Post-copulatory sexual selection is associated with sperm aggregate quality in *Peromyscus* mice

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Abstract: In some species, sperm form coordinated groups that are hypothesized to improve their swimming performance in competitive contexts or to navigate through the viscous fluids of the female reproductive tract. Here we investigate sperm aggregation across closely-related species of *Peromyscus* mice that naturally vary by mating system to test the predictions that sperm aggregates (1) are faster than solitary sperm in species that females mate multiply to aid cells in sperm competition, and (2) outperform solitary sperm cells in viscous environments. We find significant variation in the size of sperm aggregates, which negatively associates with relative testis mass, a proxy for sperm competition risk, suggesting that post-copulatory sexual selection has a stabilizing effect on sperm group size. Moreover, our results show that sperm aggregates are faster than solitary sperm in some, but not all, species, and this can vary by fluid viscosity. Of the two species that produce the largest and most frequent groups, we find that sperm aggregates from the promiscuous *P. maniculatus* are faster than solitary sperm in every experimentally viscous environment but aggregation provides no such kinematic advantage under these same conditions for the monogamous *P. polionotus*. The reduced performance of *P. polionotus* aggregates is associated with less efficient aggregate geometry and the inclusion of immotile or morphological abnormal sperm. Our cross-species comparison yields insight into the evolution of sperm social behaviors, provides evidence of extensive variation in the *Peromyscus* lineage, and reveals that differences in sperm aggregate quality associate with post-copulatory sexual selection.

Keywords: mating systems; sexual selection; sperm competition; sperm conjugation; sperm motility

Introduction

Sperm cells are one of the most diverse cell types in nature and exhibit striking variation both within and across taxa (Pitnick et al. 2009). 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 (Pitnick et al. 2009; Higginson and Pitnick 2011; Schoeller et al. 2020). Although this phenomenon is relatively rare, sperm aggregation has evolved multiple times across independent lineages of internally fertilizing species (reviewed in Immler 2008; Pitnick et al. 2009; Higginson and Pitnick 2011; Umezu et al. 2020), suggesting that these unique sperm-sperm interactions may be a convergent adaptation driven by a shared selective force.

The primary selective force that has been hypothesized to drive the evolution of sperm aggregation is post-copulatory sexual selection and, more specifically, sperm competition (Moore et al. 2002; Immler et al. 2007; Fisher and Hoekstra 2010). Sperm competition occurs when females mate with multiple males within a reproductive cycle (i.e., polyandry), allowing the sperm produced by those males to compete for fertilization of her ova (Parker 1970; Simmons 2001). Within these competitive contexts, collective sperm groups are posited to be advantageous if the combined force generated by their multiple flagella enable them to swim faster (Moore et al. 2002; Immler et al. 2007) or to migrate more efficiently through the viscous or viscoelastic secretions of the female reproductive tract (Moore and Taggart 1995; Suarez 2016), thus enhancing their fertilization success (Birkhead et al. 1999; Gage et al. 2004). Among the relatively few studies that have attempted to elucidate the advantages of collective sperm behavior, only some have provided empirical support that sperm aggregates are faster than solitary cells in competitive or complex environments (Hayashi 1998; Moore et al. 2002; Fisher and Hoekstra 2010; Pearcy et al. 2014; but see Ishijima et al. 1999). However, given the mixed results yielded by interspecific analyses (i.e., Moore and Taggart 1995; Immler et al. 2007; Pitnick et al. 2009; Woolley et al. 2009; Tung et al. 2017), the benefits of sperm conjugation are likely to be taxon- and context-dependent.

54 One potential reason for inconsistencies regarding the kinematic benefits of sperm aggregates
55 across taxonomic groups is that they vary in when and how they form (Higginson and Pitnick
56 2011; Monclus and Fornes 2016) and, consequently, often differ in the number of cells that are
57 present within them (Pizzari and Foster 2008). For example, some mammalian sperm conjugates
58 are derived from spermatogenic processes and assemble during epididymal transport (Higginson
59 and Pitnick 2011), forming groups with very little intraspecific variation; these sperm are often
60 molecularly "glued" to one another, such as the bi-flagellate pairs in grey short-tailed opossums
61 (*Monodelphis domestica*, Taggart et al. 1993), sperm 'rouleaux' of five or more cells in guinea
62 pigs (*Cavia porcellus*, McGlinn et al. 1979), or bundles of roughly 100 cells in monotremes
63 (Nixon et al. 2016). Conversely, some mammalian sperm conjugates are derived from post-
64 spermatogenic processes and assemble after ejaculation (Higginson and Pitnick 2011), thus
65 forming more variably-sized groups. For example, sperm may form temporary clusters of up to
66 sixteen cells in bulls (*Bos Taurus*, Tung et al. 2017) or more fixed groups of 3-30 cells in house
67 mice (Immler et al. 2007), 5-50 cells in the Norway rat (Immler et al. 2007), or hundreds to
68 thousands of cells in the wood mouse (*Apodemus sylvaticus*, Moore et al. 2002). These systems
69 with post-spermatogenic aggregation are ideal for assessing the kinematic consequences of
70 aggregation because their ejaculates contain both solitary and aggregated sperm cells, thereby
71 enabling a direct comparison of these naturally occurring sperm types produced by the same male.
72 Furthermore, these systems allow for investigations into the functional significance of aggregate
73 size variation upon which selective pressures like sperm competition can act.
74 Closely-related species of mice in the genus *Peromyscus* naturally vary in their mating
75 systems (Turner et al. 2010; Bedford and Hoekstra 2015), and at least two species are known to
76 produce sperm cells that form temporary, motile groups after ejaculation (Fisher and Hoekstra
77 2010). Of these, sperm produced by the promiscuous deer mouse (*P. maniculatus*), selectively
78 group with the most closely-related sperm to form aggregates of up to 30 cells (Fisher and
79 Hoekstra 2010), but there is a non-monotonic association between aggregate size and swimming

80 velocity, indicating that only aggregates of a certain size are faster than solitary cells (Fisher et al.
81 2014). In comparison, sperm from its monogamous congener, the oldfield mouse (*P. polionotus*),
82 indiscriminately group with both related and unrelated cells (Fisher and Hoekstra 2010), and their
83 aggregates are less likely to be optimally-sized (Fisher et al. 2014). These studies provide
84 compelling evidence that post-copulatory sexual selection has influenced the evolution of sperm
85 aggregation within this system, but the broader extent of sperm aggregation within the *Peromyscu*s
86 lineage remains unknown.

87 In this study, we examine the function of sperm aggregation and the role of post-copulatory
88 sexual selection in shaping the evolution of these collective sperm behaviors in *Peromyscus* mice.
89 We compared sperm from three monogamous species (*P. californicus*, *P. eremicus*, *P. polionotus*)
90 and three species in which females mate with multiple males (*P. maniculatus*, *P. leucopus*, *P.*
91 *gossypinus*), under consistent, controlled *in vitro* conditions that we experimentally altered to
92 modify the viscosity of the fluid. From these observations, we (1) describe the naturally occurring
93 intra- and inter-specific variation in sperm aggregation in these species, (2) test the prediction that
94 sperm aggregates are faster than solitary cells in the species with more intense sperm competition,
95 and (3) test the prediction that sperm aggregates are faster than solitary cells in viscous
96 environments.
97

98 **Materials and Methods**

99 (a) Sperm collection and standardization

100 We obtained captive *Peromyscus maniculatus bairdii, P. polionotus subgriseus, P. leucopus*,
101 *P. eremicus*, and *P. californicus insignis* from the Peromyscus Genetic Stock Center at the
102 University of South Carolina, and *P. gossypinus gossypinus* from Dr. Hopi Hoekstra at Harvard
103 University and housed them in same-sex cages at 22ºC on a 16L:8D cycle in accordance with
104 guidelines established by the Institutional Animal Care and Use Committee at the University of
105 Maryland in College Park (protocol R-Jul-18-38). We sought samples from all available captive

106 *Peromyscus* species, avoiding wild-caught specimens to control for variation due to life
107 experience. All males were sexually mature and we accounted for relatedness by assigning siblings
108 a unique 'Family' ID.
109 To collect sperm, we euthanized males via isoflurane overdose and cervical dislocation,
110 recorded their body and testes weight, and then removed a single caudal epididymis. We made
111 several small incisions in the epididymis and submersed it in a centrifuge tube containing medium
112 (Modified Sperm Washing Medium, Irvine Scientific, USA) that was pre-warmed at 37ºC. To help
113 standardize sperm concentrations across males based on qualitative assessments of the epididymal
114 tissue size, we varied the media volume (50µl - 1000µl) by male and accounted for these
115 volumetric differences when estimating sperm counts. We agitated the tube containing tissue and
116 media at 300rpm (ThermoMixer F1.5, Eppendorf, Germany) at 37ºC for ten minutes, inverting it
117 at the five- and ten-minute marks and then incubating it for two minutes undisturbed. Using pipette
118 tips with a cut tip to create a wider opening and avoid breaking up aggregates, we collected live
119 sperm for analysis from just below the meniscus of the solution to enrich for the most motile
120 sperm (Magdanz et al. 2019). We used a computer-assisted sperm analysis (CASA) system (Ceros
121 II™ Animal, Hamilton Thorne, USA) to rapidly assess sperm concentrations within each sample,
122 which we later verified via manual sperm counts using a hemocytometer. We then diluted samples
123 as needed with pre-warmed sperm medium to reach a standard concentration of cells across all
124 samples (Supplementary Materials).
125
126 (b) Live sperm analysis
127 To prepare live sperm samples for *in vitro* observations in a 'low-viscosity' environment, we
128 gently reverse pipetted 4µl of the sperm solution into 12µl of pre-warmed medium on a plastic
129 slide within a 9mm x 0.12mm imaging spacer (Grace Bio-Labs, USA) and covered it with a plastic
130 cover slip (Supplementary Materials). To prepare the same sperm samples for *in vitro* observations
131 in a 'high-viscosity' environment, we mixed 4µl of sperm solution with 12µl of pre-warmed

132 medium enriched with methylcellulose (Sigma Aldrich M 7140; 15cP, 2% in water; Suarez and
133 Dai 1992) to produce three experimental treatment groups with increasing viscosity levels of
134 0.75%, 1.5%, or 2.25% methylcellulose solutions (see Supplementary Materials). We recorded a
135 minimum of five 5-second videos at 60 frames/second per male, except in one case in which we
136 were only able to obtain four videos. The mean (±SE) duration of time between tissue dissection
137 and video observations (i.e., 'post-harvest time') was 59 (±1) minutes, which varied slightly
138 depending on how long it took to properly dilute each sample.

139 From the sperm videos obtained using the CASA system, we manually scored the number of
140 sperm cells present within each track (i.e., aggregate size) by observing at least three different
141 frames per track. When tracking sperm, CASA automatically excluded any tracks in which cells
142 left the field within the first 10 frames or entered the field or started moving after the first 10
143 frames. Through our manual evaluation of each track, we further removed tracks if they involved
144 cells or aggregates that were attached to debris or the slide surface, too out of focus to count the
145 number of cells, interacted with other cells or aggregates (e.g., colliding, joining, separating, or
146 impeding movement), or had a distance average path < 30 μm (often repeats of longer, more
147 informative tracks). From the remaining tracks, we categorized each track as either "solitary" or
148 "aggregated". We calculated mean *sperm aggregate size* (i.e., sperm cells per aggregate) by
149 dividing the number of aggregated sperm cells by the total number of aggregates. We further
150 scored aggregates that were comprised of at least one cell that was immotile (i.e., unmoving or
151 stuck), misaligned (i.e., cells opposed one another, interacted loosely, or formed via attachment
152 with the flagella rather than at the head region), or morphologically abnormal (i.e., kinked at the
153 midpiece or missing a head or flagella) to determine the proportion of aggregates that featured
154 these abnormalities ('abnormal') compared to those that did not ('normal'). We pooled these data
155 either across all tracks per male or across all males per species to characterize sperm aggregates by
156 males and species, respectively (Crawley 2013). Last, we calculated the coefficient of variation
157 (CV) for aggregate size within each species using the following formula: (standard

158 deviation/mean) x 100.

159 From the same CASA video recordings used to calculate the size and composition of sperm
160 aggregates, we collected swimming performance data for all motile sperm tracks. To create a
161 dataset of only motile sperm tracks, we added additional restrictions to those outlined above for
162 our aggregate analysis, including the removal of cells and aggregates that were immotile (i.e.,
163 unmoving) or poorly tracked by CASA (i.e., featuring a low number of detection points, points
164 that jumped suddenly, or points that picked up other regions of the cell). We additionally removed
165 any solitary cells that were morphologically abnormal (e.g., featuring a kinked midpiece section
166 that resulted in a backwards swimming pattern). From these data, we determined the speed
167 (curvilinear velocity, VCL) for both solitary and aggregated sperm cells for each male by pooling
168 their motile tracks within the different media treatment groups (i.e., low-viscosity or high-
169 viscosity).

170

171 (c) Statistical Analyses

172 We performed all statistical analyses using R version 3.4.2 (R Core Team 2016) and created
173 all figures using the 'ggplot2' package with R (Wickham 2016). To validate model normality, we
174 visually inspected diagnostic plots (qqplots and plots of the distribution of the residuals against
175 fitted values). Only the best fitting models are reported here.

176 To investigate differences in sperm aggregate size across our focal *Peromyscus* species, we
177 used a linear model (LM) and mean values for each male. We excluded one *P. californicus* male
178 whose measurement represented a clear outlier; the removal of this individual did not qualitatively
179 change the results (see Supplementary Materials). We initially used a linear mixed model (LMM)
180 using the lmer function from the "lme4" R package (Bates et al. 2015) with 'Family ID' as a
181 random factor, but this variable did not significantly contribute to the residual variability in the
182 response variable. Predictors that were considered for the initial LMM included male age, pairing
183 status, post-harvest time, total sperm cells, number of recorded videos, and the ratio of sperm cells

184 per video. We considered predictors with *p*-values < 0.20 for the final model, but first screened
185 each for collinearity with other significant predictors using a linear model. Whenever collinearity
186 was present, only the predictor with the greater relative significance was included in the model.
187 We dropped non-significant explanatory variables one at a time based on model comparisons
188 using an analyses of variance test to determine the minimal adequate model, which included total
189 sperm cells and species as predictors. We performed post-hoc pairwise species comparisons using
190 Tukey HSD adjustments for multiple comparisons from the "LSmeans" R package (Lenth 2016).
191 We next examined the relationship between mean sperm aggregate size and sperm aggregate
192 size CV with the mean relative testis size using mean values for each species. We accounted for
193 the evolutionary relationships among the species used in this study by adopting a phylogenetic
194 generalized least squares (PGLS) approach (Pagel 1999; Freckleton et al. 2002) using the "caper"
195 (Orme et al. 2013) and "APE" (Paradis et al. 2004) packages in R. To do so, we used an ultra-
196 metric phylogenetic tree of *Peromyscus* (provided by Dr. Roy Neal Platt II, Texas Biomedical
197 Research Institute) based on sequence variation in the mitochondrial gene, cytochrome B, as a
198 covariate in regression analyses. This phylogeny was similar to other previously established
199 *Peromyscus* phylogenies (Bradley et al. 2007; Turner et al. 2010). Predictors that were considered
200 for both regression models included testis mass and body mass – a method better suited to
201 estimating relative testis weight rather than using the ratio of testis to body mass or residuals
202 (García-Berthou 2001; Lüpold et al. 2009).
203 To test whether sperm aggregates offer a kinematic benefit, we used a paired student's t-test
204 for each species separately and mean values per male to control for inter-male variation; we
205 compared the VCL of sperm aggregates to the VCL of solitary sperm cells. We performed these
206 statistical tests for motility data collected when sperm were suspended in either low- or high-
207 viscosity media to examine whether the kinematic benefits of aggregation are context dependent.
208 To compare the composition of sperm aggregates, we used the number of abnormal aggregates
209 and total sperm aggregates summed across all males within each species (Crawley 2013) within a

210 multiple proportions test to examine whether species differ in the proportion of sperm aggregates
211 that feature abnormalities. We then compared the VCL of normal sperm aggregates to abnormal
212 sperm aggregates using paired student's t-test for each species separately and mean values per
213 male to control for inter-male variation.
214
215 **Results**
216 We found that sperm aggregate size differs among *Peromyscus* species (LM: $F_{6,126} = 56.31$, p
217 < 0.001; Figure 1A, Table 1), indicating substantial trait variation within this lineage. We found
218 greater variance in the number of aggregated cells between than within species (proportion: s^2
219 across species = 1.96; s^2 within species < 1.00, except for *P. polionotus* [$s^2 = 2.15$]; Figure S1).
220 Pairwise comparisons adjusted for multiple comparisons using LSmeans revealed that *P.*
221 *gossypinus*, *P. leucopus*, and *P. californicus* produce the smallest aggregates ($p > 0.05$ for pairwise
222 comparisons), the latter of which produces sperm cell aggregates that are statistically similar in
223 size to those produced by *P. eremicus* ($p = 0.92$; Figure 1A). Conversely, the largest aggregates
224 were observed in two sister species, *P. maniculatus* and *P. polionotus*, which produced similarly
225 sized aggregates that did not statistically differ ($p = 0.88$).
226 Moreover, we found a negative association between the coefficient of variation (CV) for
227 sperm aggregate size and testis weight (PGLS: $F_{2,3} = 6.96$, $p = 0.034$) across these focal species
228 (Figure 1B, Table 2), although there was no such association between aggregate size itself and
229 testis weight (PGLS: $F_{2,3} = 3.058$, $p = 0.153$; Table 2). The sperm aggregate size CV for each
230 species were as follows: 27.6% *P. californicus*, 19.5% *P. eremicus*, 29.5% *P. polionotus*, 18.2% *P.*
231 *maniculatus*, 14.9% *P. leucopus*, and 7.3% *P. gossypinus*.
232 When comparing the mean curvilinear velocity (VCL) of aggregated to solitary sperm cells,
233 we found that sperm aggregates are significantly faster in all experimental media (0%, 0.75%,
234 1.5%, or 2.25% methylcellulose) in only one species, the promiscuous deer mouse (*P.*
235 *maniculatus*; Table 3, Figure 2, Table S1). In the low-viscosity environment (0% methylcellulose),

236 we found that sperm aggregates are slower in two species, *P. polionotus* and *P. gossypinus*; the
237 speed of sperm aggregates did not statistically differ from solitary cells in *P. californicus, P.*
238 *eremicus*, and *P. leucopus* (Table 3, Figure 2). Within all of the high-viscosity environments
239 (0.75%, 1.5%, or 2.25% methylcellulose), we found that sperm aggregates were faster that solitary
240 sperm in *P. californicus* (Table 3, Figure 2, Table S1). We also found that for at least one higher
241 viscosity environment, sperm aggregates were relatively faster than solitary sperm in *P. eremicus*
242 (1.5% methylcellulose), *P. gossypinus* (1.5% methylcellulose), and *P. leucopus* (2.25%
243 methylcellulose); otherwise, there were no significant differences between the speed of sperm
244 aggregates and solitary sperm cells (Table 3, Figure 2, Table S1).
245 When comparing the composition of sperm aggregates, we observed the highest number of
246 abnormal aggregates in the three species with promiscuous mating systems (*P. maniculatus* =107*,*
247 *P. leucopus* = 128; *P. gossypinus* = 58) and the lowest number of abnormal aggregates in the three
248 species with monogamous mating systems (*P. eremicus* = 266, *P. polionotus* = 273, *P. californicus*
249 = 187; Figure 3a). When comparing abnormal aggregates to the total number of aggregates
250 observed, we found that species differ in the proportion of 'abnormal' aggregates that they
251 produce (multiple proportions test: $\chi^2 = 270.12$, df = 5, $p < 0.001$), with *P. maniculatus* producing
252 the lowest proportion of abnormal sperm aggregates (abnormal/total: 107/822) and *P. eremicus*
253 producing the highest proportion of abnormal aggregates (266/513) compared to all other species
254 (*P. californicus* 187/858; *P. polionotus* 273/950; *P. leucopus* 128/361; *P. gossypinus* 58/254;
255 Figure 3a). Importantly, we found that abnormality can negatively impact aggregate speed;
256 abnormal sperm aggregates have a significantly lower VCL compared to normal sperm aggregates
257 in all species except *P. leucopus* and *P. gossypinus* (Table 4, Figure 3b).
258
259 **Discussion**
260 Collective sperm behaviors have evolved independently in a number of taxa (Higginson and
261 Pitnick 2011; Schoeller et al. 2020), yet their functional significance remains poorly understood.

262 Here we examine the evolution of sperm aggregation in closely-related species of *Peromyscus*
263 mice that vary by mating strategy. In *Peromyscus*, males simultaneously produce solitary and
264 aggregated sperm cells, allowing us to directly compare these naturally occurring sperm types.
265 These comparisons revealed interspecific differences in the size of sperm aggregates that form,
266 and importantly, that variability in aggregate size negatively associates with relative testis weight,
267 a robust proxy for level of sperm competition in rodents (Ramm et al. 2005), suggesting that this
268 trait may be under stabilizing selection. We also found that collective sperm groups enhance
269 motility in some, but not all, species and in some, but not all, environments. Our data revealed that
270 these differences in aggregate speed are associated with the orientation and composition of the
271 cells within the group (Fisher et al. 2014), and that those species that evolved under high levels of
272 sperm competition produced the fewest number of abnormal aggregates. Together, our findings
273 suggest that post-copulatory sexual selection can play an important role in shaping overall sperm
274 aggregate quality, including motility, but that relaxed selection in monogamous taxa may have
275 enabled less efficient strategies to persist (van der Horst and Maree 2014), thereby generating
276 diversity in collective sperm behaviors within and between closely-related species.
277 Under controlled *in vitro* conditions, we found distinct differences in the size of sperm
278 aggregates produced by males across six closely related species of *Peromyscus* mice, with two
279 sister species (*P. maniculatus* and *P. polionotus*) forming large sperm aggregates, two sister
280 species (*P. californicus* and *P. eremicus*) forming moderately sized aggregates, and two sister
281 species (*P. leucopus* and *P. gossypinus*) forming small sperm aggregates. Moreover, we found that
282 that species with relatively larger testes, which positively associates with sperm competition risk
283 (Ramm et al. 2005), exhibit less variation in aggregate size. This result supports the prediction that
284 relaxed sperm competition allows for greater intermale variation to persist in a population (Calhim
285 et al. 2007) and suggests that post-copulatory sexual selection may be stabilizing sperm aggregate
286 size for a species-specific 'optimum' (Fisher et al. 2014). Other studies have shown that the
287 strength of sexual selection regulates variance in sperm morphology across taxa and at multiple

288 levels of organization, including within- and between-males (Immler et al. 2008; Fitzpatrick and
289 Baer 2011; Carballo et al. 2019) and within- and between-species (Calhim et al. 2007; Rowley et
290 al. 2019). Similarly, a study on sperm bundles across ten *Carabus* ground beetles also found
291 intense selection on bundle size, which are dimorphic and either small or large; the large, but not
292 small, sperm bundles are positively correlated with measures of sperm competition risk, including
293 copulatory piece length and mate guarding, suggesting that diversity of large sperm bundles is
294 associated with sexual selection (Takami and Sota 2007). In contrast to these findings that sperm
295 competition drives sperm-sperm interactions, a study on the evolution of these interactions in
296 diving beetles found that variation in sperm conjugation associates more with female reproductive
297 tract architecture (Higginson et al. 2012). Therefore, while our results show that the uniformity of
298 sperm aggregate size associates with an increase in sperm competition risk, mechanisms of female
299 control (Eberhard 1996) may also play an important evolutionary role as a stabilizing force on this
300 trait.

301 We found that aggregation enhances sperm motility in some, but not all, species and in some,
302 but not all, environments; thus, our results provide mixed support for the hypotheses that the
303 combined force generated by an aggregate's multiple flagella improves motility in the context of
304 sperm competition (Moore et al. 2002; Immler et al. 2007) and/or may allow them to migrate more
305 efficiently through the viscous or viscoelastic secretions of the female reproductive tract (Moore
306 and Taggart 1995; Suarez 2016). It is unclear if sperm grouping within an ejaculate is random or if
307 certain cells (e.g., slower sperm) tend to group together, thereby forming slower aggregates.
308 Moreover, because the viscosity female reproductive fluids in *Peromyscus* are unknown, it is not
309 clear how they compare to our artificial suspension media or if they differ among species, thus we
310 are limited in our interpretation of how sperm aggregation influences movement in the female
311 reproductive fluids (Gasparini et al. 2020). In addition, a recent study reported that cell-cell
312 interactions may be important for sperm migration through environments that mimic the highly-
313 folded epithelium of the mammalian female reproductive tract (Bukatin et al. 2020). We can say

314 with confidence, however, that sperm aggregates are not always faster than solitary cells, nor do
315 they necessarily always perform better in viscous environments. Overall, we observed that sperm
316 cells, including sperm aggregates, are slower as the viscosity of their environment is increased.
317 In only the promiscuous *P. maniculatus* did we find that sperm aggregates are relatively faster
318 than solitary sperm in every environmental context regardless of viscosity, supporting the
319 functional hypothesis that sperm aggregates evolved to be faster in the face of sperm competition
320 (Moore et al. 2002; Immler et al. 2007). Its monogamous congener, *P. polionotus*, produces
321 similarly large sperm aggregates, which are significantly bigger than the aggregates of other
322 species, but *P. polionotus* sperm aggregates are slower in low viscosity media and no faster than
323 solitary cells in more viscous environments. Together these findings suggest that the emergence of
324 sperm aggregation likely evolved prior to the divergence of these two present-day species, which
325 have been under divergent selective pressures due to differences in mating systems (Bedford and
326 Hoekstra 2015). Our findings support that relaxed sexual selection and lack of intense sperm
327 competition in *P. polionotus* has allowed for the degeneration of sperm aggregate motility (van der
328 Horst and Maree 2014), despite the persistence of high levels of sperm aggregation. Given that *P.*
329 *polionotus* aggregates were no faster than solitary sperm in any of the experimentally viscous
330 environments, this suggests a loss of functional significance, although we cannot rule out a
331 different function for sperm aggregation in this species that we failed to explore in this study. For
332 example, agglutinated bovine sperm have increased motility and more functional mitochondria,
333 suggesting that they maintain viability and motility for a longer period of time *in vitro*, thus may
334 be important in mammalian fertilization (Umezu et al. 2020).
335 Our finding that the relative motility advantages of sperm aggregation differs depending on the
336 environment for the other focal *Peromyscus* species corroborates studies in more disparate
337 taxonomic groups, which have quantified collective sperm motility and found inconsistent results.
338 For example, sperm trains exhibit greater progressive motility in the wood mouse (Moore et al.
339 2002), and greater velocity than single sperm in the Norway rat, but not the house mouse (Immler

340 et al. 2007). In invertebrates, the swimming velocity of fishfly sperm increases with number of
341 sperm in a bundle (Hayashi 1998), but in a marine snail, there is no differences in swimming speed
342 between paired and single sperm (Ishijima et al. 1999). Although it was beyond the scope of this
343 study, additional work is needed to elucidate the conditions within the female reproductive tract
344 (e.g., fluid viscosity) so that they can be approximated *in vitro*, or observed *in vivo* (Ishikawa et al.
345 2016; Wang and Larina 2018; Qu et al. 2020), to determine if sperm aggregation does indeed
346 allow for more efficient migration through viscous female secretions (Moore and Taggart 1995;
347 Suarez 2016).

348 Sperm cells are predicted to be faster if they generate increased force with proportionally less
349 drag; such effects may also be true for sperm aggregations in which cells conjoin head-to-tail,
350 thereby increasing the length of the collective unit (Higginson and Pitnick 2011). In *Peromyscus,*
351 sperm conjoin head-to-head to form aggregates (Fisher et al. 2014) and thus are wider, not longer,
352 yet can still offer a motility advantage in some instances. This kinematic benefit, however, was not
353 observed in all species, which led us question whether aggregate motility is dependent on the
354 orientation of the cells within a group, or the inclusion of immotile or morphologically abnormal
355 sperm (e.g., kinked or broken cells). When sperm heads and flagella are not oriented in the same
356 direction, the cells within an aggregate are expected to exert opposing forces on one another,
357 thereby reducing the overall motility of the group (Fisher et al. 2014; Pearce et al. 2018).
358 Similarly, the inclusion of immotile or morphologically abnormal sperm, which typically display
359 reduced motility (reviewed in Hook and Fisher 2020), is expected to reduce overall group
360 kinematics. Indeed, we found that aggregates with misaligned, immotile and/or morphologically
361 abnormal sperm were slower than aggregates without these abnormalities in all but one species.
362 Perhaps not surprisingly, the species with the fewest abnormal aggregates, the promiscuous deer
363 mouse (*P. maniculatus*), also showed the greatest kinematic improvement when sperm formed
364 groups. These findings provide evidential support for the hypothesis that the orientation of sperm
365 within the group is critical to its kinematics (Fisher et al. 2014) and that, in addition to uniformity

366 of aggregate size, post-copulatory sexual selection may additionally act upon the architecture of
367 sperm aggregates to improve their motility in a competitive context.

368 In contrast to our findings in *P. maniculatus*, sperm from their monogamous sister-species, *P.*
369 *polionotus*, often forms aggregates that contain misaligned, immotile or morphologically abnormal
370 cells, and overall *P. polionotus* aggregates are slower or no different in their speed than single
371 cells. This finding is consistent with a previous report that *P. polionotus* sperm form optimal-sized
372 aggregates less often than in *P. maniculatus* sperm (Fisher et al. 2014). Together, these
373 observations are consistent with the theory that sperm aggregation evolved prior to the divergence
374 of this species pair, and when monogamy evolved secondarily in *P. polionotus* (Greenbaum et al.
375 1978; Turner et al. 2010), relaxed sexual selection allowed for the persistence of less motile sperm
376 traits (van der Horst and Maree 2014). In an examination of sperm conjugate presence or absence
377 in 42 species of carabid beetles across nine subfamilies, results of the ancestral state reconstruction
378 showed that sperm conjugation is ancestral and that the loss of conjugation evolved independently
379 in several lineages (Sasakawa 2020). While sperm conjugation traits were not linked to mating
380 systems per se, they do suggest that the evolutionary loss of these characters is prevalent. Based on
381 our findings, we suggest that relaxed sexual selection may allow the persistence of less optimal
382 sperm traits in the *Peromyscus* system.

383 In conclusion, our study highlights the diversity of sperm aggregation within a single
384 taxonomic lineage and how post-copulatory sexual selection has likely shaped the formation and
385 performance of these cellular groups. We show that in species expected to experience higher levels
386 of sperm competition, sperm aggregates are less variable in size and males produce a fewer
387 number of aggregates with inefficient alignment or composition. Moreover, we find that sperm
388 aggregation can improve sperm motility, but this is not consistent across all species or contexts.
389 Theoretical predictions (Fisher et al. 2014; Pearce et al. 2018) suggests that motility benefits may
390 only be realized if cells maintain optimal alignment within the groups and, if achieved, collective
391 motility may provide these sperm with a competitive advantage in the female reproductive tract

392 (Higginson et al. 2012). Future work investigating *Peromyscus* sperm aggregates *in vivo* will help
393 shed light on the co-evolution of collective sperm behaviors and the complex female reproductive
394 tract through which sperm must navigate.
395
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Figures

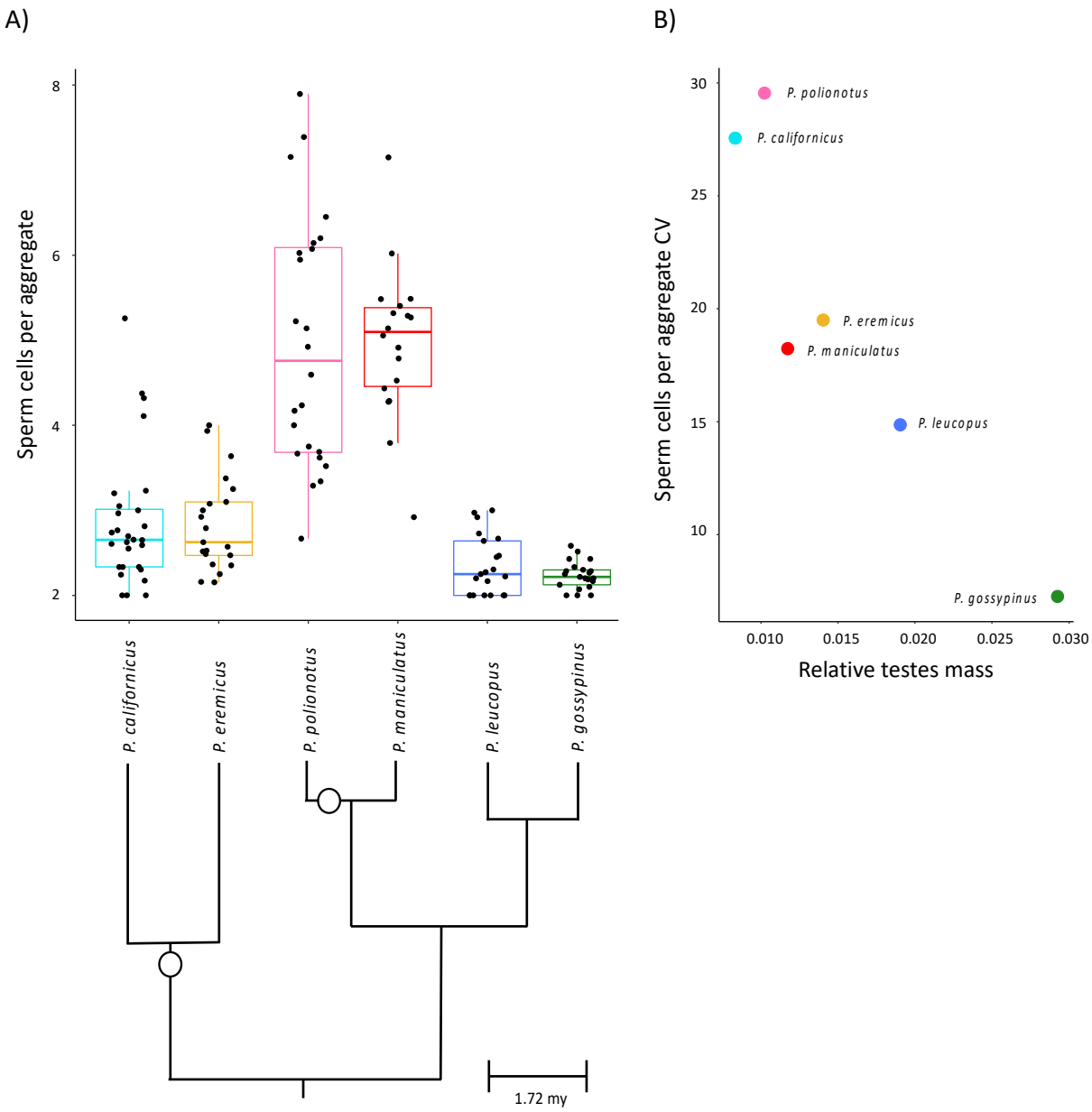

Figure 1. Sperm aggregate size differs within and among *Peromyscus* mice, and variability in this trait negatively associates with relative testis size. (A) Observed variation in the number of sperm cells per aggregate produced by males under controlled, *in vitro* conditions. Box-plots represent median and interquartile ranges with mean values per male overlaid as black dots. Species relationships are indicated within the phylogeny (adapted from Bradley et al. 2007), and open circles represent the evolutionary transitions to monogamy (Bedford & Hoekstra 2015). (B) When controlling for phylogenetic relationships across these species using a PGLS model, the coefficient of variation (CV) for the number of sperm cells in aggregate negatively correlates with relative testes mass. Note truncated y-axes.

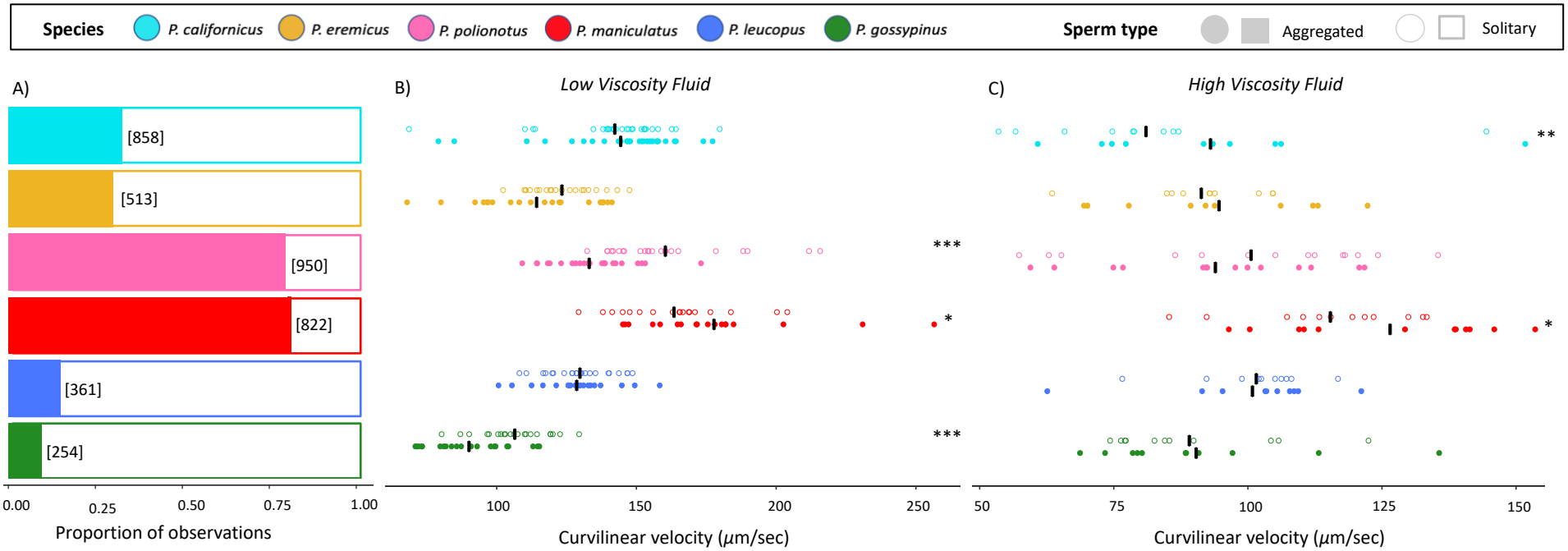

Figure 2. The frequency of sperm aggregates varies across focal *Peromyscus* species and can affect sperm swimming performance. (A) Sperm aggregates (solid bars) and solitary sperm (open bars) are simultaneously produced by males of all focal species, allowing for a direct comparison of their speeds while controlling for inter-male variation to test the hypothesis that sperm aggregates provide a competitive advantage. The summed number of sperm aggregates observed within each species is indicated in brackets. The curvilinear velocity of aggregated (closed circles) and solitary sperm (open circles) observed in either low (B) or high (C) viscosity media to test the hypothesis that sperm aggregates are faster in viscous environments. In both environments, sperm aggregates are faster in *P. maniculatus*. Aggregates are also faster in *P. californicus* in the high-viscosity environment, but aggregates are slower in *P. polionotus* and *P. gossypinus* in the low-viscosity environment. All other species produced aggregates that did not differ in speed from solitary sperm. Circles represent mean values per male, and black dashes represent species mean per sperm type. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Note the truncated x-axes.

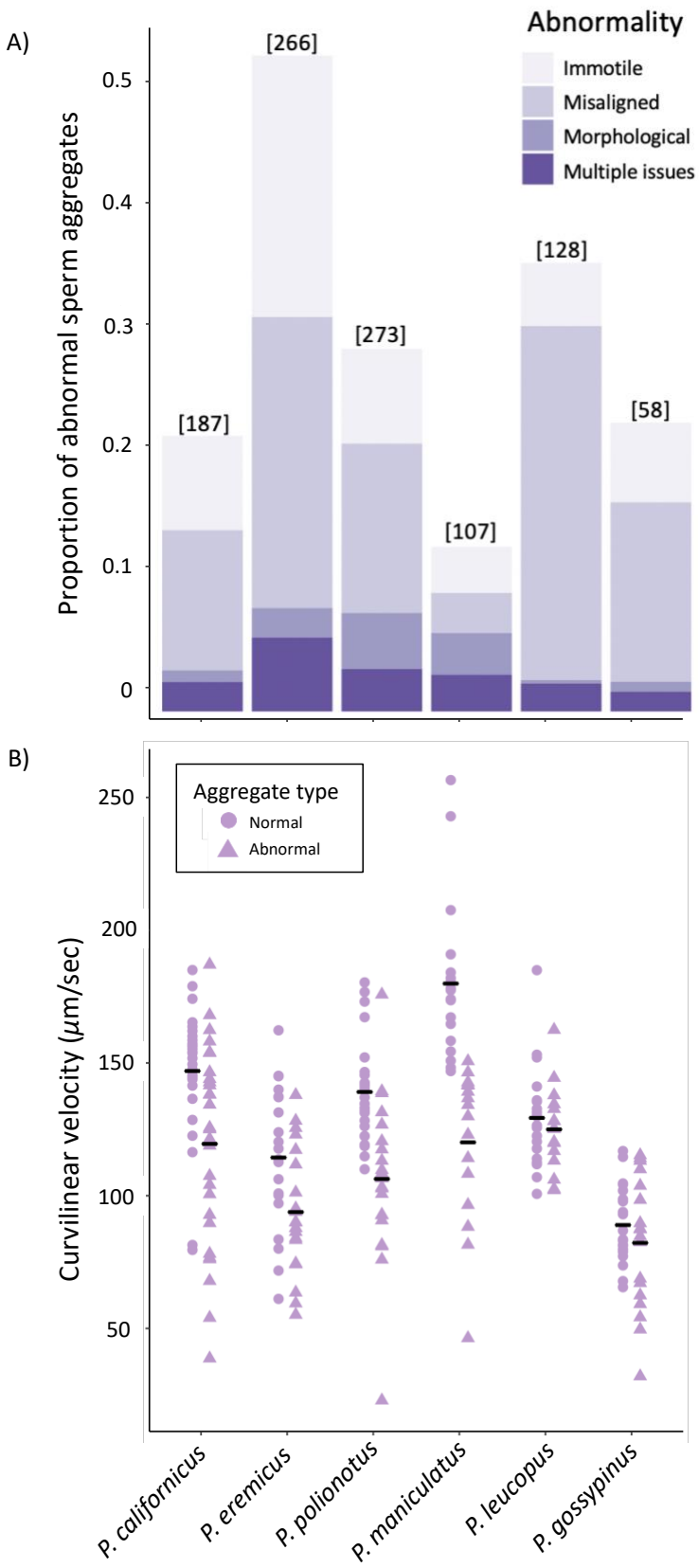

Figure 3. The alignment of sperm within aggregates and the inclusion of abnormal or immotile cells varies across focal *Peromyscus* species and impacts aggregate speed. (A) Sperm aggregates were scored as 'abnormal' if they featured one or more cells that were immotile, misaligned, morphologically abnormal, or had a combination of these issues. The total number of abnormal aggregates observed per sperm are indicated in brackets. (B) Abnormal aggregates (triangles) are slower than normal aggregates (circles) in all but two species (*P. leucopus* and *P. gossypinus*). Symbols represent mean values per male, and black dashes represent species mean per aggregate type.

Tables

TABLE 1. Fixed effects from a linear model examining differences in sperm aggregate size across focal *Peromyscus* species.

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)	5.82	**< 0.001**
P. eremicus	-1.97 (0.24)	0.12	(0.08, 0.18)	-8.14	**< 0.001**
P. gossypinus	-2.77 (0.24)	0.06	(0.04, 0.09)	-11.53	**< 0.001**
P. californicus	-2.19 (0.23)	0.10	(0.07, 0.15)	-9.67	**< 0.001**
P. leucopus	-2.68 (0.24)	0.06	(0.04, 0.10)	-11.18	**< 0.001**
P. polionotus	0.04 (0.23)	0.51	(0.40, 0.62)	0.15	0.88

All rows are compared with the intercept – *P. maniculatus*. 95% confidence intervals (CI) were calculated for each effect size.

TABLE 2. Results from PGLS models examining the number of cells in aggregate or the coefficient of variation (CV) of this trait, in relation to relative testis mass for all focal *Peromyscus* species

Response	Predictor	*t*	*P*	R^2	λ
Number of cells in aggregate	Testis mass (mg) Body mass (mg)	-1.9032 -1.0037	0.153 0.389	0.45	0.000 (1.00, 0.50)
Number of cells in aggregate CV	Testis mass (mg) Body mass (mg)	-3.7267 1.1829	**0.034** 0.322	0.70	0.000 (1, 0.01)

In all models, the branch length transformations for lambda, λ, were set using maximum likelihood ('ML'). Lower and upper bounds for this term are noted within parentheses.

TABLE 3. Results from an intra-male analysis for all focal *Peromyscus* species comparing the curvilinear velocity (VCL) of solitary and aggregated sperm in low- and high- viscosity environments to test whether sperm aggregates confer a kinematic advantage (shaded in gray)

Peromyscus Species	Low Viscosity (0% methylcellulose)					High Viscosity (0.75% methylcellulose)				
	Mean (±SE) VCL (μm/sec)		Student's paired t-test			Mean (±SE) VCL (μm/sec)		Student's paired t-test		
	Solitary cells	Aggregated cells	*t*	df	*p*	Solitary cells	Aggregated cells	*t*	df	*p*
californicus	142.3 ± 3.8	144.4 ± 4.2	-1.0545	28	0.301	81.0 ± 8.0	93.0 ± 8.0	-3.9495	9	**< 0.01**
eremicus	123.4 ± 2.6	114.3 ± 4.7	1.7308	20	0.099	91.3 ± 3.9	94.6 ± 5.9	-0.7174	9	0.491
polionotus	160.4 ± 4.5	133.1 ± 3.3	9.4575	23	**< 0.001**	100.6 ± 6.6	93.9 ± 5.2	2.0349	13	0.063
maniculatus	163.5 ± 4.7	177.7 ± 6.8	-2.2482	17	**0.038**	115.4 ± 4.3	126.5 ± 5.6	-2.9397	11	**0.014**
leucopus	129.8 ± 2.5	128.6 ± 2.9	0.24811	20	0.807	101.6 ± 3.4	100.8 ± 5.0	0.3243	9	0.753
gossypinus	106.5 ± 2.7	90.1 ± 3.2	5.4048	20	**< 0.001**	89.1 ± 4.6	90.4 ± 5.8	-0.6384	10	0.538

TABLE 4. Results from an intra-male analysis for all focal *Peromyscus* species comparing the curvilinear velocity of sperm aggregates featuring one or more cells that were immotile, misaligned, or morphologically flawed with aggregates that lacked abnormalities

Peromyscus Species	Student's paired t-test		
	t	df	*p*
californicus	3.967	27	**< 0.001**
eremicus	2.816	19	**0.011**
polionotus	4.996	20	**< 0.001**
maniculatus	5.354	15	**< 0.001**
leucopus	0.807	14	0.433
gossypinus	1.165	17	0.260