Collective sperm movements in *Peromyscus* mice are shaped by phylogenetic history and post-copulatory sexual selection

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Abstract

In some species, sperm form motile, coordinated groups as they migrate through the female reproductive tract. Collective sperm migration is posited to have evolved to improve sperm swimming performance, and thus may be beneficial in a competitive context, but limited evidence supports this theory. Here we investigate sperm aggregation across closely-related species of *Peromyscus* mice that naturally vary by mating system. We find that phylogenetic history predicts the likelihood that sperm will aggregate but that variation in aggregate size negatively associates with relative testis size, suggesting that sperm competition has a stabilizing effect on this trait. Moreover, we show that sperm aggregation is not kinematically beneficial for all species, and we hypothesize that swimming performance is dependent on the orientation and composition of sperm groups. To test this, we compared sperm from the two sister-species that aggregate most frequently and find that sperm of the species that evolved under intense sperm competition forms aggregates with efficient geometry more frequently than sperm from its monogamous congener. Together, our results are consistent with the hypothesis that sperm aggregation evolved to improve motility in a competitive context; however, when monogamy evolved secondarily, relaxed sexual selection allowed for less efficient strategies to persist.

Introduction

Sperm cells are one of the most diverse cell types in nature and exhibit striking variation both within and across taxa [1]. In addition to being morphologically diverse, sperm may exhibit complex, emergent behaviors, including sperm conjugation, which occurs when two or more cells join together for motility or transport through the female reproductive tract before dissociating prior to fertilization [1,2]. Although relatively rare, these sperm-sperm interactions have evolved multiple times across independent lineages of internally fertilizing species [1–3], yet the adaptive significance of these gametic interactions remains unclear for many taxa.

One functional hypothesis posits that sperm aggregates may be advantageous if the combined force generated by multiple flagella enable sperm conjugates to swim faster than single cells [4,5]. Improved sperm motility can be beneficial if it enables cells to move quickly through hostile environments within the female tract [6,7], and in competitive environments in which females mate with multiple males, as sperm velocity is often correlated with competitive fertilization success [7–9]. While some studies that have quantified the motility of sperm aggregates have found empirical support for this hypothesis [4,10–13], others have not [14,15]. For example, sperm groups swim faster than solitary cells in the Norway rat (*Rattus norvegicus*), yet house mouse (*Mus musculus*) sperm swim slower as groups under identical experimental conditions [5]. Another non-mutually exclusive hypothesis is that sperm aggregation facilitates migration through viscous or viscoelastic secretions of the female reproductive tract [16,17], including cervical mucus [18], which has received some empirical support [4,10,16; but see 15]. In the grey short-tailed opossum (*Monodelphis domestica*), for example, sperm pairs swim with greater motility than single sperm in viscous fluids; however, these sperm pairs were artificially induced to uncouple [16] and therefore the comparison may not be biologically informative [1]. Additionally, sperm aggregation may protect sensitive regions of the sperm, such as the acrosome, from damage and preserve sperm functionality during passage through the male or female reproductive tracts [19–22], or enhance egg penetration during fertilization [23; but see 24]. While it is often assumed

52 that coordinated sperm movements are adaptive, inconsistent findings and a wide diversity of
53 naturally variable sperm behavior have limited our understanding of the functional advantages of
54 sperm aggregation.

55 Multiple independent origins of sperm conjugation suggest that the functional consequences,
56 as well as mechanisms that regulate these cellular interactions, are likely to vary throughout nature
57 [2]. Indeed, the formation of sperm aggregates and the number of grouped cells varies widely
58 across taxa [7]. In mammals alone, sperm of some species assemble during epididymal transport
59 and are molecularly "glued" to one another as bi-flagellate pairs in grey short-tailed opossums
60 [25], as bundles of roughly 100 cells in monotremes [26], or as organized rouleaux of five or more
61 cells in guinea pigs (*Cavia porcellus*; [24]). Conversely, mammalian sperm may also assemble
62 after ejaculation to form variably-sized groups. For instance, sperm may form temporary clusters
63 of up to sixteen cells in bulls (*Bos taurus*; [15]) or more fixed groups of up to 30 cells in house
64 mice [5], 50 cells in the Norway rat [5], or thousands of cells in the wood mouse (*Apodemus*
65 *sylvaticus*; [4]) whereby the hook-shaped heads interlock or attach to the flagella of other sperm.
66 For these latter species in which sperm groups form after ejaculation, both single and aggregated
67 sperm typically co-occur, thus allowing for direct comparisons between collective and solitary
68 sperm movements within an ejaculate while controlling for within-male variability.

69 Closely-related species in the rodent genus, *Peromyscus,* produce sperm that naturally vary in
70 their collective behavior. Sperm of some species assemble temporary groups after ejaculation by
71 adhering to one another at their head region [27] and disassemble prior to fertilization [12]. In *P.*
72 *maniculatus*, sperm selectively group with the most closely-related cells to form motile groups of
73 up to 30 cells [12], but there is a non-monotonic association between group size and swimming
74 velocity, indicating that some groups are faster than single cells but others are not [28].
75 Conversely, sperm produced by their sister-species, *P. polionotus*, also form aggregates but do so
76 indiscriminately with related and unrelated cells [12], are less likely to be optimally-sized, and
77 overall move in a less linear trajectory [28]. Intriguingly, sperm competition is predicted to be

more intense in *P. maniculatus* than in *P. polionotus* due to their different mating systems. In *P. maniculatus*, both sexes mate with multiple partners, often in overlapping series just minutes apart [29], and females frequently carry multiple-paternity litters in the wild [30], whereas both behavioral [31] and genetic data [32] indicate that *P. polionotus* is strictly monogamous. Evidence suggests that monogamy has evolved at least twice within the *Peromyscus* lineage [33,34], thus enabling us to investigate if post-copulatory sexual selection, driven by female mating behavior, has shaped the evolution of sperm aggregation [4] more broadly across the *Peromyscus* lineage. In this study, we quantify intra- and inter-specific differences in the size and performance of sperm aggregates under consistent, controlled conditions to examine the evolution of collective sperm behavior and empirically test whether sperm aggregation improves swimming performance.

Materials and Methods

(a) Sperm collection

We obtained captive *Peromyscus maniculatus bairdii, P. polionotus subgriseus, P. leucopus*, *P. eremicus*, and *P. californicus insignis* from the Peromyscus Genetic Stock Center at the University of South Carolina, and *P. gossypinus gossypinus* from Dr. Hopi Hoekstra at Harvard University and housed them in same-sex cages at 22ºC on a 16L:8D cycle in accordance with guidelines established by the Institutional Animal Care and Use Committee at the University of Maryland in College Park (protocol R-Jul-18-38). We sought samples from all available captive *Peromyscus* species and avoided wild-caught specimens to control for variation due to life experience. We obtained sperm samples from sexually mature males and accounted for relatedness among the focal males by assigning siblings a unique 'Family' ID. We euthanized males via isoflurane overdose and cervical dislocation, then weighed each male and both testes (Mettler Toledo, Switzerland). Next, we removed a single caudal epididymis, made several small incisions in the tissue, and submersed it in sperm medium (Modified Sperm Washing Medium, Irvine Scientific, USA) that was pre-warmed at 37ºC; to reduce differences in sperm density despite

104 natural variation in epididymal sizes, we varied the volume (50µl - 1000µl) based on tissue size
105 and accounted for these differences when estimating final sperm counts for each male. To collect
106 sperm, we agitated the tissue at 300rpm (ThermoMixer F1.5, Eppendorf, Germany) at 37ºC for ten
107 minutes, inverting the tube at the five- and ten-minute mark, then incubated the tissue undisturbed
108 for two minutes. Using pipette tips cut to create a wider opening, we collected live sperm cells for
109 analysis from just below the meniscus of the solution to enrich for the most motile sperm [35].
110 Next we estimated sperm density using a computer-assisted sperm analysis (CASA) system (Ceros
111 II Animal, Hamilton Thorne, USA) and verified with a Neubauer-improved hemocytometer
112 (Marienfeld, Germany) then diluted samples with pre-warmed medium to reach a standard
113 concentration of 300-400 cells summed across the five 5-second videos at 100X magnification for
114 cell tracking optimality and efficiency.
115
116 (b) Live sperm analysis
117 To conduct live sperm observations, we gently reverse pipetted 4µl of the sperm solution into
118 12µl of pre-warmed medium on a plastic slide within a 9mm x 0.12mm imaging spacer (Grace
119 Bio-Labs, USA) and covered by a plastic cover slip. This set-up served as a control and represents
120 a 'low-viscosity' environment. To test sperm motility in a 'high-viscosity' environment, we
121 followed the same procedures except that we mixed 4µl of sperm solution with 12µl of pre-
122 warmed medium enriched with methylcellulose (Sigma Aldrich M 7140; 15cP, 2% in water; [36]).
123 We recorded a minimum of five 5-second videos at 60 frames/second per male, except in one case
124 we were only able to record four videos yet were able to acquire an adequate number of observed
125 cells per male. Videos were recorded at 59±1 minutes post-harvest from the epididymal tissue,
126 dependent on dilutions. The number of cells analyzed are reported in Table 1.
127 We characterized sperm aggregation by scoring videos using direct observations because
128 CASA tracks particles, and thus each track may represent a single cell or an aggregate. We
129 counted the number of cells represented by each track on at least three different frames/track.

130 From these data, we calculated the proportion of cells that aggregated for each male by dividing
131 the total number of aggregates by the total number of motile cells across all tracks [37]. Then we
132 calculated the mean number of cells in aggregate (i.e., aggregate size) by dividing the sum of cells
133 in aggregate by the sum of aggregates, both across all tracks for each male and across all males for
134 each species. Finally, we calculated the coefficient of variation (CV) for both the proportion and
135 number of cells that aggregated within each species using the following formula: (standard
136 deviation/mean) x 100.

137 During video scoring, we identified aggregates that contained cells that were facing different
138 directions within the group, immotile, morphologically abnormal, or stuck to the microscope slide.
139 We used this dataset to characterize differences in sperm aggregation for *P. maniculatus* and *P.*
140 *polionotus*, the two species whose sperm were observed to aggregate the most extensively. We
141 characterized aggregates as: 'aligned' if all sperm adhered to one another in a head-to-flagella
142 orientation and contained no immotile, morphologically abnormal or damaged cells; 'defective' if
143 one or more sperm cell was marked as morphologically abnormal (kinked or broken), immotile, or
144 stuck to the slide; or 'opposed' if sperm were otherwise morphologically normal and motile but
145 were not oriented in the same direction, which included star-shaped aggregates [28]. We then used
146 these data to calculate the proportion of aggregates scored for these criteria within each male for
147 these two species.

148 We recorded the following metrics for each recorded track (i.e., single sperm and aggregates):
149 *straight-line velocity* (VSL; calculated using the straight-line distance between the first and last
150 detected positions of the sperm head, divided by the time taken to swim the track; also known as
151 average velocity), *curvilinear velocity* (VCL; calculated using the summed distance between the
152 sperm head positions in each frame divided by the time taken to travel the track; also known as
153 speed), *average path velocity* (VAP; the time-averaged velocity of the sperm head along its
154 average path), and *linearity* (LIN; the ratio of VSL to VCL to measure the straightness of the
155 trajectory; [38]). We calculated the mean of each kinematic parameter for both single cells and

156 sperm aggregates separately for each of three populations of sperm cells: all cells, motile cells
157 (i.e., devoid of visually inspected tracks in which cells were unmoving, stuck, or featured an
158 obvious morphological abnormality such as a kinked midpiece), and progressively motile cells
159 (i.e., motile cells with a VSL $\geq$ 25μm/sec). We used these data to calculate the proportion of
160 motile aggregates by dividing the sum of motile aggregates by the total number of sperm
161 aggregates, and the proportion of progressively motile aggregates by dividing the sum of
162 progressive aggregates by the total number of sperm aggregates for each male [37]. For our
163 kinematic analyses, we focused on the motile sperm dataset to eliminate artifacts from damaged or
164 dead cells, and the total sperm population to quantify frequency of aggregation and aggregate size
165 in an effort to capture natural aggregation rates (results from other cell populations are reported in
166 Table S1, Figure S1).
167
168 (c) Statistical Analyses
169 We performed all statistical analyses using R version 3.4.2 [39] and created all figures using
170 the 'ggplot2' package with R [40]. Generalized linear mixed models (GLMM) were analyzed
171 using the glmer function from the "lme4" R package and a logit link function [41]. We also used
172 this package to analyze linear mixed models (LMM) using the lmer function and generalized linear
173 models (GLM) using the glm function. We screened for collinearity using linear models and
174 variance inflation factor (VIF) values and used model selection to drop non-significant explanatory
175 variables one at a time based on model comparisons (using Akaike information criterion changes <
176 2 for GLMMs or an analysis of variance test for LMMs) to determine the minimal adequate
177 model. To validate model normality, we visually inspected diagnostic plots (qqplots and plots of
178 the distribution of the residuals against fitted values). Only the best fitting models are reported
179 here. Post-hoc pairwise comparisons of final models concerning aggregate frequency and size
180 were performed using Tukey HSD adjustments for multiple comparisons from the "LSmeans" R
181 package [42].

182 We used multiple data sets and statistical approaches to analyze the frequency and size of
183 sperm aggregates. For the mean proportion of aggregated cells by male, we used a GLMM in
184 which the binomial response was the number of sperm cells in aggregate, and the total number of
185 sperm cells was the binomial denominator. We used an observation-level random effect (OLRE) to
186 control for overdispersion [37,43] as well as family ID as random factors in these analyses. For the
187 mean proportion of aggregated cells by species, we included an ultra-metric phylogenetic tree of
188 *Peromyscus* as a covariate in our regression analyses using a phylogenetic generalized least
189 squares (PGLS) approach [44,45] using the "caper" [46] and "APE" [47] packages in R. For the
190 mean number of aggregated cells by male, excluding one *P. californicus* male whose measurement
191 represented a clear outlier, we initially used a LMM but then reverted to using a linear model (LM)
192 because the family ID random factor did not significantly contribute to the residual variability in
193 the response variable. For the mean number of aggregated cells by species, we used a PGLS
194 approach. We also conducted similar PGLS analyses using the within-species CV for each of these
195 parameters. When assessing the relationship between relative testis size and aggregation, our
196 models included testis mass with body mass as a separate fixed factor, a method better suited to
197 estimating relative testis weight is size rather than using the ratio of testis to body mass or
198 residuals [48,49].
199 We used multiple data sets and statistical approaches to further analyze sperm aggregation.
200 We used generalized linear models (GLM) to analyze the composition and orientation of sperm
201 aggregates in *P. maniculatus* and *P. polionotus,* paired student's t-test to compare the VCL of
202 aggregates based on their composition or orientation within species, and GLMMs to assess species
203 differences in the proportion of aggregates that were motile or progressively motile. To determine
204 if sperm aggregates have motility or force benefits over single cells, we initially performed a
205 principal component analysis (PCA) using three related swimming performance measures (VSL,
206 VCL, and VAP) to reduce dimensionality and obtain a composite measure for motile solitary and
207 aggregated sperm cells in both low- and high-viscosity media. Because we found that species

208 distributions overlapped for this composite measure for both single sperm and aggregates in low-
209 and high-viscosity media (Figures S2 and S3), we focused on individual kinematic parameters
210 (VSL, VCL, VAP, LIN) within each species separately. To determine if sperm aggregates have
211 motility benefit over single cells in either low- or high-viscosity media, we used paired student's t-
212 tests to compare each kinematic parameter between solitary and aggregated sperm cells within
213 males for each species within each environment.
214
215 **Results**
216 We found significant differences in the proportion of aggregated cells among *Peromyscus*
217 species (binomial GLMM: $n = 134$, $P < 0.001$; Table 1, 2), with more variance across species than
218 within species (s^2 across species = 0.10; s^2 within species < 0.10). Pairwise comparisons adjusted
219 for multiple comparisons revealed that species that are most closely related within the phylogeny
220 do not significantly differ from one another, whereas all other pairwise species comparisons do
221 (Figure 1). *P. maniculatus* and *P. polionotus* both have the highest frequencies of aggregation,
222 despite having different mating systems, which corresponds with our finding that relative testis
223 mass does not significantly associate with the proportion of cells aggregated (binomial GLMM: n
224 = 127, $P = 0.163286$). We also found no association between the frequency of sperm aggregation
225 and sperm velocity (VCL: binomial GLMM: $n = 130$, $P = 0.1435$). In examining the coefficient of
226 variation (CV) for the proportion of cells aggregated within each species, we found 68.8% for *P.*
227 *californicus*, 50.6% for *P. eremicus*, 14.4% for *P. polionotus*, 10.8% for *P. maniculatus*, 125.7%
228 for *P. leucopus*, and 60.5% for *P. gossypinus*. In a cross-species analysis controlling for
229 phylogenic relationships and body mass, we found no effect of testis size on the variation present
230 within species for the proportion of aggregated cells ($F_{2,3} = 0.3603$, $P = 0.5604$).
231 For the number of aggregated cells, we found significant differences among species (LM:
232 $F_{6,126} = 56.37$, $P < 0.001$; Table 1, 2), with more variance observed across species ($s^2 = 1.96$) than

233 within species ($s^2 < 1.00$, except for *P. polionotus* [$s^2 = 2.15$]). *P. maniculatus* and *P. polionotus*
234 produce the largest sperm aggregates, whereas *P. gossypinus*, *P. leucopus*, and *P. californicus*
235 produce the smallest aggregates ($P < 0.05$ for pairwise comparisons), the latter of which produces
236 sperm cell aggregates that are statistically similar in size to those produced by *P. eremicus* ($P =$
237 0.9203; Figure 2A). In a cross-species analysis controlling for phylogenic relationships and body
238 mass, we found a significant negative effect of a species' mean testis weight on the species'
239 variation in the number of cells in aggregate ($F_{2,3} = 8.398$, $P = 0.02655$; Figure 2B). The CV for
240 the number of cells aggregated within each species were as follows:: 26.5% for *P. californicus*,
241 19.2% for *P. eremicus*, 27.5% for *P. polionotus*, 18.4% for *P. maniculatus*, 13.3% for *P. leucopus*,
242 and 7.2% for *P. gossypinus*.

243 Within the two species that produce the largest and most frequent aggregates, sperm cells
244 within aggregates are significantly more aligned in a head-to-flagella orientation in *P. maniculatus*
245 (99.1%, 731/738) than in *P. polionotus* (87.5%, 720/826; GLM: $n = 42$, $P = 3.92e-12$), a feature
246 that significantly positively correlates with VCL in both species (Figure 3; paired t-tests: *P.*
247 *maniculatus* $t = 5.9627$, $df = 4$, $P = 0.003972$; *P. polionotus* $t = 11.247$, $df = 11$, $P = 2.257e-07$).
248 We also found that there are significantly fewer sperm aggregates in *P. maniculatus* with
249 immotile, stuck, or morphologically abnormal cells (8.1%, 64/795) than in *P. polionotus* (11.5%,
250 94/814; binomial GLM: $n = 40$, $P = 0.00503$), a feature that we found significantly reduces VCL
251 in both species (*P. maniculatus* $VCL_{aligned} = 179.60 \pm 6.88$ μm/sec, $VCL_{defective} = 132.47 \pm 6.18$
252 μm/sec, paired t-test: $t = 20.627$, $df = 14$, $P = 7.075e-12$; *P. polionotus* $VCL_{aligned} = 135.48 \pm 3.51$
253 μm/sec, $VCL_{defective} = 115.62 \pm 5.22$ μm/sec, paired t-test: $t = 16.312$, $df = 20$, $P = 5.079e-13$).

254 When comparing the proportion of motile and progressively motile aggregates across all
255 species, we found *P. eremicus* produced the smallest proportion of motile aggregates ($P < 0.05$ for
256 all *P. eremicus* pairwise comparisons; $P > 0.05$ for all other pairwise comparisons). Fitted values
257 of the proportion of motile aggregates using LSmeans were 0.77 ± 0.04 for *P. eremicus*, $0.91 \pm$
258 0.02 for *P. californicus*, 0.91 ± 0.02 for *P. gossypinus,* 0.92 ± 0.02 for *P. polionotus,* 0.93 ± 0.02

259 for *P. leucopus*, and 0.95 ± 0.01 for *P. maniculatus*. Similarly, *P. eremicus, P. polionotus,* and *P.*
260 *gossypinus* all had the smallest proportions of progressively motile aggregates ($P < 0.05$ for all
261 pairwise comparisons; fitted values using LSmeans were 0.64 ± 0.06, 0.65 ± 0.05, and 0.75 ± 0.05,
262 respectively), the latter species of which did not significantly differ from *P. californicus* ($P =$
263 0.1981; 0.87 ± 0.02 for LSmeans fitted values). Conversely, *P. maniculatus* and *P. leucopus* had
264 similarly greater proportions of progressively motile aggregates ($P = 0.3417$; fitted values using
265 LSmeans were 0.94 ± 0.02 and 0.91 ± 0.02, respectively), the latter of which did not differ from *P.*
266 *californicus* ($P = 0.9109$).

267 Overall, we found species-specific differences in the effect of sperm aggregation on motility,
268 regardless of environmental complexity (Table 3, Figure 4). In low-viscosity medium, we found
269 that sperm aggregates have a significantly greater VCL in *P. maniculatus*, VSL in *P. maniculatus*,
270 *P. leucopus*, and *P. californicus*, LIN in *P. leucopus*, and VAP in *P. californicus* compared to
271 single cells but a significantly lower VCL, VSL, and VAP velocity in *P. polionotus* and *P.*
272 *gossypinus* than single sperm (Figure 4). In the high-viscosity medium, we found that sperm
273 aggregates have a significantly greater VCL in *P. maniculatus* and *P. californicus* and a higher
274 VSL and VAP in *P. californicus* compared to single sperm but a significantly lower LIN in *P.*
275 *californicus*, *P. eremicus*, *P. polionotus*, and *P. maniculatus* as well as a reduced VSL and VAP in
276 *P. polionotus* than single cells (Figure 4).

277

278 **Discussion**

279 While it is known that collective sperm behaviors have evolved independently in a number of
280 taxa [2], it remains unclear how sperm aggregation evolves among closely-related species. Our
281 comparative study reveals that sperm aggregating behaviors vary across mice in the genus
282 *Peromyscus,* and that the evolution of this trait is complex. We observed an effect of phylogenetic
283 history on the frequency of sperm aggregation, indicating that collective sperm behavior likely
284 evolved prior to the divergence of present-day species. Additionally, we find a negative

285 association between relative testis weight, a robust proxy for intensity of sperm competition in
286 rodents [50], and the coefficient of variation for the number of cells in aggregate, suggesting that
287 sexual selection has a stabilizing effect on sperm aggregate size. We then compared the motility of
288 single and aggregated sperm across all species under low- and high-viscosity conditions, and show
289 that aggregation is not kinematically beneficial for all species, regardless of environmental
290 complexity. Next, by examining sperm from the species that aggregate the most frequently, we
291 found support for the theoretical hypothesis [28] that cellular orientation within the group is
292 critical to its kinematics. Moreover, we observed more aggregates with efficient geometry in the
293 species that evolved under strong post-copulatory sexual selection compared to its monogamous
294 sister-species. These findings support the prediction that sperm aggregation likely evolved in
295 *Peromyscus* to improve motility in a competitive context but that relaxed selection may have
296 enabled less efficient strategies to persist, thereby generating diversity in collective sperm
297 behaviors within these closely-related species.

298 Our results reveal distinct species-specific differences across *Peromyscus* mice in the
299 frequency of sperm aggregation and the average size of these cellular groups. Multiple
300 *Peromyscus* species produce sperm that aggregate more extensively than other studied muroid
301 rodents [51], with the notable exception of the wood mouse (*Apodemus sylvaticus*; [4]). In general,
302 the proportion of sperm cells that aggregate is most similar within each sister-species pair.
303 Specifically, *P. maniculatus* and *P. polionotus* produce sperm that aggregate the most (>80% of
304 sperm), but the pair they are most closely-related to, *P. gossypinus* and *P. leucopus*, are the species
305 least likely to produce sperm that aggregate (<10% of sperm); the most distantly-related species
306 pair we assessed, *P. californicus* and *P. eremicus*, both produce sperm with a moderate propensity
307 to aggregate (~25% of sperm). These findings support one of two possibilities for the evolution of
308 sperm aggregates within these species: (a) a genus-wide ancestral trait of moderate sperm
309 aggregation with subsequent diversification leading to an increase in *P. maniculatus* and *P.*
310 *polionotus* and a decrease in *P. gossypinus* and *P. leucopus*, or (b) the independent evolution of

311 aggregation in the ancestors of the *P. californicus*-species pair and the *P. maniculatus*-species pair.
312 Such complex evolutionary histories with losses and recurrences of sperm conjugation, and
313 subsequent species divergence, have also been demonstrated in diving beetles (Dytiscidae [52]),
314 consistent with the evolutionary lability that we observe. Our experimental results may explain
315 selection against sperm aggregation if forming groups reduces sperm swimming performance,
316 which we find in at least one species, *P. gossypinus*. However, we found that sperm produced by
317 their sister-species, *P. leucopus*, also rarely aggregate, despite our observation that these rare
318 collective groups have a greater average velocity and are more linear than single cells.
319 Interestingly, we observed that these two promiscuous species that rarely aggregate, *P. gossypinus*
320 and *P. leucopus*, have the largest relative testes of the species studied, suggesting that they may
321 have evolved increased sperm production to improve competitive fertilization success [53], rather
322 than adaptations that influence motility [54]. Moreover, within these species (and *P. polionotus*)
323 we found a negative association between sperm speed and aggregation, even after controlling for
324 sperm density, indicating that differences in aggregate formation are not simply a by-product of
325 increased encounter rates. Together these results suggest different strategies employed by
326 divergent species across the *Peromyscus* genus in response to sperm competition.
327 In contrast to our results on the frequency of sperm aggregation within species, we found that
328 the average size of sperm groups does not align as closely with phylogenetic relationships. In
329 addition, we found that species with relatively larger testes, which is positively associated with
330 increased sperm competition [50], exhibit less variation (CV) in aggregate size. This result
331 supports the prediction that relaxed sperm competition allows for greater intermale variation to
332 persist in a population [55] and suggests that this post-copulatory sexual selection may be
333 stabilizing sperm aggregate size for a species-specific 'optimum' [28]. Similarly, other studies
334 have shown that the strength of sexual selection regulates variance in sperm morphology across
335 taxa and at multiple levels of organization, including within- and between-males [56–58] and
336 within- and between-species [55,59]. A study on sperm bundles across ten *Carabus* ground beetles

337 also found intense selection on bundle size, which are dimorphic and either small or large; the
338 large, but not small, sperm bundles are positively correlated with measures of sperm competition
339 risk, including copulatory piece length and mate guarding, suggesting that diversity of large sperm
340 bundles is associated with sexual selection [60]. In contrast to these findings that competition
341 drives sperm-sperm interactions, a study on the evolution of such sperm traits in diving beetles
342 found that variation in sperm conjugation is more associated with female reproductive tract
343 architecture [61]. Therefore, while our results suggest that stabilizing selection on sperm aggregate
344 size is associated with an increase in sperm competition given the correlation with relative testis
345 weight, mechanisms of female control [62] may also play an important evolutionary role.

346 We compared the motility of single and aggregated sperm sampled from the same male to test
347 whether sperm aggregates swim faster or more efficiently than single sperm, which is predicted if
348 the combined force of multiple flagella enhances their motility [4]. We found improved kinematic
349 measures in half of the species studied (*P. maniculatus, P. californicus*, and *P. leucopus*), thus
350 supporting this functional hypothesis; however, in several species we found aggregation had a
351 negative (*P. polionotus* and *P. gossypinus*) or no (*P. eremicus*) impact on motility relative to single
352 sperm cells. While we found support for a theoretical prediction that sperm aggregates achieve
353 greater straight-line velocity because they move in a more linear path of travel rather than at a
354 faster speed (i.e., curvilinear velocity; [28]) in two of our six focal species (*P. californicus* and *P.*
355 *leucopus*), we did not find this kinematic benefit for sperm aggregation in all *Peromyscus* species.
356 These results corroborate other studies in more disparate taxonomic groups that have quantified
357 sperm aggregation motility and found inconsistent results. For example, sperm trains exhibit
358 greater swimming progressive motility in the wood mouse [4], and greater velocity than individual
359 sperm in the Norway rat, but not the house mouse [5]. In invertebrates, the swimming velocity of
360 fishfly sperm increases with number of sperm in a bundle [10], but in a marine snail, there is no
361 differences in swimming speed between paired and single sperm [14]. One possible explanation

362 for these differences in sperm aggregate swimming performance across taxa is that cell orientation
363 within a group is critical for its collective motility.
364 Sperm cells are predicted to be faster if they generate increased force with proportionally less
365 drag; such effects may also be true for sperm aggregations in which cells conjoin head-to-tail,
366 thereby increasing the length of the collective unit [2]. In *Peromyscus,* sperm conjoin head-to-head
367 to form aggregates and are thus wider, not longer, yet can still offer a motility advantage for some
368 species. This kinematic benefit, however, was not observed in all species, which led us to
369 hypothesize that aggregate motility is dependent on the orientation of sperm within a group and
370 whether it contains abnormal or immotile cells. When sperm heads and flagella are not oriented in
371 the same direction, the cells within an aggregate are expected to exert opposing forces on one
372 another, thereby reducing the overall motility of the group [27,28]. To test this hypothesis, we
373 quantified aggregate geometry and composition in the two species that form the most frequent and
374 largest sperm aggregates. We found that in *P. maniculatus*, a promiscuous species, sperm were
375 more likely form aggregates with optimal geometry, compared to sperm from the monogamous *P.*
376 *polionotus*, which frequently included misaligned, opposing or morphologically abnormal cells.
377 Consistent with our prediction, *P. maniculatus* aggregates exhibit greater straight-line and
378 curvilinear velocity compared to single cells, but the opposite was true for *P. polionotus*. This
379 finding is consistent with a previous report that *P. polionotus* sperm tend to form optimal-sized
380 aggregates less often than in *P. maniculatus* [28]. Together, these observations further support the
381 hypothesis that sperm aggregation evolved prior to the divergence of the species pair [12], and
382 when monogamy evolved secondarily in *P. polionotus* [34,63], relaxed sexual selection allowed
383 for the persistence of less motile sperm traits. In line with this prediction, we observed the smallest
384 proportion of motile and progressively motile sperm aggregates in another monogamous species,
385 *P. eremicus,* but the largest proportion of progressively motile aggregates in two promiscuous
386 species, *P. maniculatus* and *P. leucopus*. Similar results have been reported in house mice, in
387 which males evolving under intense sperm competition produced a greater proportion of motile

388 sperm, compared to males from lineages subject to relaxed selection [64]. Based on these findings,
389 we suggest that relaxed sexual selection may allow the persistence of less optimal strategies in
390 *Peromyscus*.

391 When we compared single and aggregated sperm in a viscous environment to test if
392 aggregation improves motility in more complex fluids, similar to the secretions or cervical mucus
393 [18] of the female reproductive tract [17,65], we found that collective motion is only beneficial in
394 some species. Compared to single sperm, aggregates swim with greater curvilinear velocity in *P.*
395 *maniculatus* and *P. californicus*, and greater straight-line and average path velocities in *P.*
396 *californicus,* but aggregates were less linear than single cells in all species except for *P. leucopus*
397 and *P. gossypinus*. While other studies have found kinematic benefits for sperm conjugates
398 compared to single cells in higher viscosities in the gray short-tailed opossum [16], the wood
399 mouse [4], and the fishfly [10], a study in bulls found that sperm were slower, exhibited less
400 organized swimming patterns, and were less likely to cluster in viscous fluids [15], both of which
401 are consistent with our analysis of *Peromyscus* aggregates. Ultimately, the benefit of sperm
402 aggregation depends on the relative importance of each kinematic parameter during sperm
403 migration *in vivo*. Although beyond the scope of this study, we predict that improved linearity
404 afforded by collective motion may help to direct the sperm through the dynamic structures and
405 fluids of the female reproductive tract [66] and that increased velocity will reduce the time it takes
406 for sperm to arrive at the fertilization site.

407 In conclusion, our study highlights the diversity of sperm aggregation within a single
408 taxonomic lineage and how selection has shaped the formation and performance of these cellular
409 groups. We show that both evolutionary history and varying levels of post-copulatory sexual
410 selection influence the frequency and size of sperm groups. Moreover, we find that sperm
411 aggregation can improve sperm motility in both simple and complex fluids, but this is not
412 consistent across all species. Theoretical predictions [27,28] and emerging empirical evidence
413 suggests that motility benefits may only be realized if cells maintain optimal alignment within the

414 groups and, if achieved, may provide these sperm with a competitive advantage in the female
415 reproductive tract [61]. Future work investigating sperm aggregates *in vivo* [e.g., 66,67] will shed
416 light on the co-evolution of these unique gametic behaviors with the remarkably variable and
417 dynamic female reproductive tracts through which sperm must navigate.
418

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FIGURES AND TABLES

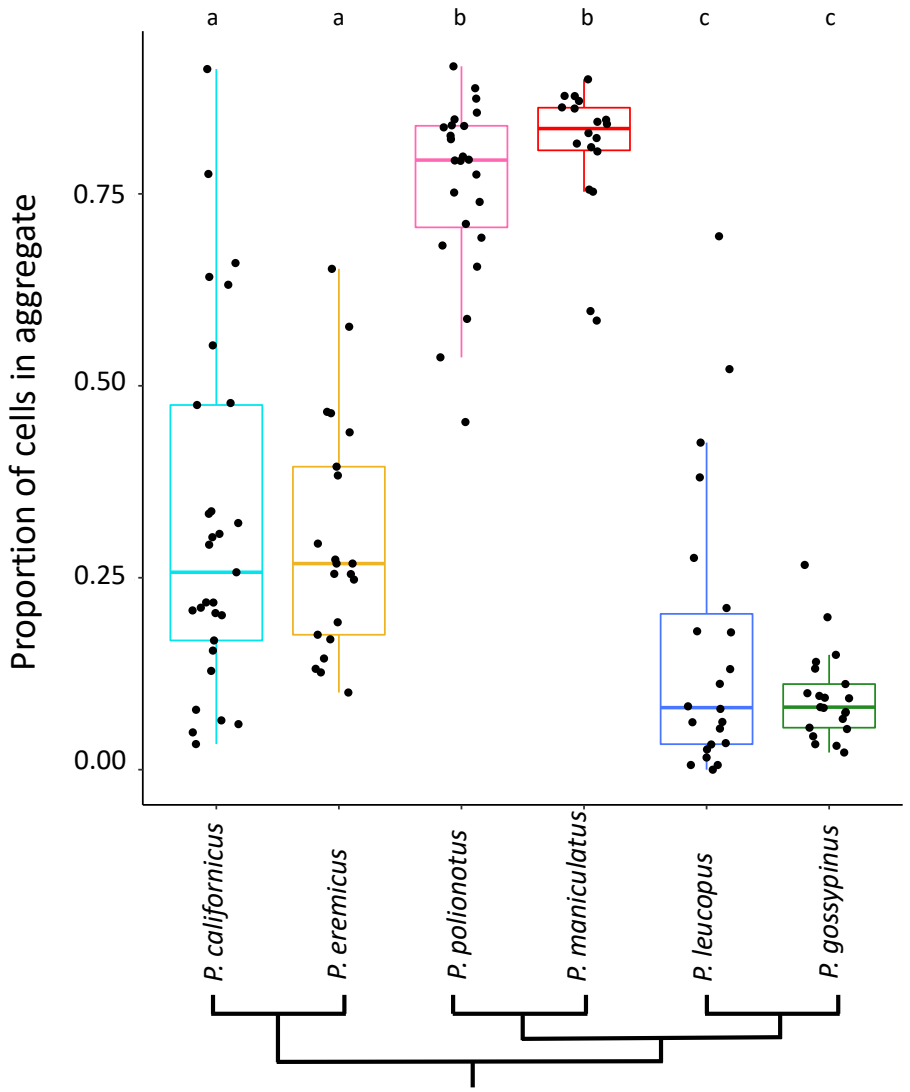

Figure 1.

The observed natural variation in the proportion of aggregated sperm cells for six closely related species of *Peromyscus* mice (phylogeny adapted from Bradley et al. 2007). Box-plots represent median and interquartile ranges with raw data overlaid. Statistically significant differences at the $P = 0.05$ level are denoted by differing letters; shared letters denote no statistical difference.

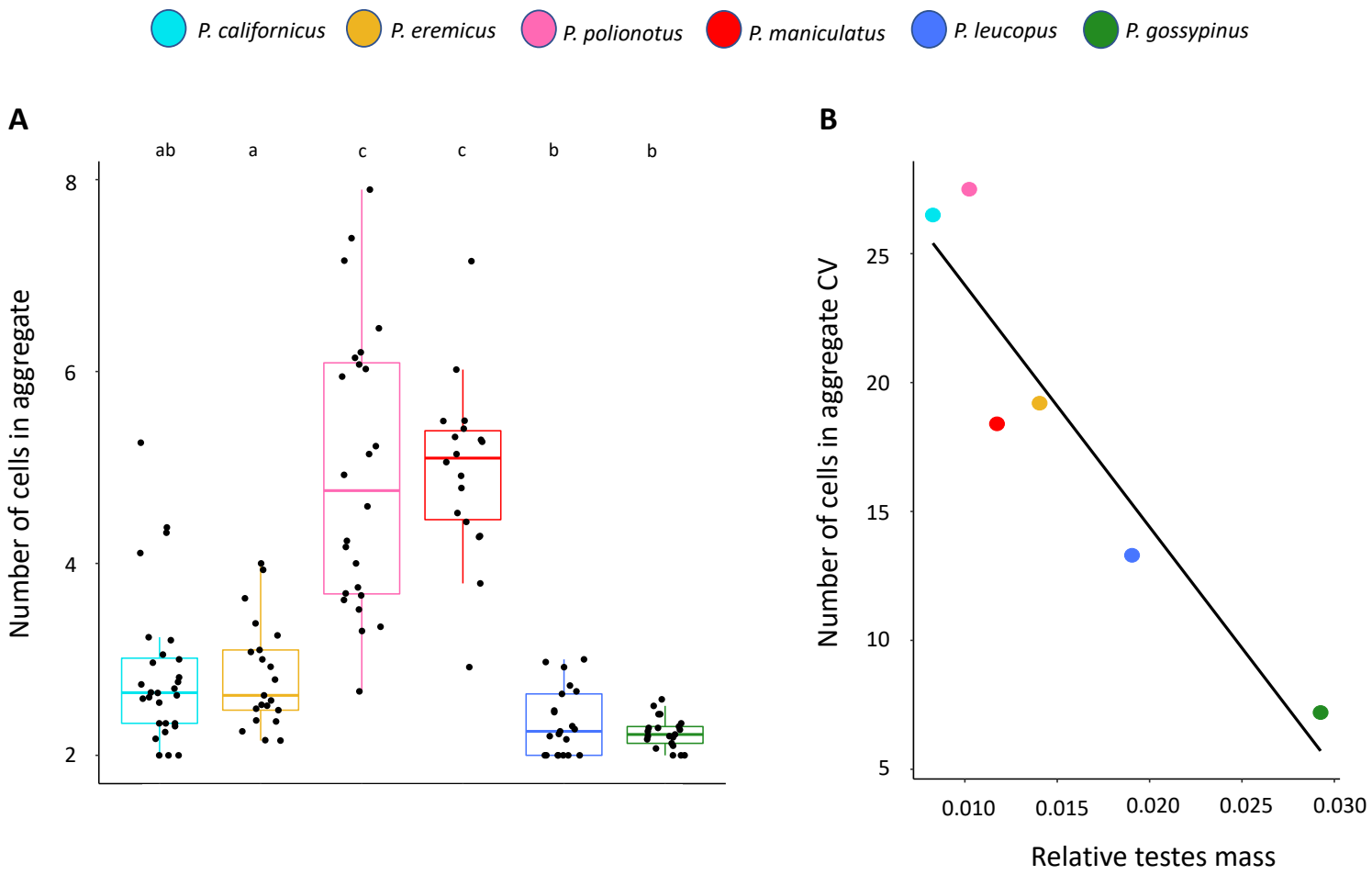

Figure 2.

Sperm aggregate size varies among species and is negatively associated with relative testes size. (A) The observed natural variation in the number of aggregated sperm cells for six closely related species of *Peromyscus* mice. Box-plots represent median and interquartile ranges with raw data overlaid. Statistically significant differences at the $P = 0.05$ level are denoted by differing letters; shared letters denote no statistical difference. (B) When controlling for phylogenetic relationships, the coefficient of variation (CV) for the number of aggregated sperm cells negatively correlates with relative testis mass across these species. Note truncated y-axes.

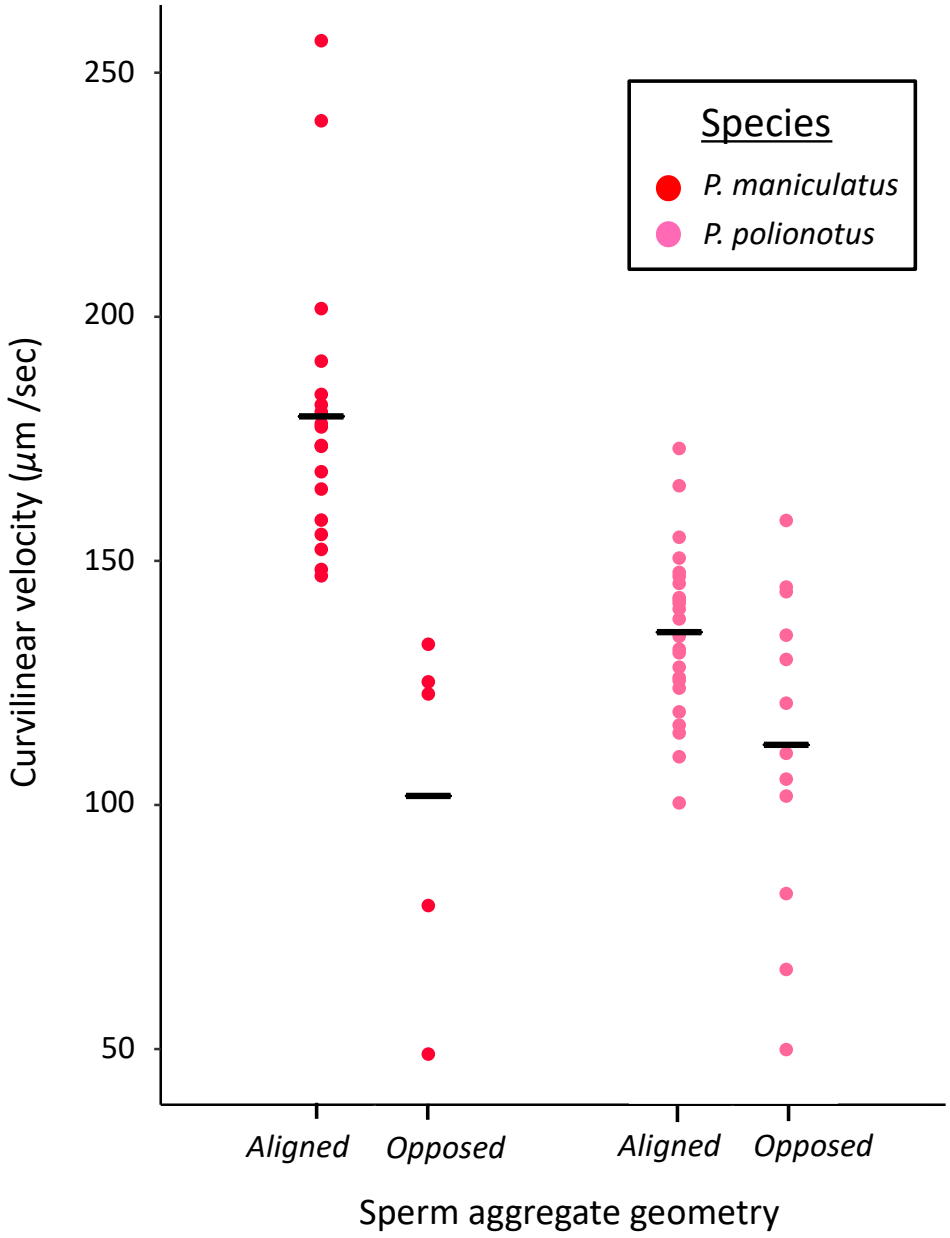

Figure 3.

The effect of sperm aggregate geometry on the curvilinear velocity (μm/sec) of sperm aggregates for two species that aggregated most – *Peromyscus maniculatus* and *Peromyscus polionotus*. Circles represent mean values per male within each species, and black lines represent the mean value within each category. Note truncated y-axis.

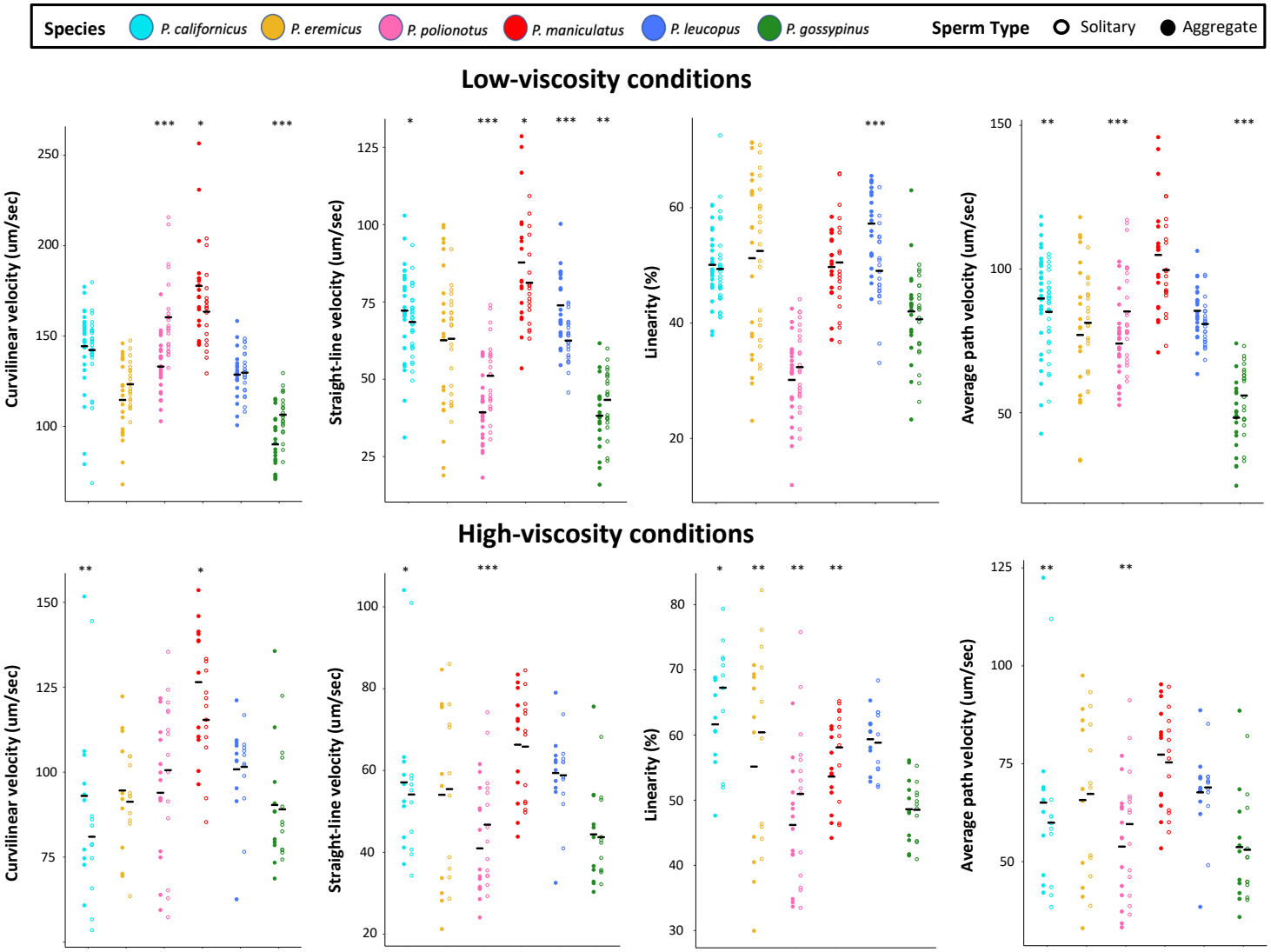

Figure 4.

Kinematic parameters of sperm aggregates (closed circles) and solitary sperm cells (open circles) for six species of *Peromyscus* mice in low- and high-viscosity conditions. Circles represent mean values per male, and black lines represent species means. Statistical significance levels comparing aggregated and solitary cells within each species are indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Note truncated y-axes.

TABLE 1.

Summary of live sperm aggregate results for mice in the genus *Peromyscus*

Peromyscus Species	No. males	No. sperm	% motile sperm	% progressive sperm	No. sperm aggregates	% cells in aggregate	% motile aggregates	% progressive aggregates
californicus	28	8235	81.2%	78.9%	823	29.7% (2446/8235)	90.5% (745/823)	86.6% (713/823)
eremicus	21	4906	67.4%	58.2%	513	30.2% (1481/4906)	77.8% (399/513)	66.7% (342/513)
polionotus	24	6360	82.4%	57.3%	949	79.5% (5059/6360)	90.9% (863/949)	66.1% (627/949)
maniculatus	18	4991	85.0%	84.3%	822	81.3% (4059/4991)	93.4% (768/822)	92.3% (759/822)
leucopus	22	6341	87.6%	84.5%	361	15.0% (949/6341)	90.3% (326/361)	88.1% (318/361)
gossypinus	21	5970	82.2%	70.3%	254	9.6% (576/5970)	88.6% (225/254)	70.1% (178/254)

Motile sperm cells include only those that exhibited movement patterns. Progressive sperm cells are motile cells with a straight-line velocity $\geq$ 25 μm/sec.

TABLE 2.

Fixed effects from a binomial generalized linear mixed model examining differences in the proportion of aggregated sperm cells and a linear model examining differences in the number of aggregated sperm cells across six species of *Peromyscus* mice

GLMM: PROPORTION OF CELLS IN AGGREGATE					
Model Term	Beta (SE)	Exp (beta)	95% CI	z	Pr(>|z|)
Intercept	1.52 (0.22)				
eremicus	-2.47 (0.30)	0.07	(0.04, 0.13)	-8.14	**< 0.001**
gossypinus	-3.94 (0.31)	0.02	(0.01, 0.03)	-12.89	**< 0.001**
californicus	-2.58 (0.29)	0.07	(0.04, 0.12)	-9.06	**< 0.001**
leucopus	-3.79 (0.30)	0.02	(0.01, 0.04)	-12.46	**< 0.001**
polionotus	-0.25 (0.29)	0.44	(0.30, 0.58)	-0.85	0.393
LM: NUMBER OF CELLS IN AGGREGATE					
Model Term	Beta (SE)	Exp (beta)	95% CI	t	Pr(>|z|)
Intercept	3.90 (0.26)				
Total Sperm Cells	0.00 (0.00)	0.50	(0.50, 0.50)	5.83	**< 0.001**
eremicus	-1.97 (0.24)	0.12	(0.08, 0.18)	-8.15	**< 0.001**
gossypinus	-2.77 (0.24)	0.06	(0.04, 0.09)	-11.53	**< 0.001**
californicus	-2.19 (0.23)	0.10	(0.07, 0.15)	-9.68	**< 0.001**
leucopus	-2.68 (0.24)	0.06	(0.04, 0.10)	-11.18	**< 0.001**
polionotus	0.04 (0.23)	0.51	(0.40, 0.62)	0.16	0.877

For both models, all rows are being compared with the intercept – *Peromyscus maniculatus*. 95% confidence intervals (CI) were calculated for each effect size.

TABLE 3.

Results from an intra-male analysis comparing motile solitary and aggregated sperm kinematics in low- and high-viscosity conditions for six species of *Peromyscus* mice to test whether sperm aggregates confer kinematic benefits (shaded in gray)

PEROMYSCUS SPECIES	df	KINEMATIC VARIABLE			
		Curvilinear Velocity (μm/sec)	Linearity (VSL/VCL)	Straight-Line Velocity (μm/sec)	Average Path Velocity (μm/sec)
LOW-VISCOSITY CONDITIONS					
californicus	*28*	t = -1.0545, p = 0.3007	t = -0.56153, p = 0.5789	t = -2.2982, **p = 0.02923**	t = - 2.8162, **p = 0.008805**
eremicus	*20*	t = 1.6225, p = 0.1204	t = 0.73517, p = 0.4708	t = 0.1312, p = 0.8969	t = 1.0464, p = 0.3079
polionotus	*23*	t = 9.4575, **p = 2.1566e-09**	t = 1.699, p = 0.1028	t = 5.8355, **p = 6.026e-06**	t = 6.0729, **p = 3.408e-06**
maniculatus	*17*	t = 2.2482, **p = 0.03812**	t = 0.48075, p = 0.6368	t = -2.2335, **p = 0.03924**	t = -1.9206, p = 0.07172
leucopus	*20*	t = 0.23337, p = 0.8178	t = -4.8385, **p = 9.973e-05**	t = -5.5521, **p = 1.959e-05**	t = -1.838, p = 0.08096
gossypinus	*20*	t = 5.4048, **p = 2.73e-05**	t = -1.0247, p = 0.3177	t = 3.715, **p = 0.001369**	t = 4.479, **p = 0.0002298**
HIGH-VISCOSITY CONDITIONS					
californicus	*9*	t = -3.7465, **p = 0.003357**	t = 2.682, **p = 0.02512**	t = -2.7743, **p = 0.0216**	t = -4.1106, **p = 0.002634**
eremicus	*9*	t = -0.71739, p = 0.4913	t = 4.2087, **p = 0.002277**	t = 0.62918, p = 0.5449	t = 0.56505, p = 0.5858
polionotus	*13*	t = 2.0349, p = 0.06278	t = 3.9242, **p = 0.001745**	t = 4.8314, **p = 0.0003279**	t = 4.18, **p = 0.001079**
maniculatus	*11*	t = -2.9397, **p = 0.01345**	t = 3.9259, **p = 0.002369**	t = -0.30835, p = 0.7636	t = -1.0543, p = 0.3114
leucopus	*9*	t = 0.32429, p = 0.7531	t = -0.31635, p = 0.759	t = -0.34159, p = 0.7405	t = 0.95055, p = 0.3667
gossypinus	*10*	t = -0.63835, p = 0.95376	t = -0.076451, p = 0.9406	t = -0.60402, p = 0.5593	t = -0.71643, p = 0.4901

Statistical results are based on paired-student t-tests.