

1 **Experimental and statistical re-evaluation provides no evidence for *Drosophila***
2 **courtship song rhythms**

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18 **Abstract**

19 From 1980 to 1992, a series of influential papers reported on the discovery, genetics, and
20 evolution of a periodic cycling of the interval between *Drosophila* male courtship song pulses.
21 The molecular mechanisms underlying this periodicity were never described. To reinitiate
22 investigation of this phenomenon, we performed automated segmentation of songs, but failed
23 to detect the proposed periodicity [Arthur BJ et al. (2013) *BMC Biol* 11:11; Stern DL (2014) *BMC*
24 *Biol* 12:38]. Kyriacou CP et al. [(2017) *PNAS* 114:1970-1975] report that we failed to detect song
25 rhythms because i) our flies did not sing enough and ii) our segmenter did not identify many of
26 the song pulses. Kyriacou et al. manually annotated a subset of our recordings and reported
27 that two strains displayed rhythms with genotype-specific periodicity, in agreement with their
28 original reports. We cannot replicate this finding and show that the manually-annotated data,
29 the original automatically segmented data, and a new data set provide no evidence for either
30 the existence of song rhythms or song periodicity differences between genotypes. Furthermore,
31 we have re-examined our methods and analysis and find that our automated segmentation
32 method was not biased to prevent detection of putative song periodicity. We conclude that
33 there is currently no evidence for the existence of *Drosophila* courtship song rhythms.

34

35 **Significance statement**

36

37 Previous studies have reported that male vinegar flies sing courtship songs with a periodic
38 rhythm of approximately 55 seconds. Several years ago, we showed that we could not replicate
39 this observation. Recently, the original authors have claimed that we failed to find rhythms
40 because 1) our flies did not sing enough and 2) our software for detecting song did not detect
41 all song events. They claimed that they could detect rhythms in song annotated by hand. We
42 report here that we cannot replicate their observation of rhythms in the hand-annotated data
43 or in any dataset and that our original methods were not biased against detecting rhythms. We
44 conclude that song rhythms cannot be detected.

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46 \body

47 **Introduction**

48 When a male vinegar fly (*Drosophila melanogaster*) encounters a sexually receptive female, he
49 performs a series of courtship behaviors, including the production of songs containing pulses
50 and hums (or sines) via unilateral wing vibration (Fig. 1a). Every parameter of song displays
51 extensive quantitative variation within a bout of singing, including the amplitude and frequency
52 of pulses and sines and the timing of individual pulse and sine events (1, 2, 4–8). Like humans
53 during conversation, *Drosophila* males modulate their song based on sensory feedback from
54 their communication partner (4, 5).

55 Visual inspection of songs reveals that the mean inter-pulse interval varies over time
56 (Fig. 1b). This observation was first made in 1980 by Kyriacou and Hall (10) and they reported
57 that the mean cycled with a periodicity of about 55 sec and was controlled, in part, by the
58 *period* gene, a gene required for circadian rhythms (11). Later papers demonstrated that
59 evolution of a short amino-acid sequence within the *period* protein caused species-specific
60 differences in this periodicity (11–14). These reports attracted considerable interest because
61 they implicated the *period* gene in ultradian rhythms, in addition to its well-known role in
62 circadian rhythms(15), and because it illustrated how genetic evolution can cause behavioral
63 evolution.

64 Despite this progress, the molecular mechanisms causing this periodicity remained
65 unknown. To further advance study of these rhythms, previously we searched for this
66 periodicity using sensitive methods and failed to find evidence for song rhythms (1). We were
67 mindful, however, that Kyriacou and Hall had argued that the presence or detectability of the
68 rhythms was sensitive to assay conditions and methods of analysis (16). One of us, therefore,

69 replicated the methods of Kyriacou and Hall as closely as possible, but, again, song rhythms
70 could not be detected (2).

71 Kyriacou et al. (3) have recently questioned our previous conclusions. Here we focus on
72 three major assertions that they claim call our conclusions into doubt. First, we examine their
73 central claim that manual analysis of songs, but not automated analysis, reveals genotype-
74 specific song rhythms. We find that re-analysis of their manually-annotated data provides no
75 statistical support for genotype-specific rhythms. We also find no evidence for song rhythms in
76 the original dataset and a new larger dataset. Second, we examined their claim that the original
77 recordings contained insufficient data to detect rhythms and find that this claim is not
78 supported by simulation studies. Third, we examine their claim that the high false negative rate
79 of the automated song segmenter decreased the probability of detecting song rhythms and find
80 no evidence that the missing pulse events biased our analysis of song rhythms. Further, we
81 identify the major sources of false negative events in automated song analysis and illustrate
82 that minor modifications to initialization parameters substantially improve performance of the
83 song segmenter. Kyriacou et al. (3) also raised a number of minor concerns—such as how to
84 choose an appropriate inter-pulse interval cutoff, whether temperature was controlled
85 appropriately in our experiments, and whether songs produced beyond the first few minutes of
86 courtship should be analyzed—that we consider peripheral to the central questions raised and
87 therefore we have addressed these concerns (which are also unsupported by re-analysis) in the
88 SI Appendix.

89

90 **Results**

91 Earlier papers that identified song cycles employed several unusual methods of data
92 analysis that it is useful to review. First, continuous inter-pulse interval data were binned into
93 10 sec intervals. We reported previously that binning the data, together with the analysis of
94 relatively short songs, creates peaks in spectrogram analysis that fall within an artificially
95 narrowed frequency range, corresponding approximately to the frequency range originally
96 reported for the periodicity, and reduces the significance of periodogram peaks (2 and see
97 below). Despite the fact that this procedure squeezes periodogram results into a narrow
98 frequency range, few songs contained peaks reaching a significance level of $p < 0.05$ (four of
99 149 songs, Fig. 3a of (2)), strongly suggesting that these peaks represent signals that cannot be
100 distinguished from noise. All of the previously reported “statistically significant” comparisons of
101 different genotypes are derived from analysis of mainly non-significant periodogram peaks. In
102 this re-evaluation, we do not discuss binning, but instead focus on other methodological issues.

103

104 **No evidence that manual song segmentation reveals genotype-specific song rhythms**

105

106 Kyriacou et al.’s (3) core finding is that different genotypes displayed different periodic
107 rhythms of the inter-pulse interval. This is also the most important discovery reported in earlier
108 papers on this subject (11–13, 17). Kyriacou et al. (3) manually annotated recordings made by
109 Stern (2) from a wild-type strain, *Canton-S*, and a strain carrying a *period* gene mutation, *per^L*,
110 for flies they categorized as singing “vigorously.” We re-analyzed these data and the
111 automatically segmented data (2). Flies homozygous for *per^L* display circadian rhythms that are
112 longer than normal (15) and earlier papers have reported that *per^L* confers longer periods on

113 the inter-pulse interval rhythm (10–13). Kyriacou et al. (3) report a difference in the mean song
114 period between *Canton-S* and *per^L* with the manually annotated data, but not with the
115 automatically segmented data, suggesting that song cycles exist and display genotype-specific
116 frequencies and that the automatically segmented data is biased against detecting the song
117 rhythm.

118 Kyriacou et al. (3) used several methods to measure periodicity in the original time
119 series, which we discuss in more detail in the next paragraph. For approximately 85% of these
120 songs, these methods do not yield statistically significant signals in the frequency range of 20-
121 150 sec. Because most songs do not yield statistically significant peaks, Kyriacou et al. (3)
122 identified the peak with maximum power in the range of 20-150 sec for each song and
123 compared these values between genotypes. This is an unorthodox approach to data analysis. It
124 is equivalent to sampling outliers from a distribution of random noise and then performing
125 further statistics with these data. Nonetheless, Kyriacou et al (3) detected genotype-specific
126 song rhythms using this method and so, below, we accept this premise and investigate whether
127 there is statistical support for genotype-specific rhythms in the data. We start by examining
128 whether there is evidence for rhythms in individual songs.

129 The general model proposed for these song rhythms is that the inter-pulse interval
130 varies, on average, with a regular periodicity (10). Therefore, it should be possible to detect this
131 rhythmicity with appropriate methods of periodogram analysis. We have previously employed
132 Lomb-Scargle periodogram analysis (18–20) because this method does not require evenly
133 spaced samples and Kyriacou et al. (3) also adopted this method. For example, the Lomb-
134 Scargle periodogram of the time series in Fig. 1b is shown in Fig. 1c. In this case, despite the

135 obvious variation in inter-pulse interval values observed in Fig. 1b, there is no significant
136 periodicity between 20 and 150 sec. Kyriacou et al. (3) also employed Cosinor (21) and CLEAN
137 (22) for periodogram analysis. CLEAN does not produce a significance value for periodogram
138 peaks, so it is difficult to interpret. We find that Cosinor exhibits a high false positive rate (SI
139 Appendix, Fig. S1), and should be avoided for this type of analysis.

140 Kyriacou et al. (3) state that wild-type *D. melanogaster* songs exhibit periodicity
141 between 20 and 150 sec. Previously they reported that rhythms occurred with 50 – 60 sec
142 periodicity (10). Increasing the width of the periodicity window from 50-60 sec to 20-150 sec
143 increases the probability of detecting significant periods, but, even given this wide frequency
144 range, we observed that only 4 of the 25 manually annotated *Canton-S* songs and 3 of the 25
145 automatically segmented songs contained periodogram peaks that reached a significance level
146 of $P < 0.05$. (When we binned data in 10 sec bins, these values declined to 0 of 25 manually
147 annotated and 1 of 25 automatically segmented songs.) These significant peaks are not
148 localized to any particular narrow frequency range (SI Appendix, Fig. S1).

149 One reason to study non-significant peaks would be if periodicity is weak and not
150 detected reliably by periodogram analysis. This seems unlikely, since simulated song rhythms
151 can be detected with high confidence ((1, 2) and see below). Nonetheless, if periodogram
152 analysis is underpowered, then we expect to observe that the major peak in most songs should
153 display nearly-significant periodicity. In fact, we observe that 72% of p-values are greater than
154 0.2 (SI Appendix, Fig. S2). There is therefore no evidence that songs contain weak periodicity.

155 An alternative possible reason to include non-significant periodogram peaks in
156 downstream analysis is that the signal to noise of the periodicity is extremely low. An analogue

157 in neuroscience is that neural signals sometimes cannot be detected with high signal to noise
158 and that only by averaging over many trials of a stimulus presentation can a neural response be
159 detected robustly. We therefore examined the power distribution averaged over all the results
160 for each genotype. These plots are essentially flat, suggesting that there is no signal hidden in
161 the fluctuations of individual periodograms (SI Appendix, Fig. S3).

162 Given these observations, further analysis of these data seems unwarranted. However,
163 Kyriacou et al. (3) compared the maximum periodogram peaks between 20-150 sec for the
164 *Canton-S* and *per^L* recordings and found that the manually-annotated data showed a
165 statistically-significant difference in the mean period, although the automatically segmented
166 data did not (Fig. 3d of Kyriacou et al. (3)). This is the key result of their paper. We therefore
167 attempted to replicate this observation. For the manually annotated data from each song we
168 identified the peak in the periodogram of maximum power falling between a period of 20 and
169 150 sec. In contrast to their published results, we found that the average of the periods with
170 maximum power (most of which were not significant) was not significantly different at $P < 0.05$
171 between the genotypes *Canton-S* and *per^L* (Fig. 1d). We have no explanation for this
172 discrepancy between our statistical analysis and theirs.

173 Since there is no biological or quantitative justification for the particular frequency
174 ranges examined in any study, we wondered whether the results were sensitive to the
175 frequency range examined. We explored a wide range of possible frequency ranges and found
176 that the test statistic was sensitive to the precise frequency range selected (Fig 1e). Most
177 frequency windows do not generate a statistically significant difference between the genotypes

178 (Fig. 1e,g) and false discovery rate correction for multiple testing (23, 24) yields no frequency
179 ranges with significant results (Fig 1f,g).

180 Thus, there is no support for the specific results reported by Kyriacou et al. (3) and there
181 is no statistical support for defining song inter-pulse interval cycle periods as occurring within
182 any particular window. Most importantly, our analysis indicates that genotype-specific analysis
183 of non-significant periodogram peaks has no justification. It is difficult to reconstruct precisely
184 what steps in the analysis led previous reports to identify statistically significant genotype-
185 specific differences, but it is possible that previous studies may have serendipitously selected
186 frequency ranges that yielded significant results and/or did not properly control for multiple
187 testing.

188

189 **New data provide no evidence for genotypic specific song periodicities**

190

191 While we could not reproduce results reported by Kyriacou et al (3), we decided to take
192 their observation at face value as a preliminary result and to test directly whether genotype
193 specific song rhythms could be detected in a new, expanded data set. We recorded song from
194 33 *Canton-S* males and 34 *period^L* males. We identified the strongest periodogram peak in the
195 frequency range of 20-150 s for each song and found no significant difference between these
196 genotypes (Fig. 1h). We then compared test statistics across a wide set of frequency ranges, as
197 described above. We identified some frequency ranges that yielded significant results in the
198 predicted direction (Fig. 1i), with *period^L* rhythms slower than *Canton-S* rhythms, but for three
199 reasons we believe these results are spurious. First, and most importantly, none of these ranges

200 are significant after false discovery rate correction (Fig. 1j). Second, multiple frequency ranges
201 support the *opposite* conclusion, that *Canton-S* rhythms are slower than *period^L* rhythms (Fig.
202 1k). Third, the frequency ranges yielding significant comparisons only partially overlap with the
203 ranges found for the original dataset (c.f. Figs. 1e & 1i). In conclusion, there is not only no
204 evidence that song rhythms exist, there is also no evidence that reported genotype specific
205 differences in a song rhythm exist.

206 Putative song cycles cannot be identified in most automatically segmented song (2) and,
207 as we showed above, in most manually annotated song. In addition, when statistically
208 significant periodicity is detected, the frequencies of this periodicity do not cluster in a specific
209 frequency range, but instead are spread randomly across the entire frequency range examined
210 (SI Appendix, Fig. S5; Fig. 4 of Stern (2)). Finally, no songs are significant after correcting for
211 multiple comparisons (Fig. 1). All together, these results imply that the few *statistically*
212 significant periodicities that can be found do not carry *biological* significance.

213

214 **No evidence that low-intensity courtship provided insufficient data to detect song rhythms**

215

216 While we found no statistical evidence for the existence of song rhythms or of genotype
217 specific rhythms, we feel it is important to rebut several other statements made by Kyriacou et
218 al. (3). They state that rhythms can be detected only in songs produced by “vigorously” singing
219 males and write: “sporadic songs could not possibly provide any test for song cycles.” It is not
220 clear if they mean that rhythms can be detected only in *songs* with many pulses or that only
221 *flies* that sing songs with many pulses (“vigorous singers”) produce rhythms. Kyriacou et al. (3)

222 manually annotated songs from flies that they categorized as vigorous and we showed above
223 that significant periodicity can be found in only a minority of these songs and that these
224 significant values are not localized to a particular frequency range (SI Appendix, Fig. S1d).
225 Therefore, it is unlikely that only *flies* that sing songs with many pulses produce periodicity. We
226 therefore performed simulations to determine whether rhythms can be detected only in *songs*
227 with many pulses.

228 We previously investigated songs from 45-minute courtship recordings that contained at
229 least 1000 inter-pulse interval measurements (2). Kyriacou et al. (3) argued that more than 180
230 inter-pulse interval measurements per minute (or approximately 5000 events in a 45-minute
231 recording) should be identified to allow identification of song rhythms. To examine this claim,
232 we performed a statistical power analysis using songs with variable numbers of inter-pulse
233 interval measurements, where statistical power corresponds to the proportion of times
234 periodicity is detected in songs where periodicity has been artificially imposed on song data
235 (Fig. 2). We started with six 45-minute recordings of *Canton-S* from Stern (2) that contained
236 more than 10,000 inter-pulse interval measurements. None of these six songs yielded
237 statistically significant power in the frequency range between 50 and 60 sec (the range
238 originally defined to contain rhythms (10)) and one song produced a marginally significant peak
239 at 31.7 sec ($P = 0.04$), which falls between 20 and 150 sec (the range used by Kyriacou et al. (3)).
240 Figure 2d and 2e illustrate the inter-pulse interval data and periodogram for one of these songs.
241 Therefore, these songs do not contain strong periodicity in the predicted range and can serve as
242 a template to examine the power of Lomb-Scargle periodogram analysis to detect simulated
243 rhythms imposed on these data.

244 The initial reports of periodic cycles in the inter-pulse interval reported rhythms with a
245 mean period of 55 sec and an amplitude of approximately 2 ms (10). Therefore, we imposed a
246 55 second rhythm with an amplitude of 2ms on the six songs containing more than 10,000
247 inter-pulse interval measurements (Fig. 2a-c). We detected the simulated 55 sec rhythm in all
248 six songs with P-values $< 10e-74$ (example shown in Fig. 2f,g). We then randomly removed data
249 points from the songs iteratively and calculated the fraction of times we could detect the
250 simulated rhythm with $P < 0.05$. We removed data randomly from the dataset to simulate the
251 effect of failing to detect individual events in the song and we also removed chunks of data (in
252 10 sec bins) to simulate large gaps between song bursts, such as might be generated during
253 low-intensity courtship. We found that in both scenarios we could randomly remove at least
254 90% of the data and still detect simulated rhythms at least 80% of the time (example shown in
255 Fig. 2h,i; summary statistics shown in Fig. 2j and SI Appendix, Fig. S4a). That is, as long as songs
256 contained at least 1000 inter-pulse interval measurements, Lomb-Scargle periodogram analysis
257 detected simulated rhythms with power greater than 0.8. Similar results were found when we
258 analyzed only the first 400 sec of songs (SI Appendix, Fig. S4c,d). Furthermore, periodicity could
259 be detected with power greater than 0.8 when the amplitude of simulated periodicity was
260 greater than at least 1 msec (SI Appendix, Fig. 4b). These results were robust to noise in the
261 original periodicity. Song with a signal to noise ratio of as low as 0.25 could be detected with
262 power > 0.7 with sample sizes of at least 1000 inter-pulse interval measurements (Fig. 2k).
263 Similarly, periodicity could be detected reliably when we simulated a non-sinusoidal rhythm (SI
264 Appendix, Fig. Fig. S4e) and when periodicity was imposed for only a fraction of the total song

265 (SI Appendix). Thus, Lomb-Scargle periodogram analysis is a sensitive method for detecting
266 simulated periodicity, even in the presence of noise or discontinuities in the waveform.

267 Songs containing at least 1000 inter-pulse intervals provide sufficient data to identify
268 putative song cycles. In fact, we find that songs can be deeply corrupted by the absence of large
269 segments of song and simulated periodicity can still be detected.

270

271 **No evidence that the automated fly song segmenter biased the results**

272

273 Kyriacou et al. (3) expressed concern that our automated fly song segmenter displayed a
274 low true positive rate (the segmenter failed to detect approximately 50% of the pulses
275 identified through manual annotation) and produced some false positive calls (approximately
276 4% of events scored as pulses by the automated segmenter appear to be noise). They suggest
277 that these incorrect pulse event assignments could bias estimation of the mean inter-pulse
278 interval and therefore decrease the signal-to-noise of the periodic cycle, making it difficult to
279 detect a periodic signal. In principle, a large sample of incorrect calls could bias results, so we
280 investigated whether this was the case for our prior analyses. We used Kyriacou et al.'s (3)
281 manually-annotated dataset to investigate the potential for bias and to evaluate performance
282 of the automated segmenter.

283 When a single pulse event is not detected, the inter-pulse interval is then calculated as
284 the sum of the two neighboring real intervals. On average, this is approximately double the
285 average inter-pulse interval. The average inter-pulse interval for the *Canton-S* recordings
286 reported in Stern (2) is approximately 35 msec with a standard deviation of approximately 7

287 msec. Therefore, skipping a single pulse event is expected to result in inter-pulse interval
288 measurements of approximately 70 msec, but with considerable variance. Following Kyriacou
289 and Hall (16), Stern (2) employed a heuristic threshold of 65 msec to reduce the number of
290 spurious inter-pulse interval values. Therefore, in the specific case when a single pulse in a
291 train is missed, approximately one third of the incorrectly scored doublet inter-pulse interval
292 measurements would be shorter than 65 msec and are expected to contaminate the original
293 dataset.

294 However, this scenario applies only when one undetected pulse is flanked by two pulses
295 that are detected. Skipping more than one pulse would always result in inter-pulse interval
296 measurements that are excluded by the 65 ms threshold. We found, however, that only 9% of
297 the pulses missed by automated segmentation were singletons (SI Appendix, Fig. S6a). These
298 incorrect inter-pulse intervals contribute to a slight excess of inter-pulse intervals with high
299 values (SI Appendix, Fig. S6b). Lowering the inter-pulse interval threshold would, therefore,
300 remove most or all spurious inter-pulse intervals. Since our power analysis, discussed above,
301 revealed that periodogram analysis was robust to random removal of inter-pulse interval
302 events, as long as songs still contained at least 1000 values, loss of a small number of inter-
303 pulse intervals is not expected to hamper detection of rhythms. After reducing the inter-pulse
304 interval threshold to 55 msec, we still found no compelling evidence for significant periodicity in
305 the original data (SI Appendix, Fig. S7). Therefore, we explored the effect of reducing the inter-
306 pulse interval cutoff even further. In this case, we used all 68 *Canton-S* songs from Stern (2) and
307 retained for analysis only those songs that contained at least 1000 inter-pulse interval
308 measurements after imposing the new inter-pulse interval threshold. We explored a range of

309 cutoff values from 25 to 65 msec. We found that we could detect the simulated rhythm in most
310 songs with at least 1000 inter-pulse interval measurements remaining after thresholding, even
311 when the threshold was as low as 25 msec (Fig. 3). Therefore, we can find no evidence that
312 pulses missed by the automated song segmenter or the specific inter-pulse interval threshold
313 used in Stern (2) prevented detection of song rhythms.

314 Although detection of putative song rhythms is robust to dropped pulses in songs that
315 retain at least approximately 1000 inter-pulse intervals, it is worth reviewing briefly why the
316 segmenter failed to detect certain pulses in recordings reported in Stern (2). The first step of
317 song segmentation involves detection of pulse-like signals and sine-like signals (1). In
318 subsequent steps, the segmenter filters out many kinds of sounds that were originally classified
319 as song pulses. Both the initial detection of pulses and subsequent filtering steps are sensitive
320 to multiple parameters. These parameters are specified prior to segmentation and can be
321 modified to enhance performance of the segmenter for different recordings. We identified two
322 primary causes for missed pulses. First, Stern (2) recorded song in larger chambers than those
323 used previously with these microphones (1), to match the chamber size used by Kyriacou & Hall
324 (10). This larger chamber with one microphone had reduced sensitivity compared to the
325 original smaller chamber. The segmenter thus tended to miss pulses of lower amplitude, which
326 are hard to automatically differentiate from noise, and this explains approximately 35% of the
327 missed pulses (SI Appendix, Fig. S8a, c).

328 The second major cause of missed pulses is that *Drosophila* males produce pulses with a
329 range of carrier frequencies (tones). The higher frequency pulses tend to resemble other non-
330 song noises, like grooming, and a user can set parameters in the segmenter to attempt to

331 exclude these non-song noises based on the carrier frequency of the event. Stern (2) used
332 parameters to minimize the false positive rate, including a relatively low carrier frequency
333 cutoff for pulses. The lower pulse frequency threshold used by Stern (2) explains approximately
334 42% of the missed pulses (SI Appendix, Fig. S8b,d). Using the same software with different
335 parameters (from Coen et al. (5)) recovers many of these high-frequency pulses without
336 substantially increasing the false positive rate (SI Appendix, Fig. S8c-f).

337 Above, we showed that including more pulse events, by manual annotation, did not
338 increase the probability of detecting song rhythms. Therefore, there is no evidence that the
339 data resulting from the song segmenter parameters used in Stern (2) generated a data set that
340 was biased against detection of song rhythms. While the song segmenter does not detect all
341 pulse events that can be detected by manual annotation, the segmenter does provide data sets
342 that are several orders of magnitude larger than those that can be generated by manual
343 annotation, which has allowed discovery of multiple new phenomena related to *Drosophila*
344 courtship song (4–6). In addition, the sensitivity of the song segmenter can be improved with
345 optimization of initial parameters, as expected of any segmentation algorithm.

346

347 **Discussion**

348 We cannot detect a periodic cycling of the inter-pulse interval in *Drosophila* courtship
349 song even in the songs manually annotated by Kyriacou et al. (3) and used as evidence for
350 periodicity in their paper. While it is impossible to prove a negative, our results agree with
351 previous analyses that have concluded that there is no statistical evidence that these rhythms
352 exist (1, 2). In particular, by exploring some of the relevant parameter space with statistical

353 tests on the song that was manually-annotated by Kyriacou et al. (3), we find that subsets of
354 parameters sometimes produce p-values lower than 0.05, but that (1) few regions of parameter
355 space generate “significant” results, (2) these “significant” regions are scattered apparently
356 randomly in parameter space, and (3) none of these “significant” results survive multiple test
357 correction (Fig. 1).

358 Previously, we offered one explanation for how apparent song rhythms may have been
359 detected. We found that binning data from short songs confined the periodogram peaks with
360 maximum power close to the range reported as the song cycle (2). While few of these peaks
361 reached statistical significance, previous authors have accepted these peaks as “signal” and
362 performed statistical analyses to compare the peaks between genotypes. All “statistically
363 significant” results from earlier papers were derived mainly from non-significant peaks in
364 periodogram analysis and from relatively small sample sizes (usually fewer than 10 flies of each
365 genotype), so it is questionable whether these derivative statistics are valid. Genotype-specific
366 periodicities reported in earlier papers may have resulted, by chance, from studies of a small
367 number of short songs that fortuitously led to occasional apparent replication of the original
368 observations.

369 There may be a more prosaic explanation for the initial discovery of song cycles. Every
370 fly produces highly variable inter-pulse intervals. In addition, a running average of these data
371 reveals that the average inter-pulse interval cycles up and down (Fig. 1b), similar to the
372 temporally-binned data first reported by Kyriacou and Hall (10). There is no debate about this
373 observation. The claim in dispute is that the average inter-pulse interval cycles regularly. We
374 can find no evidence for this claim. It is easy to imagine, however, that visual examination of

375 short recordings of song would make it appear as if the mean inter-pulse interval cycled
376 regularly.

377 The extraordinary within-fly variation in the inter-pulse interval and in the mean inter-
378 pulse interval may result from multiple causes, including the possibility that male flies respond
379 to ever-changing cues during courtship and modulate their inter-pulse interval to optimize their
380 chances of mating. Individual *Drosophila* males modulate specific aspects of their courtship
381 song based on their own patterns of locomotion and in response to feedback from females,
382 including the transition between sine and pulse song (5) and the amplitude of pulse song (4).
383 There is additional evidence that males modulate the carrier frequency of sine song (1). We
384 hypothesize that male flies also modulate their inter-pulse interval in response to specific
385 internal or external cues.

386 We can find no statistical evidence for periodicity of the inter-pulse interval in individual
387 courtship songs and no evidence that comparisons of the strongest periodogram peaks from
388 each song identify genotype-specific rhythms. These results hold *both* for the songs manually
389 annotated by Kyriacou et al. (3) and for two independent large datasets automatically
390 annotated with FlySongSegmenter using optimized parameters. At this time, a conservative
391 assessment of the problem is that *Drosophila* courtship song rhythms and genotype-specific
392 effects on these rhythms cannot be replicated.

393

394 **Methods**

395 Courting fruit flies of Oregon-R and *per^L* were recorded as described previously (2). All analyses
396 were performed in Matlab. All data and code are freely available, as described in the Software
397 and Data Availability section. Further methods can be found in SI Appendix.

398

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400 We thank Elizabeth Kim for recording the new samples of flies.

401

402 **Software and data availability**

403

404 Computer code for all analyses described in this paper is available at

405 <https://github.com/murthylab/noIPcycles>. Code for the version of FlySongSegmenter used in

406 Cohen et al. (5) is available at <https://github.com/murthylab/songSegmenter>. The raw and

407 segmented song data for the new song recordings is available at

408 <https://www.janelia.org/lab/stern-lab/tools-reagents-data>.

409

410 **References**

- 411 1. Arthur BJ, Sunayama-Morita T, Coen P, Murthy M, Stern DL (2013) Multi-channel
412 acoustic recording and automated analysis of *Drosophila* courtship songs. *BMC Biol*
413 11:11.
- 414 2. Stern DL (2014) Reported *Drosophila* courtship song rhythms are artifacts of data
415 analysis. *BMC Biol*.
- 416 3. Kyriacou CP, Green EW, Piffer A, Dowse HB, Takahashi JS (2017) Failure to reproduce

- 417 period-dependent song cycles in *Drosophila* is due to poor automated pulse-detection
418 and low-intensity courtship. *PNAS*. doi:10.1073/pnas.1615198114.
- 419 4. Coen P, Xie M, Clemens J, Murthy M (2016) Sensorimotor Transformations Underlying
420 Variability in Song Intensity during *Drosophila* Courtship. *Neuron* 89(3):629–644.
- 421 5. Coen P, et al. (2014) Dynamic sensory cues shape song structure in *Drosophila*. *Nature*.
422 doi:10.1038/nature13131.
- 423 6. Ding Y, Berrocal A, Morita T, Longden KD, Stern DL (2016) Natural courtship song
424 variation caused by an intronic retroelement in an ion channel gene. *Nature*
425 536(7616):329–332.
- 426 7. Shirangi TR, Wong AM, Truman JW, Stern DL (2016) Doublesex Regulates the
427 Connectivity of a Neural Circuit Controlling *Drosophila* Male Courtship Song. *Dev Cell*
428 37(6):533–544.
- 429 8. Shirangi TR, Stern DL, Truman JW (2013) Motor Control of *Drosophila* Courtship Song.
430 *Cell Rep* 5(3):678–686.
- 431 9. Bennet-Clark HCC, Ewing AW, Bennet-Clark HCC (1968) The courtship songs of
432 *Drosophila*. *Behaviour* 31(3):288–301.
- 433 10. Kyriacou CP, Hall JC (1980) Circadian rhythm mutations in *Drosophila melanogaster* affect
434 short-term fluctuations in the male's courtship song. *PNAS* 77(11):6729–6733.
- 435 11. Zehring WA, et al. (1984) P-element transformation with period locus DNA restores
436 rhythmicity to mutant, arrhythmic *Drosophila melanogaster*. *Cell* 39(2 Pt 1):369–376.
- 437 12. Wheeler DA, et al. (1991) Molecular transfer of a species-specific behavior from
438 *Drosophila simulans* to *Drosophila melanogaster*. *Science* 251(4997):1082–5.

- 439 13. Kyriacou CP, Hall JC (1986) Interspecific genetic control of courtship song production and
440 reception in *Drosophila*. *Science (80-)* 232:494–497.
- 441 14. Ritchie MG, Halsey EJ, Gleason JM (1999) *Drosophila* song as a species-specific mating
442 signal and the behavioural importance of Kyriacou & Hall cycles in *D. melanogaster* song.
443 *Anim Behav* 58:649–657.
- 444 15. Konopka RJ, Benzer S (1971) Clock Mutants of *Drosophila melanogaster*. *Pnas*
445 68(9):2112–2116.
- 446 16. Kyriacou CP, Hall JC (1989) Spectral analysis of *Drosophila* courtship song rhythms. *Anim*
447 *Behav* 37:850–859.
- 448 17. Kyriacou CP, van den Berg MJ, Hall JC (1990) *Drosophila* courtship song cycles in normal
449 and period mutant males revisited. *Behav Genet* 20(5):617–644.
- 450 18. Lomb NR (1976) Least-squares frequency analysis of unequally spaced data. *Astrophys*
451 *Space Sci* 39(1964):447–462.
- 452 19. Scargle JD (1982) Studies in astronomical time series analysis. II - Statistical aspects of
453 spectral analysis of unevenly spaced data. *Astrophys Journal, Part 1* 263:835–853.
- 454 20. Ruf T (1999) The Lomb-Scargle periodogram in biological rhythm research: Analysis of
455 incomplete and unequally spaced time-series. *Biol Rhythm Res* 30(2):178–201.
- 456 21. Refinetti R, Lissen GC, Halberg F (2013) *Procedures for numerical analysis of circadian*
457 *rhythms* doi:10.1080/09291010600903692.Procedures.
- 458 22. Roberts DH, Lehár J, Dreher JW, Lehar J (1987) Time series analysis with CLEAN. I.
459 Derivation of a spectrum. *Astron J* 93(4):968–989.
- 460 23. Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and

- 461 powerful approach to multiple testing. *J R Stat Soc B* 57(1):289–300.
- 462 24. Colquhoun D, London C (2014) An investigation of the false discovery rate and the
- 463 misinterpretation of P values. *R Soc Open Sci* 1:1–15.

464

465

466 Figure Legends

467

468 Figure 1. Genotype-specific periodicity cannot be detected in *Drosophila* courtship song. (A)

469 *Drosophila* males produce courtship song, composed of pulses (red) and sines (blue), by

470 extending and vibrating a wing. The inter-pulse interval is the time between consecutive pulses

471 within a single train of pulses. (B) The average inter-pulse interval varies over time. (Purple line

472 is loess fit with sliding window of 200 samples). (C) Lomb-Scargle periodogram analysis of the

473 inter-pulse interval data from panel (B) plotted for the range of 20 -150 sec. None of the peaks

474 are significant at $p < 0.05$. (D) Comparison of the peak power between 20-150 sec from the

475 Lomb-Scargle periodograms for the song data for the genotypes periodL (perL) and Canton-S

476 (CS) manually-annotated by Kyriacou et al. (3). Red points and lines represent mean ± 1 SD for

477 each genotype. (Right-tailed T-test $p = 0.06$. Rank Sum $p = 0.10$.) (E) P-values for period

478 windows with different lower and upper bounds. (F) False discovery rate q values for the

479 windows shown in (E). (G) Fraction of ranges with significant (p or $q < 0.05$) for either the test of

480 Canton-S less than periodL or periodL less than Canton-S. (H-K) Same as (D-G) for newly

481 collected song data from the same genotypes annotated using FlySongSegmenter. (H) (Right-

482 tailed T-test $p = 0.06$. Rank Sum $p = 0.45$.)

483

484 Figure 2. Simulations to explore power to detect rhythms, should they exist. (A-C) Example of

485 how a periodic cycle was added to raw inter-pulse interval (IPI) data. Purple line in (A) illustrates

486 the running mean of the raw data. Blue line in (B) shows a periodic rhythm with an amplitude of

487 2 msec and a period of 55 sec. Original data with simulated periodicity is shown in (C). (D) One

488 example of 45 minutes of inter-pulse interval data. Purple line shows running mean. (E) Lomb-
489 Scargle periodogram of data in (D) does not detect periodicity. (F) Data from (D) with a 55 sec
490 periodicity imposed. (G) Lomb-Scargle periodogram of data in (F) now reveals a highly
491 significant peak at 55 sec, consistent with the simulated periodicity. (H) Random removal of
492 95% of the inter-pulse interval data from (F). (I) Lomb-Scargle periodogram of the data in (H)
493 detects significant periodicity. (J) Power analysis of six songs (each song a different color)
494 containing more than 10,000 inter-pulse interval events after 55 sec periodicity was added and
495 individual inter-pulse interval events were removed randomly. Power equals the fraction of
496 times out of 100 that a song contained a rhythm with significant periodicity between 50 and 60
497 sec at $P < 0.05$. (K) Power to detect simulated noisy periodicity versus number of IPIs remaining
498 after random removal of IPIs. Means of simulations for six songs containing more than 10,000
499 inter-pulse interval measurements are shown. Examples of simulated noisy rhythms are shown
500 to the right. Colorbar shows power to detect simulated rhythm.

501

502 Figure 3. The specific inter-pulse interval threshold does not influence the statistical power to
503 detect putative song rhythms. (A) Example of one original song with 55 sec periodicity
504 artificially imposed on the original inter-pulse interval data. (B) Lomb-Scargle periodogram of
505 data in panel (A), revealing strong signal at 55 sec. (C) Same simulated data as in panel (A) with
506 all inter-pulse interval values greater than 25 sec removed. (D) Lomb-Scargle periodogram
507 reveals strong signal of the simulated periodicity at 55 sec, even though the data were
508 thresholded at 25 sec. (E) Power to detect simulated periodicity versus inter-pulse interval
509 threshold for songs retaining at least 1000 inter-pulse interval values after thresholding.





