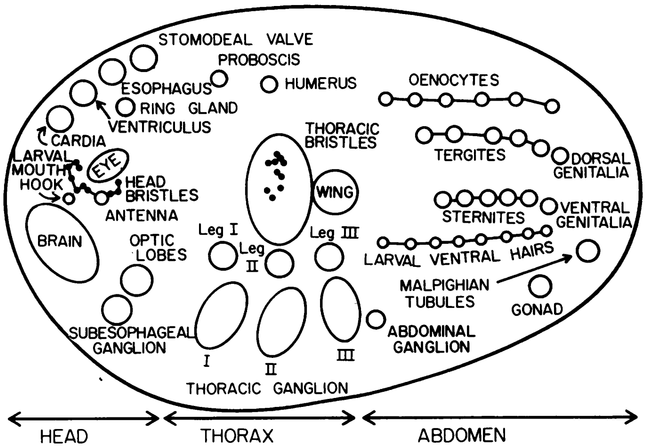

FIG. 1. Fate map of *Drosophila* embryo, showing right half of blastoderm seen from inside. Approximate locations of primordia of various larval and adult structures are shown, as determined via mosaic mapping by various authors (5–9).

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plate; the shallow space prevented copulation. Courtship was observed under a binocular microscope at 23–25° for 5 min under dim light. Each mosaic was tested three times and scored positive if it displayed the action in a sustained or repetitive manner in one or more trials; brief or dubious display was discounted. As controls, 120 male-female pairs of normal flies (30 each of 4, 5, 6, and 7 days age) were tested; 98% of the males showed persistent following and wing vibration. Among 30 female pairs tested, no male courtship behavior was observed. The marker genes did not interfere significantly with courtship; 50 *y cho* Hk^1 males, each tested daily at 4–7 days of age, were positive for both following and wing vibration in 98% of the trials.

Of the 477 mosaic flies, 252 displayed both following and wing vibration (on 3/3 trials for 230 flies, 2/3 for 14, 1/3 for 8); none displayed one action without the other. Fig. 2 illustrates the cuticle sex patterns of a group of mosaics chosen (by random numbers) from the group that performed, compared with ones similarly chosen from the group that did not. Those performing were manifestly more likely to have male cuticle, particularly in the anterior parts. Table 1 shows the correlation of wing vibration with body surface. Among mosaics having the entire head male, the vast majority (162/167) performed vibration; when head cuticle was entirely female, the reverse was true (2/135). Nevertheless, there were five cases of head-all-male that did not vibrate and two cases of head-all-female that did. This would be expected if the determining focus were not on the head surface, but in an internal organ; in some mosaics, the internal focus could have a genotype different from the overlying cuticle. The correlation of male cuticle with wing vibration was weaker for the thorax and even more so for the abdomen. These data indicate that the relevant focus is much closer to the head than to the thorax or abdomen, but is not in the head cuticle itself. Among mosaics with head cuticle split along the midline into male and female halves, no corresponding laterality was observed in the use of the right or left wing to perform vibration. In the great majority of cases, both wings were used.

The general location of the focus may be obtained by a contour mapping procedure. If a structure (e.g., a bristle) is close on the fate map to the wing vibration focus, mosaic dividing lines will rarely separate the two sites, so that it will be rare that a mosaic is female for the structure but male in respect to wing vibration (or vice versa). For more distant structures this will occur more frequently. By scoring these probabilities for various structures, one can draw contours such that lowest values indicate the position of the focus. Table 2 illustrates the procedure.

Table 1. Male wing vibration compared with sex of cuticle

	Head		Thorax		Abdomen	
Vibrate	Male	Female	Male	Female	Male	Female
Yes	162	2	91	11	22	26
No	5	133	33	47	36	19

Data are for mosaic flies having uniform sex for entire cuticle of head, thorax, or abdomen. Proboscis and humerus were not included because they arise from parts of the embryo distant from other head and thoracic structures.

Table 2. Contour mapping of wing vibration command focus

	A	B	$\frac{B}{A}$ %
Head cuticle			
AN	54.2	20.0	36.9
VB	55.5	21.8	39.3
PA	56.3	22.4	39.8
PT	56.1	22.2	39.6
OV	51.9	11.3	21.8
IV	51.6	11.8	22.9
PO	53.6	15.6	29.1
AO	53.8	16.7	31.0
OC	52.3	14.7	28.1
PV	50.4	10.7	21.2
OCC	50.9	11.1	21.8
PR	58.9	37.1	63.0
Thorax cuticle			
HU	59.1	41.9	70.9
Leg I	61.1	43.7	71.5
Leg II	62.8	49.0	78.0
Leg III	62.5	51.3	82.1
PST	62.1	47.8	77.0
ANP	61.6	47.3	76.8
PNP	61.5	46.7	75.9
ASA	62.2	47.3	76.0
PSA	61.3	46.4	75.7
APA	61.0	45.8	75.1
PPA	60.5	46.0	76.0
Thorax cuticle (cont.)			
ADC	62.2	47.3	76.0
PDC	60.8	46.2	76.0
SCT	60.1	46.0	76.5
SP	63.9	49.9	78.1
SN	63.2	48.7	77.1
W	60.3	46.4	76.9
Leg shaking			
Hk I	63.2	46.0	72.8
Hk II	60.6	45.7	75.4
Hk III	62.2	49.8	80.1
Abdomen cuticle			
2 t	55.3	48.0	86.8
3 t	56.3	51.8	92.0
4 t	54.4	52.4	96.3
5 t	54.4	54.1	99.4
6 t	51.2	52.9	103.3
G t	49.7	52.2	105.0
2 s	55.3	48.7	88.1
3 s	54.9	50.7	92.3
4 s	53.5	50.3	94.0
5 s	54.6	52.9	96.9
6 s	51.6	50.6	98.1
G s	47.6	49.3	103.6

Column A gives % of the entire ensemble of 477 mosaics in which the specified landmark is male. Column B gives the % maleness among the group of 225 mosaics that *did not* perform male wing vibration. Data from both sides of the fly are combined. Cuticular landmarks and the Hk foci controlling shaking of the 3 legs are designated as in Hotta and Benzer (6).

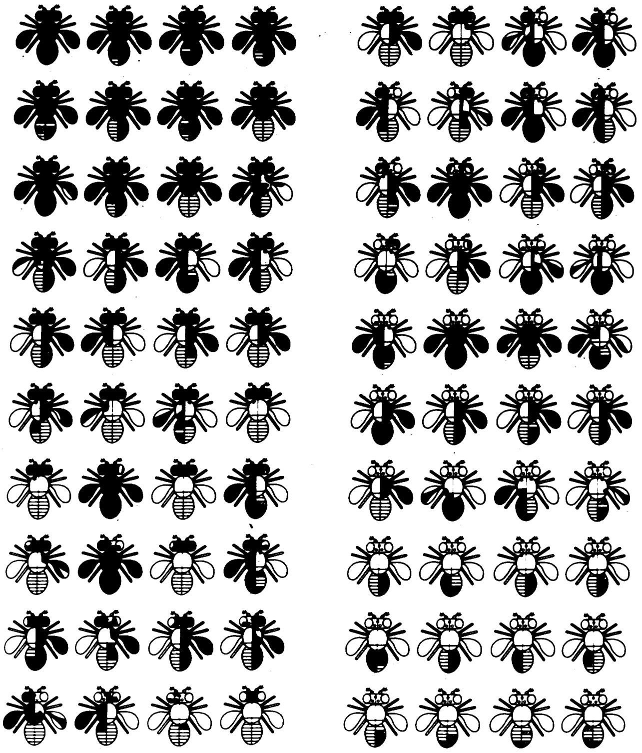

FIG. 2. Mosaic flies. Female cuticle is indicated by solid color; light areas are male. Set of 40 on the left was chosen randomly from the group that *did not* display wing vibration; set on the right from group that *did* display wing vibration. Within each group, mosaics are arranged in order of anterior female parts first.

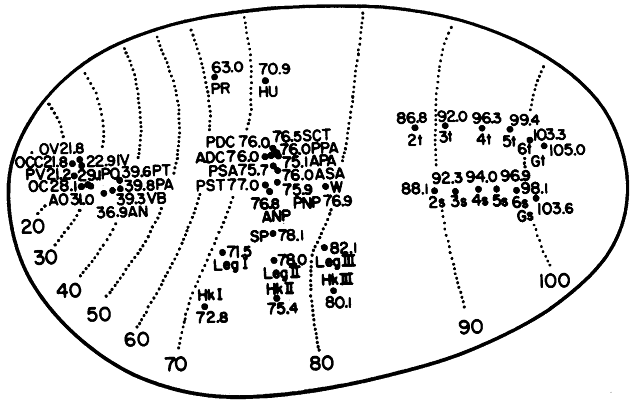

FIG. 3. Contour map of the focus for male wing vibration. In mosaics that *did not* display wing vibration, the focus is taken to be female. Probability of each landmark being male among these mosaics is given (data from Table 2). Contours represent constant probability values; lowest values occur near the focus. Positions of landmarks were established by fate mapping as previously described (6).

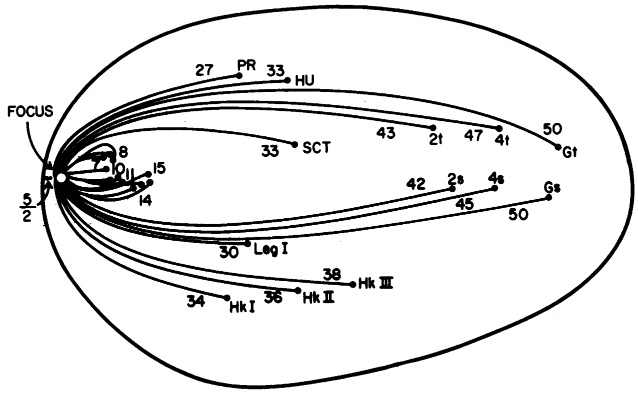

FIG. 4. Position of wing vibration focus determined by fate mapping. The focus is domineering, i.e., a male focus on either side of the animal is sufficient. Distances are given in sturts, one sturt representing a distance between two sites such that the probability of a mosaic dividing line passing between the points is 1%.

Note (column A) that the probability of a surface landmark being male among the entire ensemble of 477 mosaics was close to 50%, with a significant increase for the thorax. Column B gives the frequency of maleness within the group of 225 mosaic flies that *did not* show wing vibration; i.e., the ones in which the wing vibration focus was female. These values were obtained by combining data from landmarks on both sides of the fly. By doing this, the contour method avoids the issue of possible interactions between homologous bilateral foci. In the next column, B is divided by A to compensate for the local percent of maleness of the structure in the entire population. In Fig. 3 these values are shown on a fate map previously obtained by measuring the distances of the various structures from each other and from the dorsal and ventral midlines (6). Contours are drawn according to frequency of maleness. In drawing contours, one must bear in mind that the map corresponds to one side of a bilaterally symmetrical blastoderm, the data from both sides having been combined. The contours should therefore be perpendicular to the boundary of the map; otherwise, singularities in slope would occur at the midline. Contours ought not to have sharp curvature, except close to a focus. Needless to say, contours may not cross; an intersection would imply two different values at the same point. The contours in Fig. 3 locate the wing vibration focus at the anterior end of the blastoderm; structures near that end are most likely to be of the same sex as the behavioral focus. A similar result was obtained by the converse calculation for the 252 mosaics that *did* show wing vibration.

Table 3. Mapping of wing vibration focus

AA′	$\overline{AA'}$	$\overline{Af}$	$\overline{ff'}$	AA′	$\overline{AA'}$	$\overline{Af}$	$\overline{ff'}$
AN	22.4	14.2	3.9	HU	33.0	33.2	6.1
VB	22.0	14.7	2.1	Leg I	37.0	29.9	0.1
PA	21.9	14.4	0.2	SCT	45.7	33.4	5.0
PT	22.6	14.5	0.7	Hk I	31.8	34.2	8.0
OV	12.2	7.8	9.0	Hk II	35.5	35.9	4.3
IV	13.6	8.3	9.2	Hk III	34.3	38.1	3.9
PO	16.6	10.3	5.5	2 t	36.8	42.7	3.3
AO	17.5	11.1	4.8	2 s	40.6	41.8	3.3
OC	16.1	9.7	7.5	4 t	39.5	46.6	5.1
PV	15.1	7.2	11.0	4 s	40.7	44.9	4.9
OCC	14.4	7.5	10.5	G t	30.1	49.9	5.6
PR	25.8	26.8	4.9	G s	18.9	49.6	5.5

AA′ designates a bilateral homologous pair of cuticular landmarks. Distances are in sturt units: $\overline{AA'}$, between landmarks; $\overline{Af}$, between landmark on one side and ipsilateral focus; $\overline{ff'}$, between foci. Domineering model (6) was used.

A more precise method of mapping, in which interactions between bilateral foci are taken into account, is the procedure of Hotta and Benzer (6). The domineering model assumes that a male focus on either side is sufficient to produce male behavior; the submissive model requires both foci to be male. By choosing a homologous pair of cuticle landmarks, one can calculate, using appropriate equations for each model, the distance of each focus from the ipsilateral landmark and the distance between the pair of foci (i.e., twice the distance of each from the midline). The equations for both models yield the same values for interfocal distance, but with opposite signs; the incorrect model, yielding negative distances, can thus be ruled out. Our data, calculated by this method, were consistent with a domineering model for wing vibration. The results are given in Table 3 and are illustrated in Fig. 4. The focus is localized at the anterior end, close to the midline, near the head, but not in the head cuticle. It is in this general area of the fate map that the brain is located (8, 12). The fact that no mosaics were observed in which following and wing vibration were dissociated implies that both actions are controlled by structures that are identical, overlapping, or very close together.

Male Attempted Copulation. A second set of mosaics was used for studying later male steps. These were generated by crossing *y* males to $w^{vC}/y\ w\ spl$ females. Cuticle genotype was scored for a limited set of landmarks chosen to represent different regions of the map (OC, VB, HU, PSC, Legs I, II, and III, 4s, 4t, and G). Each mosaic fly was maintained in a separate vial and aged 7 days before testing. The test apparatus was a rotary multiple "mating wheel" containing a plastic disc with 10 holes for 10 mosaics and a second disc with 10 holes, out of register with the first set, for females. After the flies were introduced, 5 min was allowed for accommodation to the apparatus. The discs were then rotated to bring the holes into register, producing chambers ⅜ inch wide × ¼ inch high, sufficient to allow copulation. The 10 chambers were observed simultaneously under dim light at 23–25°. Each mosaic fly was tested with two *y f* XX virgin females for 60 min. The actions scored were persistent following, persistent wing vibration, attempted copulation (i.e., the mosaic curling its abdomen under while following close behind a female), and successful copulation (i.e.,

Table 4. Male courtship steps performed by mosaic flies

mosaics	→	follow	→	vibrate	→	attempt copulate	→	copulate
208		130		130		99		23†
	78				31		76*	

* Genitalia 5 male, 12 female, 59 mixed.

† Genitalia all male.

sustained joining of genitalia, typically lasting for about 20 min). In control experiments, 20 *y* males tested performed all the steps through successful copulation. Table 4 shows the number of mosaics performing each successive step. The foci for following and wing vibration, calculated by the same methods as in the previous experiment, mapped to the same anterior location. Analysis of the data by Jeffrey Hall, using the maximum likelihood method of Merriam and Lange (18) gave essentially the same results.

The foci for later steps cannot be calculated from the entire ensemble of mosaics; a later step must be considered within the subgroup that performed the preceding ones. Within each subgroup one can ask which parts must be male to go on to the next step. The results for mosaics in which the head, thorax, or abdomen landmarks were male are shown graphically in Fig. 5. In panel A, for the entire ensemble of mosaics, wing vibration occurred most frequently when the head was male. Panel B shows, among the subgroup that performed wing vibration, what fraction attempted copulation. That fraction was highest when the thorax landmarks were male, in spite of the fact that the subgroup was biased in favor of male head cuticle, since head cuticle is close on the fate map to the focus for wing vibration. This suggests that the thoracic region might contain the focus for attempted copulation. In panel C, the subgroup that attempted copulation is graphed for successful copulation. The dotted lines indicate the fraction in each group with entirely male genitalia (i.e., dorsal and ventral components on left and right sides, as judged under a stereo microscope before the behavior was tested). Almost all mosaics with male genitalia that attempted copulation were successful.

To calculate the fate map position of the attempted copulation focus, the method of Hotta and Benzer (6) was applied to the subset that performed wing vibration. (The distances thus obtained are not, strictly speaking, sturts because they are not calculated from the entire ensemble of mosaics. The subset is enriched for mosaics having the anterior region male. Therefore, the measurements are subject to some distortion of anterior distances. To correctly map a focus such as this, a theory is needed that takes into account interactions between foci on the same side as well as opposite sides of the animal.) Since the data of Fig. 5 indicated that the attempted copulation focus is posterior to the one for wing vibration, the more posterior landmarks were used as reference points. Fig. 6 shows the calculated position of the focus, based on the male domineering model, that gave positive interfocal distances. It maps to the general region of the thoracic ganglion (8, 12). Thus, the focus for this courtship step is in a very different location from the ones for following and wing vibration.

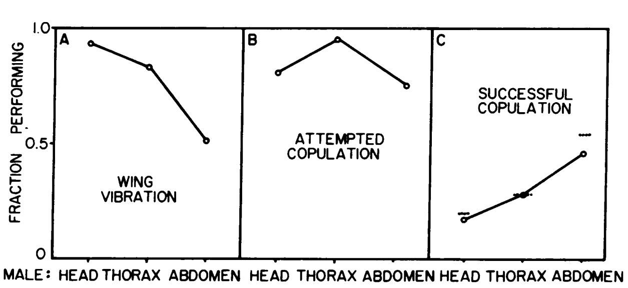

FIG. 5. Correlation of steps in courtship with maleness of the three main body sections. Mosaics considered are three classes: male in all head landmarks scored, or all thorax landmarks, or all abdomen landmarks, in each case irrespective of sex of other parts. (A) Fraction of each type that performed wing vibration; (B) among those performing wing vibration, fraction that attempted copulation; (C) fraction of those attempting copulation that were successful. Dotted lines indicate fraction having male genitalia.

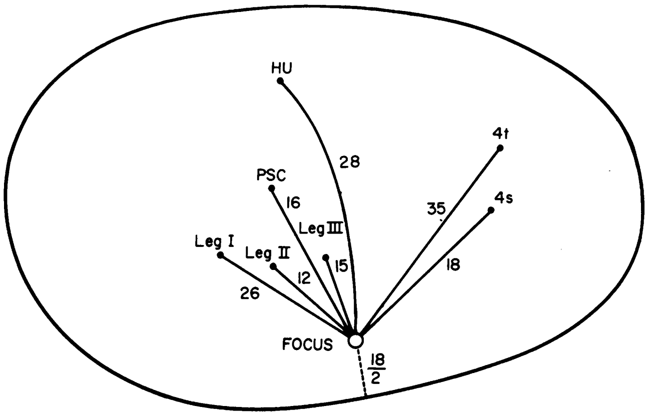

FIG. 6. Fate map of focus for attempted copulation, based on the group of mosaics that performed wing vibration. Results indicate domineering focus in general region of thoracic ganglion.

Female Components of Courtship. The mosaics of the previous experiment were also used to study the female-specific foci for induction of courtship by males and for receptivity to copulation. As controls, 20 *y* males (age 7 days) were tested in the mating wheel for 60 min (each with two normal males) for courtship by the normal males. Under these conditions, the usual abortive pursuit and tentative wing vibration displayed by males toward each other occurred, but persistent following, wing vibration, or attempts at copulation were not observed. Each mosaic was tested in the mating wheel with two normal males for 60 min. If either or both of the normal males exhibited a sustained courtship action clearly more pronounced than the behavior typically displayed toward control males, the mosaic fly was scored as positive for the ability to provoke that action. The results were consistent with the observations of others (12–14) that female structures in the posterior region, on or in the abdomen, are able to provoke sustained courtship by males. A detailed analysis of this "sex appeal" will appear separately (Jallon and Hotta, in preparation).

The role of female receptivity became evident by examining the group that provoked male attempts at copulation. One requirement for successful copulation was, of course, that the mosaic have female genitalia, but not all such mosaics copulated. Among 24 with female head landmarks, there were 19 successes. Among 20 with male head landmarks, there was only one success. These results indicate that receptivity (or lack of rejection) is controlled by an anterior focus distinct from the posterior structures that provoke courtship.

DISCUSSION

Fig. 7 summarizes the sequential interplay between sex-specific foci. Evidently, to induce sustained courtship by a male, some female tissue in the posterior region is required. An anterior focus in the male comes into play and initiates following; the

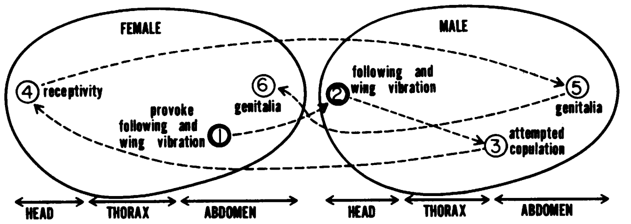

FIG. 7. Interplay of male and female sex-specific foci in sequential courtship steps, as delineated by analysis of mosaics.

same focus, or one very close by, commands wing vibration. Given this, a second male focus located in the mid-region leads to attempted copulation. A mosaic fly may thus attempt to copulate with females even if it has female abdomen and genitalia. Receptivity to copulation seems to depend upon an anterior focus being female; successful copulation requires reasonably normal male and female genitalia. Additional foci must surely exist for other detailed steps, such as opening of the female genital plates, erection of the male penis, and disengagement to terminate copulation.

The mosaic experiments show that it is possible to have a fly in which the entire external cuticle of the head, which contains the sense organs and gives rise to the sensory neurons, is female, but which nevertheless can produce typical male wing extension if the critical focus inside the head is male. This indicates that, in *Drosophila*, male or female receptors may transmit similar signals to the central nervous system; if it were required that the antenna, say, be male, the focus would map to that structure. Initiation of wing vibration depends upon the internal focus; given this, typical male pursuit and wing display can be performed by a fly with a female thorax and a female thoracic ganglion, vibrating female wings. This suggests that a female thoracic ganglion is capable of generating the neural patterns for wing vibration, when properly activated. It may be another of many examples in invertebrates where a trigger stimulus can initiate a complex firing pattern in a genetically determined neural circuit, which then functions autonomously to produce a coordinated action (15). In mosaics, the male wing vibration focus is domineering; a male focus on one side is apparently sufficient to trigger circuits for vibration of both left and right wings. The ability of females to recognize the species-specific male courtship song may be due to the presence of these same circuits, which fail to emit the song due to lack of appropriate command (16) signals from the brain.

Fate mapping alone does not identify the nature of the sex-specific difference at the foci. The wing vibration focus could, for instance, be a cluster of neurons that develops only in male brain tissue, or a group of neuroendocrine cells that modify local brain activity. The mapping does indicate where to look for the differences, while avoiding the pitfalls inherent in mutilation experiments.

While fate mapping with respect to cuticular structures can roughly localize internal foci on the blastoderm map, for definitive identification of the corresponding organs, the internal tissues should also be tagged with genetic markers that permit direct identification of their sex in mosaics. To do this, Hall (12) has repeated and extended these experiments using an acid phosphatase null mutant gene to mark the nervous system. His findings indicate that the focus for wing vibration is the brain (or some other structure within a few sturts on the fate map); the focus for attempted copulation appears closely linked to the thoracic ganglion.

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