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Drosophila females have an acoustic preference for symmetric males

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In many species, including humans and *Drosophila*, symmetric individuals secure more matings, suggesting that bilateral symmetry signals the quality of potential mates and is subject to sexual selection. However, this idea remains controversial, largely because obtaining conclusive experimental evidence has been hindered by confounding effects arising from the methods used to increase asymmetry in test subjects. Here, we show that altering gravity during development increases asymmetry in *Drosophila melanogaster* without a detrimental effect on survival, growth, and behavior. Testing males with altered-gravity–induced asymmetry in female mate-choice assays revealed symmetry-based discrimination of males via auditory cues. Females similarly discriminated against males with genetically induced asymmetry, suggesting that their preference for symmetry is not specific to altered gravity. By segmenting the male courtship song into left and right wing-generated song-bouts, we detected asymmetry in the courtship song of altered-gravity males with asymmetric wings that experienced rejection. Females experimentally evolved in the absence of mate choice lacked this preference for symmetry, suggesting that symmetry is maintained by sexual selection. Our data provide evidence for the role of symmetry in sexual selection and reveal how nonvisual cues can flag mate asymmetry during courtship.

sexual selection | developmental instability | fluctuating asymmetry | altered gravity | courtship song

Evolution of bilateral symmetry contributed greatly to the diversification of life on Earth (1), and its canalization is attributed to natural selection on either directed locomotion or internal circulation, or both (2, 3). However, it remains unclear whether optimizing traits like locomotion and internal circulation exerts enough selection required to maintain the astounding level of bilateral precision achieved during development. On the other hand, nondirectional deviation from perfect symmetry, commonly referred to as fluctuating asymmetry (FA), is considered to be a consequence of developmental instability and an indicator of individual genetic quality (4) that might be subjected to sexual selection via mate choice (5). FA is different from the individual-level nondirectional lateralization and the taxonomic-level systematic morphological and neuroanatomical asymmetries (directional asymmetry), both of which are known to be modulated by environmental factors to some extent (6, 7). Experimentally, the idea of relating FA with sexual selection is highly contested (8). Despite numerous studies across taxa, conclusive evidence for discrimination against asymmetric individuals during mate choice has been challenging to obtain, largely because experimentally inducing FA is seldom independent of confounding effects on survival, growth, and behavior (9, 10). While some studies increase bilateral asymmetry artificially by adding ornaments (e.g., colored rings on bird feet) or by surgical manipulations (e.g., clipping of wings/feathers) (11), others use biotic and abiotic stressors (e.g., parasites and temperature) (4) to induce asymmetry by increasing developmental noise. Yet, most of these studies are criticized for improper controls and reproducibility that are often traced back to competing experimentally manipulated males with unmanipulated control males (9, 10). Furthermore, expression of asymmetry across morphological traits and preference of symmetric mates are both highly variable, even within a given species, depending on the intensity of selection operating and their evolved canalization mechanisms (12–14).

A key assumption that the FA/sexual selection idea makes is that animals can assess symmetry of potential mates during mate choice, and consequently use this information to discriminate against mates with higher asymmetries. However, the perceptual basis of assessing asymmetry is neither clearly defined nor rigorously tested, but strongly presumed to involve visual cues (15). This presumption largely neglects the multimodal nature of sexual communication across species and also the plausibility that animals can perceive asymmetry through irregularities in certain nonvisual cues

Significance

Theoretically, symmetry in bilateral animals is subject to sexual selection, since it can serve as a proxy for genetic quality of competing mates during mate choice. Here, we report female preference for symmetric males in *Drosophila*, using a mate-choice paradigm where males with environmentally or genetically induced wing asymmetry were competed. Analysis of courtship songs revealed that males with asymmetric wings produced songs with asymmetric features that served as acoustic cues, facilitating this female preference. Females experimentally evolved in the absence of mate choice lost this preference for symmetry, suggesting that it is maintained by sexual selection.

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(e.g., auditory, mechanosensory, and chemosensory) that are also signaled during courtship (15). Thus, both female preference for symmetric mates and their ability to assess mate symmetry during courtship, remain largely unclear.

The genetic and sensory basis of courtship behavior, mate choice, and sexual selection has been extensively investigated in *Drosophila melanogaster*. However, the association between mating success and symmetry of bilateral traits has been so far inconsistent across studies that have used *D. melanogaster* and other *Drosophila* species (13, 16–18). *Drosophila* males court females in an elaborate ritual, providing several sensory cues (e.g., visual, auditory, and chemical) that are known to affect female preference (19, 20). But to our knowledge, whether females can use these cues to assess male symmetry in morphological traits (e.g., wing size, sex comb, body bristles) has never been tested.

In this study, we investigated the importance of male wing symmetry during mate choice in *D. melanogaster* and its implications for sexual selection. To assess this, we competed males with varying wing asymmetries that were induced either by rearing them under altered gravity or by genetic manipulation (21, 22). Our investigations demonstrate that females discriminate against wing asymmetry among competing males using acoustic cues.

Results

Altered Gravity Perturbs Bilateral Symmetry. Considering that asymmetry might be generated by perturbing physical forces for which no canalization mechanism could have evolved, we sought to manipulate an environmental factor that has remained unaltered. At the time scale of the evolution of life, Earth's gravity has remained constant, but its role in establishing or maintaining bilateral symmetry has not been fully elucidated (23). Comparative analysis of *D. melanogaster* subjected to altered gravity during their development on a vertically rotating clinostat (24) and control animals reared either under uniform gravity on horizontally rotating clinostats or under still condition revealed that altered gravity increases FA in wing area (Fig. 1 *A* and *B*, *SI Appendix*, Fig. S2*A*, and Movie S1). FA, the nondirectional deviation from bilateral symmetry (4), was measured as FA-index 6 (FAi), which estimates the variance of the signed (±) difference between right and left measurements normalized to trait size of each individual (25). The effect of altered gravity on wing area was also recapitulated in flies reared on a microgravity simulator (random positioning machine, RPM), which, unlike the clinostat, rotates in random directions at random speed to alter the direction of gravity vector (24) (Fig. 1 *A* and *B* and Movie S1). This method was not used further due to logistical reasons (*SI Appendix*, Fig. S1).

Rearing flies under altered gravity increased the FAi in wing area of both sexes (*SI Appendix*, Fig. S2*B*). While altered gravity increased the FAi in some bilateral traits, like wing width and sex comb tooth number (Fig. 1 *C* and *D*), this effect was not observed for the number of aristal branches (Fig. 1*E*). Apart from the increased FA, no detrimental effect of altered gravity on egg-to-adult viability (Fig. 1*F*) or adult body weight at eclosion (Fig. 1*G*) was found in comparison with uniform gravity and still. Rotation on clinostats in both altered- and uniform-gravity conditions accelerated larval development by 20 h (Fig. 1*H*), indicating that this effect on larval development is not responsible for the increased FA observed under altered gravity. Upon assaying female reproductive fitness, we found that females reared under altered and uniform gravity were more fecund than still females when crossed with still males (Fig. 1*I*). When males from the three treatments were crossed with still females, the number of offspring sired did not differ among them (*SI Appendix*, Fig. S2*C*). Compared with control males (uniform gravity and still), altered-gravity males were faster at initiating copulation (Fig. 1*J*) but copulated for shorter durations (Fig. 1*K*). Taken together, these results suggest that in altered-gravity conditions the overall fitness of individuals is not decreased. In addition, the effect of altered gravity on *Drosophila* symmetry was in a manner comparable to FA induced when genes known to buffer against developmental instability (e.g., *Drosophila* insulin-like peptide 8, *Dilp8*) are genetically perturbed (21, 22).

Females Prefer Symmetric Males. We exploited this altered-gravity–induced variability in wing area symmetry to test female preference for symmetric males. To this end, randomly selected pairs of altered-gravity males were used in a mate-selection assay with a single still virgin female. The mate-choice assays were carried out under controlled climatic conditions. The copulating "winner" and the noncopulating "loser" males were separated for each contest (Fig. 2*A*) and wing asymmetry was evaluated for all males of the winner and loser groups. In contests where symmetry of competing males differed (assessed posthumously), 81% of winner males showed higher wing symmetry than their loser counterpart (*SI Appendix*, Fig. S3*A*). Consistent with this, the FAi of the winner group was significantly lower than that of the loser (shown as percent difference in FAi between groups) (Fig. 2*B*). The overall number of offspring sired by winners and losers, when individually remated with new still virgins, did not differ (Fig. 2*C*), indicating that loser males are as able to mate as winners. In 74% of contests, winners sired more offspring than losers (*SI Appendix*, Fig. S3*B*), indicating increased fertility of symmetric males.

Our assay vials are large, allowing females to move away from suitor males. We tested if size of the assay arena affects the outcome of the contest by repeating our assays in 25-fold smaller contest vials (*SI Appendix*, Fig. S3*C*). Similar FAi differences between winner and losers were observed in these smaller vials, suggesting that assay arena size is not important (Fig. 2*D*). Conversely, we also assayed mate choice in a more natural setting using a large cage (*SI Appendix*, Fig. S3*D*) with increased male density (25 virgin still females, 50 altered-gravity males). Copulating pairs were removed from the cage to constitute winners, while the remaining males were declared losers. Under these conditions, the winners' FAi was still significantly lower than that of the losers' (Fig. 2*E*), suggesting that symmetry-based segregation can operate beyond paired competition assays. While these results indicate that sexual selection leads to symmetry-based mate segregation, both female mate choice and male–male competition seem to be equally plausible mechanisms (26).

Recent studies suggest that *Drosophila* males could also exhibit mate preference (27). We assayed male preference for symmetric females in the same experimental paradigm by using one naive still male and two altered-gravity virgin females (note: altered gravity also increases FA in females) (*SI Appendix*, Fig. S2*B*). In this case, the female that copulated with the male was considered winner and the other female loser. Unlike females, males did not show a significant preference for symmetric females (*SI Appendix*, Fig. S3*E*).

Sensory Perception of Asymmetry. Mating behavior in *D. melanogaster* involves multimodal sensory signals exchanged

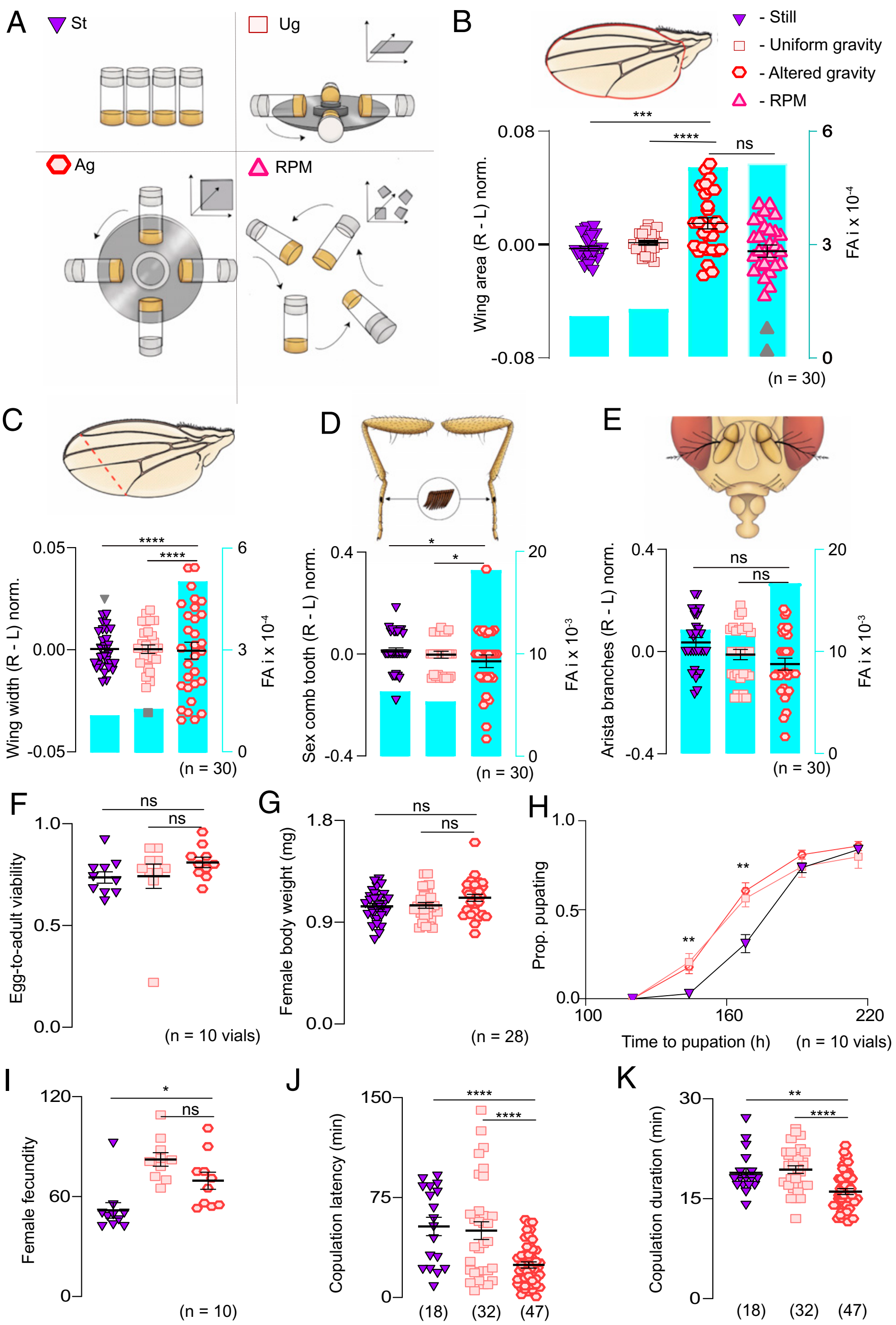

Fig. 1. Altered gravity during development increases FA. (*A*) Schematic of the four rearing conditions and their axis of rotation: still, uniform gravity, altered gravity, and RPM. (*B*–*E*) Schematic of bilateral trait measured: female wing area (*B*), female wing width (*C*), number of male sex comb teeth (*D*), and number of male aristal branches (*E*). Normalized difference (right − left) for each trait and treatment is plotted as dots (left *y* axis) and the FAi 6 calculated as variance of this data for each treatment (cyan bars, right *y* axis); statistical differences between treatments were tested using Levene's test to evaluate heterogeneity of variance. (*F*–*K*) Life history and behavioral traits of flies reared in still, uniform-gravity, and altered-gravity conditions, with differences between the treatments tested using *t* tests. Proportion of eggs surviving to eclose as adults was assayed in 10 vials per treatment, each seeded with 50 eggs (*F*); dry body-weight of females at eclosion (*G*); and time to pupation was assayed in 10 vials per treatment each seeded with 50 eggs (*H*). Average number of eggs laid per day (over a 5-d period) by still, uniform-gravity, and altered-gravity females that were mated to still males for 24 h (*I*). Time taken to initiate copulation (*J*) and duration of copulation (*K*) in males while individually courting a still virgin female. Gray symbols in dot plots represent excluded outliers that inflate FA; ns, not significant; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. Sample size (number of flies) is shown in parentheses.

between the courting individuals and competing mates (19, 20). To identify the sensory cues that signal male asymmetry during courtship, we modified our assay in ways that altered perception of visual, olfactory, and auditory cues while courting. Visual cues were abrogated by conducting mate-choice assays under red light (28). A reduction in FA-based male segregation was seen, but the FAi of winners still remained significantly lower than that of losers (Fig. 2*F*). We next ablated the antennae of still virgin females (removal of both antennae and the arista positioned onto the antenna), making them simultaneously anosmic (smell-blind) and deaf (29). When used for the mate-preference assay, these animals showed no preference for symmetric males, with the winners' FAi not being statistically different from that of losers' FAi (Fig. 2*G*).

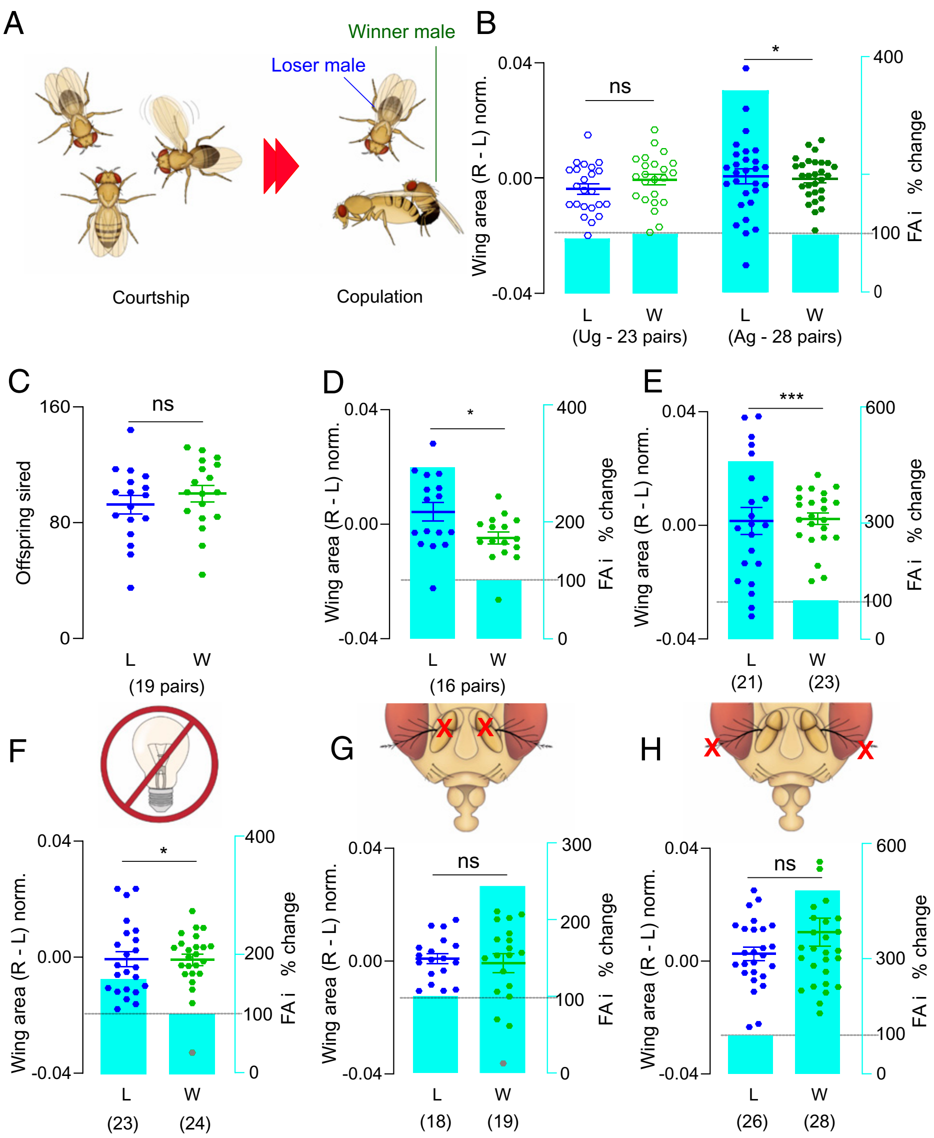

Fig. 2. Symmetric males have higher mating success. (*A*) Schematic representation of mate-choice assay to segregate winner and loser males: the focal female was always a still virgin and the competing males were always from the same treatment. (*B* and *D*–*H*) Graphs show normalized difference ($R - L$) in wing area of winner (green) and loser (blue) males plotted as dots (along left y axis); fold-change in the FAi value as cyan bars (along right y axis) with lower value set at 100%; and Levene's tests were used to compare the variance of the two groups. Wing area difference and FAi fold-change in winner and loser males reared in uniform- and altered-gravity conditions (*B*). Number of offspring borne by still females over 4 d when mated with winner and loser altered-gravity males for 24 h (*C*). Winner and loser FA (fold-change) when mate choice among altered-gravity males was done in 25-fold smaller vials (*D*). FA (fold-change) in winner and loser altered-gravity males segregated based on copulation success during mass mating in a large cage (*E*). (*F*–*H*) Schematics of mate-choice assay variants to determine the sensory basis of choosing symmetric mates, and consequential effect on winner and loser FA (fold-change) in altered-gravity males. (*F*) The assay was performed under red light to abrogate visual cues. (*G* and *H*) the assays were done using focal still virgin females with either both aristae and antennae (*G*) or only aristae (*H*) surgically removed 24 h prior to the assay; this diminishes olfactory and auditory or only auditory perception in females, respectively, during mate choice. Gray symbols in dot plots represent excluded outliers that inflate FA; ns, not significant; $^{*}P < 0.05$, $^{***}P < 0.001$. Sample size (number of flies) is shown in parentheses.

Ablating only the aristae, making females deaf (30) but capable of olfaction, was sufficient to abolish symmetry-based mate segregation (Fig. 2*H*), indicating that sound provides stronger cues for proper symmetry-based segregation, relative to vision and smell. Notably, the loss of the bias toward low FA males in assays involving deaf females indicates that female mate choice, rather than male competition, is a major determinant of selection.

Females Discriminate Genetically or Surgically Induced Asymmetry. We next explored whether female preference for symmetric males could be observed in the context of other types of FA-inducing manipulations. Intriguingly, genetic manipulations offer an alternative method for inducing FA and recent work indicates that blocking the function of the *Dilp8* increases FA in bilateral traits, including wings (21, 22). We set up mate-choice assays where *Dilp8* mutant males with increased wing FA (*SI Appendix*, Fig. S4*A*) replaced altered-gravity males. When pairs of $dilp8^{ko/ko}$ mutant males competed for mating with a still-reared female, we observed that symmetric $dilp8^{ko/ko}$ males were more successful at mating than asymmetric ones (Fig. 3*A*). This confirms our observation of a preference for symmetric mates, and indicates that this preference is not a specific response to altered-gravity–induced FA. Furthermore, to exclude the possibility that other features correlating with altered-gravity– or genetically induced wing asymmetry would influence female preference, we tested whether restricting male asymmetry to wings would be sufficient to induce a female's

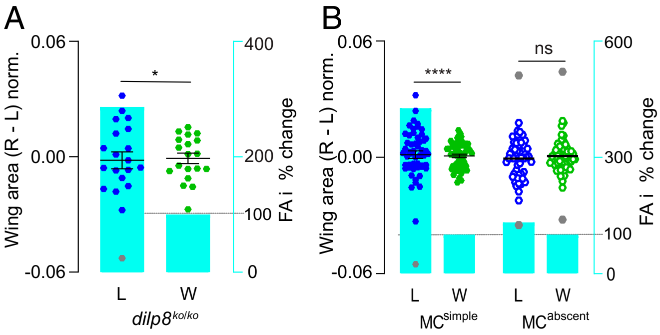

Fig. 3. (*A*) FA-based segregation of *dilp8*$^{ko/ko}$ mutant males into winners and losers by still females in the mate-choice assay; normalized difference ($R - L$) in wing area plotted as scatter plot along the left *y* axis and fold-change in FAi as cyan bars along the right *y* axis with lower value set at 100% ($n = 20$; Levene's test). (*B*) Wing area difference ($R - L$) and fold-change in FAi (represented as dots and cyan bars, respectively) of winner and loser *MC*^{simple} males reared under altered gravity when subjected to mate choice by virgins from *MC*^{simple} and *MC*^{absent} populations ($n = 53$ to 56 females/regime; Levene's test). FA-based segregation of males observed in assays with virgins from *MC*^{simple} populations but not with virgins from *MC*^{absent} populations. Gray symbols in dot plots represent excluded outliers that inflate FA; ns, not significant; $*P < 0.05$, $****P < 0.0001$.

mate choice. For this, we genetically overexpressed the cyclin G (*CycG*) gene exclusively in the pouch area of the wing imaginal disk during wing development using the driver *PDM2-Gal4* (31), a condition described to induce adult wing FA (32) (*SI Appendix*, Fig. S4*B*). We then competed these males with asymmetric wings in our mate-choice assay and found again that females prefer males with symmetric wings (*SI Appendix*, Fig. S4*C*). Similarly, when females were courted by males with wings that were damaged surgically to be either symmetric or asymmetric (*SI Appendix*, Fig. S4*D*), females preferentially mated with symmetrically damaged males (*SI Appendix*, Fig. S4*E*). While this latter data may not be definitive on their own, the data are nevertheless informative within the context of this study. Altogether, these data indicate that female's choice is based on the wing symmetry of their mates.

Asymmetric Wings Affect Song Symmetry. *Drosophila* males woo females by vibrating their unilaterally extended wing and alternating between sides to produce a song while courting a female (30). This courtship song has been extensively analyzed as a single song with several bouts composed of three distinguishable song types: sine, P-fast, and P-slow pulse (30, 33, 34, 35) (Fig. 4*B*). We hypothesized that in males with asymmetric wings, the song produced by each wing would differ in ways that could be less attractive to the female. Using audiovisual recordings of fly courtship in a large arena (30, 36) coupled to the tracking of the extended wings using the DeepPoseKit tracking software (37) (Fig. 4*A*), we segmented the courtship song into bouts produced by the right and left wings, such that asymmetries in acoustic characteristics of all song types could be assessed. In these experiments, winner and loser males from altered gravity were first separated using our mate-choice assay. As expected, losers had higher FAi than winners (*SI Appendix*, Fig. S5*A*), while their average wing sizes did not differ (*SI Appendix*, Fig. S5*B*). Each segregated male was subsequently allowed to court a new virgin female in a recording chamber and its song recorded and segmented into left and right components. We calculated the amplitude, frequency, and duration of the sine song, as well as the amplitude, frequency, interpulse intervals, and pulse amount for the P-fast and P-slow songs. Average song parameters of winner and loser males did not differ and thus cannot explain female preference (*SI Appendix*, Fig. S5 *C–M*). Asymmetries in the sine song parameters were low and not different between winner and loser males (*SI Appendix*, Fig. S6 *A–C*). In contrast, winner males had lower FAi compared with that of the loser males for certain pulse song parameters, like P-slow frequency and interpulse interval, (Fig. 4 *C–F*). Wing asymmetry positively correlated with asymmetry in amplitude and frequency of P-fast song, suggesting a link between morphological asymmetry and the FA of the song (Fig. 4 *G* and *H* and *SI Appendix*, Fig. S6 *D–G*). Males did not exhibit preference for a specific wing while singing, based on the side (right vs. left) (*SI Appendix*, Fig. S7*A*) or the size of the wing (larger vs. smaller) (*SI Appendix*, Fig. S7*B*). Together with our observations of perturbed mate selection by deaf females, these results suggest an active female mate-choice segregation based on preference for the most symmetrical song among rival males.

Mate Choice Maintains Female Preference. Our results above establish that female *Drosophila* assess male courtship songs to preferentially choose symmetric mates, but how such preference for symmetry is evolutionarily maintained remained unclear. We hypothesized that populations in which females are deprived of the opportunity to choose mates should have reduced or even lack such a preference. We tested this hypothesis in replicate *D. melanogaster* populations that had experimentally evolved with or without mate choice (*MC*^{simple} and *MC*^{absent}, respectively) for 86 generations (38). When presented with *MC*^{simple} males reared under altered gravity in our mate-choice assay, *MC*^{simple} polygamous females exhibited preference for symmetric mates, with winner males having lower FA than losers (Fig. 3*B* and *SI Appendix*, Fig. S8). In contrast, *MC*^{absent} monogamous females showed no preference for symmetric males (Fig. 3*B* and *SI Appendix*, Fig. S8), demonstrating that selection imposed by mate choice is essential for the maintenance of this female preference for symmetry.

Discussion

The role of FA as a reliable signal of mate quality assessed during courtship has been highly debated due to inconclusive empirical evidence (4). Here we have presented a robust experimental paradigm that can be exploited to explicitly test several tenets (39) of this hypothesis. In addition to drawing attention to the inescapable role of gravity in affecting organismal development, our data demonstrate improved success of symmetric individuals in sexual endeavors, irrespective of how individual symmetry was perturbed (environmentally or genetically). We extend the classic analyses of courtship song as a solo by instead considering it as a duet sung by bilateral wings. Using bilateral segmentation, we show that a male's morphological asymmetry affects his reproductive fitness through FA in the courtship song. It remains to be determined as to whether and how *Drosophila* females compare such dynamic auditory cues coming either from a single male or rival males to make informed decisions (40). Interestingly, such plausibility is empirically supported by a recent study showing how a male positions itself during courtship on either side of the female's posterior and sings with the wing of the respective side to stimulate the female's ipsilateral antenna (41). Furthermore, *D. melanogaster* females are known to discriminate against males adapted to microecological conditions on opposite canyon slopes causing assortative mating (42). This female discrimination was speculated to be mediated by changes in features of the male

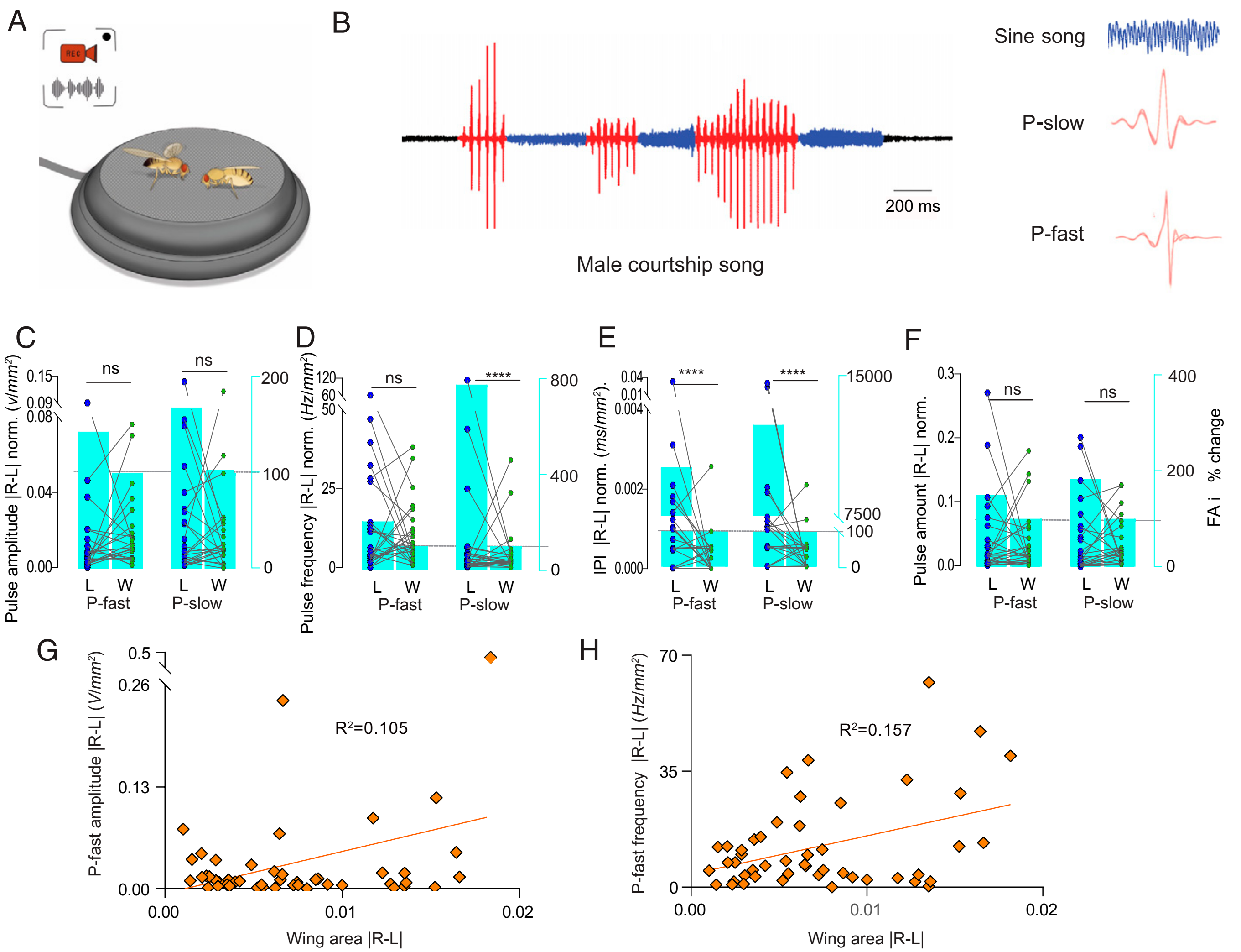

Fig. 4. Loser males' courtship songs are asymmetric in their attributes. (*A*) Schematic representation of simultaneous recordings of song and video during *D. melanogaster* courtship. Male wing extension was tracked, to identify the wing that produced each song bout. (*B*) *D. melanogaster* courtship song comprises three song modes (sine [blue], P-fast and P-slow [red]). (*C–F*) Absolute difference $|R - L|$ for P-fast and P-slow song parameters normalized to wing size is plotted along the left *y* axis for loser (blue) and winner (green) altered-gravity males in each competition and the fold-change in FA index [FA_4 = variance (right − left)] as cyan bars along the right *y* axis with lower value set at 100%. Pulse amplitude (V/μm^2) (*C*); pulse frequency (Hz/μm^2) (*D*); interpulse interval (ms/μm^2) (*E*); and amount (*F*). Dots correspond to the median value for each individual, line connect males that competed for the same female. ns, not significant; $^{****}P < 0.0001$. Differences in variance between winners and losers compared using Levene's test; $n = 25$ pairs. (*G* and *H*) Correlation between wing area asymmetry and P-fast song characteristics (amplitude and frequency) of winner and loser altered-gravity males (see *SI Appendix*, Fig. S6 *D–G* for other song parameters). Slopes were significantly from 0 for both parameters: (*G*) P-fast amplitude ($F_{1,\,46} = 5.394$; $P = 0.0247$); and (*H*) P-fast frequency ($F_{1,\,46} = 5.394$; $P = 0.0053$).

courtship song (43), suggesting that changes in preference to male songs can drive reproductive isolation toward speciation. More broadly, and consistent with the recent recognition of courtship as a multimodal behavior (44), we conjecture that the current emphasis on visual cues (45), as well as the neglect of nonvisual cues in mate-asymmetry perception, might partly explain previous inconsistencies regarding the relation between FA and sexual selection.

Materials and Methods

***Drosophila* Strains and Rearing.** *D. melanogaster* were maintained at 25 °C in 12-h light:12-h dark conditions, on a standard yeast-cornmeal diet (46). Wild-type Canton S strain of *D. melanogaster* was used (47), unless noted otherwise. $dilp8^{ko/ko}$ mutant (48) were used for the experiment shown in Fig. 3*A*; w^{1118} was used as a control strain *SI Appendix*, Fig. S4*A*. The UAS/Gal4 system involving *UAS::mRFP-CycG (RCG76)* (31) and $pdm2^{R11F02}$-*Gal4* (32) was used to drive overexpression of *CycG* exclusively in the wing pouch (*SI Appendix*, Fig. S4*B*). Published replicate *D. melanogaster* populations that were experimentally evolved with or without mate choice (MC^{simple} and MC^{absent}) for 86 generations (38) at University of Ottawa were procured and reared on our standard yeast-cornmeal diet for two generations before using them for experiments shown in Fig. 3*B* and *SI Appendix*, Fig. S8. For more details on this evolutionary experiment, please see Yun et al. (38).

Rearing *Drosophila* under Altered Gravity. For all experiments, unless stated otherwise, we used a simple two-dimensional (2D) rotating clinostat to alter gravity during *Drosophila* development. Tube rotors (Stuart, SB3 –variable speed) rotating at 35 rpm served as our clinostats (24), wherein on each clinostat 10 *Drosophila* culture vials could be simultaneously mounted and aligned 4 cm from the center to rotate either perpendicular (altered gravity) or parallel (uniform gravity) to the earth surface as in Fig. 1*A* (rotation axis was regularly checked using a sprit level). Each of these vials had 5-mL standard yeast-cornmeal diet (46) and were seeded with 50 eggs collected from collective egg laying by females for 2 h (to avoid females laying retained or older eggs they were allowed to oviposit for 12 h prior to the 2-h collection period). The clinostats were rotated continuously throughout larval and pupal development, and was only stopped when most of the flies had eclosed. Additional nonrotating control (still) was set up by placing similarly seeded vials in a vial rack adjacent to the rotating clinostats. To confirm our findings, we used another state-of-the-art ground-based microgravity simulator, the random positioning machine (RPM 2.0) (24) commercially supplied by Airbus and the European Space Agency. This equipment overcomes the drawback of habituation of test animals to the constant direction and speed of rotation experienced on a 2D clinostat, by automated randomization of the speed and direction of the rotation along its two axes (Movie S1) that's remotely controlled through a customized software. We used the RPM set to rotate in a manner where zero-*g* is simulated. However, the FA levels in flies reared on the RPM varied with the distance of the culture vial from the center of the platform (*SI Appendix*, Fig. S1), this position effect was likely caused by differences in shear forces experienced within these vials. For this reason, we use a clinostat in preference to RPM for all experiments in this study. Both clinostat and RPM were housed within an incubator (pHcbi cooled incubator, MIR-554) maintained at 25 °C, 60% humidity, and 12:12-h dark:light cycle.

Life History Traits. Survival and development of flies reared under altered gravity, uniform gravity, and still conditions were assayed. Time taken to pupate (Fig. 1*H*) was determined by scoring number of larvae that pupated within each vial every 24 h since egg-laying. Adult flies eclosing out in each vial were counted to determine egg-to-adult viability in each treatment (Fig. 1*F*). These adults were dried (72 °C for 24 h) and weighed on a microbalance (company) to determine adult body weight (Fig. 1*G*). Fecundity of females reared in the three treatments was assayed by crossing virgins with still males and counting the number of eggs they laid over the next 5 d (Fig. 1*I*). Similarly, the reproductive fitness of male flies in these treatments was assessed by crossing them with still virgins for 24 h, and counting the number of offspring sired across the first 4 d of egg-laying (*SI Appendix*, Fig. S2*C*). We also compared two male mating-behavior parameters: time to copulation and copulation duration among the three treatments. Both parameters were assessed by placing a still virgin with individual males from each treatment (Fig. 1 *J* and *K*). Time to copulation was measured from the time females were introduced into the mating vial to the start of copulation, the duration from this moment forward until the couple separated was measured as copulation duration. The data for life history traits–egg to adult viability, time to pupation, adult body weight, fecundity, and behaviors–were analyzed using a *t* test.

FA: Measurement, Analysis, and Graphical Representation. Flies for FA measurements were always stored in 70% ethanol, and unless mentioned explicitly, wing area was the focal trait we assessed for all experiments. Pair of wings of each fly was dissected and mounted in fixative (5:6, lactic acid:glycerol) on a glass slide, orienting them such that the right and left wing were clearly distinguishable, and covered with a coverslip (46). The wings were imaged at 36× magnification using a Leica fluorescence stereomicroscope MZ16 FA fitted with a Leica digital camera DFC 490. The perimeter of the wing was traced manually using ImageJ software (49) to determine the wing area. To quantify FA, we use index $\mathrm{FA6} = var\,[2(R - L)/(R + L)]$ that estimates the variance of the signed difference between right (R) and the left (L) side measurements normalized to the average trait size of each individual (25, 50). FA6 is a preferred index in our context because: 1) it uses signed differences and is thus not affected by directional asymmetry; and 2) it normalizes the difference relative to trait size and, thus, being dimensionless can be compared among traits (25, 50).

Nonetheless, to detect FA reliably it is necessary that the error in measuring (measurement error) the trait is small; we estimated this for a subset of our samples where area of right and left wings were measured independently by two experimenters. These measurements were examined using a mixed-model ANOVA with sides (left vs. right) and the experimenter as fixed factors, and individual and individual × side interaction as random factors. The interaction between individual and side was significant, indicating that nondirectional asymmetry (FA) was greater than measurement error. The measurement error estimated as the residual variance in this analysis only accounted for a small fraction of the total variance. FA was also assessed in a few other bilateral traits, like wing-width, sex comb tooth number, and number of aristal branches (Fig. 1 *C*–*E*).

The FA indices (the variance of signed difference between right and left), between treatments were compared using Levene's test to evaluate heterogeneity of variance (4). Outliers that could confound the estimates of FA were identified by inspecting the scatterplots. For data containing outliers, Levene's tests both including and excluding the outliers were performed and compared. The outliers were retained in the data if the two analyses gave the same outcome, but removed if they were the lone values that inflated FA measurements.

Complete data are presented in graphical representation as a scatterplot along the left *y* axis, with outliers marked in gray when removed during analysis. FA index is presented as cyan bars plotted along the right *y* axis as either absolute value (Fig. 1) or as percent fold-change (Figs. 2–4) where the lower value is set at 100%.

Mate-Choice Assay and Its Variants. Mate-choice assays were conducted in regular culture vials (95 mm × 25 mm) with 10-mL fly food, allowing enough room for females to exercise mate choice by evading harassing suitors. The mate-choice assays were carried out under controlled climatic conditions at the same time of the day to remove confounding effects of temperature, light, and photoperiod on male courtship and female mate choice. Males and virgin females required for the assay were reared at a constant density of 50 eggs per vial, collected upon eclosion every 4 h and maintained in groups of 10 flies per vial for 3 to 4 d. A day before the assay, randomly chosen pairs of males were transferred into fresh vials. At the start of the assay a single virgin female was transferred into each of these vials with males and observed until copulation occurred. The copulating male and his rival male were separated into new vials, designated as winner and loser of the given contest, and used for the different experiments.

Slight variations of this assay were used to test several hypotheses. 1) To assess reproductive fitness of winner and loser males sorted in the assay, the males were allowed to mate with new virgin females for 24 h and the number of offspring they sired over a 4-d egg-laying period was quantified (Fig. 2*C*). 2) In Fig. 2*D* the assay was performed in a glass vial that was 25-fold smaller (length × diameter: 4 × 1 cm) than the regular culturing vial (length × diameter: 9 × 2.5 cm) we use throughout. 3) In Fig. 2*E*, the assay was conducted in large cage (length × width × height: 41 × 17.5 × 22 cm), wherein 50 altered gravity males simultaneously competed for mating with 25 still virgins. 4) For the data presented in Fig. 2*F*, the assay was carried out in dark room dimly lit with a red light; a control assay in light was also done. 5) In Fig. 2*G*, the still virgins were antennectomized (antennae and arista were removed) 24 h prior to the assay, while in Fig. 2*H* only the aristae of the females were removed to make them deaf. 6) For *SI Appendix*, Fig. S4*C*, male offspring of cross between *UAS::mRFP-CycG (RCG76)/+* males and *pdm2*^{R11F02}*-Gal4* females were used in the assay. 7) For Fig. 3*A*, still reared *Dilp8*$^{ko/ko}$ males were competed for mating with a still-reared CantonS virgin female. 8) For *SI Appendix*, Fig. S4*E*, 24 h prior to the mate-choice assay, wings of still-reared males were surgically damaged to be either symmetric and asymmetric (*SI Appendix*, Fig. S4*D*), and were competed in pairs. The assay was performed under red light to exclude visual cues that could influence female choice. 9) Finally, for Fig. 3*B*, the males from the (*MC*^{simple}) regime were reared under altered gravity and tested in the mate-choice assay with virgin females from the seven replicates, experimentally evolved *MC*^{simple} and *MC*^{absent} populations. The data from the experimentally evolved mate-choice lines were analyzed using a mixed-model nested-ANOVA, with replicate populations nested within evolutionary regime.

Courtship Song Recording. Altered-gravity males were segregated after the initial winner–loser assay (Fig. 2*A*). Virgin still females were group-housed. After the winner–loser assay, flies were shipped overnight to the recording facility at the European Neuroscience Institute, Göttingen, Germany. The flies were allowed to accustom to the new environment for 2 to 6 d prior to the courtship assays. Flies were kept on a 12:12-h dark:light cycle, at 25 °C and 60% humidity.

The behavioral chamber was constructed as previously described (30, 51); the floor was lined with a double layer of white plastic mesh and equipped with 16 recording microphones. A male and a female fly were introduced gently into the chamber using an aspirator. Recordings were performed within the first 150 min after the start of the light phase to catch the flies' morning activity peak. Recordings were stopped after 20 or 30 min. We recorded videos at 100 frames per second and audio data at 10 kHz.

Quantification and Statistical Analysis of Courtship Song. Data analysts were blind to the group (winner/loser) of each male.

Analysis of wing angles during song production. The center position of flies was tracked in the videos using custom routines written in Python. The position of head, thorax, and both wing tips was determined using DeepPoseKit (37). Wing angles were defined as the angle spanned by two lines connecting the thorax with the wing tips and the head. Wing angles were normalized to fall in the range [−180°, 180°] with angles >0° denoting wing positions away from the center line. The side of the male showing the larger wing angle at the time of a pulse was considered to be the extended wing that produced the song. For sine song, we only considered sine trains during which the male did not switch the wing.

Annotation of pulse and sine song. Pulse and sine song were labeled using DAS, which detects pulse and sine song with high accuracy: pulses with a precision of 97% and a recall of 96% and sine with a precision of 92% and a recall of 98% (52). Detections of sine song shorter than 6 ms were excluded from our analysis as false positives. Pulse waveforms were extracted from the recordings by taking 25 ms (250 samples at 10 kHz) around the pulse center from the audio channel on which the pulse amplitude was the highest.

Classification of pulses. *D. melanogaster* males are known to produce two types of pulses, termed P-fast and P-slow . The pulses detected by DAS were classified into these two pulse types using methods modified from Clemens et al. (35).

To reduce variability in the waveforms arising from the distance and position of the singing male from the microphone, we centered waveforms to their peak energy by smoothing the squared pulse waveform with a rectangular window spanning 15 samples (1.50 ms). Afterward, we flipped the waveform's sign such that the average of the 10 samples (1.00 ms) prior to the pulse center was positive. The amplitude of each waveform $x(t)$ was then scaled to unit-norm: $x(t)/\sqrt{\sum x(t)^2}$.

The normalized pulses were then classified into P-fast and P-slow, by learning an embedding and clustering using a subset of 10,000 pulses randomly selected from all animals. For embedding, we used UMAP (Uniform Manifold Approximation and Projection for Dimension Reduction) (53) and for clustering, we used HDBSCAN (hierarchical density-based clustering) (54). The full set of pulses was then embedded and clustered, and P-fast and P-slow were identified based on the asymmetrical shape and the carrier frequency of cluster centroids.

Pulse amount is given by the time spent singing a specific song type (P-fast or P-slow) out of the total time spent singing any song type (P-slow, P-fast, sine). For pulses, we took the interval to the next pulse in a train as the duration of a single pulse event if that interval was shorter than 200 ms. Interpulse intervals are given by the median of all interpulse intervals shorter than 200 ms.

Calculation of pulse and sine carrier frequencies. Because song pulses are transient signals, their raw magnitude spectra are relatively broad (35). Accordingly, the peak frequency of the spectrum is no reliable measure of the pulse carrier frequency. Therefore, we used the center of mass of the magnitude spectra, thresholded at 0.5, to calculate the carrier frequencies of pulse and sine song.

Calculation of pulse and sine amplitudes. The amplitude of pulse waveforms was given by the maximum of the absolute waveform. The amplitude of each sine song was calculated as the root-mean square, where $x(t)$ is a sine song of duration T: $\left(\sqrt{1/T\sum_{t}^{T} x(t)^2}\right)$.

Determining FA in courtship song parameters. We separated the data for the different song parameters depending on the wing side involved (right or left) and accumulated it over the entire recording period for each fly to determine the median value of the parameter for each side. The difference between the median value of each parameter for right and left side ($R - L$) was calculated to determine FA [*FA indTex 4 = variance of (R – L)*] in courtship song parameters of winner and loser males. We also used the unsigned asymmetry $|R - L|$ values of wing area and the courtship song parameters that were normalized to average wing size, to explore using multiple linear regression the extent to which functional asymmetry in the song could be explained by structural asymmetry in the wing.

Software Availability. Software is available as follows:

Song segmentation: DAS https://github.com/janclemenslab/das (52);
Centroid tracking: https://github.com/janclemenslab/ethotracker;
Pose tracking: DeepPoseKit: https://github.com/jgraving/DeepPoseKit (37);
Pulse classification: https://github.com/janclemenslab/das_unsupervised (35).

Data Availability. All relevant data referred to in the paper will be deposited in the open-access data repository Dryad (https://doi.org/10.5061/dryad.kh189327c). Raw data for courtship recordings are available at Göttingen Research Online: https://data.goettingen-research-online.de/dataverse/asymmetric_males. All other study data are included in the main text and supporting information.

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