Substrate-Borne Vibratory Communication during Courtship in *Drosophila melanogaster*

Caroline C.G. Fabre, 1,4,* Berthold Hedwig,1
Graham Conduit,2 Peter A. Lawrence,1 Stephen F. Goodwin,3 and José Casal1

1Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 2EJ, UK
2King’s College, 21 King’s Parade, Cambridge CB2 1ST, UK
3Department of Physiology, Anatomy and Genetics, University of Oxford, Sherrington Building, Parks Road, Oxford OX1 3PT, UK

Summary

Courtship in *Drosophila melanogaster* has become an iconic example of an innate and interactive series of behaviors [1–11]. The female signals her acceptance of copulation by becoming immobile in response to a male’s display of stereotyped actions. The male and female communicate via vision, air-borne sounds, and pheromones [1, 2], but what triggers the female’s immobility is undetermined. Here, we describe an overlooked and important component of *Drosophila* courtship. Video recordings and laser vibrometry show that the male abdomen shakes (‘quivers’), generating substrate-borne vibrations at about six pulses per second. We present evidence that the female becomes receptive and stops walking because she senses these vibrations, rather than as a response to air-borne songs produced by the male fluttering the wings [1, 2, 12]. We also present evidence that the neural circuits expressing the sex-determination genes *fruitless* and *doublesex* [8] drive quivering behavior. These abdominal quivers and associated vibrations, as well as their effect on female receptivity, are conserved in other *Drosophila* species. Substrate-borne vibrations are an ancient form of communication that is widespread in animals. Our findings in *Drosophila* open a door to study the neuromuscular circuitry responsible for these signals and the sensory systems needed for their reception.

Results and Discussion

Characteristics of Male Quivering during Courtship

Pairs of flies were placed in a chamber and filmed at 30–150 frames per second. The behaviors of both the male and female were annotated and analyzed from initiation of courtship until copulation. In addition to well-known courtship behaviors, we observed frequent bouts of abdominal movements in the male that we refer to as ‘quivering’ (Movie S1 available online). Quivering consists of up-and-down movements of the abdomen (Movie S2) with a frequency of 6.64 ± 0.78 beats per second (n = 12 bouts/12 flies). Quivering is a behavior specific to male courtship: we find that females and isolated males do not quiver, nor do males placed with other males (data not shown).

We quantified 30 ethograms of completed courtships (Experimental Procedures) in wild-type Oregon-R flies (Figures 1 and S1) for courtship behaviors including wing fluttering alone (about one-third of total courtship time), abdominal quivering alone (one-seventh of total courtship time), and simultaneous wing fluttering and abdominal quivering (one-seventh of total courtship time). We also recorded whether females were moving or stationary (Figure 1); they were immobile for one-third of the total courtship time (Figure S1B).

The most interesting results come from when the two behaviors of the males are compared relative to the simultaneous behavior of the female (Figure 1). Male quivering occurs 69% of the time that females are stationary but only 10% of the time that females are moving (Figure 1). In contrast, males flutter their wings about as often, independently of whether females are stationary or moving (53% and 42%, respectively; Figure 1). Although males flutter their wings for approximately half of the time that the females are stationary (53%), we note that males are also quivering for two-thirds of this 53%. However, when males flutter during female movement (42%), the males quiver for only one-seventh of this 42% (Figure 1).

Thus, the data show that female immobility can coincide with male fluttering but mainly occurs when he is also quivering. Statistical analysis of the data shows that male quivering behavior (quivering or not) and female behavior (stationary or moving) are strongly associated, whereas the comparable association between male fluttering and female behavior also exists but is weaker (Figure 1 and S1C). Similar results were found for male and female pairs of another wild-type strain, Canton S (Figures S1E–S1G).

Our results do not support the general perception that signals generated by male wing fluttering act alone to diminish movement of females [7]. They show instead that quivering of the abdomen coincided with female immobility much more than wing fluttering did (Figure 1 and Movie S1). Also, and consistent with this finding, bouts of quivering vary in duration and depend on whether the female is moving (average duration only 1 s; Figure S1B) or stationary (average duration about 3 s; Figure S1B). In contrast, bouts of fluttering were longer when the female was moving than when she was immobile (Figure S1B).

If the wings of the wild-type male were amputated, males quivered more frequently than the wild-type (t test, p = 0.011) and for longer periods (p = 0.029); females placed with these males stopped moving more than when paired with intact males (p = 0.017) (Figures S2A–S2C). We also used males carrying mutations in the sex-determination genes *doublesex* (*dsx*) and *fruitless* (*fru*) because neurons expressing these genes drive male-specific behavior [5, 13, 14]. These mutations had no clear effects on the percentage of the courtship time that males fluttered their wings (*dxs* p = 0.45 and *fru−* p = 0.24), although the pattern of fluttering was different from the wild-type (data not shown). However, *dxs* (Figures S2D–S2F) and *fru−* (Figures S2G–S2I) mutant males quivered less than normal males (*dxs* p = 9.27 × 10−5 and *fru−* p = 2.17 × 10−5). Importantly, we observed that their wild-type female partners stopped less than when courted by wild-type males (*dxs* p = 0.026 and *fru−* p = 0.006) (Figures S2D–S2I, compare...
Thus, if the male quivering is increased or decreased by intervention, the wild-type females stop more or less often, respectively (Figures S2A–S2I). These findings argue (but do not prove) that female stopping is a response to quivering and not a cause of quivering.

A mild activation of dsx-expressing neurons or fru-expressing neurons by forcing expression of Drosophila TRPA1 (Movie S3) triggered quivering in solitary males—as well as a mélange of other courtship behaviors (Movie S3) [15]. When stronger conditions were used to activate the fru-expressing neurons, quivering was induced also in females, arguing that appropriate neurons and circuitry are present but latent in the female. It follows that some of these neurons direct the abdominal quivering of the male during courtship and that the neuronal circuitry differs between normal males and females.

How Might Females Sense Male Quivering?
Our observations suggest that quivering of the male abdomen is sensed by the female and causes her to stop walking. We therefore asked how the tremor of the male abdomen might be transmitted to the female. One possibility is that the female could see quivering—vision is known to be used during courtship [7, 9]. To investigate, we performed courtship assays in the “dark” using infrared light that flies cannot detect [16]. Males quivered normally and again there was a strong coincidence between quivering and female immobility, suggesting that vision is not an important component (Figures S2J–S2L).

Next, we asked whether quivering might be associated with release of male-specific pheromones via the cuticle. In Drosophila, pheromones are low-volatility hydrocarbons and are produced by the abdominal oenocytes of both male and female [7]. By using RNA interference, we reduced the expression of the sex-determination gene transformer (tra), but only in the female nervous system [17]. The result was neuronally masculinized females that showed male-like behavior directed toward normal females; these masculinized females exhibited abdominal quivering, which wild-type females never do (Figures 2 and S3 and Movie S4). Their wild-type female partners tend to become immobile when the masculinized females exhibit abdominal quivering (for quantitation, see Figures 2 and S3). We have presented evidence that, in normal courtship and as a response to the male quivering, the females tend to stop.
stop. But these neuronally masculinized females have a female anatomy, and accordingly their oenocytes produce only female pheromones [18]. Yet, when they quiver, their wild-type females appear to respond by stopping. Data are presented as in Figure 1. See also Figure S3 and Movie S4.

We found both abdominal quivering and associated substrate-borne vibrations are conserved in other Drosophila species (Figure 4). In D. sechellia and D. yakuba (Figures 4A, 4B, S4A, and S4B, and Movie S6), we observed vibrations in the substrate with a pulse repetition rate of 7.13 ± 0.96 (D. sechellia; n = 50 pulses/5 flies) and 6.80 ± 0.49 (D. yakuba; n = 19 pulses/5 flies) (Figure 4C). The pulse interval is 157.56 ± 11.13 ms (n = 50 pulses/5 flies) for D. yakuba and 173.37 ± 8.70 ms (n = 19 pulses/5 flies) for D. sechellia. The frequency and length of the quivering bouts varied (data not shown). In both species, males simultaneously quiver the abdomen and flutter their wings more frequently than D. melanogaster (Figures 4, S4A, and S4B; compare with Figures 1 and S1). Importantly, in both species, quivering was again strongly associated with female immobility (Figure 4 and Movie S6). Less-detailed, yet similar, observations were made on different Drosophila species: some from the same group as D. melanogaster (D. biarmipes, D. mauritiana, and D. simulans), and others from more distant groups (D. mojavensis and D. willistoni).

It is strange that substrate-borne signals have so far been overlooked in D. melanogaster, particularly as substrate-borne vibrations are well known in small invertebrates [26–28]. Tremulatory signals were detected during courtship of other arthropods, for example pentatomid bugs [20] and salticid spiders [22, 29, 30]. Such signals may be generated by upward-and-down movements of the abdomen, similar to the quivering we observe, or by shaking of appendages [28, 29, 31–35]. Substrate-borne vibrations were thought to be unusual in Diptera; exceptions were the male and female reed fly, Lipara,
which exchange signals as vibrations transmitted within the
reed stems. The male signal appears to originate from tremu-
lar movements of the abdomen [36]. Even two decades ago, abdominal
movements were observed in D. silvestris, but this was then
reported as a behavior unique to these flies of the Hawaiian
islands [37]. Later, a repeated movement of the male abdomen
that tapped the substrate was noted as part of a broad
description of courtship behavior in D. melanogaster but not
associated with any particular female behavior [38]. We have
now characterized a male behavior, which we call quivering,
that does not appear to include contact with the substrate
and that generates substrate-borne vibrations. We do not
know exactly how quivering produces these vibrations, but
notice that the pulses themselves are short, suggesting
some instantaneous element within the quiver beat.

The characteristics of substrate-borne signals depend on
the material in which they are transmitted; they are robust
and can propagate with little attenuation [21]. The frequency,
amplitude, and modulation of these vibrations may carry
information to the receiver about the sender [27, 39–41].
Substrate-borne signals may not be detectable by predators,
as the latter may not possess suitable receptors [42]. It has
not escaped our notice that vertebrates also use substrate-
borne vibratory signaling [43].

Experimental Procedures
Fly Mutant and Wild-Type Stocks
Flies were raised on standard cornmeal medium under a 12:12 hr light:dark
cycle and kept and tested at 25 ºC with 65% humidity. For the analysis of
wild-type behavior, we used Oregon R (OrR) and Canton S (Cs). fru.Gal4
(tu[5–8],cr) and elav.Gal4 (elav[1–5]) flies were obtained from the Bloomington
Stock Center. UAS.dTRPA1 flies were kindly provided by Stefan Pulver.
UAS.trail flies were obtained from the VDRC Stock Center. The dsx.Gal4
dsx[7,14–20] line used was that described in [44]. For the analysis of the effect
of mutations in the sex-determination genes, two allelic combinations
were used: dsx[7,14–20]/Df(3R)Dsx15 and fru[7,14–20]/Df(3R)4-40. For details of mutant
alleles, see FlyBase [45]. Drosophila simulans, D. yakuba, D. mauritiana,
D. sechellia, D. biarmipes, and D. willistoni flies were obtained from the
University of California, Drosophila Species Stock Center. D. mojavensis flies
were kindly provided by Darren Parker. Adult flies were collected upon
eclosion with light CO2 anesthesia. Before mating, individual males or small
groups of five to ten virgin females were kept isolated in vials with fresh food.
For some experiments, courting pairs were kept under infrared light
[16] or the wings of collected males were cut off with microscissors and
under anesthesia.

Behavioral Recording
Pairs of flies were tested in a single trial when they were 4–6 days old. Their
behavior was recorded with a 10× macro lens and a Firewire Stingray F-033B camera (Allied Vision Technologies; Stadtrada, Germany) and
acquired with “Astro IDCC” (Aupperle Services and Contracting; Calgary,
Canada) into a laptop computer. For analysis of the wild-type, 30 courting
pairs were recorded and analyzed. For other studies, a minimum of four
pairs of flies was tested. Transparent plexiglass courtship chambers
(10 mm diameter and 6 mm height) were assembled from two half chambers
each of 3 mm height. Each fly was collected with a mouth aspirator and
introduced into one half chamber. After a recovery period of 5 min, both
halves were fused, and filming of the pair was commenced. Recording
was started at the initiation of courtship and for approximately 600 s, or until
copulation occurred. Each pair was tested only once. Before each test,
chambers were washed with ethanol and dried.

Heat-Activation Experiments
Ectopic expression of the heat-activatable cation channel TRPA1 (dTRPA1)
was obtained with the fru.Gal4 and dsx.Gal4 drivers in both males and
females. The courtship chamber was inserted into a metal heating block
set to produce a temperature of 26 ± 27 ºC inside the chamber; at this
temperature, we observed an effect on male but not female behavior with
both drivers (Movie S3). We noted that at 29.5 ºC and only using the fru.Gal4
driver, the females began to quiver; however, the male’s behavior became
even more frenetic [19].

Behavior Annotations and Analysis
Movies were annotated with the “Annotation” software version 1.3, regist-
ering all standard male courting behaviors (such as orientating toward the
female, following the female, proboscis extension, licking, tapping), in partic-
ular when males showed wing fluttering (this behavior comprises wing exten-
sion/vibration and scissoring) and/or abdominal quivering, and also whether
the female was moving or immobile. The data for each movie were imported
into Excel files. For statistical analysis and generation of diagrams, we used
the R programming language and software environment [46]. All intervals
shown in the paper are for 95% confidence level. We tested for associations
between the three behavioral variables: female mobility, male quivering, and
male fluttering; using the number of bouts, \( N = \) a fitted series of generalized
linear models with a Poisson error structure, \( \alpha = 1 \) or male
quivering + (or) - male fluttering + (or) - male quivering [47] (see legends to Figures S1C and S1D).

Recording Vibrational Signals with Laser Vibrometry
Video and laser vibrometer recordings were conducted on a vibration-
damped table in a soundproof room. Flies were placed into cylindrical
chambers of approximately 10 mm in diameter and 6 mm in height, made
of plastic. The top of this cylinder was a transparent film through which
the flies were recorded using the Stingray F-33B camera with an attached
blue filter (cutoff wavelengths at 395 and 480 nm). The bottom of the cylinder
consisted of a piece of thermal foil, a membrane made of silver metalized

Figure 3. Substrate-Borne Vibrations Generated during Abdominal Quiv-
ering of Courting Males

(A) Scheme of the video and laser vibrometer recording system.
(B) Oscillogram of substrate-borne vibrations generated during a single
bout of quivering of about 7 s; the wings of the male were amputated. There
is some variation in the amplitudes of the substrate vibrations.
(C) Details of (B) above to show higher resolution.
See also Movie S5.
polyester material, with an albedo of approximately 0.8 (Sub Zero Technology; Leicester, UK). The beam of a PSV-400 laser vibrometer (Polytec. Waldbronn, Germany) was directed perpendicular to the surface of this membrane (Figure 3). Signals were digitized with 12 bit amplitude resolution with a PCI MIO-16-E4 card (Analog Devices; Norwood, MA) and digitized with LabView (National Instruments; Austin, TX) on a PC. Signals were transformed into .wav data with the Neurolab software [48]. Video and laser vibrometer recordings were synchronized at the start by brief interruption of the laser path; this produces both a momentary peak in the oscillogram and a black frame in the video. Oscillograms were analyzed with the Raven software [49]. Neither an electret microphone (frequency response, 50 Hz to 13k Hz; sensitivity, $60 \pm 3$ dB) nor a piezoelectric transducer (resonant frequency, $2.8 \pm 0.5$ kHz) registered any air-propagated sound emitted during abdominal quivering. We do not know whether wing fluttering of Drosophila produces vibrational signals in the substrate.

Supplemental Information

Supplemental Information includes six movies and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2012.09.042.

Acknowledgments

We thank L. Arnoult and P. Conduit for crucial discussions and the reviewers for constructive criticism; W. Amos for help with statistics; M. Bate, J. Berni, A. Bluon, S. Flynn, N. Gompel, H. ter Hofstede, B. Hudry, C. O’Kane, M. Landgraf, M. Neville, L. de Nerestang, H. Pavlou, B. Prud’homme, S. Pulver, C. Rezaval, C. Skjöldebrand, and B. Tiepman for advice and support; EPSRC for loan of the laser vibrometer to B.H., Wellcome Trust grants WT085297 and WT085291 to S.F.G., BBSRC grant RG54425 to B.H.; and Wellcome Trust grant WT086986MA to P.A.L. C.C.G.F. was supported by an EMBO long-term fellowship during part of this work.

Received: August 6, 2012
Revised: September 7, 2012
Accepted: September 25, 2012
Published online: October 25, 2012

References

1. Greenspan, R.J., and Ferveur, J.F. (2000). Courtship in Drosophila. Annu. Rev. Genet. 34, 205–232.
2. Tauber, E., and Eberl, D.F. (2003). Acoustic communication in Drosophila. Behav. Processes 64, 197–210.
3. Billeter, J.C., Rideout, E.J., Dornan, A.J., and Goodwin, S.F. (2006). Control of male sexual behavior in Drosophila by the sex determination pathway. Curr. Biol. 16, R766–R776.
4. Dahanukar, A., and Ray, A. (2011). Courtship, aggression and avoidance: pheromones, receptors and neurons for social behaviors in Drosophila. Fly (Austin) 5, 58–63.
5. Dauwalder, B. (2011). The roles of fruitless and doublesex in the control of male courtship. Int. Rev. Neurobiol. 99, 87–105.
6. Dickson, B.J. (2008). Wired for sex: the neurobiology of Drosophila mating decisions. Science 322, 904–909.
7. Ferveur, J.F. (2010). Drosophila female courtship and mating behaviors: sensory signals, genes, neural structures and evolution. Curr. Opin. Neurobiol. 20, 764–769.
8. Siwicki, K.K., and Kravitz, E.A. (2009). Fruitless, doublesex and the genetics of social behavior in Drosophila melanogaster. Curr. Opin. Neurobiol. 19, 200–206.
9. Yamamoto, D., Jallon, J.M., and Komatsu, A. (1997). Genetic dissection of sexual behavior in Drosophila melanogaster. Ann. Rev. Entomol. 42, 551–585.
10. Yamamoto, D. (2008). Brain sex differences and function of the fruitless gene in Drosophila. J. Neurogenet. 22, 309–332.
11. Kohatsu, S., Koganeezawa, M., and Yamamoto, D. (2011). Female contact activates male-specific interneurons that trigger stereotypic courtship behavior in Drosophila. Neuron 69, 498–508.

12. Tompkins, L., Gross, A.C., Hall, J.C., Gailey, D.A., and Siegel, R.W. (1997). Extended reproductive roles of the fruitless gene in Drosophila melanogaster revealed by behavioral analysis of new fru mutants. Genetics 147, 1107–1130.

13. Pan, Y., Robinett, C.C., and Baker, B.S. (2011). Turning males on: activation of male courtship behavior in Drosophila melanogaster. PLoS ONE 6, e21144.

14. Ewing, A.W. (1964). The influence of wing area on the courtship behavior of Drosophila melanogaster. J. Comp. Physiol. 118, 397–402.

15. Boake, C.R.B., and Poulsen, T. (1997). Correlates versus predictors of reproductive isolation in spiders. In Spider Communication: Mechanisms and Ecological Significance, P.N. Wittn and J.S. Rovner, eds. (Princeton, NJ: Princeton University Press), pp. 123–129.

16. Kulkarni, S.J., Steinlauf, A.F., and Hall, J.C. (1988). The dissonance cue that triggers courtship behavior in Drosophila melanogaster. Proc. Natl. Acad. Sci. USA 104, 19577–19582.

17. Virant-Doberlet, M., and Cokl, C. (2004). Vibrational communication in insects. Neotrop. Entomol. 33, 121–134.

18. Bart, F.G. (2002). Spider sensens - technical perfection and biology. Zoology (Jena) 105, 271–285.

19. Rovner, J.S. (1980). Vibration in treefrogs. Curr. Biol. 20, 1012–1017.

20. Rideout, E.J., Dornan, A.J., Neville, M.C., Eadie, S., and Goodwin, S.F. (2010). Control of sexual differentiation and behavior by the doublesex gene in Drosophila melanogaster. Nat. Neurosci. 13, 458–466.

21. McQuillen, P., St Pierre, S.E., and Thurmond, J.; FlyBase Consortium. (2012). FlyBase 101—the basics of navigating FlyBase. Nucleic Acids Res. 40(Database issue), D706–D714.

22. Hedwig, B., and Knepper, M. (1992). NEUROLAB, a comprehensive program for the analysis of neurophysiological and behavioural data. J. Neurosci. Methods 45, 135–148.

23. Bioacoustics Research Program. (2004). Raven Pro: Interactive Sound Analysis Software. Version 1.2 Edition (Ithaca, New York: The Cornell Lab of Ornithology).

24. Bart, F.G. (2001). A Spider’s World: Senses and Behavior (New York: Springer).

25. Cocroft, R., and Rodriguez, R. (2010). Host shifts and signal divergence: mating signals covary with host use in a complex of specialized plant-feeding insects. Biol. J. Linn. Soc. Lond. 99, 60–72.

26. Uetz, G.W., and Stratton, G.E. (1982). Acoustic communication and reproductive isolation in spiders. In Spider Communication: Mechanisms and Ecological Significance, P.N. Wittn and J.S. Rovner, eds. (Princeton, NJ: Princeton University Press), pp. 123–129.

27. Mook, J.H., and Bruggemann, C.G. (1968). Acoustical communication by Zygogramma bicolorata (Hemiptera, Lygaeidae). J. Exp. Biol. 57, 397–402.

28. Hoy, R.R., Holikakula, A., and Kaneshiro, K. (1988). Hawaiian courtship songs: evolutionary innovation in communication signals of Drosophila. Science 240, 217–219.