Male evolution under relaxed selection: Evidence for degeneration in sperm produced by male snails from asexual lineages

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Abstract

How drastic changes in selective regimes affect trait evolution is an important open biological question. We take advantage of naturally occurring and repeated transitions from sexual to asexual reproduction in *Potamopyrgus antipodarum*, a New Zealand freshwater snail to address how relaxed selection on male-specific traits influences sperm morphology. The occasional production of male offspring by the otherwise all-female asexual lineages allows a unique and powerful opportunity to assess the fate of sperm traits in a context where males are unnecessary. These comparisons revealed that the sperm produced by "asexual" males are markedly distinct from sexual counterparts. In particular, the asexual male sperm harbored markedly higher phenotypic variation and was much more likely to be grossly abnormal. Together, these data suggest that transitions to asexual reproduction might be irreversible at least in part because male function is likely to be compromised. More broadly, our results are consistent with a scenario where relaxed selection translates into rapid trait degeneration.

41 Evolution by natural selection generates adaptation and often underlies the initial appearance and
42 subsequent elaboration of complex traits. While Charles Darwin was one of the very first to link
43 selection and adaptation, he also recognized the importance of variation away from presumed optimal
44 phenotypes (Darwin 1859). One of the most striking examples of this phenomenon is the eye of
45 *Astyanax mexicanus*, a freshwater fish characterized by both surface and cave ecotypes. One of the
46 main differences between the *A. mexicanus* surface and cave forms is that the cave dwellers have lost
47 eye structures, likely linked to the perpetual absence of light in their habitat (Jeffery 2005, Cartwright *et*
48 *al.* 2017). The process that yields the dismantling of once useful traits, so-called regressive (or
49 degenerative) evolution, is generally thought to be a consequence of a shift in selection regime and can
50 result in the degeneration of ancestrally important traits. Examples of trait loss *via* regressive evolution
51 are common in nature and across diverse taxonomic groups, including humans (Prout 1964, Wilson
52 1982, Remko *et al.* 2005, Ansaloni & Catena 2009; Stanley 2014). Transitions to relaxed selection are
53 often attributed to environmental or life-history alterations that render historically important
54 phenotypes irrelevant (Lahti *et al.* 2009; Ellers *et al.* 2012; Helliwell 2013).

55 We investigate how shifts in reproductive mode (here, sexual to asexual reproduction) alter the
56 selective regime experienced by traits that are used only for sexual reproduction. Our particular focus is
57 on the nature and extent of the loss of "maleness" in asexual lineages where females reproduce without
58 any need for males. In this scenario, males, and the genes underlying male-specific traits, are
59 presumably extraneous (Carson *et al.* 1982). This is the basis for the expectation that in these asexual
60 lineages, traits needed only for sexual reproduction ("sex-specific traits"; *e.g.*, genes used only for
61 meiosis or male-specific traits like sperm (Schurko & Logsdon 2008; Tvedte *et al.* 2017) will experience a
62 shift from purifying to relaxed selection at the onset of asexuality. If maintenance of these now-neutral
63 sex-specific traits is not otherwise costly, and if the genes underlying the sex-specific traits are not
64 pleiotropic with respect to traits important outside the context of sex, the sex-specific traits will

65 subsequently evolve primarily *via* genetic drift and mutation instead of selection (van der Kool &
66 Schwander 2014). These expectations have been evaluated with respect to sexual signaling in bush
67 crickets (Lehmann *et al.* 2007) and wasps (Pannebakker *et al.* 2005), sperm-storage organs and
68 fertilization success of males produced by otherwise all-female asexual lineages in stick insects
69 (Schwander *et al.* 2013), and sperm formation in oribatid mites (Taberly 1988; Maraun 2003; Heethoff
70 2007). While these studies indicate that transitions to asexuality often but not always (*e.g.* Lehmann *et*
71 *al.* 2007) underlie sex-specific trait degeneration, the conclusions that can be drawn are limited by the
72 inability to directly compare the sexual and asexual forms (*e.g*., sexuals and asexuals are allopatric, are
73 not closely related, or the asexuals are hybrids).

74 How sperm and seminal fluid proteins evolve under relaxed selection is of particularly broad interest
75 because these traits are among the most important mediators of fertilization, sperm competition, sexual
76 conflict, and speciation (Swanson & Vacquier 2002). Accordingly, characterizing sperm evolution in the
77 context of asexuality will provide a rare glimpse into the consequences of instantaneous shifts in
78 selection for traits that are usually critically important determinants of evolutionary trajectories.

79 There are two major barriers facing the characterization of the evolution of sperm and related traits in
80 an asexual background. First, obligately asexual lineages derived from dioecious taxa are typically all or
81 nearly all females (*e.g.*, Gottlieb & Zchori-Fein 2001; Schon *et al.* 2009; Neiman *et al.* 2012). Second,
82 even if male asexuals exist, powerful and rigorous characterization of how relaxed selection influences
83 sperm evolution requires the direct comparisons that can only come from sperm produced by
84 ecologically and phenotypically similar males from closely related sexual lineages. Perhaps because
85 many asexual taxa are either of hybrid origin or do not have close sexual relatives (reviewed in Neiman
86 & Schwander 2011), this type of study has yet to be conducted.

87 We are able to overcome both of these barriers by taking advantage of the occasional production of
88 male offspring by obligately asexual *Potamopyrgus antipodarum* (Neiman *et al.* 2012). This New Zealand
89 freshwater snail provides a powerful means of directly evaluating the impact of shifts in reproductive
90 mode because otherwise similar obligately sexual and obligately asexual snails regularly coexist in
91 natural populations, enabling direct comparisons between sexual and asexual individuals, genomes, and
92 populations. Asexual *P. antipodarum* are the product of multiple separate transitions from sexual
93 ancestors, allowing us to treat distinct asexual lineages as replicated experiments into asexuality
94 (Neiman *et al.* 2011; Paczesniak *et al.* 2013). What makes *P. antipodarum* particularly well suited for this
95 study is that asexual female lineages occasionally (~1.5% of all offspring, depending on the asexual
96 lineage involved; Neiman *et al.* 2012) produce males, perhaps *via* loss of sex chromosomes during
97 oogenesis (Soper *et al.* 2013). We leverage this unusual phenomenon to characterize morphological
98 variation of sperm in sexually *vs.* asexually produced males, with the expectation that the latter have
99 experienced a relatively recent history of relaxed selection on male-specific traits and genes. Based on
100 this logic, we predict that the sperm produced by asexual males will harbor more variation and be more
101 likely to be abnormal than sperm from their sexual counterparts.

102

103 *Materials and Methods*

104 **Snail collection and ploidy/reproductive mode determination**

105 *Potamopyrgus antipodarum* used in this study were collected either from New Zealand lake populations
106 (11 lakes; 33 sexual males) or from individual and separately derived asexual lineages (8 lineages; 24
107 asexual males). The latter are recently descended (<5 generations) from asexual snails collected from
108 New Zealand lake populations that were subsequently cultured in our laboratory at the University of
109 Iowa. All snails were housed in plastic 10L tanks filled with oxygenated carbon-filtered tap water in a

110 16C room with a 16:8-hr light:dark cycle and fed dried *Spirulina* algae *ad libitum* (following Zachar &
111 Neiman 2013). We focused our search for asexual males in lineages that had been previously
112 characterized as relatively likely to harbor asexual males (Neiman *et al.* 2012). We also emphasized
113 asexual lineages descended from lake populations that population genetic studies (Paczesniak *et al.*
114 2013) demonstrated harbor asexual subpopulations that represent separate *in situ* transitions from
115 sexual to asexual reproduction. For the sexual males, we used field collections from lakes that were
116 known to contain relatively high frequencies (>10-20%) of sexual males (Neiman *et al.* 2011).

117 We identified males by sexing each individual from each field collection (N = 1370 snails) or lineage (N =
118 4505 snails) that was large enough (>/=3mm shell length) to have reached sexual maturity (*e.g.*, Larkin
119 *et al.* 2016). Sexual *P. antipodarum* are diploid and asexuals are either triploid or tetraploid (Neiman *et*
120 *al.* 2011), allowing us to use comparisons between triploid and tetraploid-produced sperm to at least in
121 part control for effects of asexuality *vs.* ploidy elevation on sperm phenotype. We used a Becton
122 Dickinson ARIA II flow cytometer on dissected head tissue (following Bankers *et al.* 2017) to assign ploidy
123 (diploid, triploid, tetraploid) and, thus, reproductive mode (diploid = sexual; triploid, tetraploid =
124 asexual) to each male. The overall incidence of males in asexual lineages was ~1% (59/5875), and
125 asexual lineages varied in the relative frequency of male production (Supp Table 1). Both observations
126 are consistent with a similar survey in Neiman *et al*. (2012). Because our goal was to replicate males
127 three times within each asexual lineage that we used, we excluded 35 males that either represented a
128 lineage in which more than three males were found (13 asexual lineages; these "extra" males were
129 excluded arbitrarily) or were from a lineage in which fewer than three males were found (six asexual
130 lineages). This left us with a total of 24 asexually produced male *P. antipodarum* for our study. For sexual
131 males, we used the first three arbitrarily selected sexual males that we identified per each of seven field
132 collections, for a total of 21 sexual male *P. antipodarum*. Hereafter, we use "set" to indicate one three-
133 male replicate from each unique lineage or lake of origin and ploidy combination: our diploid *P.*

134 *antipodarum* sample represents seven male sets (each from a different field collection), triploid *P.*
135 *antipodarum* are represented by six male sets (each from a different asexual lineage and lake of origin),
136 and tetraploid *P. antipodarum* are represented by two male sets (from two different asexual lineages,
137 each from a different lake), for a total of 15 *P. antipodarum* sets and 45 males (Figure 1).

138 We provided a separate control for nuclear DNA content, which can have major influences on sperm
139 phenotype (Alavi *et al.* 2013), by including males and sperm from the closely related (Haase 2008) and
140 diploid obligate sexual species *P. estuarinus* (three sets, for a total of nine males) and *Halopyrgus*
141 *pupoides* (one set, three males). Because *P. estuarinus* and *H. pupoides* have only about 60% of the
142 nuclear genome DNA content of diploid *P. antipodarum* (Logsdon *et al.* 2017), we used comparisons
143 across these three diploid taxa to account for the effect of nuclear genome DNA content on sperm
144 phenotype. These outgroup snails were collected from their native New Zealand estuary habitat
145 (Waikuku Beach, New Zealand) in January 2018.

146 **Sperm extraction and imaging**

147 We used forceps to extract the bodies of male snails from their shells and then dissected away the
148 sperm duct, which transports sperm and seminal fluid from the testis to the penis. We centered the
149 sperm duct tissue on a glass microscope slide and added ~5µl of DAPI staining solution (following Soper
150 *et al.* 2013). We used repeated pipetting of this solution to release sperm from the ruptured duct and
151 distribute the sperm across the slide. We then mounted the slide on an Olympus IX71 fluorescence
152 microscope and used the DAPI filter to locate the fluorescent sperm heads and the polarized filter to
153 photograph sperm for subsequent analysis. We arbitrarily selected and then photographed a minimum
154 of 12 whole sperm cells for every male. We excluded sperm cells that were entangled with other sperm
155 or were obscured by non-sperm cellular detritus. We used sperm from at least three males (one male
156 set) for each unique lake and ploidy combination, for a total of at least 36 sperm per set.

157 **Phenotype measurements**

158 We imported photographs into ImageJ (Rueden *et al.* 2017) and measured head length (HL; head tip to
159 head base), head width (HW; width at midpoint of head), head circumference (HC; perimeter of head),
160 and tail length (TL; length from head base to tail end) for each sperm cell. We also used Mahalanobis
161 distance (MD; distance between a distribution and a focal point) to provide a multi-dimensional
162 generalization that represents a composite of all four of the sperm traits that we measured (De
163 Maesschalck *et al.* 2000). Finally, we used our consistent observations of a number of qualitatively
164 distinct phenotypic classes of sperm abnormalities to sort sperm into "normal" (most common
165 phenotype of smooth cylindrical head and smooth tail of consistent width; 79% of all sperm) *vs.* one of
166 six "abnormal" ultrastructural categories: >1 tail, distorted neck, oblong head, tail bump, two heads,
167 wrinkled head (*e.g.*, Menkveld *et al.* 1990). This approach allows us to distinguish large-scale differences
168 in complex sperm morphology that might not be evident from our measurements of individual sperm
169 traits.

170 **Statistical analyses**

171 We used a RAxML tree generated from Illumina whole-genome sequencing data from 26 *P. antipodarum*
172 (10 sexual individuals, 16 asexual individuals (11 triploid and 5 tetraploid)) to provide a phylogenetic
173 framework needed to control for potential effects of phylogenetic relationship on sperm phenotype
174 (Supp Figure 1). This tree also demonstrates that we have likely captured a broad sample of sexual and
175 asexual *P. antipodarum* genetic diversity, including a number of separately derived asexual lineages. We
176 used this tree in Phylosignal (Keck *et al.* 2016), an R package that tests for phylogenetic signal, to assess
177 whether sperm morphology was determined at least in part by shared ancestry. We attached mean trait
178 values for each of the sperm traits measured below to a subsampled phylogenetic tree, which
179 represented directly (same asexual lineage) or indirectly (shared lake of origin, justified by multiple lines

180 of evidence for a predominant effect of source population on *P. antipodarum* genotype, *e.g.*, Dybdahl &
181 Lively 1995; Paczesniak *et al.* 2013; Bankers *et al*. 2017) nine of the 15 *P. antipodarum* sets from which
182 we sampled males (*i.e.*, sets with both genetic data and sperm measurements). We did not find any
183 significant phylogenetic correlations underlying these sperm traits (Supp Figure 2). Together, the
184 outcome of these analyses indicate that phylogenetic relatedness is not a main driver of variation in
185 sperm morphology. We also used hierarchical clustering of sperm morphology data to produce a
186 cladogram that we used to assess whether phylogenetic relatedness is the primary driver of across-
187 lineage/lake sperm variation (Figure 2, Supp Figure 3).

188 We applied ANOVA and MANOVA models to evaluate how lake/lineage of origin affected each of the
189 four individual sperm traits that we measured (HL, HW, HC, TL) along with the composite MD trait. We
190 first used M/ANOVA for the trait values estimated for each set to assess whether the three different
191 males within each set varied in sperm morphology. We then applied M/ANOVA to groupings of diploid,
192 triploid, and tetraploid *P. antipodarum* to evaluate whether ploidy affects sperm morphology. Finally,
193 we used an unpaired *t*-test to compare means for sexual and asexual *P. antipodarum* sperm traits to
194 assess the effect of reproductive mode on sperm morphology. Prior to each of the M/ANOVA analyses,
195 we used a Shapiro-Wilk test applying a Bonferroni-corrected threshold *p*-value of 0.0026 to account for
196 multiple comparisons (19 within-set comparisons per each of the 95 Shapiro-Wilk tests) for each of the
197 five traits to evaluate whether the data met the assumption of a normal distribution. For the 35 tests
198 that violated this assumption, we applied a Box-Cox transformation to attempt to achieve normality,
199 which was successful in 14 cases. For the 21 cases that still violated normality assumptions, we used
200 Kruskal-Wallis tests to compare population medians. We then used principal component analysis (PCA)
201 to visualize among-species (*P. antipodarum*, *P. estuarinus*, *H. pupoides*) differences that maximize
202 variance for the four multidimensional sperm trait values in order to detect species-specific differences.
203 Finally, we used Linear Discriminate Analysis (LDA) by grouping diploid, triploid, and tetraploid *P.*

204 *antipodarum* into predefined classes. This analysis minimizes within-ploidy variance and maximizes
205 across-ploidy variance to find linear combinations of variables that best explain the data. We used this
206 analysis to assess how differing ploidy level translates to differences in sperm morphology. All statistical
207 analyses were implemented in R 3.4.1 (R Core Team 2013) or SPSS 24.0 (IBM Corp. 2016)

208 *Results*

209 We evaluated a total of 750 sperm from each of 57 male snails (21 diploid sexuals, 18 triploid asexuals,
210 six tetraploid asexuals, nine *P. estuarinus*, three *H. pupoides* (Figure 1). representing 12 lakes and three
211 estuaries of origin as well as three species. Because we did not find any asexual male in field collections
212 despite evaluating >380 field-collected male *P. antipodarum* from eight lake populations, we had to
213 compare field-collected sexual individuals to laboratory-reared asexual males. While we cannot formerly
214 exclude the possibility that these differences in rearing environment between sexually and asexually
215 produced males could influence sperm production, we felt that it was important to only use field-
216 collected sexual males because of the distinct possibility that the inbreeding that is a necessary
217 component of lab culture of sexual lineages of these snails would influence snail phenotype in a manner
218 that does not reflect natural variation. Because asexual *P. antipodarum* reproduce via apomictic
219 parthenogenesis (Phillips & Lambert 1998), inbreeding, and inbreeding depression, is not a concern for
220 laboratory-reared asexuals. Because male-specific traits that are encoded in the genomes of asexual
221 lineages presumably experience weak or even no selection, we also believe that it is unlikely - though
222 not impossible - that laboratory-imposed selection would affect these male traits in a manner that is
223 qualitatively different than male traits harbored in asexual lineages in the field.

224 Our analyses revealed extensive qualitative (Supp Figure 4) and quantitative variation (Supp Table 2) in
225 sperm morphology. Multi-dimensional hierarchical clustering of primary sperm traits (HL, HW, HC, and
226 TL) suggested that reproductive mode (*vs.* ploidy or shared phylogenetic history) is the primary driver of

227 sperm morphology in *P. antipodarum* (Figure 2, Supp Figure 3). This conclusion is bolstered by the fact
228 that diploid sexual *P. antipodarum* sperm clustered with the two diploid sexual outgroup species instead
229 of with the asexual polyploid conspecifics.

230 **Sperm morphology is primarily driven by reproductive mode**

231 We used comparisons across the three ploidy levels represented by *P. antipodarum* and sperm from the
232 diploid outgroup species *P. estuarinus* and *H. pupoides* to account for effects of nuclear genome DNA
233 content on sperm morphology. That nuclear genome DNA content *per se* is not the sole driver of sperm
234 morphology in these snails is demonstrated by our findings that 1) sperm produced by *H. pupoides* and
235 *P. estuarinus*, which seem to share the same relatively small nuclear DNA content, were nevertheless
236 morphologically distinct (Figure 2, Supp Figure 5), 2) head width and tail length were statistically
237 indistinguishable between triploid and tetraploid *P. antipodarum* (Supp Figure 6), 3) the CV for triploid
238 and tetraploid *P. antipodarum* is statistically indistinguishable for all traits except tail length (Supp Figure
239 7) and 4) different ploidies overlap in morphometric space (Figure 3). We did detect some evidence for
240 ploidy effects on sperm trait means and variances: the LDA revealed ploidy-based clustering (Figure 3),
241 and the ANOVA demonstrated a main effect of ploidy on all sperm traits (Supp Table 2 B). Our CV-based
242 analyses indicated that sperm produced by diploid male *P. antipodarum* harbors significantly lower
243 variance (*e.g.*, HL mean = 4.48µm, SD = 0.17) than sperm produced by triploid (HL mean = 5.29µm, SD =
244 0.44) and tetraploid (HL mean = 5.99µm, SD = 0.38) males (Supp Figure 7).

245 **Asexual *P. antipodarum* often produce abnormal sperm**

246 Visual inspection of *P. antipodarum* sperm revealed substantial variation. Most (N=429; 76%) of these
247 sperm had an overtly normal phenotype, represented by a smooth and cylindrical head and a flagellum
248 of consistent width from the neck to tip (Supp Figure 4 A). We sorted the most common deviations we
249 observed from this normal phenotype into six discrete categories that emerged during our imaging and

250 analysis stages: >1 tail (3.5% of all sperm), distorted neck (2.5%), oblong head (1.5%), tail bump (10%),
251 two heads (1%), and wrinkled head (6.5%) (see Supp Figure 4 B-D). There was markedly higher incidence
252 of abnormal sperm produced by triploid (N=95; 43% of all triploid sperm) and tetraploid (N=24; 27%) *P.*
253 *antipodarum* relative to the sperm produced by diploid *male P. antipodarum* (N=16; 6%) (Fisher's exact
254 test $p = < 0.001$) (Figure 4). Misshapen heads were the only abnormality shared across all three ploidies,
255 while the most common abnormality, tail bump, is restricted to triploid asexual *P. antipodarum*.

256 **Among- and within-species variation in sperm morphology**

257 We used a principal component analysis to visualize and compare multivariate sperm phenotypes across
258 sexual *P. antipodarum* and the two closely related sexual outgroup species. This analysis revealed a
259 distinct species-level signal, with no overlap between the clusters of sperm data corresponding to each
260 species (Supp Figure 5). Our ANOVA and MANOVA analyses demonstrated statistically significant
261 differences in each of the five sperm traits amongst these three species (HL: $p = 0.0005$, HW: $p = 0.001$,
262 HC: $p = 0.01$, TL: $p = 7.6e-08$, MD: $p = 4.5e-13$).

263 We used a MANOVA to address whether there was intraspecific (individual level) variation
264 within each of the 45 *P. antipodarum* male sets. This analysis revealed significant across-male variation
265 within four diploid and four triploid sets (Supp Table 2). Sperm tail length was the trait most often
266 variable across sets (5/19 populations), and sperm head circumference was the least likely to feature
267 across-set variation (1/19). The MD analyses revealed only two cases of significant within-population
268 variation (asexual Haupiri and asexual Brunner). The CV analyses indicated that sperm head length and
269 head circumference are the least variable traits and head width and tail length are the most variable
270 traits across all *P. antipodarum* sperm (Supp Figure 8).

271

272 *Discussion*

273 We used the powerful *Potamopyrgus antipodarum* system to perform a direct comparison of male-
274 specific traits across otherwise similar males that differ in ploidy level and reproductive mode. We found
275 evidence for a major effect of sexual *vs*. asexual reproduction on sperm morphology, with sperm
276 produced by asexual males harboring substantially more variation and much more likely to feature
277 large-scale structural abnormalities. That a substantial fraction of asexual-produced sperm is abnormal
278 is consistent with a scenario where male-specific traits are no longer useful in an asexual lineage. We
279 also used comparisons between the three different *P. antipodarum* ploidies and three different diploid
280 sexual taxa included in our study to assess the extent to which increased nuclear genome DNA content
281 in the asexual *P. antipodarum*, *versus* asexuality *per se*, might affect sperm morphology. That asexual-
282 produced sperm are generally larger, and that these size differences to some extent scale with ploidy
283 (Supp Figure 6) is consistent with this possibility. Even so, the significant differences in sperm trait values
284 (*e.g.*, head length) between *P. estuarinus* and *H. pupoides*, which have virtually identical genome sizes
285 (Logsdon *et al.* 2017), indicates that other factors besides nuclear genome content are also important in
286 determining sperm morphology.

287 Our study is especially novel in comparing sperm phenotypes across reproductive modes as well as
288 ploidy level, allowing us to at least in part decouple effects of polyploidy from those of asexuality. We
289 can draw parallels and contrasts with other ploidy-focused sperm studies: Alavi *et al.* (2013) showed
290 that tetraploid European weatherfish (*Misgurnus fossilis*) produce sperm that are 4.8% larger than
291 sperm produced by their triploid counterparts, and Flajshans *et al.* (2008) found that triploid male
292 Prussian carp (*Carassius gibelio*) make sperm with larger volume than sperm generated by diploid and
293 tetraploid males. Considered together along with these two earlier studies, we can use our results to
294 conclude that nuclear genome DNA content is not the sole driver of the patterns we observed. Our
295 study is especially novel in allowing us to compare both across different ploidy levels as well as among
296 lineages with different nuclear genome content but the same ploidy level. We were also able to exclude

297 a major role of phylogeny in driving any of the patterns that our analyses revealed. Together,
298 comparisons provide a powerful means of disentangling effects of reproductive mode from nuclear
299 genome DNA content from ploidy elevation.

300 The abnormal phenotypes that often characterized sperm from asexual male *P. antipodarum* do appear
301 to be affected in part by ploidy: sperm from triploid asexual males spanned a wider range of abnormal
302 phenotypes and were more than twice as likely to be abnormal than sperm from tetraploid
303 counterparts. Cytogenetic characterization of some components of spermatogenesis in diploid sexual *vs.*
304 triploid asexual *P. antipodarum* have demonstrated that sperm production in the latter is associated
305 with aberrant meiotic pairing (Soper *et al.* 2013). Meiosis is disrupted when odd number of
306 chromosomes pair (Gupta 1985; Comai 2005; Henry *et al.* 2005), hinting that meiotic disruption is one
307 possible explanation for the differences between triploid and tetraploid-produced sperm in *P.*
308 *antipodarum*.

309 The morphologically distinct and often abnormal sperm produced by asexual *P. antipodarum* could also
310 be at least in part a consequence of mutation accumulation in the genes underlying sperm production,
311 which are expected to be under relaxed selection in all (or nearly all)-female asexual populations
312 (Schurko & Logsdon 2008; Lahti *et al.* 2009). Under this scenario, genes that influence sperm traits in
313 asexuals should be accumulating mutations at a higher rate than sexuals. This mutation accumulation
314 process would be evidenced by the degeneration and eventual pseudogenization of genes and traits
315 related to sperm function in asexual populations.

316 Our discovery that asexual-produced sperm harbor more morphological variation and are more likely to
317 be abnormal than sexually produced sperm is consistent with this expectation. The fact that the male
318 offspring occasionally produced by female asexual *P. antipodarum* are still investing in sperm suggests
319 that the time period over which the genes underlying sperm production have been subject to relaxed

320 selection is relatively short and/or that selection favoring the loss of this trait is weak. The latter would
321 not be surprising in light of the fact that male production is quite rare (~<1.5% of all offspring) in asexual
322 *P. antipodarum*. A powerful test of the former could come from comparisons of males produced by
323 different asexual *P. antipodarum* lineages, which are often recently derived (Paczesniak *et al.* 2013) but
324 also vary in the time since derivation from sexual *P. antipodarum* (Neiman *et al.* 2005). If time since
325 derivation to asexuality is a key determinant of the extent of male trait degeneration, we predict that
326 males from older asexual lineages should tend to produce sperm that harbors relatively high variance
327 and is abnormal at a higher frequency than sperm produced by males from younger asexual lineages.

328 While we cannot formally exclude the possibility that ploidy elevation *per se* is a major driver of sperm
329 abnormality, the fact that diploid male sexual *P. antipodarum* produce normal sperm at a similar
330 frequency to the two outgroup species suggests that nuclear genome DNA content alone does not
331 account for the high frequency of sperm abnormalities observed in asexual male *P. antipodarum*. The
332 wide sperm phenotype variation that we observed across asexual males also hints at a process (*i.e.*
333 mutation accumulation) that is stochastic compared to a direct consequence of ploidy elevation.
334 Together, our results indicate that asexual male *P. antipodarum* are likely experiencing decay of male-
335 specific traits, highlighting the likelihood that transitions to asexual reproduction results in striking
336 differences in selective regimes between sexual and asexual populations. This finding is of direct
337 relevance to evaluating how relaxed selection influences phenotypes in natural populations and
338 suggests that transitions back to sexual reproduction in asexual populations are likely to face challenges
339 associated with degeneration of sex-specific traits. Critical next steps include the characterization of
340 molecular evolution of sperm-related genes in sexual *vs.* asexual populations and assessment of
341 whether older asexual populations have experienced a relatively high level of trait decay.

342

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References Cited

Alavi, Sayyed Mohammad Hadi, *et al.* "Sperm morphology, motility, and velocity in naturally occurring polyploid European weatherfish (*Misgurnus fossilis* L.)." *Theriogenology* 80.2 (2013): 153-160.

Altmann, Thomas, *et al.* "Easy determination of ploidy level in *Arabidopsis thaliana* plants by means of pollen size measurement." *Plant Cell Reports* 13.11 (1994): 652-656.

Ansaloni, Luca, F. Catena, and A. D. Pinna. "What is the function of the human vermiform appendix?." *European Surgical Research* 43.2 (2009): 67-71.

Bankers, Laura, *et al.* "Genomic evidence for population-specific responses to co-evolving parasites in a New Zealand freshwater snail." *Molecular Ecology* 26.14 (2017): 3663-3675.

Bast, Jens, *et al.* "Consequences of asexuality in natural populations: insights from stick insects." *Molecular Biology and Evolution* 35.7 (2018): 1668-1677.

365 Berg, Johannes, Stana Willmann, and Michael Lässig. "Adaptive evolution of transcription factor binding
366 sites." *BMC Evolutionary Biology* 4.1 (2004): 42.

367 Carson, Hampton L., L. S. Chang, and T. W. Lyttle. "Decay of female sexual behavior under
368 parthenogenesis." *Science* 218.4567 (1982): 68-70.

369 Cartwright, Reed A., *et al.* "The importance of selection in the evolution of blindness in cavefish." *BMC*
370 *Evolutionary Biology* 17.1 (2017): 45.

371 Comai, Luca. "The advantages and disadvantages of being polyploid." *Nature Reviews Genetics* 6.11
372 (2005): 836.

373 Darwin, Charles. "On the origin of the species by natural selection." (1859).

374 De Maesschalck, Roy, D. Jouan-Rimbaud, and D. L. Massart. "The mahalanobis distance." *Chemometrics*
375 *and Intelligent Laboratory Systems* 50.1 (2000): 1-18.

376 Doniger, Scott W., J. Huh, and J. C. Fay. "Identification of functional transcription factor binding sites
377 using closely related *Saccharomyces* species." *Genome Research* 15.5 (2005): 701-709.

378 Dybdahl, Mark F., and C. M. Lively. "Diverse, endemic and polyphyletic clones in mixed populations of a
379 freshwater snail (*Potamopyrgus antipodarum*)." *Journal of Evolutionary Biology*8.3 (1995): 385-398.

380 Ellers, Jacintha, *et al.* "Ecological interactions drive evolutionary loss of traits." *Ecology Letters* 15.10
381 (2012): 1071-1082.

382 Fong, Daniel W., T. C. Kane, and D. C. Culver. "Vestigialization and loss of nonfunctional
383 characters." *Annual Review of Ecology and Systematics* 26.1 (1995): 249-268.

384 Flajšhans, Martin, *et al.* "Characteristics of sperm of polyploid Prussian carp *Carassius gibelio." Journal of*
385 *Fish Biology* 73.1 (2008): 323-328.

386 Foulkes, M. Raine. "Pollen size as an indicator of ploidy level in rhexia, a small genus of flowering plants
387 endemic to the North American coastal plain, in north Florida and coastal Georgia." *Georgia Journal of*
388 *Science* 76.1 (2018): 93.

389 Gottlieb, Yuval, and E. Zchori-Fein. "Irreversible thelytokous reproduction in *Muscidifurax*
390 *uniraptor*." *Entomologia Experimentalis et Applicata* 100.3 (2001): 271-278.

391 Gould, Frank W. "Pollen size as related to polyploidy and speciation in the *andropogon saccharoides—A.*
392 *barbinodis* complex." *Brittonia* 9.2 (1957): 71-75.

393 Gupta, Prabhat, and G. Fedak. "Genetic control of meiotic chromosome pairing in polyploids in the
394 genus *Hordeum*." *Canadian Journal of Genetics and Cytology* 27.5 (1985): 515-530.

395 Heethoff, Michael, *et al.* "High genetic divergences indicate ancient separation of parthenogenetic
396 lineages of the oribatid mite *Platynothrus peltifer* (Acari, Oribatida)." *Journal of Evolutionary*
397 *Biology* 20.1 (2007): 392-402.

398 Helliwell, Katherine E., G. L. Wheeler, and A. G. Smith. "Widespread decay of vitamin-related pathways:
399 coincidence or consequence?." *Trends in Genetics* 29.8 (2013): 469-478.

400 Henry, Isabelle M., *et al.* "Aneuploidy and genetic variation in the *Arabidopsis thaliana* triploid
401 response." *Genetics* 170.4 (2005): 1979-1988.

402 IBM Corp. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp.

403 Jeffery, William R. "Regressive evolution in *Astyanax* cavefish." *Annual Review of Genetics* 43 (2009): 25-
404 47.

405 Lahti, David C., *et al.* "Relaxed selection in the wild." *Trends in Ecology & Evolution* 24.9 (2009): 487-496.

Larkin, Katelyn, C. Tucci, and M. Neiman. "Effects of polyploidy and reproductive mode on life history trait expression." *Ecology and Evolution* 6.3 (2016): 765-778.

Law, Jennifer H., and B. J. Crespi. "The evolution of geographic parthenogenesis in *Timema* walking-sticks." *Molecular Ecology* 11.8 (2002): 1471-1489.

Lehmann, Gerlind, U.C. Johannes Strauß, and R. Lakes-Harlan. "Listening when there is no sexual signalling? Maintenance of hearing in the asexual bushcricket *Poecilimon intermedius*." *Journal of Comparative Physiology A* 193.5 (2007): 537-545.

Maraun, Mark, *et al.* "Radiation in sexual and parthenogenetic oribatid mites (Oribatida, Acari) as indicated by genetic divergence of closely related species." *Experimental & Applied Acarology* 29.3-4 (2003): 265-277.

Marinho, Ricardo, *et al.* "Pollen and stomata morphometrics and polyploidy in Eriotheca (Malvaceae-Bombacoideae)." *Plant Biology* 16.2 (2014): 508-511.

Muller, Hermann Joseph. "Some genetic aspects of sex." *The American Naturalist* 66.703 (1932): 118-138.

Neiman, Maurine, J. Jokela, and C. M. Lively. "Variation in asexual lineage age in *Potamopyrgus antipodarum*, a New Zealand snail." *Evolution* 59.9 (2005): 1945-1952.

Neiman, Maurine, S. Meirmans, and P. G. Meirmans. "What can asexual lineage age tell us about the maintenance of sex?." *Annals of the New York Academy of Sciences* 1168.1 (2009): 185-200.

Neiman, Maurine, and T. Schwander. "Using parthenogenetic lineages to identify advantages of sex." *Evolutionary Biology* 38.2 (2011): 115-123.

426 Neiman, Maurine, *et al.* "Wide variation in ploidy level and genome size in a New Zealand freshwater
427 snail with coexisting sexual and asexual lineages." *Evolution* 65.11 (2011): 3202-3216.

428 Neiman, Maurine, *et al.* "Male offspring production by asexual *Potamopyrgus antipodarum*, a New
429 Zealand snail." *Heredity* 109.1 (2012): 57.

430 Neiman, Maurine, and T. Schwander. "Using parthenogenetic lineages to identify advantages of sex."
431 *Evolutionary Biology* 38 (2011):115-123.

432 Ohta, Tomoko. "Near-neutrality in evolution of genes and gene regulation." *Proceedings of the National*
433 *Academy of Sciences* 99.25 (2002): 16134-16137.

434 Paczesniak, Dorota, *et al.* "Discordance between nuclear and mitochondrial genomes in sexual and
435 asexual lineages of the freshwater snail *Potamopyrgus antipodarum*." *Molecular Ecology* 22.18 (2013):
436 4695-4710.

437 Pannebakker, Bart, *et al.* "Sexual functionality of *Leptopilina clavipes* (Hymenoptera: Figitidae) after
438 reversing *Wolbachia*-induced parthenogenesis." *Journal of Evolutionary Biology*1 8.4 (2005): 1019-1028.

439 Phillips, Ngaire R., and D. M. Lambert. "Genetics of *Potamopyrgus antipodarum* (Gastropoda:
440 Prosobranchia): evidence for reproductive modes." *New Zealand Journal of Zoology* 16.3 (1989): 435-
441 445.

442 Prout, Timothy. "Observations on structural reduction in evolution." *The American Naturalist* 98.901
443 (1964): 239-249.

444 Leys, Remko, *et al.* "Regressive evolution of an eye pigment gene in independently evolved eyeless
445 subterranean diving beetles." *Biology Letters* 1.4 (2005): 496-499.

Rueden, Curtis T., *et al.* "ImageJ2: ImageJ for the next generation of scientific image data." *BMC Bioinformatics* 18.1 (2017): 529.

Schwander, Tanja, and B. J. Crespi. "Multiple direct transitions from sexual reproduction to apomictic parthenogenesis in *Timema* stick insects." *Evolution* 63.1 (2009): 84-103.

Schurko, Andrew M., and J. M. Logsdon. "Using a meiosis detection toolkit to investigate ancient asexual "scandals" and the evolution of sex." *Bioessays* 30.6 (2008): 579-589.

Schön, Isa, G. Rossetti, and K. Martens. "Darwinulid ostracods: ancient asexual scandals or scandalous gossip?." *Lost Sex*. Springer, Dordrecht (2009): 217-240.

Schwander, Tanja, *et al.* "Neutral and selection-driven decay of sexual traits in asexual stick insects." *Proceedings of the Royal Society of London B: Biological Sciences* 280.1764 (2013): 20130823.

Soper, Deanna M., *et al.* "Spermatozoa production by triploid males in the New Zealand freshwater snail *Potamopyrgus antipodarum*." *Biological Journal of the Linnean Society* 110.1 (2013): 227-234.

Stanley, Shane Gareth. *Vestigial hind limbs in the appendicular skeleton of three genera and four species of African skink (Squamata, Scincidae)*. Diss. Fayetteville State University, 2014.

Taberly, Georges. "Researchs on the thelytokous parthenogenesis of two species of Oribatid mites: *Trhypochthonius tectorum*(Berlese) and *Platynothrus peltifer*(Koch). IV. Observations on atavic males." *Acarologia* 29.1 (1988): 95-112.

Tvedte, Eric S., A. A. Forbes, and J. M. Logsdon Jr. "Retention of core meiotic genes across diverse Hymenoptera." *Journal of Heredity* 108.7 (2017): 791-806.

van der Kooi, Casper J., and Tanja Schwander. "On the fate of sexual traits under asexuality." *Biological Reviews* 89.4 (2014): 805-819.

467 Villeneuve, Anne M., and Kenneth J. Hillers. "Whence meiosis?." *Cell* 106.6 (2001): 647-650.

468 Wilson, Carl L. "Vestigial structures and the flower." *American Journal of Botany* (1982): 1356-1365.

469 Wright, Sewall. "Pleiotropy in the evolution of structural reduction and of dominance." *The American*
470 *Naturalist* 98.899 (1964): 65-69.

471 Zachar, Nicholas, and M. Neiman. "Profound effects of population density on fitness-related traits in an
472 invasive freshwater snail." *PloS One* 8.11 (2013): e80067.

473

474 *Figure Legends*

475 Figure 1. Schematic representing our experimental design, which included sperm from 57 individual
476 males (each male represented by small colored boxes) from three different species, two reproductive
477 modes, three ploidy levels, 12 lakes, and three estuaries. We characterized at least 12 sperm per male
478 and three males per set, for a total of at least 36 sperm per set. Each set is represented by the boxed
479 lake of origin and three small colored boxes representing the three males in the set. *P. est* = *P.*
480 *estuarinus* from three different New Zealand estuaries (CC = Waikuku Beach; Hutt = Hutt River, NZ; Fox =
481 Foxton). *H. pupoides* was collected from Waikuku Beach.

482

483 Figure 2. Hierarchical clustering dendrogram of sperm head morphology. Each square represents the
484 mean trait values of the >/= 36 sperm taken from each of the three males used for each unique
485 ploidy/reproductive mode/population set. Labeling follows Figure 1.

486

487 Figure 3. Linear discriminant function analysis of transformed sperm traits (head length, head width,
488 head circumference, tail length) for diploid, triploid, and tetraploid *P. antipodarum*. Histogram
489 represents the cumulative numbers of sperm in each bin for LD1. Arrows represent each variable (HL,
490 HW, HC, TL), length of arrows represents degree of discrimination, and arrow angle is the direction of
491 the variables in 2-D space.

492

493 Figure 4. Proportion and characterization of sperm phenotypes that we categorized as ”abnormal”.

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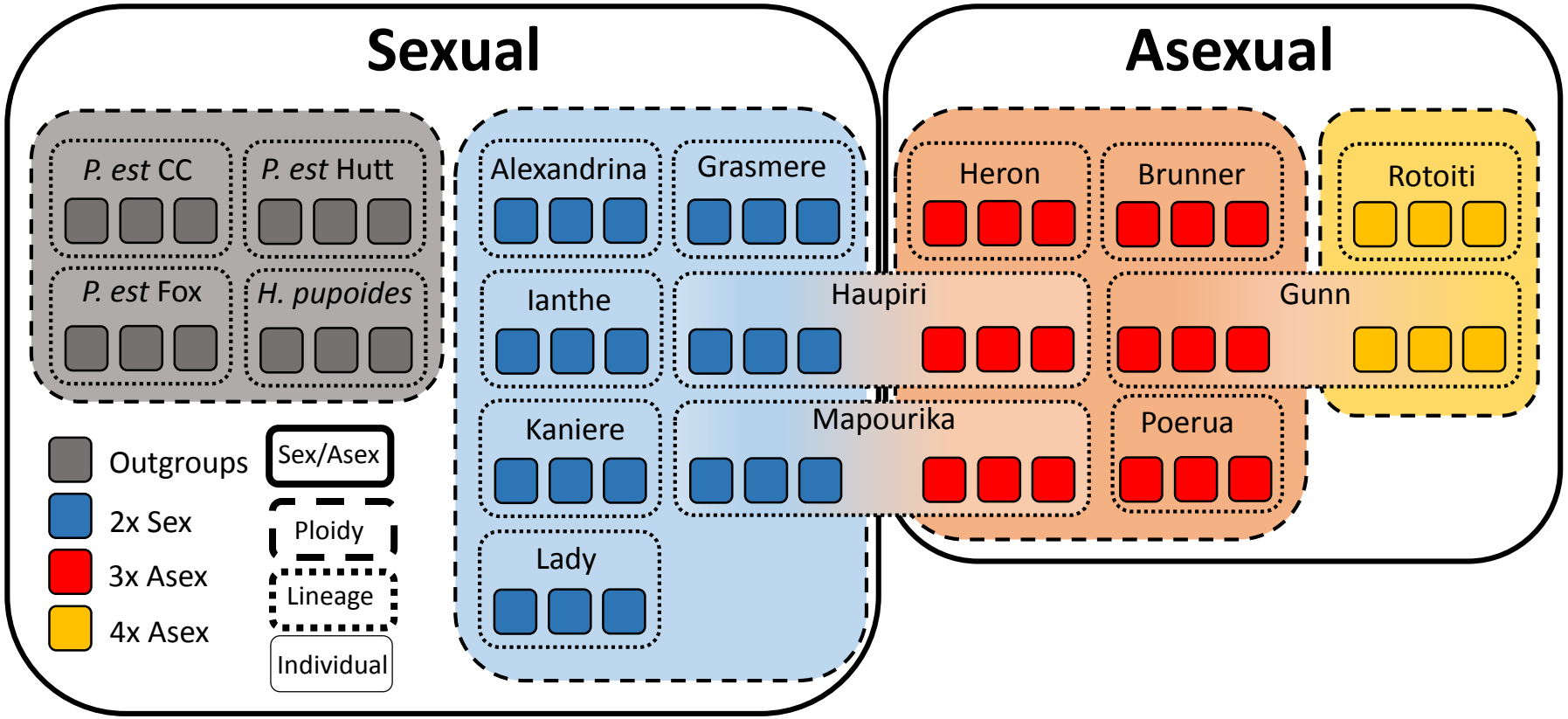

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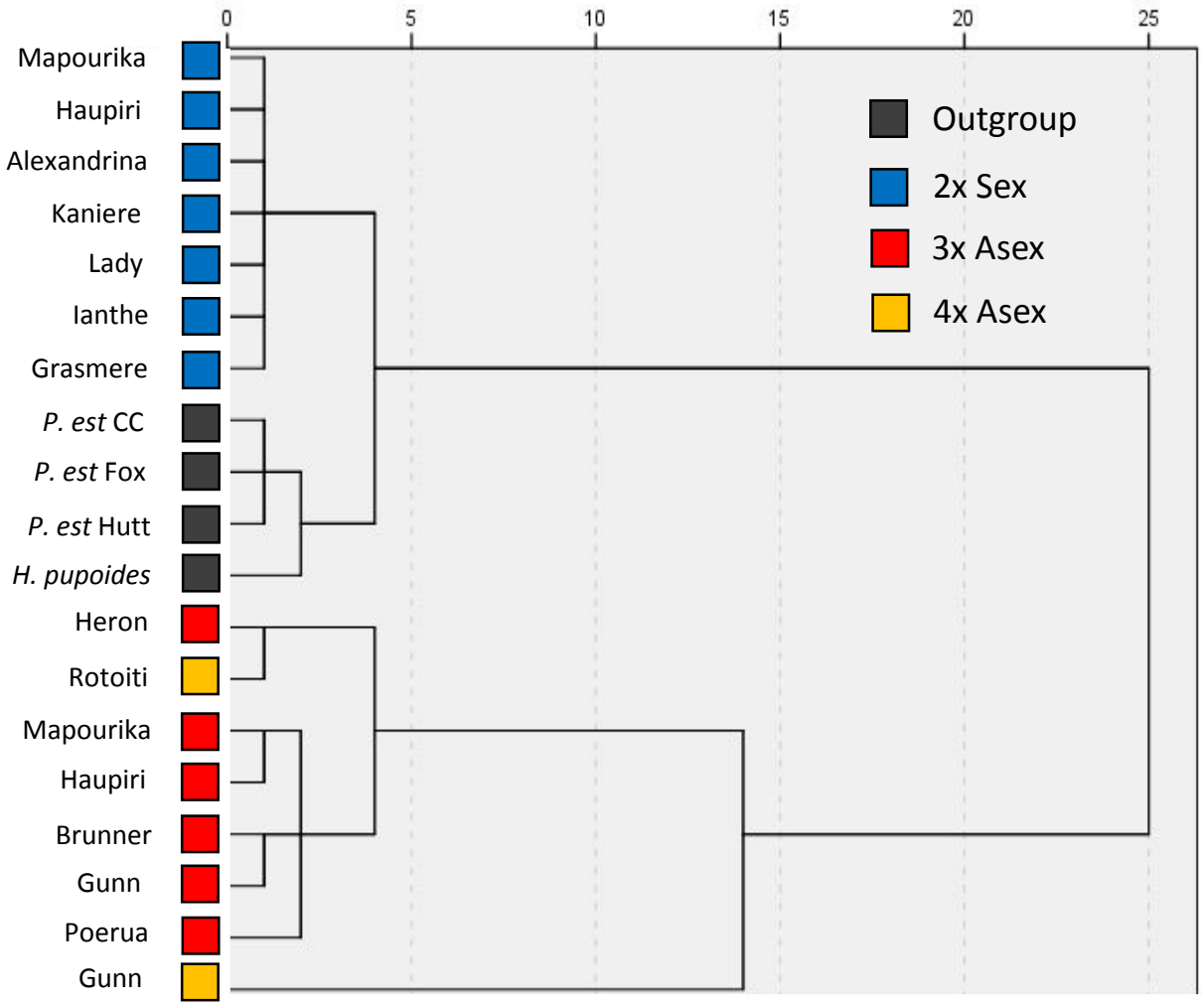

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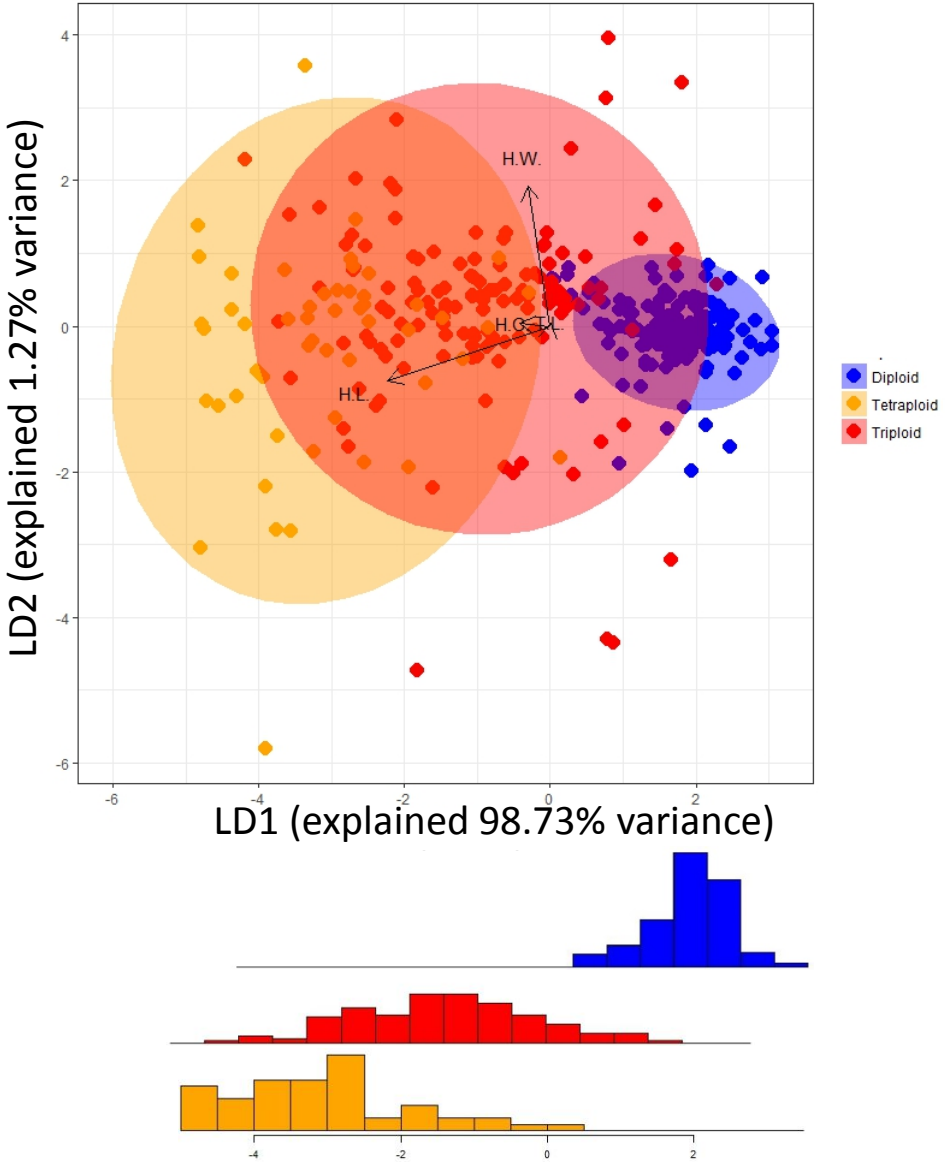

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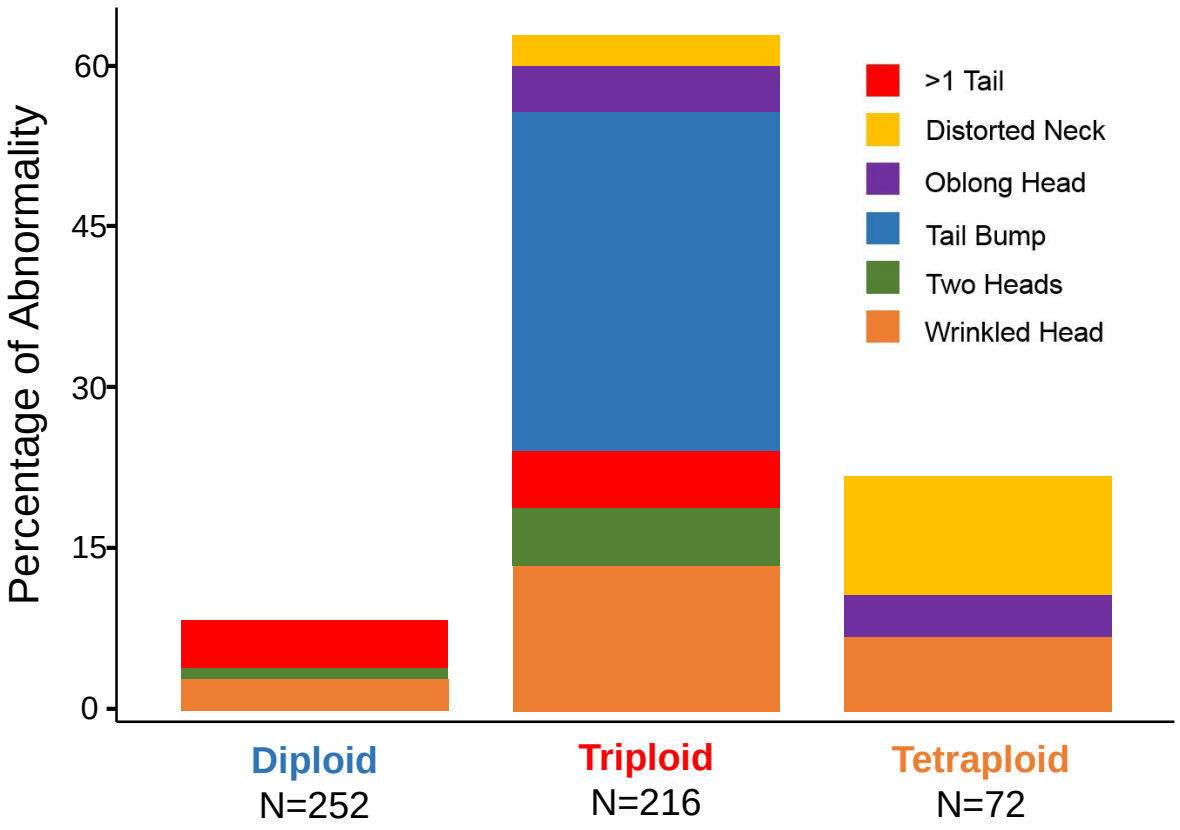

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